(12) **United States Patent**
Little et al.

(10) **Patent No.:** US 11,090,363 B2
(45) **Date of Patent:** Aug. 17, 2021

(54) **VASOACTIVE INTESTINAL PEPTIDE RELEASE FROM MICROPARTICLES**

(71) Applicant: **University of Pittsburgh—Of the Commonwealth System of Higher Education**, Pittsburgh, PA (US)

(72) Inventors: **Steven R. Little**, Allison Park, PA (US); **Andrew Jason Glowacki**, Pittsburgh, PA (US)

(73) Assignee: **UNIVERSITY OF PITTSBURGH—OF THE COMMONWEALTH SYSTEM OF HIGHER EDUCATION**, Pittsburgh, PA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 451 days.

(21) Appl. No.: **14/162,418**

(22) Filed: **Jan. 23, 2014**

(65) **Prior Publication Data**

US 2014/0142039 A1   May 22, 2014

Related U.S. Application Data

(63) Continuation-in-part of application No. 13/383,122, filed as application No. PCT/US2010/041463 on Jul. 9, 2010, now Pat. No. 8,846,098.

(60) Provisional application No. 61/224,571, filed on Jul. 10, 2009, provisional application No. 61/755,829, filed on Jan. 23, 2013.

(51) **Int. Cl.**
     A61K 38/22   (2006.01)
     A61K 9/50   (2006.01)

(52) **U.S. Cl.**
     CPC *A61K 38/2278* (2013.01); *A61K 9/5031* (2013.01)

(58) **Field of Classification Search**
     CPC A61K 38/2278; A61K 9/5031
     See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,685,883	A	8/1987	Jernberg	433/215
5,059,123	A	10/1991	Jernberg	433/215
5,197,882	A	3/1993	Jernberg	433/215
5,290,271	A	3/1994	Jernberg	604/891.1
5,364,634	A	11/1994	Lew et al.	424/451
5,885,829	A	3/1999	Mooney et al.	435/325
5,939,047	A	8/1999	Jernberg	424/49
6,123,957	A	9/2000	Jernberg	424/435
6,576,226	B1	6/2003	Jernberg	424/49
6,703,037	B1 *	3/2004	Hubbell	A61K 9/1635
				424/422
6,726,898	B2	4/2004	Jernberg	424/49
8,053,235	B2	11/2011	Buckner et al.	435/372.3
8,128,934	B2	3/2012	Suzumura et al.	435/158.1
2010/0247562	A1	9/2010	Gong et al.	424/193.1
2015/0079026	A1 *	3/2015	Little	A61K 31/436
				424/85.2

FOREIGN PATENT DOCUMENTS

DE	602004029444	11/2010
EP	2288372 B1	2/2012
WO	WO/2004/006951	1/2004
WO	WO/2008/150868	12/2008
WO	WO/2010/017220	2/2010
WO	WO/2011/004264	1/2011
WO	WO/2011/006029	1/2011
WO	WO/2011/014871	2/2011
WO	WO/2011/017303	2/2011
WO	WO/2011/085343	7/2011

OTHER PUBLICATIONS

RG502H—Sigma Catalog at www.sigmaaldrich.com Product #719897. Last accessed Jun. 29, 2016. 4 pages.*
RG505—Sigma Catalog at www.sigmaaldrich.com Product #739960. Last accessed Jun. 29, 2016. 4 pages.*
Rothstein et al., (J Mater Chem. 2008. 18:1873-1880).*
Fernandez-Martin (Eur J Immunol. Feb. 2006;36(2):318-26. Abstract Only).*
Ishioka et al., (Clin Exp Immunol. 1992. 87, 504-508).*
20080063652, Mar. 13, 2008, Pykett and Rosenzweig, U.S. Appl. No. 10/594,806, filed Sep. 18, 2007.
20090028876, Jan. 29, 2009, Teng, U.S. Appl. No. 11/814,515, filed Apr. 16, 2008.
20090208471, Aug. 20, 2009, Yun, U.S. Appl. No. 12/296,386, filed Apr. 6, 2007.
20110178502, Jul. 21, 2011, Poznansky and Chronos, U.S. Appl. No. 13/031,431, filed Feb. 21, 2011.
20110123502, May 26, 2011, Barry, et al., U.S. Appl. No. 11/814,515, filed Feb. 8, 2011.
20110123590, May 17, 2010, Iwashima and Singh, U.S. Appl. No. 12/781,536, filed May 26, 2011.
20110229448, Sep. 22, 2011, Kelleher, et al., U.S. Appl. No. 12/678,853, filed Dec. 29, 2010.
20110059125, Mar. 10, 2011, Tzianabos and Kasper, U.S. Appl. No. 12/754,948, filed Apr. 6, 2010.
Abe, T. et al. (2006) Osteoblast differentiation is impaired in SOCS-1-deficient mice, *Journal of Bone and Mineral Metabolism* 24(4), 283-290.

(Continued)

Primary Examiner — Michael D Pak
(74) *Attorney, Agent, or Firm* — Baker Botts L.L.P.

(57) **ABSTRACT**

Controlled release of VIP from PLGA microparticles was accomplished and varied through use of different polymer molecular sizes, addition of solutes to the inner aqueous phase, and use of our computer model. Released VIP from microparticles appeared to be bioactive and caused DCs to produce more CCL22 than DCs treated with blank particles at 7 and 24 hours. Additionally, DCs treated with VIP microparticle releasates recruited higher percentages of FoxP3+ T-cells in in vitro chemotaxis studies. Testing in a mouse model in vivo indicated that VIP microparticles have significant therapeutic potential to treat periodontal disease by reducing the bone loss in infected mice relative to the blank group.

9 Claims, 41 Drawing Sheets
(32 of 41 Drawing Sheet(s) Filed in Color)

(56) **References Cited**

OTHER PUBLICATIONS

Baatar, D. et al. (2007) Human Peripheral Blood T Regulatory Cells (Tregs), Functionally Primed CCR4+ Tregs and Unprimed CCR4– Tregs, Regulate Effector T Cells Using FasL, *The Journal of Immunology* 178(8), 4891-4900.

Backes, J. M. et al. (2004) Role of C-Reactive Protein in Cardiovascular Disease, *The Annals of Pharmacotherapy* 38(1), 110-118.

Baker, P. J. (2000) The role of immune responses in bone loss during periodontal disease, *Microbes and Infection* 2(10), 1181-1192.

Barrat, F. J. et al. (2002) In Vitro Generation of Interleukin 10-producing Regulatory CD4+ T Cells is Induced by Immunosuppressive Drugs and Inhibited by T Helper Type 1 (Th1)- and Th2-inducing Cytokines, *The Journal of Experimental Medicine* 195(5), 603-616.

Belghith, M. et al. (2003) TGF-[beta]-dependent mechanisms mediate restoration of self-tolerance induced by antibodies to CD3 in overt autoimmune diabetes, *Nature Medicine* 9(9), 1202-1208.

Boggess, K. A. et al. (2006) Maternal periodontal disease in early pregnancy and risk for a small-for-gestational-age infant, *American Journal of Obstetrics and Gynecology* 194(5), 1316-1322.

Brocker, T. et al. (1997) Targeted Expression of Major Histocompatibility Complex (MHC) Class II Molecules Demonstrates that Dendritic Cells Can Induce Negative but Not Positive Selection of Thymocytes in Vivo, *The Journal of Experimental Medicine* 185(3), 541-550.

Bromley, S. K. et al. (2008) Orchestrating the orchestrators: chemokines in control of T cell traffic, *Nature Immunology* 9(9), 970-980.

Brusko, T. M. et al. (2008) Human regulatory T cells: role in autoimmune disease and therapeutic opportunities, *Immunological Reviews* 223(1), 371-390.

Burns, E. et al. (2006) Cutting Edge: TLR2 is Required for the Innate Response to Porphyromonas gingivalis: Activation Leads to Bacterial Persistence and TLR2 Deficiency Attenuates Induced Alveolar Bone Resorption, *The Journal of Immunology* 177(12), 8296-8300.

Campanelli, A. P. et al. (2006) CD4+CD25+ T Cells in Skin Lesions of Patients with Cutaneous Leishmaniasis Exhibit Phenotypic and Functional Characteristics of Natural Regulatory T Cells, *Journal of Infectious Diseases* 193(9), 1313-1322.

Cannizzaro, S. M. et al. (1998) A novel biotinylated degradable polymer for cell-interactive applications, *Biotechnology and Bioengineering* 58(5), 529-535.

Cardoso, C. R. et al. (2008) Characterization of CD4+CD25+ natural regulatory T cells in the inflammatory infiltrate of human chronic periodontitis, *Journal of Leukocyte Biology* 84(1), 311-318.

Chen, D. and Bromberg, J. S. (2006) T Regulatory Cells and Migration, *American Journal of Transplantation* 6(7), 1518-1523.

Chen, H.-Y. et al. (2007) Colloids with high-definition surface structures, *Proceedings of the National Academy of Sciences* 104(27), 11173-11178.

Chen, M.-L. et al. (2005) Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF-β signals in vivo, *Proceedings of the National Academy of Sciences of the United States of America* 102(2), 419-424.

Chorny, A. et al. (2006) Vasoactive intestinal peptide induces regulatory dendritic cells that prevent acute graft-versus-host disease while maintaining the graft-versus-tumor response, *Blood* 107(9), 3787-3794.

Claudino, M. et al. (2008) The broad effects of the functional IL-10 promoter-592 polymorphism: modulation of IL-10, TIMP-3, and OPG expression and their association with periodontal disease outcome, *Journal of Leukocyte Biology* 84(6), 1565-1573.

Curiel, T. J. et al. (2004) Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival, *Nature Medicine* 10(9), 942-949.

Curtsinger, J. et al. (1997) Artificial cell surface constructs for studying receptor-ligand contributions to lymphocyte activation, *Journal of Immunological Methods* 209(1), 47-57.

de Smedt, T. et al. (1997) Effect of interleukin-10 on dendritic cell maturation and function, *European Journal of Immunology* 27(5), 1229-1235.

Delgado, M. et al. (2004) VIP/PACAP preferentially attract Th2 effectors through differential regulation of chemokine production by dendritic cells, *The FASEB Journal*.

Doh, J. and Irvine, D. J. (2006) Immunological synapse arrays: Patterned protein surfaces that modulate immunological synapse structure formation in T cells, *Proceedings of the National Academy of Sciences* 103(15), 5700-5705.

Dutzan, N. et al. (2009) Over-expression of forkhead box P3 and its association with receptor activator of nuclear factor-κ B ligand, interleukin (IL)-17, IL-10 and transforming growth factor-β during the progression of chronic periodontitis, *Journal of Clinical Periodontology* 36(5), 396-403.

Eke, P. I. and Genco, R. J. (2007) CDC Periodontal Disease Surveillance Project: Background, Objectives, and Progress Report, *Journal of Periodontology* 78(7s), 1366-1371.

Ernst, C. W. O. et al. (2007) Diminished forkhead box P3/CD25 double-positive T regulatory cells are associated with the increased nuclear factor-kB ligand (RANKL+) T cells in bone resorption lesion of periodontal disease, *Clinical & Experimental Immunology* 148(2), 271-280.

Falany, M. L. et al. (2001) Osteoclasts Secrete the Chemotactic Cytokine Mim-1, *Biochemical and Biophysical Research Communications* 281(1), 180-185.

Fan, X. et al. (2004) Donor-specific B-cell tolerance after ABO-incompatible infant heart transplantation, *Nature Medicine* 10(11), 1227-1233.

First, M. R. (2002) Immunosupressive agents and their actions, *Transplantation Proceedings* 34(5), 1369-1371.

Fisher, M. A. et al. (2008) Periodontal Disease and Other Nontraditional Risk Factors for CKD, *American Journal of Kidney Diseases* 51(1), 45-52.

Fontenot, J. D. et al. (2003) Foxp3 programs the development and function of CD4+CD25+ regulatory T cells, *Nature Immunology* 4(4), 330-336.

Frasca, L. et al. (2002) Human Anergic CD4+ T Cells Can Act as Suppressor Cells by Affecting Autologous Dendritic Cell Conditioning and Survival, *The Journal of Immunology* 168(3), 1060-1068.

Fu, S. et al. (2004) TGF-β Induces Foxp3+ T-Regulatory Cells from CD4+ CD25– Precursors, *American Journal of Transplantation* 4(10), 1614-1627.

Garlet, G. P. et al. (2005) Actinobacillus actinomycetemcomitans-induced periodontal disease in mice: patterns of cytokine, chemokine, and chemokine receptor expression and leukocyte migration, *Microbes and Infection* 7(4), 738-747.

Garlet, G. P. et al. (2006) Expression of suppressors of cytokine signaling in diseased periodontal tissues: a stop signal for disease progression?, *Journal of Periodontal Research* 41(6), 580-584.

Garlet, G. P. et al. (2010) Regulatory T cells attenuate experimental periodontitis progression in mice, *Journal of Clinical Periodontology* 37(7), 591-600.

Garlet, G. P. et al. (2006) Cytokine pattern determines the progression of experimental periodontal disease induced by Actinobacillus actinomycetemcomitans through the modulation of MMPs, RANKL, and their physiological inhibitors, *Oral Microbiology and Immunology* 21(1), 12-20.

Garlet, G. P. et al. (2007) The dual role of p55 tumour necrosis factor-α receptor in Actinobacillus actinomycetemcomitans-induced experimental periodontitis: host protection and tissue destruction, *Clinical & Experimental Immunology* 147(1), 128-138.

Garlet, G. P. et al. (2008) The essential role of IFN-γ in the control of lethal Aggregatibacter actinomycetemcomitans infection in mice, *Microbes and Infection* 10(5), 489-496.

Garlet, G. P. et al. (2003) Patterns of chemokines and chemokine receptors expression in different forms of human periodontal disease, *Journal of Periodontal Research* 38(2), 210-217.

Geissmann, F. et al. (1999) TGF-β1 Prevents the Noncognate Maturation of Human Dendritic Langerhans Cells, *The Journal of Immunology* 162(8), 4567-4575.

(56)   **References Cited**

OTHER PUBLICATIONS

Gershon, R. K. and Kondo, K. (1971) Infectious immunological tolerance, *Immunology* 21(6), 903-914.

Graca, L. et al. (2004) Donor-specific transplantation tolerance: the paradoxical behavior of CD4+CD25+ T cells, *Proceedings of the National Academy of Sciences of the United States of America* 101(27), 10122-10126.

Graca, L. et al. (2002) Both CD4+CD25+ and CD4+CD25- Regulatory Cells Mediate Dominant Transplantation Tolerance, *The Journal of Immunology* 168(11), 5558-5565.

Grakoui, A. et al. (1999) The Immunological Synapse: A Molecular Machine Controlling T Cell Activation, *Science* 285(5425), 221.

Graves, D. T. and Cochran, D. (2003) The contribution of interleukin-1 and tumor necrosis factor to periodontal tissue destruction, *Journal of Periodontology* 74(3), 391-401.

Graves, D. T. et al. (2008) The use of rodent models to investigate host-bacteria interactions related to periodontal diseases, *Journal of Clinical Periodontology* 35(2), 89-105.

Green, E. A. et al. (2003) CD4+CD25+ T regulatory cells control anti-islet CD8+ T cells through TGF-β-TGF-β receptor interactions in type 1 diabetes, *Proceedings of the National Academy of Sciences* 100(19), 10878-10883.

Gregori, S. et al. (2001) Regulatory T Cells Induced by 1α,25-Dihydroxyvitamin D3 and Mycophenolate Mofetil Treatment Mediate Transplantation Tolerance, *The Journal of Immunology* 167(4), 1945-1953.

Gurgel, B. C. d. V. et al. (2005) Selective COX-2 inhibitor reduces bone healing in bone defects, *Brazilian Oral Research* 19(4), 312-316.

Haining, W. N. et al. (2004) pH-Triggered Microparticles for Peptide Vaccination, *The Journal of Immunology* 173(4), 2578-2585.

Hanes, J. et al. (1995) Polymer microspheres for vaccine delivery, *Pharmaceutical Biotechnology* 6, 389-412.

Hariharan, S. et al. (2000) Improved Graft Survival after Renal Transplantation in the United States, 1988 to 1996, *New England Journal of Medicine* 342(9), 605-612.

Hasturk, H. et al. (2007) Resolvin E1 Regulates Inflammation at the Cellular and Tissue Level and Restores Tissue Homeostasis in Vivo, *The Journal of Immunology* 179(10), 7021-7029.

Herold, K. C. et al. (2002) Anti-CD3 Monoclonal Antibody in New-Onset Type 1 Diabetes Mellitus, *New England Journal of Medicine* 346(22), 1692-1698.

Hoffmann, P. et al. (2004) Large-scale in vitro expansion of polyclonal human CD4+CD25high regulatory T cells, *Blood* 104(3), 895-903.

Hori, S. T. S. (2003) Control of Regulatory T Cell Development by the Transcription Factor Foxp3, *Science* 299(5609), 1057.

Iellem, A. et al. (2001) Unique Chemotactic Response Profile and Specific Expression of Chemokine Receptors Ccr4 and Ccr8 by Cd4+Cd25+ Regulatory T Cells, *The Journal of Experimental Medicine* 194(6), 847-854.

Inaba, K. et al. (1992) Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/ macrophage colony-stimulating factor, *The Journal of Experimental Medicine* 176(6), 1693-1702.

Itoh, K. et al. (2001) Bone Morphogenetic Protein 2 Stimulates Osteoclast Differentiation and Survival Supported by Receptor Activator of Nuclear Factor-κB Ligand, *Endocrinology* 142(8), 3656-3662.

Jenkins, M. K. et al. (1990) Inhibition of antigen-specific proliferation of type 1 murine T cell clones after stimulation with immobilized anti-CD3 monoclonal antibody, *The Journal of Immunology* 144(1), 16-22.

Kajita, K. et al. (2007) Quantitative messenger RNA expression of Toll-like receptors and interferon-α1 in gingivitis and periodontitis, *Oral Microbiology and Immunology* 22(6), 398-402.

Karim, M. et al. (2005) CD25+CD4+ regulatory T cells generated by exposure to a model protein antigen prevent allograft rejection: antigen-specific reactivation in vivo is critical for bystander regulation, *Blood* 105(12), 4871-4877.

Keegan, M. E. et al. (2004) Biodegradable Microspheres with Enhanced Capacity for Covalently Bound Surface Ligands, *Macromolecules* 37(26), 9779-9784.

Kempen, D. H. R. et al. (2004) Development of biodegradable poly(propylene fumarate)/poly(lactic-co-glycolic acid) blend microspheres. II. Controlled drug release and microsphere degradation, *Journal of Biomedical Materials Research Part A* 70A(2), 293-302.

Kingsley, C. I. et al. (2002) CD25+CD4+ Regulatory T Cells Prevent Graft Rejection: CTLA-4- and IL-10-Dependent Immunoregulation of Alloresponses, *The Journal of Immunology* 168(3), 1080-1086.

Köhler, G. and Milstein, C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity, *Nature* 256(5517), 495-497.

Lee, I. et al. (2005) Recruitment of Foxp3+ T regulatory cells mediating allograft tolerance depends on the CCR4 chemokine receptor, *The Journal of Experimental Medicine* 201(7), 1037-1044.

Levings, M. K. et al. (2001) Human Cd25+Cd4+ T Regulatory Cells Suppress Naive and Memory T Cell Proliferation and Can Be Expanded in Vitro without Loss of Function, *The Journal of Experimental Medicine* 193(11), 1295-1302.

Li, Y. et al. (1999) Blocking both signal 1 and signal 2 of T-cell activation prevents apoptosis of alloreactive T cells and induction of peripheral allograft tolerance, *Nature Medicine* 5(11), 1298-1302.

Lim, H. W. et al. (2006) Regulation of Trafficking Receptor Expression in Human Forkhead Box P3+ Regulatory T Cells, *The Journal of Immunology* 177(2), 840-851.

Little, S. R. et al. (2004) Poly-β amino ester-containing microparticles enhance the activity of nonviral genetic vaccines, *Proceedings of the National Academy of Sciences of the United States of America* 101(26), 9534-9539.

Lorentzon, M. et al. (2005) Reduced Bone Mineral Density in SOCS-2-Deficient Mice, *Pediatric Research* 57(2), 223-226.

Lu, L. et al. (2000) Controlled release of transforming growth factor β1 from biodegradable polymer microparticles, *Journal of Biomedical Materials Research* 50(3), 440-451.

Mahnke, K. et al. (2003) Induction of CD4+/CD25+ regulatory T cells by targeting of antigens to immature dendritic cells, *Blood* 101(12), 4862-4869.

Martin, T. J. and Sims, N. A. (2005) Osteoclast-derived activity in the coupling of bone formation to resorption, *Trends in Molecular Medicine* 11(2), 76-81.

Matzinger, P. (2002) The Danger Model: A Renewed Sense of Self, *Science* 296(5566), 301.

Matzinger, P. and Guerder, S. (1989) Does T-cell tolerance require a dedicated antigen-presenting cell?, *Nature* 338(6210), 74-76.

Merck, E. et al. (2006) Ligation of the FcRγ Chain-Associated Human Osteoclast-Associated Receptor Enhances the Proinflammatory Responses of Human Monocytes and Neutrophils, *The Journal of Immunology* 176(5), 3149-3156.

Mescher, M. F. (1992) Surface contact requirements for activation of cytotoxic T lymphocytes, *The Journal of Immunology* 149(7), 2402-2405.

Monks, C. R. F. et al. (1998) Three-dimensional segregation of supramolecular activation clusters in T cells, *Nature* 395(6697), 82-86.

Mundy, G. R. (1999) Cellular and molecular regulation of bone turnover, *Bone* 24(5, Supplement 1), 35S-38S.

Nakamura, H. et al. (2008) Lack of Toll-like receptor 4 decreases lipopolysaccharide-induced bone resorption in C3H/HeJ mice in vivo, *Oral Microbiology and Immunology* 23(3), 190-195.

Nonnenmacher, C. et al. (2003) DNA from Periodontopathogenic Bacteria is Immunostimulatory for Mouse and Human Immune Cells, *Infection and Immunity* 71(2), 850-856.

O'Donnell, P. B. and McGinity, J. W. (1997) Preparation of microspheres by the solvent evaporation technique, *Advanced Drug Delivery Reviews* 28(1), 25-42.

Offenbacher, S. and Beck, J. D. (2005) A perspective on the potential cardioprotective benefits of periodontal therapy, *American Heart Journal* 149(6), 950-954.

(56)   **References Cited**

OTHER PUBLICATIONS

Ouyang, X. et al. (2006) SOCS-2 interferes with myotube formation and potentiates osteoblast differentiation through upregulation of JunB in C2C12 cells, *Journal of Cellular Physiology* 207(2), 428-436.

Pardoll, D. (2003) Does the Immune System See Tumors as Foreign or Self?, *Annual Review of Immunology* 21(1), 807-839.

Pennsylvania Department of Health. (2002) Status of Oral Health in Pennsylvania, (Pennsylvania Department of Health, Ed.).

Perez, V. L. et al. (1997) Induction of Peripheral T Cell Tolerance in Vivo Requires CTLA-4 Engagement, *Immunity* 6(4), 411-417.

Pfeilschifter, J. and Mundy, G. R. (1987) Modulation of type beta transforming growth factor activity in bone cultures by osteotropic hormones, *Proceedings of the National Academy of Sciences* 84(7), 2024-2028.

Piccirillo, C. A. et al. (2002) CD4+CD25+ Regulatory T Cells Can Mediate Suppressor Function in the Absence of Transforming Growth Factor β1 Production and Responsiveness, *The Journal of Experimental Medicine* 196(2), 237-246.

Pietra, B. A. and Boucek, M. M. (2000) Immunosuppression for pediatric cardiac transplantation in the modern era, *Progress in Pediatric Cardiology* 11(2), 115-129.

Pischon, N. et al. (2008) Association among rheumatoid arthritis, oral hygiene, and periodontitis., *Journal of Periodontology* 79(6), 979-986.

Ragazzo, J. L. et al. (2001) Costimulation via lymphocyte function-associated antigen 1 in the absence of CD28 ligation promotes anergy of naïve CD4+ T cells, *Proceedings of the National Academy of Sciences* 98(1), 241-246.

Raimondi, G. et al. (2006) Regulated Compartmentalization of Programmed Cell Death-1 Discriminates CD4+CD25+ Resting Regulatory T Cells from Activated T Cells, *The Journal of Immunology* 176(5), 2808-2816.

Ribeiro, F. V. et al. (2006) Selective Cyclooxygenase-2 Inhibitor May Impair Bone Healing Around Titanium Implants in Rats, *Journal of Periodontology* 77(10), 1731-1735.

Riley, J. L. et al. (2009) Human T Regulatory Cell Therapy: Take a Billion or So and Call Me in the Morning, *Immunity* 30(5), 656-665.

Rosenberg, A. S. (1991) in *Current Protocols in Immunology* (Coligan, J. E., Kruisbeek, A. M., Margulies, D. H., Shevach, E. M., and Strober, W., Eds.), pp. 4.4.01-04.04.12, John Wiley and Sons, New York.

Rothstein, S. N. et al. (2008) A simple model framework for the prediction of controlled release from bulk eroding polymer matrices, *Journal of Materials Chemistry* 18(16), 1873-1880.

Rothstein, S. N. et al. (2009) A unified mathematical model for the prediction of controlled release from surface and bulk eroding polymer matrices, *Biomaterials* 30(8), 1657-1664.

Sakaguchi, S. et al. (1995) Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases, *The Journal of Immunology* 155(3), 1151-1164.

Sakhalkar, H. S. et al. (2003) Leukocyte-inspired biodegradable particles that selectively and avidly adhere to inflamed endothelium in vitro and in vivo, *Proceedings of the National Academy of Sciences* 100(26), 15895-15900.

Salomon, B. et al. (2000) B7/CD28 Costimulation Is Essential for the Homeostasis of the CD4+CD25+ Immunoregulatory T Cells that Control Autoimmune Diabetes, *Immunity* 12(4), 431-440.

Sather, B. D. et al. (2007) Altering the distribution of Foxp3+ regulatory T cells results in tissue-specific inflammatory disease, *The Journal of Experimental Medicine* 204(6), 1335-1347.

Seymour, G. J. et al. (2007) Relationship between periodontal infections and systemic disease, *Clinical Microbiology & Infection* 13(S4), 3-10.

Simon, A. M. et al. (2002) Cyclo-Oxygenase 2 Function Is Essential for Bone Fracture Healing, *Journal of Bone and Mineral Research* 17(6), 963-976.

Steinman, R. M. and Nussenzweig, M. C. (2002) Avoiding horror autotoxicus: The importance of dendritic cells in peripheral T cell tolerance, *Proceedings of the National Academy of Sciences* 99(1), 351-358.

Steinman, R. M. et al. (2003) Tolerogenic Dendritic Cells, *Annual Review of Immunology* 21(1), 685-711.

Suda, T. et al. (1992) Modulation of Osteoclast Differentiation, *Endocrine Reviews* 13(1), 66-80.

Taams, L. S. et al. (2002) Antigen-specific T cell suppression by human CD4+CD25+ regulatory T cells, *European Journal of Immunology* 32(6), 1621-1630.

Takahashi, N. et al. (1988) Osteoblastic Cells are Involved in Osteoclast Formation, *Endocrinology* 123(5), 2600-2602.

Takahashi, N. et al. (1991) Deficiency of Osteoclasts in Osteopetrotic Mice is Due to a Defect in the Local Microenvironment Provided by Osteoblastic Cells, *Endocrinology* 128(4), 1792-1796.

Tang, Q. and Bluestone, J. A. (2008) The Foxp3+ regulatory T cell: a jack of all trades, master of regulation, *Nature Immunology* 9(3), 239-244.

Taubman, M. A. et al. (2005) Immune Response: The Key to Bone Resorption in Periodontal Disease, *Journal of Periodontology* 76(11-s), 2033-2041.

Taylor, P. A. et al. (2002) Tolerance induction of alloreactive T cells via ex vivo blockade of the CD40:CD40L costimulatory pathway results in the generation of a potent immune regulatory cell, *Blood* 99(12), 4601-4609.

Taylor, P. A. et al. (2002) The infusion of ex vivo activated and expanded CD4+CD25+ immune regulatory cells inhibits graft-versus-host disease lethality, *Blood* 99(10), 3493-3499.

Teitelbaum, S. L. (2000) Bone Resorption by Osteoclasts, *Science* 289(5484), 1504.

Thomas, T. T. et al. (2004) Microparticulate formulations for the controlled release of interleukin-2, *Journal of Pharmaceutical Sciences* 93(5), 1100-1109.

Udagawa, N. et al. (2000) Osteoprotegerin Produced by Osteoblasts is an Important Regulator in Osteoclast Development and Function, *Endocrinology* 141(9), 3478-3484.

van Maurik, A. et al. (2002) Cutting Edge: CD4+CD25+ Alloantigen-Specific Immunoregulatory Cells That Can Prevent CD8+ T Cell-Mediated Graft Rejection: Implications for Anti-CD154 Immunotherapy, *The Journal of Immunology* 169(10), 5401-5404.

Vignali, D. A. A. et al. (2008) How regulatory T cells work, *Nature Reviews Immunology* 8(7), 523-532.

Volkmann, A. et al. (1997) Antigen-presenting cells in the thymus that can negatively select MHC class II-restricted T cells recognizing a circulating self antigen, *The Journal of Immunology* 158(2), 693-706.

Vuolteenaho, K. et al. (2008) Non-Steroidal Anti-Inflammatory Drugs, Cyclooxygenase-2 and the Bone Healing Process, *Basic & Clinical Pharmacology & Toxicology* 102(1), 10-14.

Walker, M. R. et al. (2003) Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25- T cells, *The Journal of Clinical Investigation* 112(9), 1437-1443.

Wang, Z. et al. (2006) Use of the Inhibitory Effect of Apoptotic Cells on Dendritic Cells for Graft Survival via T-Cell Deletion and Regulatory T Cells, *American Journal of Transplantation* 6(6), 1297-1311.

Williams, R. C. et al. (2001) Treatment of Periodontitis by Local Administration of Minocycline Microspheres: A Controlled Trial, *Journal of Periodontology* 72(11), 1535-1544.

Yamagiwa, S. et al. (2001) A Role for TGF-β in the Generation and Expansion of CD4+CD25+ Regulatory T Cells from Human Peripheral Blood, *The Journal of Immunology* 166(12), 7282-7289.

Yu, J. J. et al. (2007) An essential role for IL-17 in preventing pathogen-initiated bone destruction: recruitment of neutrophils to inflamed bone requires IL-17 receptor-dependent signals, *Blood* 109(9), 3794-3802.

Zhang, N. et al. (2009) Regulatory T Cells Sequentially Migrate from Inflamed Tissues to Draining Lymph Nodes to Suppress the Alloimmune Response, *Immunity* 30(3), 458-469.

Zhang, X. et al. (2004) Short- and Long-Term Effects of IL-1 and TNF Antagonists on Periodontal Wound Healing, *The Journal of Immunology* 173(5), 3514-3523.

(56) **References Cited**

OTHER PUBLICATIONS

Zhang, Z. et al. (1996) Pattern of liver, kidney, heart, and intestine allograft rejection in different mouse strain combinations, *Transplantation* 62(9), 1267-1272.

Zhao, C. et al. (2006) Bidirectional ephrinB2-EphB4 signaling controls bone homeostasis, *Cell Metabolism* 4(2), 111-121.

Zhao, X. et al. (2005) Directed cell migration via chemoattractants released from degradable microspheres, *Biomaterials* 26(24), 5048-5063.

Benson, et al., "All-Trans Retinoic Acid Mediates Enhanced T Reg Cell Growth, Differentiation, and Gut Homing in the Face of High Levels of Co-Stimulation." *J Exp Med*, 204(8):1765-1774 (2007).

Beyersdorf, et al., "Selective Targeting of Regulatory T Cells with CD28 Superagonists Allows Effective Therapy of Experimental Autoimmune Encephalomyelitis." *J Exp Med*, 202(3):445-455 (2005).

Bour-Jordan and Bluestone, "Regulating the Regulators: Costimulatory Signals Control the Homeostasis and Function of Regulatory T Cells." *Immunol Rev*, 229(1):41-66 (2009).

Brunstein, et al., "Alternative Donor Transplantation after Reduced Intensity Conditioning: Results of Parallel Phase 2 Trials Using Partially HLA-Mismatched Related Bone Marrow or Unrelated Double Umbilical Cord Blood Grafts." *Blood*, 118(2):282-288 (2011a).

Brunstein, et al., "Infusion of Ex Vivo Expanded T Regulatory Cells in Adults Transplanted with Umbilical Cord Blood: Safety Profile and Detection Kinetics." *Blood*, 117(3):1061-1070 (2011b).

Campbell and Koch, "Phenotypical and Functional Specialization of Foxp3+ Regulatory T Cells." *Nat Rev Immunol*, 11(2):119-130 (2011).

Chen, et al., "Conversion of Peripheral CD4+CD25– Naive T Cells to CD4+CD25+ Regulatory T Cells by TGF-Beta Induction of Transcription Factor Foxp3." *J Exp Med*, 198(12):1875-1886 (2003).

Cobbold, et al., "Infectious Tolerance via the Consumption of Essential Amino Acids and MTOR Signaling." *Proc Natl Acad Sci U S A*, 106(29):12055-12060 (2009).

de Kleer, et al., "CD4+CD25bright Regulatory T Cells Actively Regulate Inflammation in the Joints of Patients with the Remitting Form of Juvenile Idiopathic Arthritis." *J Immunol*, 172(10):6435-6443 (2004).

Delgado, et al., (2001) "Vasoactive intestinal peptide prevents experimental arthritis by downregulating both autoimmune and inflammatory components of the disease." *Nat. Med.* 7:563-568.

DePaolo, et al., "Co-Adjuvant Effects of Retinoic Acid and IL-15 Induce Inflammatory Immunity to Dietary Antigens." *Nature*, 471(7337):220-224 (2011).

Fernandez-Martin, et al., (2006) "Vasoactive intestinal peptide induces regulatory T cells during experimental autoimmune encephalomyelitis" Eur. J. Immunol. 36:318-326.

Floess, et al., "Epigenetic Control of the Foxp3 Locus in Regulatory T Cells." *PLoS Biol*, 5(2):e38 (2007).

Glowacki, et al., "Prevention of Inflammation-Mediated Bone Loss in Murine and Canine Periodontal Disease via Recruitment of Regulatory Lymphocytes." *Proc Natl Acad Sci U S A*, 110(46):18525-18530 (2013).

Gonzalez-Rey, et al., (2007) "Vasoactive intestinal peptide and regulatory T-cell induction: a new mechanism and therapeutic potential for immune homeostasis" Trends in Molecular Medicine 13:241-251.

Haxhinasto, et al., "The AKT-MTOR Axis Regulates De Novo Differentiation of CD4+Foxp3+ Cells." *J Exp Med*, 205(3):565-574 (2008).

Hippen, et al., "Generation and Large-Scale Expansion of Human Inducible Regulatory T Cells That Suppress Graft-Versus-Host Disease." *Am J Transplant*, 11(6):1148-1157 (2011).

Kopf, et al., "Rapamycin Inhibits Differentiation of Th17 Cells and Promotes Generation of Foxp3+ T Regulatory Cells." *Int Immunopharmacol*, 7(13):1819-1824 (2007).

Lu, et al., "Characterization of Protective Human CD4CD25 Foxp3 Regulatory T Cells Generated with IL-2, TGF-Beta and Retinoic Acid." *PLoS One*, 5(12):e15150 (2010).

Maroof, "Chapter 17. Generation of Murine Bone-Marrow-Derived Dendritic Cells." In Robinson, Stagg and Knight, eds., vol. 64, Methods in Molecular Medicine: *Dendritic Cell Protocols*, 191-98 (2001).

Misaka, et al., (2010) Inhalable powder formulation of a stabilized vasoactive intestinal peptide (VIP) derivative: Anti-inflammatory effect in experimental asthmatic rats. *Peptides* 31:72-78.

Park, et al., (2007) Three Dimensional Micro-Computed Tomographic Imaging of Alveolar Bone in Experimental Bone Loss or Repair. *J. Periodontol.* 78:273-281.

Pozo, et al., (2007) Tuning immune tolerance with vasoactive intestinal peptide: A new therapeutic approach for immune disorders. *Peptides* 28:1833-1846.

Raimondi, et al., "Mammalian Target of Rapamycin Inhibition and Alloantigen-Specific Regulatory T Cells Synergize to Promote Long-Term Graft Survival in Immunocompetent Recipients." *J Immunol*, 184(2):624-636 (2010).

Robinson, et al., "Tregs and Allergic Disease." *J Clin Invest*, 114(10):1389-1397 (2004).

Suntharalingam, et al., "Cytokine Storm in a Phase 1 Trial of the Anti-CD28 Monoclonal Antibody TGN1412." *N Engl J Med*, 355(10):1018-1028 (2006).

Thomson, et al., "Immunoregulatory Functions of MTOR Inhibition." *Nat Rev Immunol*, 9(5):324-337 (2009).

Trzonkowski, et al., "First-in-Man Clinical Results of the Treatment of Patients with Graft Versus Host Disease with Human Ex Vivo Expanded CD4+CD25+CD127– T Regulatory Cells." *Clin Immunol*, 133(1):22-26 (2009).

Wang, et al., "TGF-Beta-Dependent Suppressive Function of Tregs Requires Wild-Type Levels of CD18 in a Mouse Model of Psoriasis." *J Clin Invest*, 118(7):2629-2639 (2008).

Webster, et al., "In Vivo Expansion of T Reg Cells with IL-2-Mab Complexes: Induction of Resistance to EAE and Long-Term Acceptance of Islet Allografts without Immunosuppression." *J Exp Med*, 206(4):751-760 (2009).

Wernig, et al., (2008) Depot formulation of vasoactive intestinal peptide by protamine-based biodegradable nanoparticles. *J of Controlled Release* 130:192-198.

Schor, H. et al., (2000) "Modulation of leukocyte behavior by an inflamed extracellular matrix," *Dev. Immunol.* 7(2-4), 227-238.

Wilkinson, P. C. and Newman, I., (1994) "Chemoattractant activity of IL-2 for human lymphocytes: a requirement for the IL-2 receptor beta-chain," *Immunology* 82(1), 134-139.

Linder, et al., "Structure and Expression of the Gene Encoding the Vasoactive Intestinal Peptide Precursor." Proc Natl Acad Sci U S A, 84(2):605-609 (1987).

Onoue, et al., "Structure-Activity Relationship of Vasoactive Intestinal Peptide (Vip): Potent Agonists and Potential Clinical Applications." Naunyn Schmiedebergs Arch Pharmacol, 377(4-6): Abstract only (2008).

Delgado, M. et al., (2005) "Vasoactive intestinal peptide generates CD4+CD25+ regulatory T cells in vivo," *J. Leukoc. Biol.* 78(6), 1327-1338.

Jhunjhunwala, et al., (2012a) "Controlled release formulations of IL-2, TGF-β1 and rapamycin for the induction of regulatory T cells," *J. Control. Release* 159(1), 78-84.

Zorn, E. et al., (2006) "IL-2 regulates FOXP3 expression in human CD4+CD25+ regulatory T cells through a STAT-dependent mechanism and induces the expansion of these cells in vivo," *Blood* 108(5), 1571-1579.

Steenblock and Fahmy, (2008) "A Comprehensive Platform for Ex Vivo T-cell Expansion Based on Biodegradable Polymeric Artificial Antigen-presenting Cells," *Mol. Ther.* 16(4), 765-772.

PCT International Search Report for WO2011006029 of International Application No. PCT/US2010/041463 dated Oct. 12, 2010.

Sigma-Aldrich Catalog for Resomer Polymers (dating back to 1977). (www.signaaldrich.com/materials-scince/polymer-scienc/resomer.html) (last accessed Jul. 8, 2016).

Jhunjhunwala, et al., (2012c) "Bioinspired Controlled Release of CCL22 Recruits Regulatory T Cells in Vivo." *Adv. Mater.* 24:4735-4738.

(56) **References Cited**

OTHER PUBLICATIONS

Abrams, et al., "Interleukin-2 Therapy in Patients with HIV Infection. Insight—Esprit Study Group and SILCAAT Scientific Committee." *N Engl J Med* 2009; 361:1548-1559.

Li D., et. al. "Interleukin 2 gene transfer prevents NKG2D suppression and enhances antitumor efficacy in combination with cisplatin for head and neck squamous cell cancer." *Cancer Research* 2002; 62(14): 4023-4028.

Rousseau F. et. al. "Local and systemic effects of an allogeneic tumor cell vaccine transgenic human lymphotactin with interleukin-2 in patients combining with advanced or refractory neuroblastoma." *Blood* 2003; 101(5): 1718-1726.

Mucida, et al., "Reciprocal Th17 and Regulatory T Cell Differentiation Mediated by Retinoic Acid." *Science*, 317(5835):256-260 (2007).
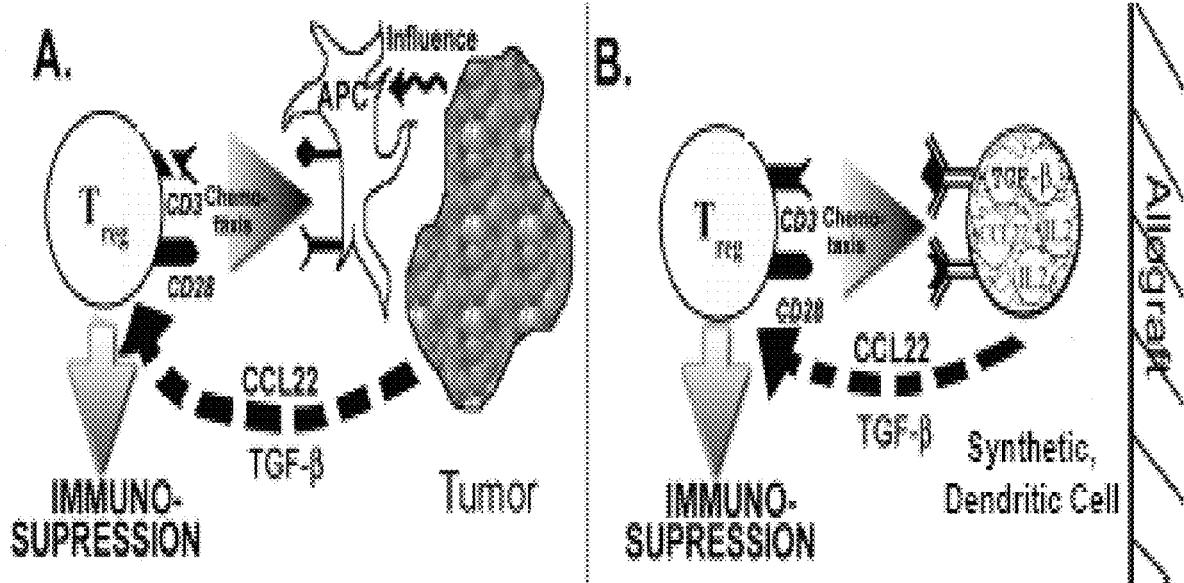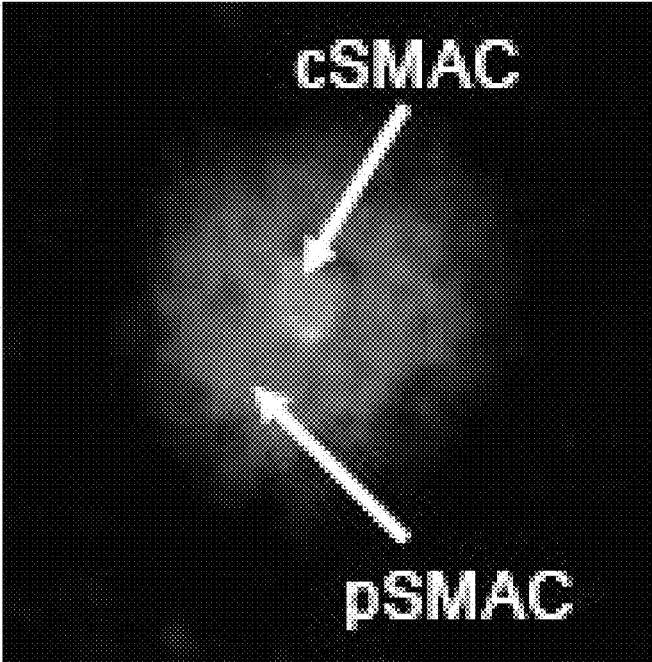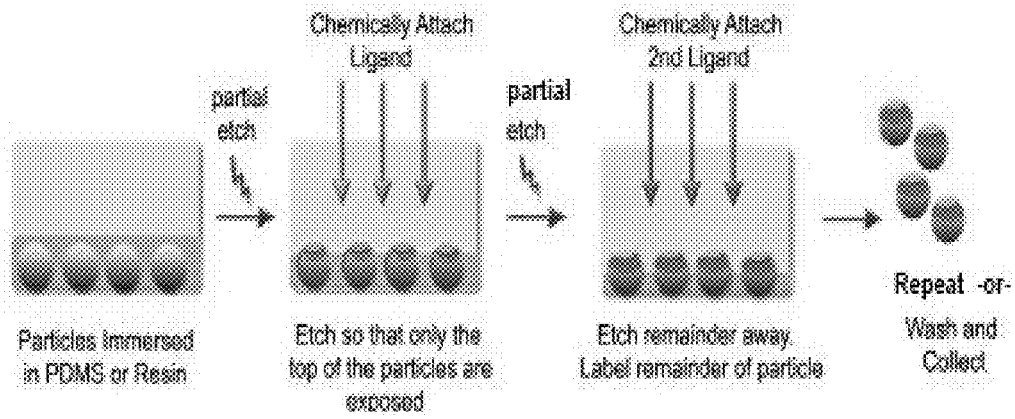
* cited by examiner
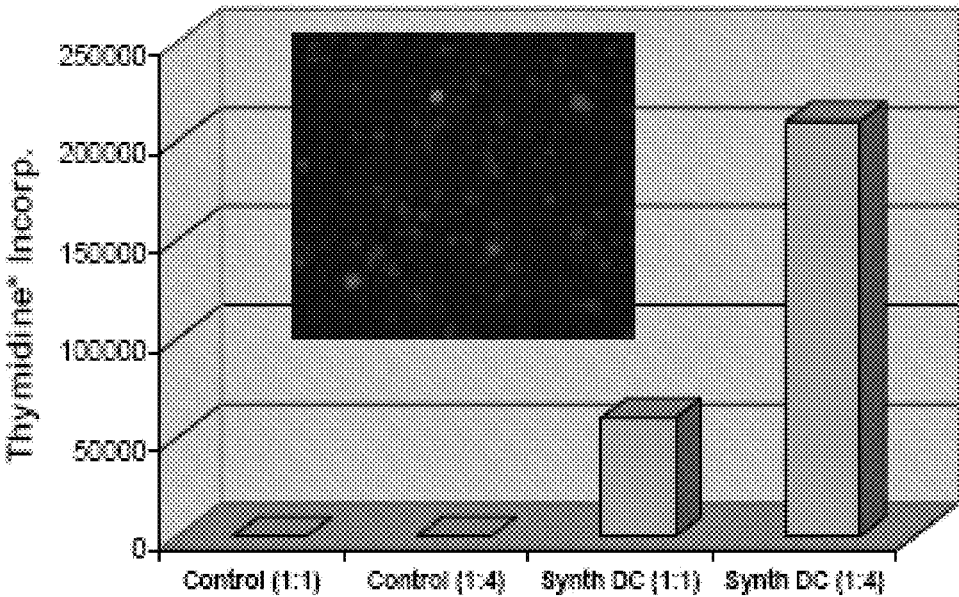
FIGURE 1

Figure 2

Figure 3

Chemically Attach Ligand

Chemically Attach 2nd Ligand

partial etch

partial etch

Particles immersed in PDMS or Resin

Etch so that only the top of the particles are exposed

Etch remainder away. Label remainder of particle

Repeat -or-

Wash and Collect

A

cSMAC

pSMAC
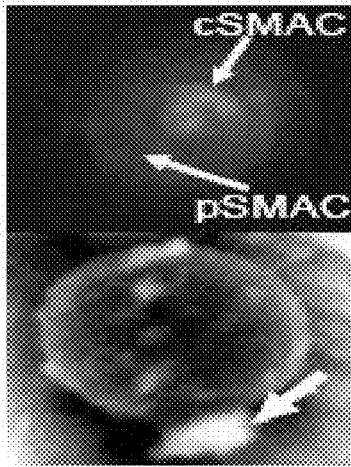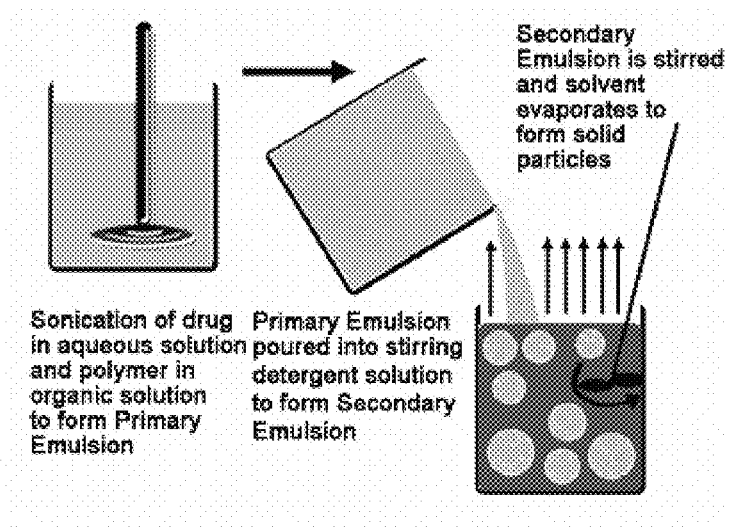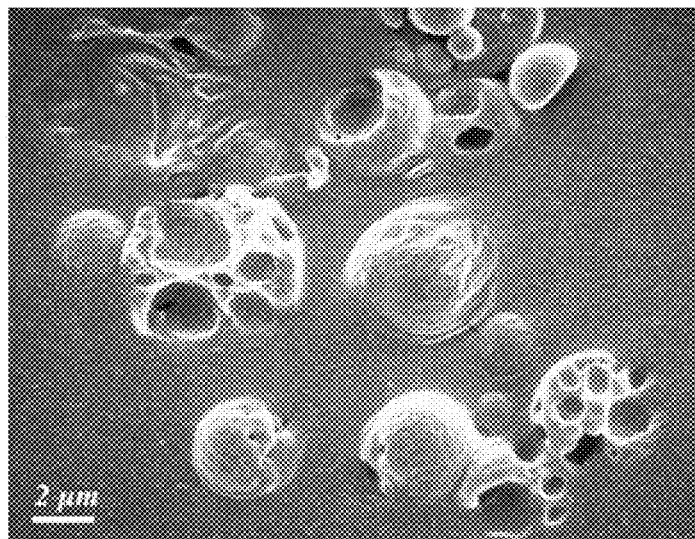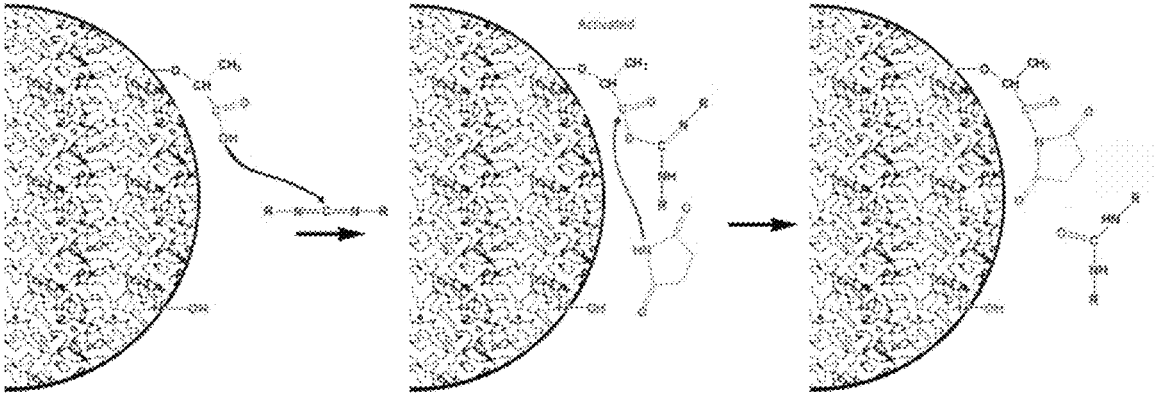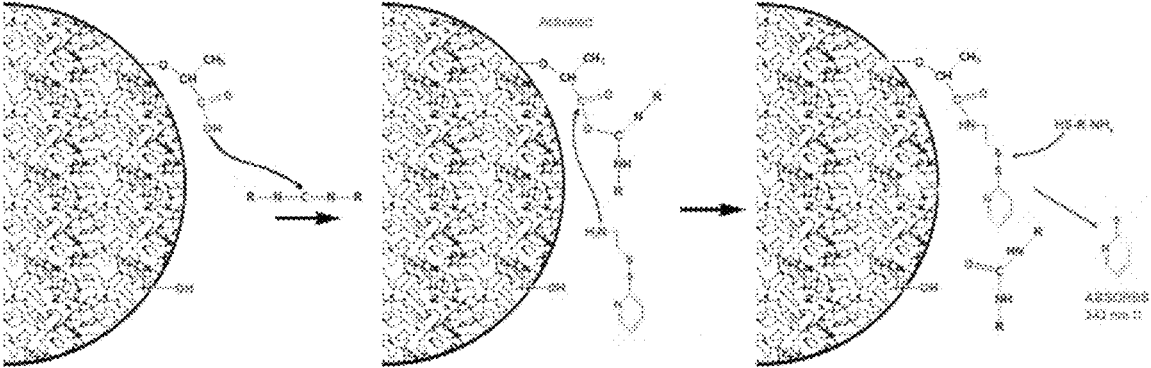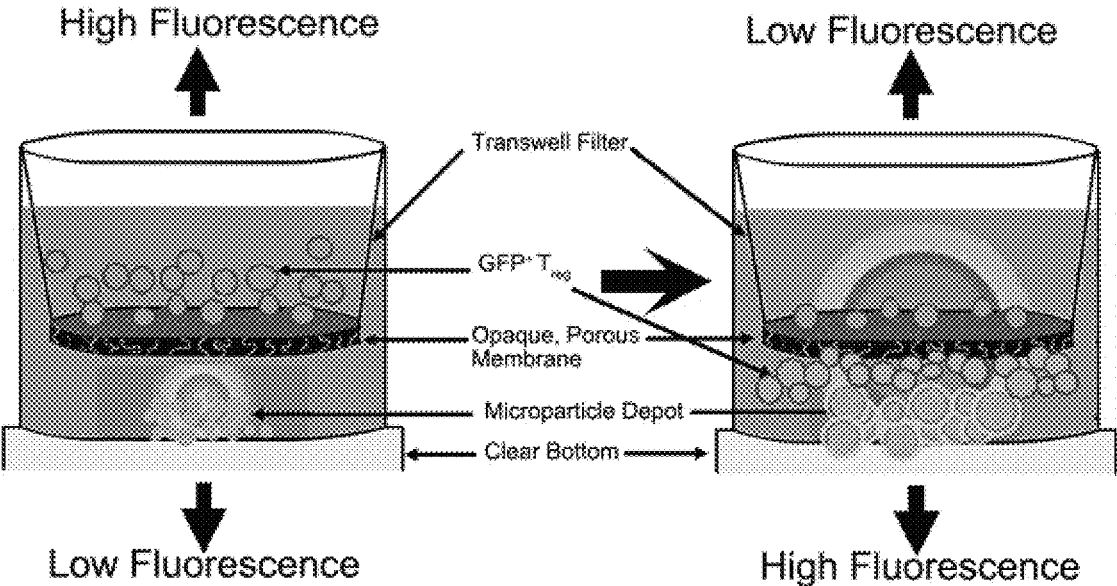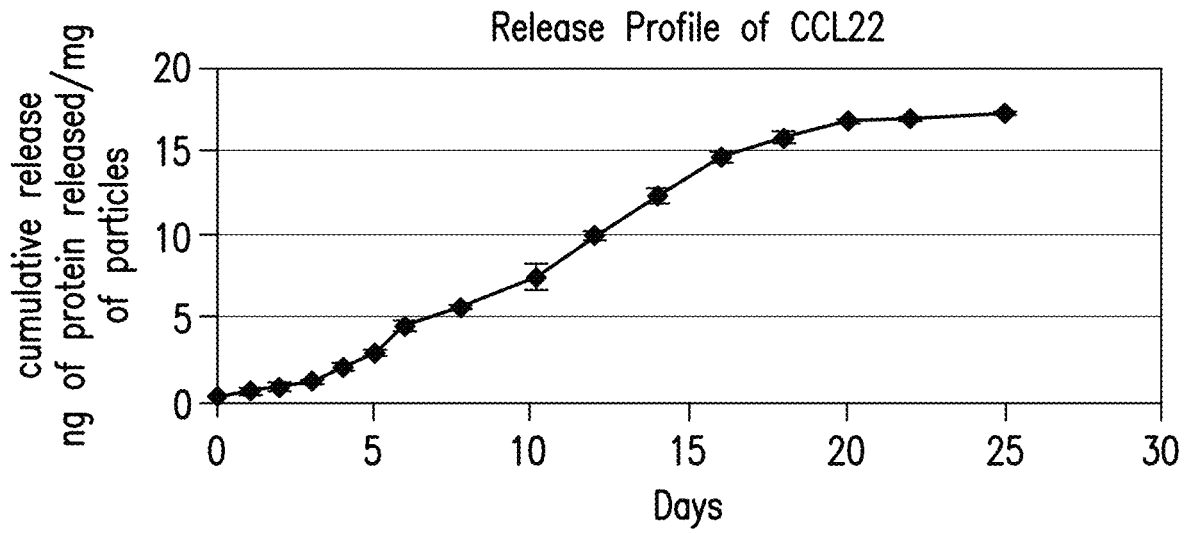
B

Figure 4

A.

B.

Figure 5

Figure 6

Figure 7

Figure 8

FIGURE 9A
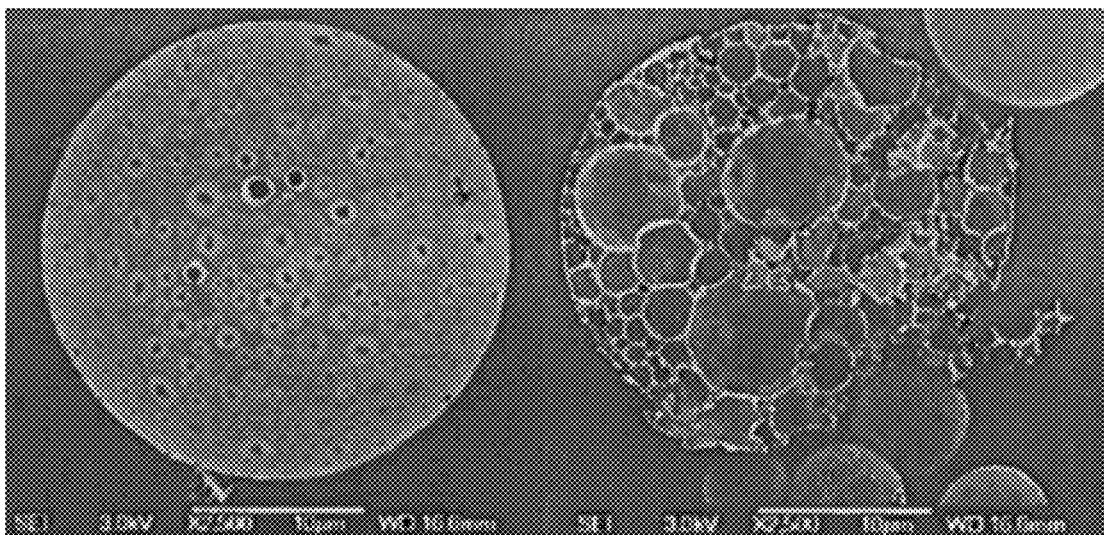
FIGURE 9B
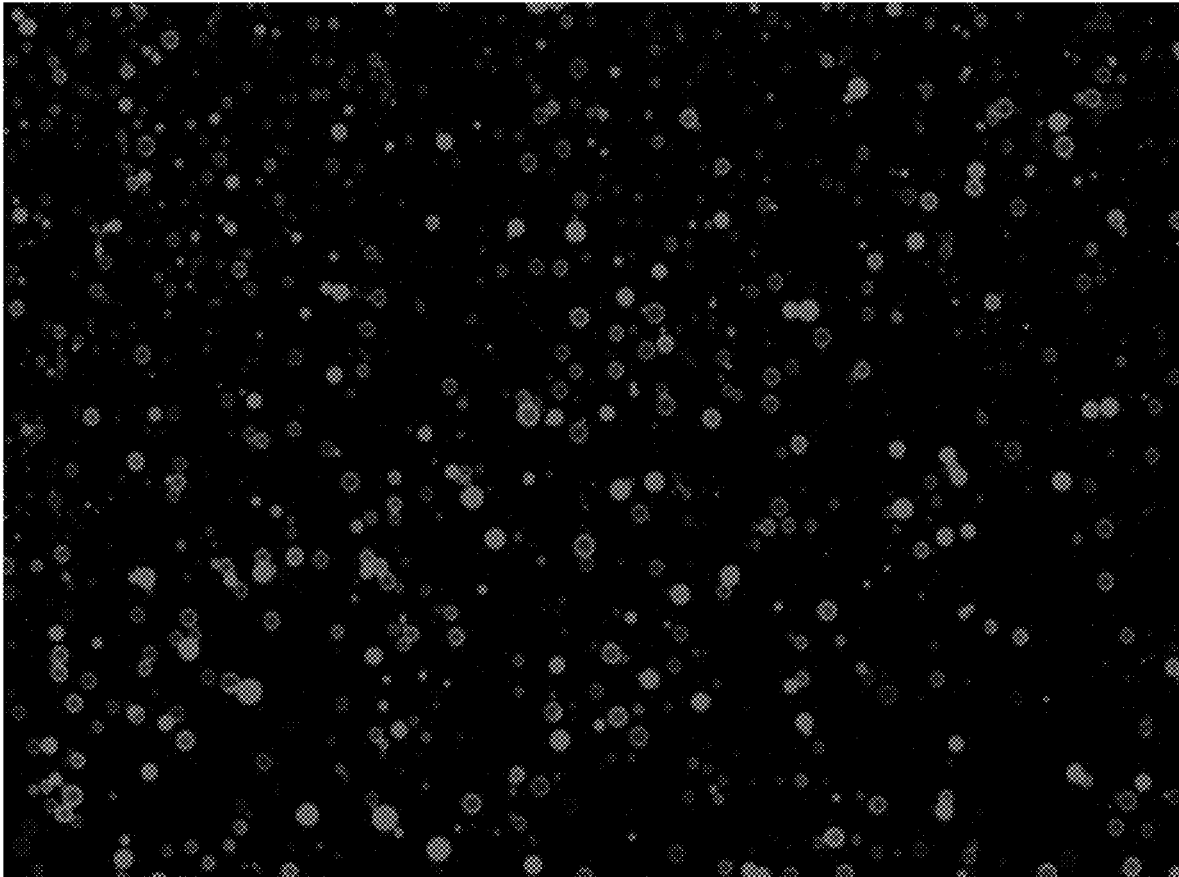
Figure 10

FIGURE 11
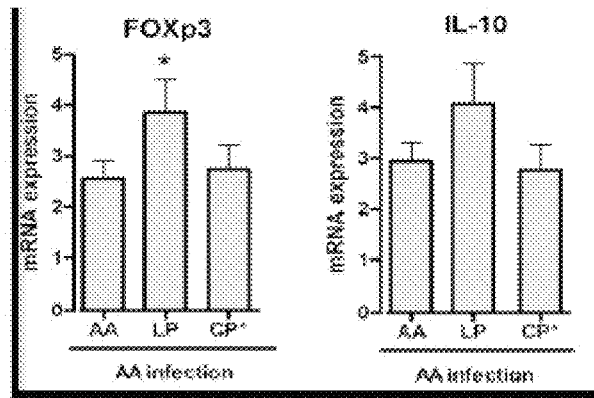
A.
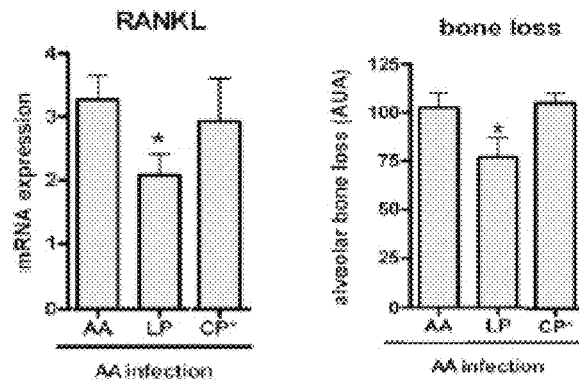
B.

FIGURE 12

FIGURE 13

FIGURE 14

Figure 15

Figure 16

Average
Volume
Diameter

Polymer Microparticle Average Sized Distribution

FIGURE 17

Figure 18

FIGURE 18C

A   Experimental Design

Day 0: Luc$^+$
Treg Injection   Imaging

Day −3:   Day 2:
Microparticle Injection   mature allo−
                                  DC injection

FIGURE 19A

B

Fluorescence   Phosphorescence

Blank
MP

CCL22
MP

FIGURE 19B

C
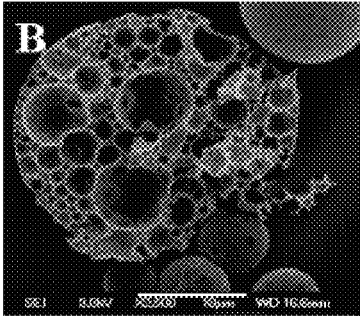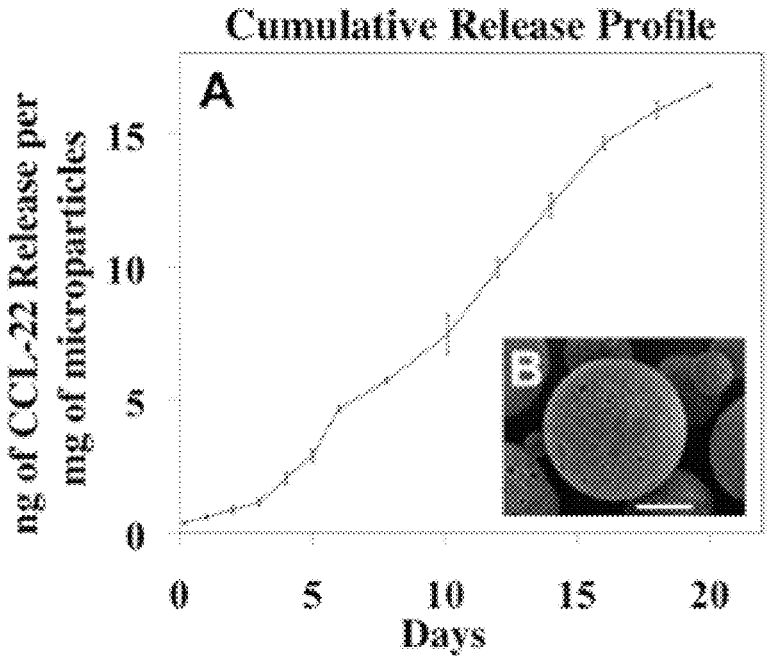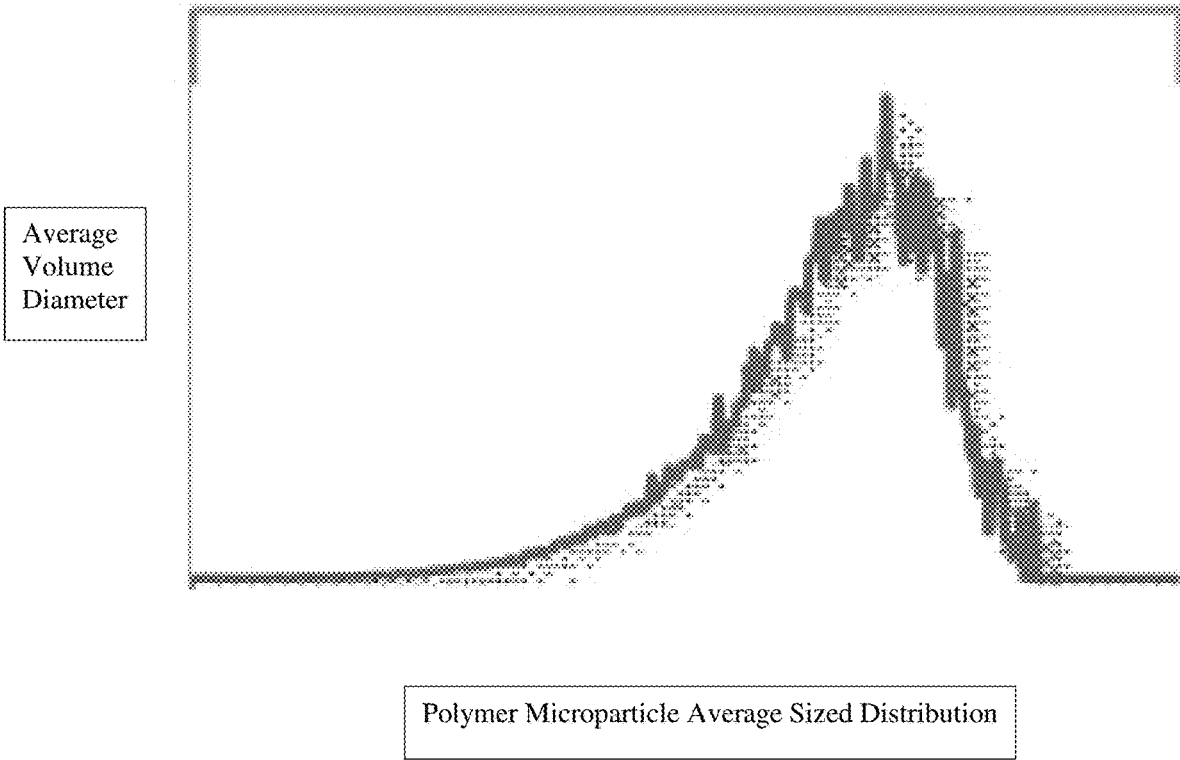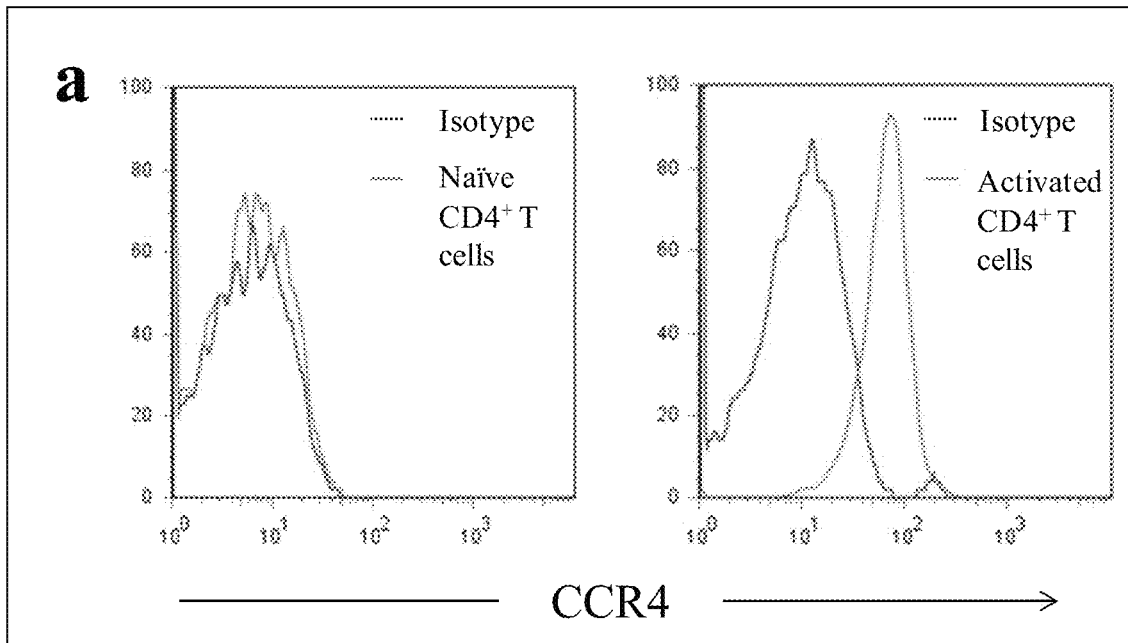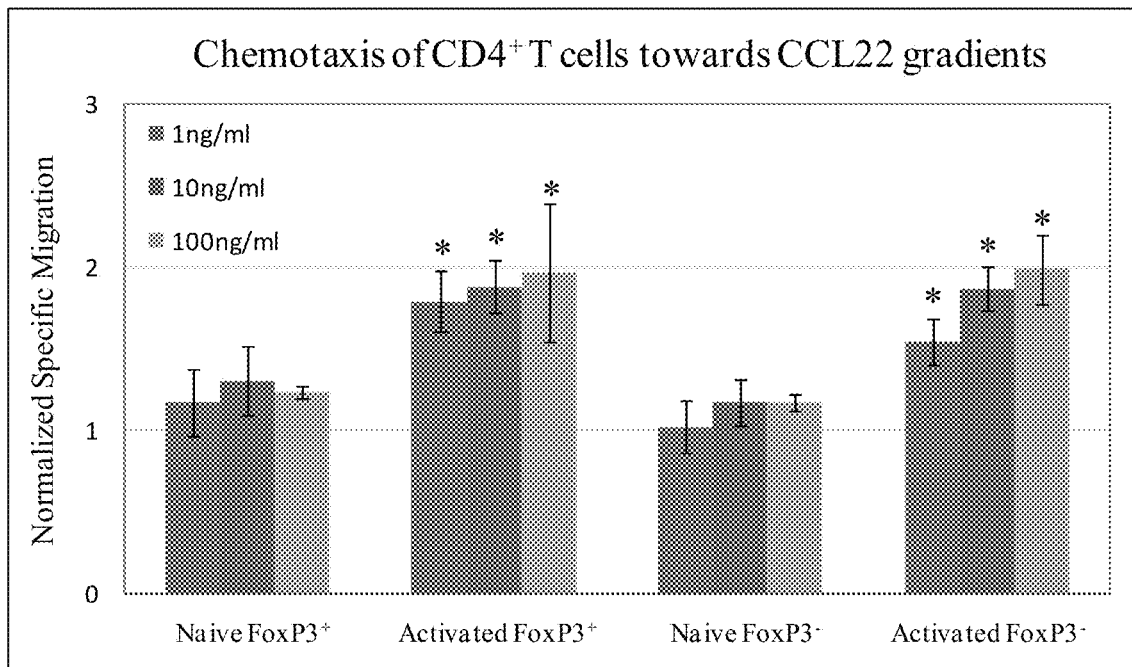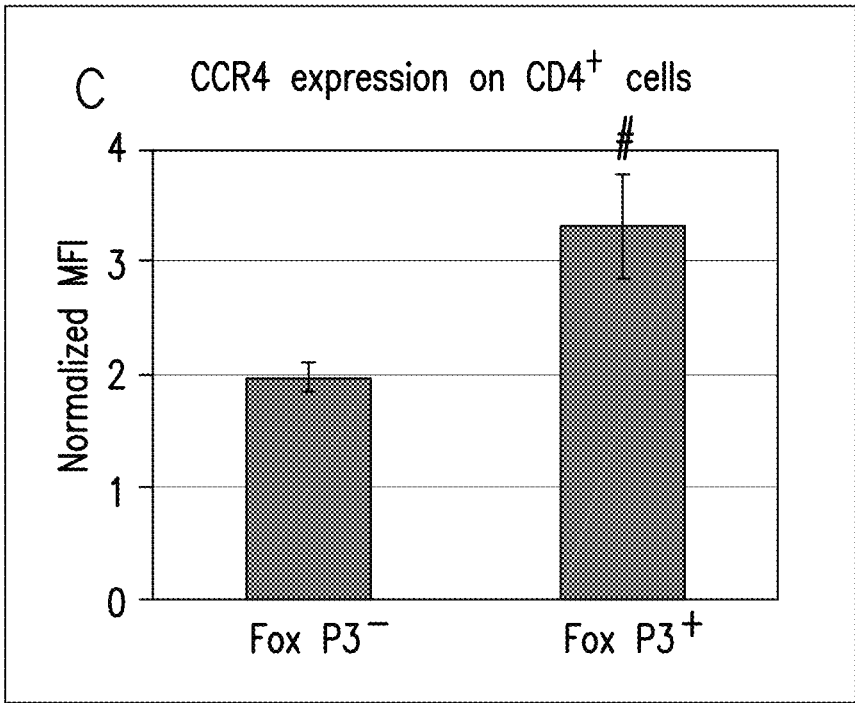
FIGURE 19C

Figure 20

Figure 21

Figure 22

Figure 23

Figure 24

Figure 25

Figure 26

A
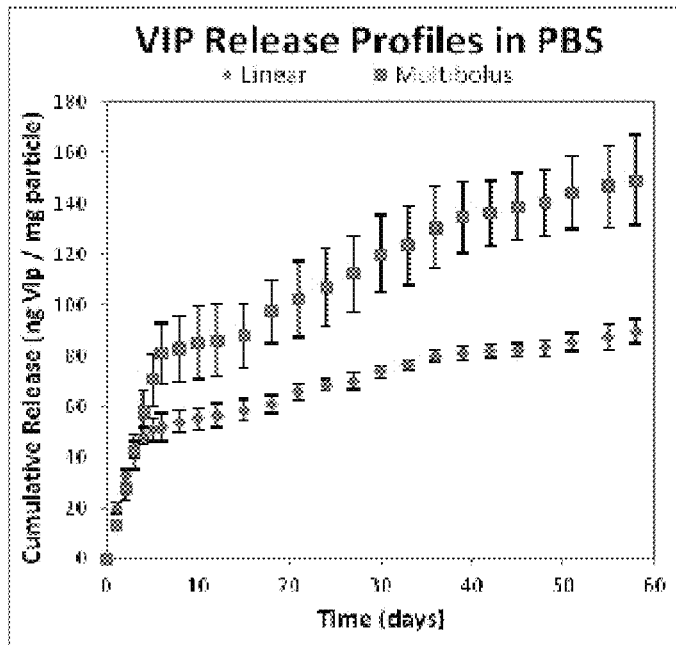
B

Figure 27

Figure 28

Figure 29

Figure 30

A.

B

Figure 31

Figure 32
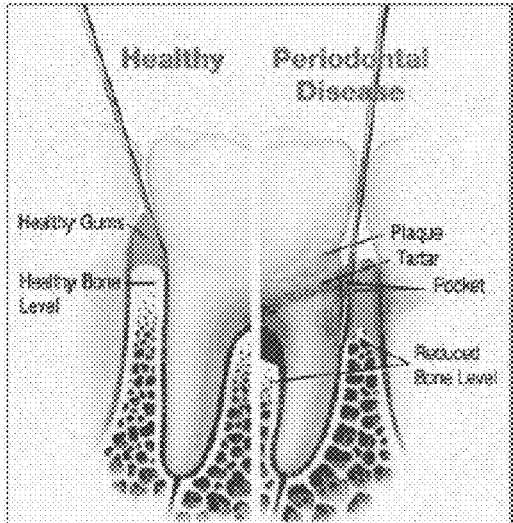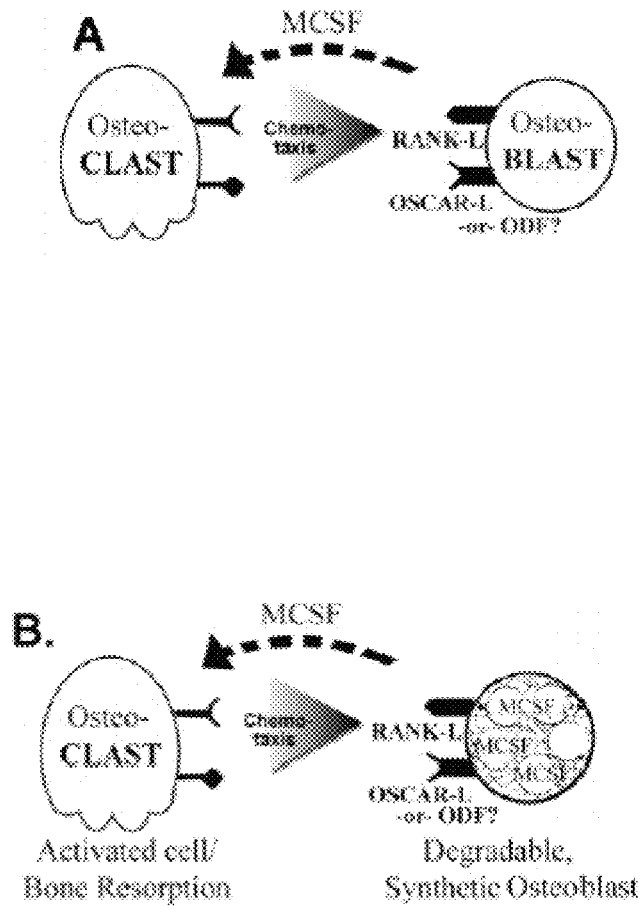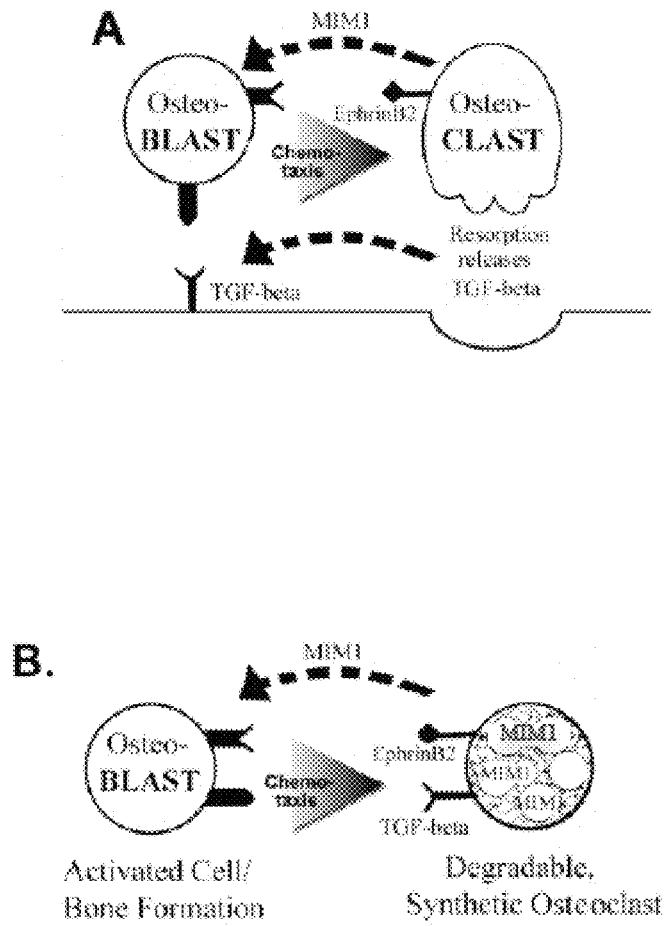
Figure 33
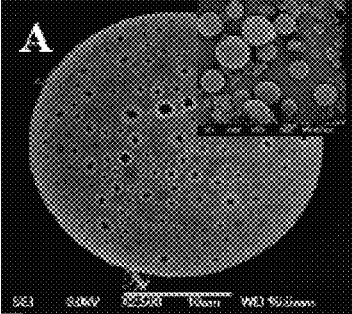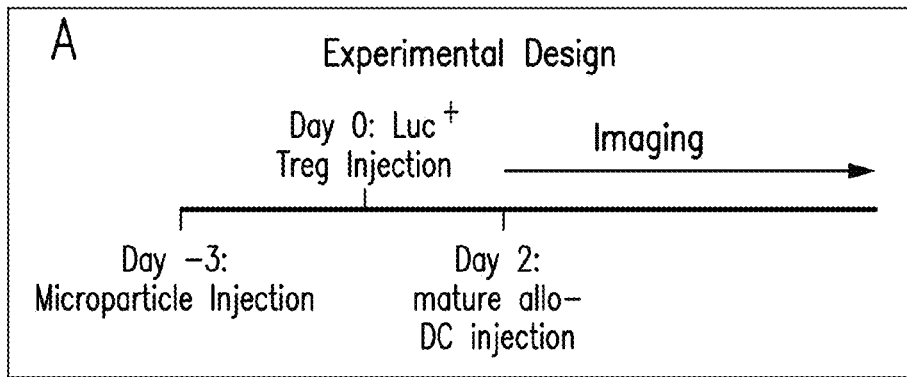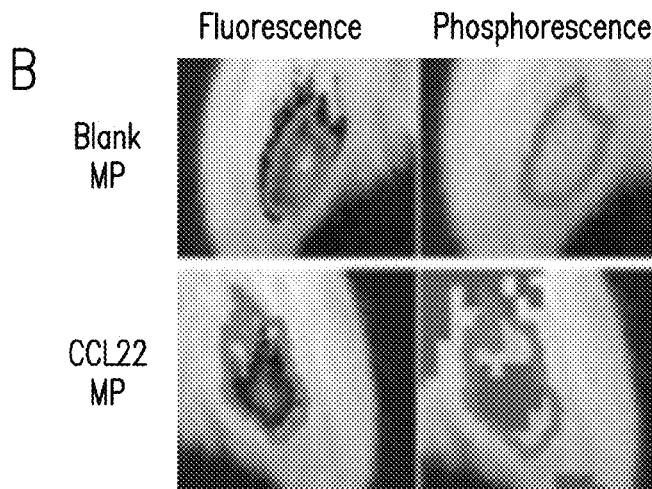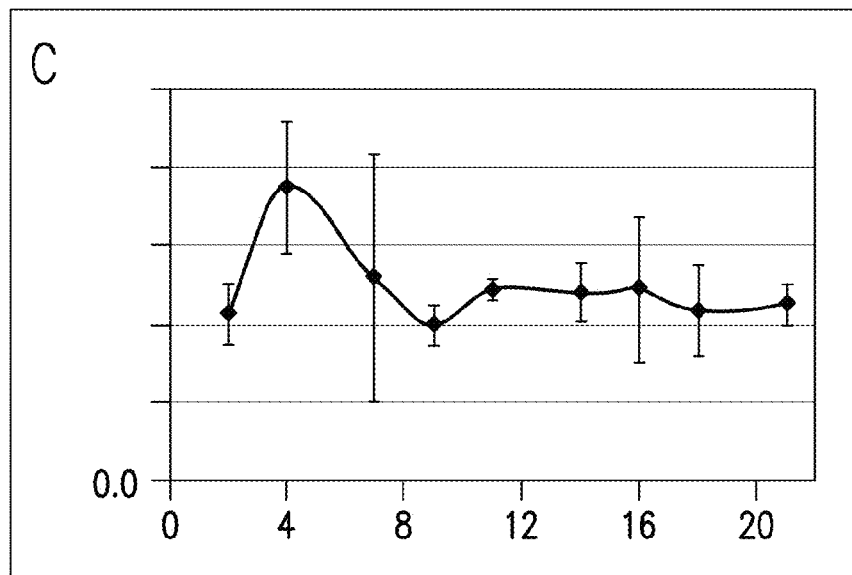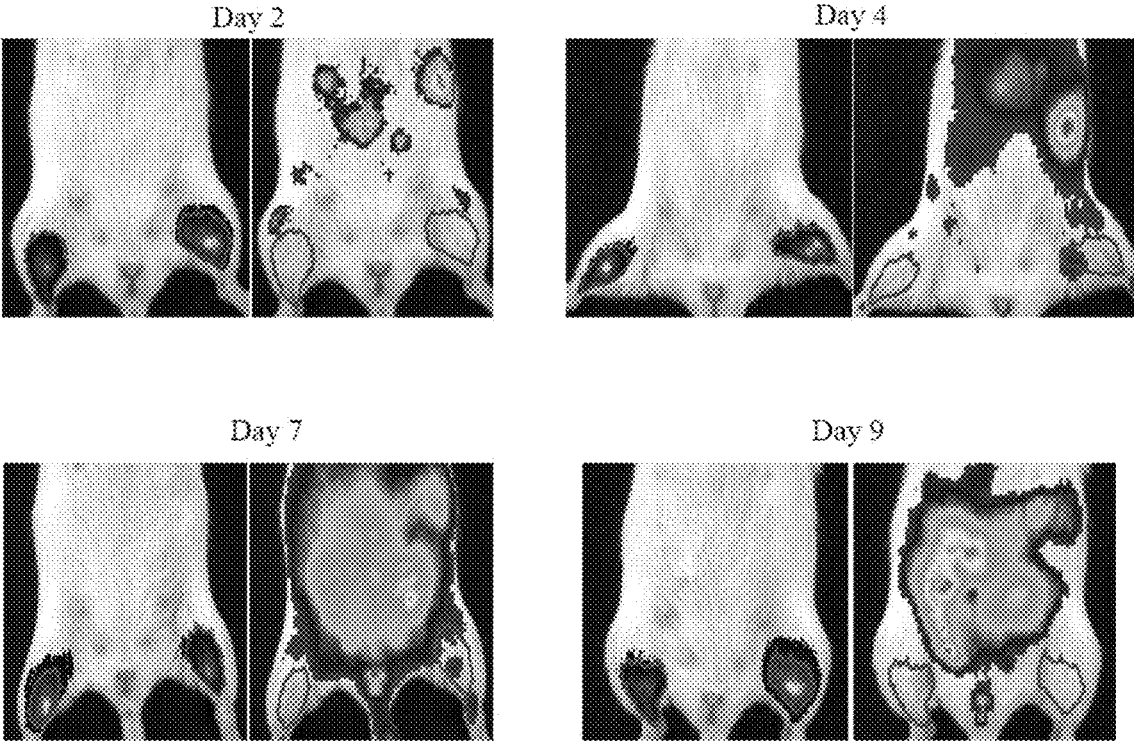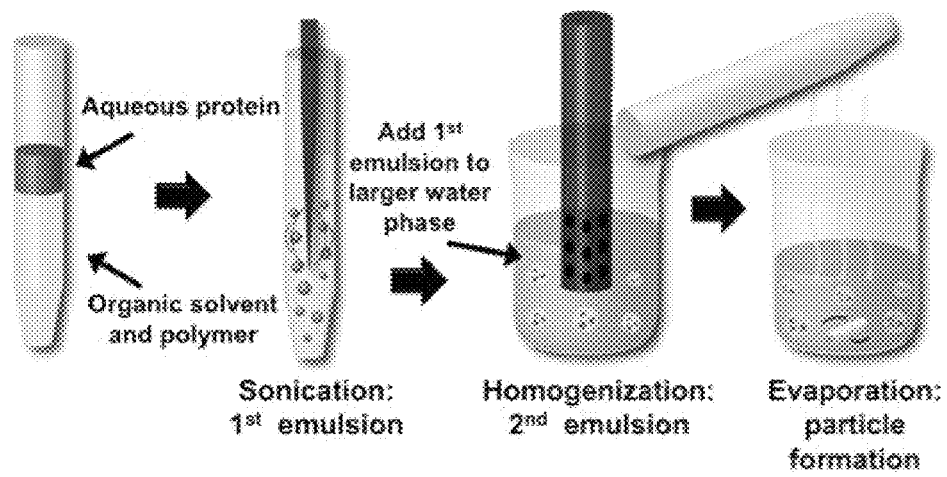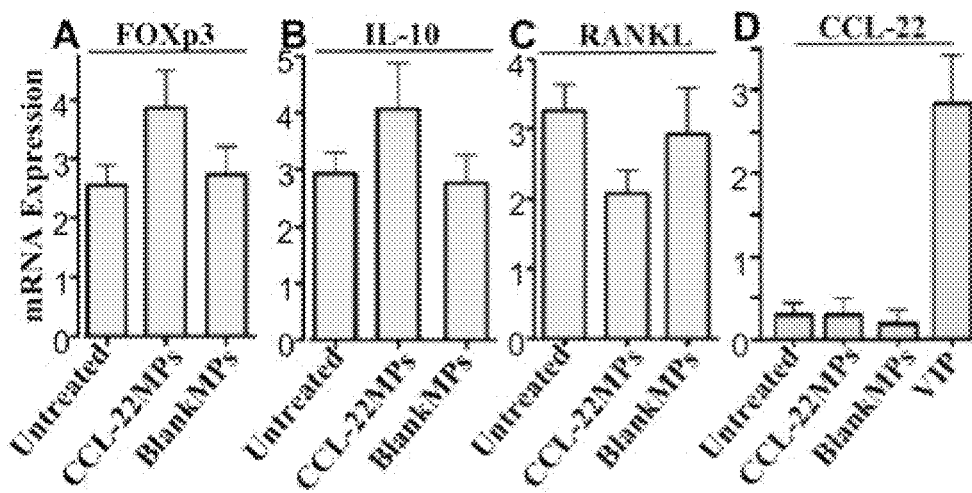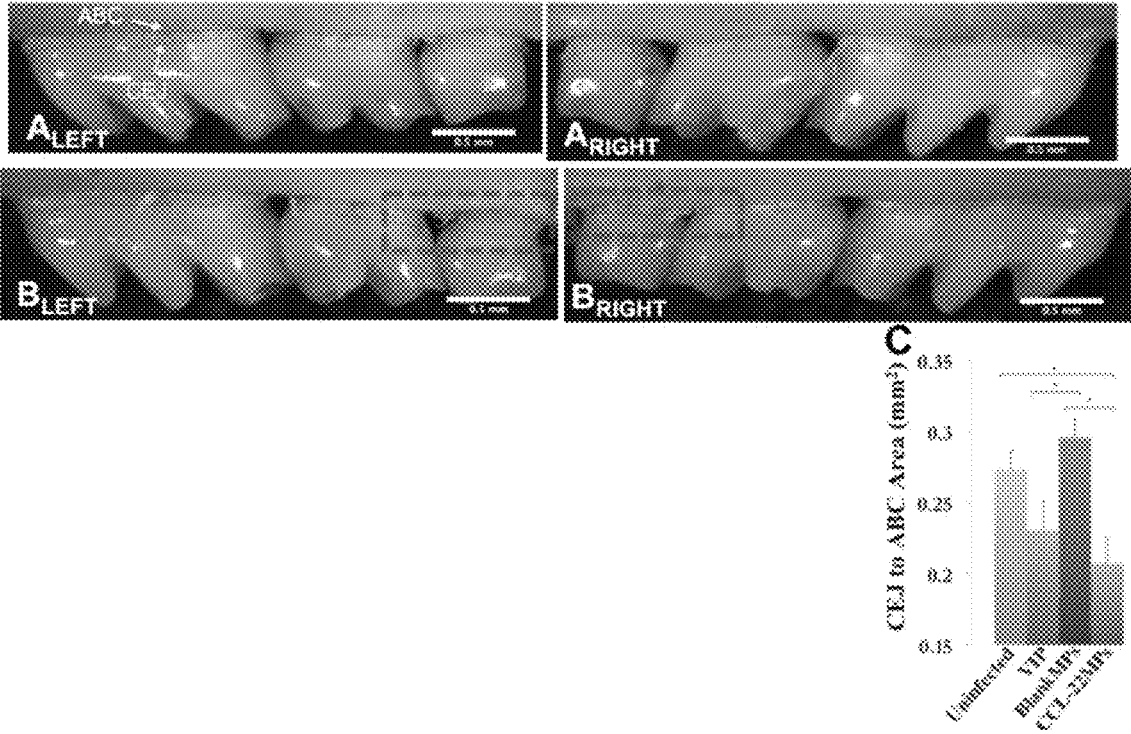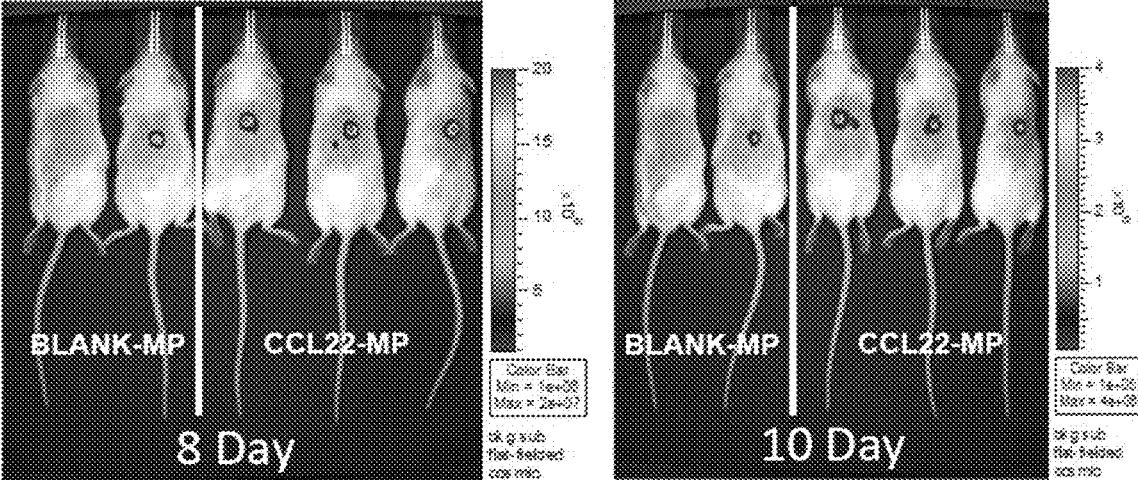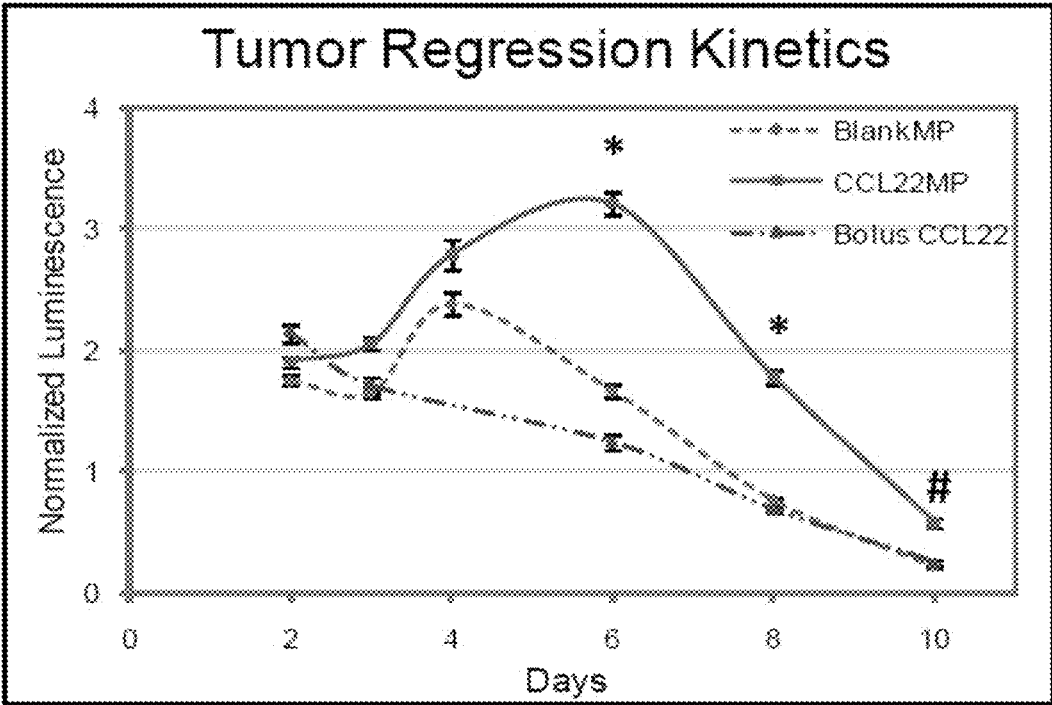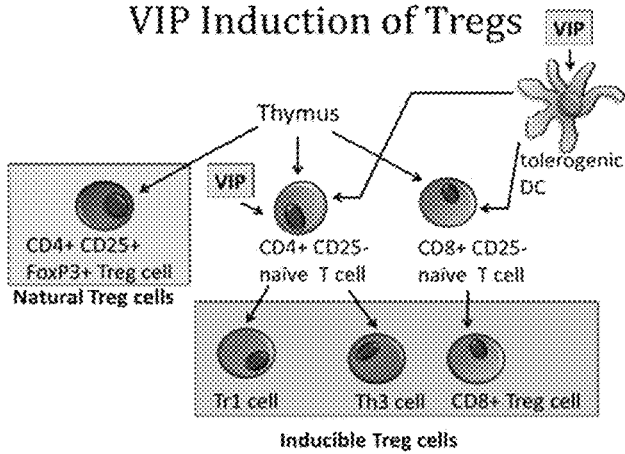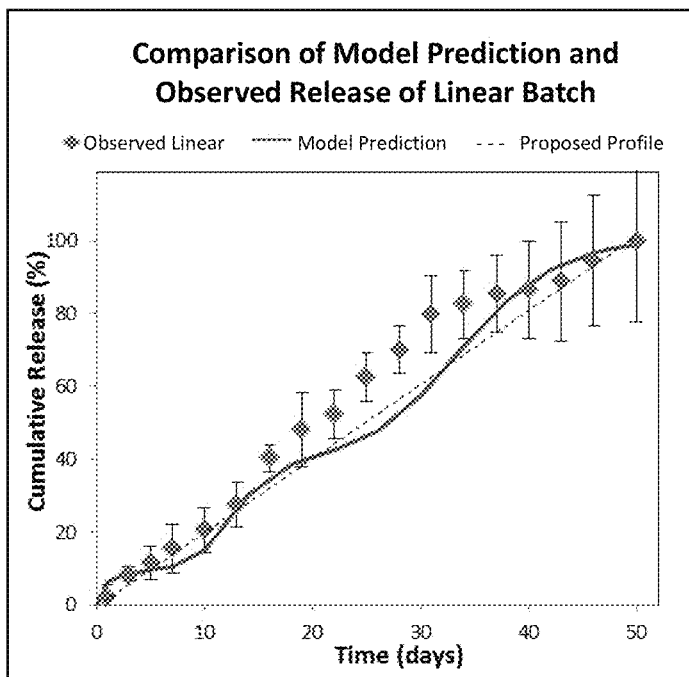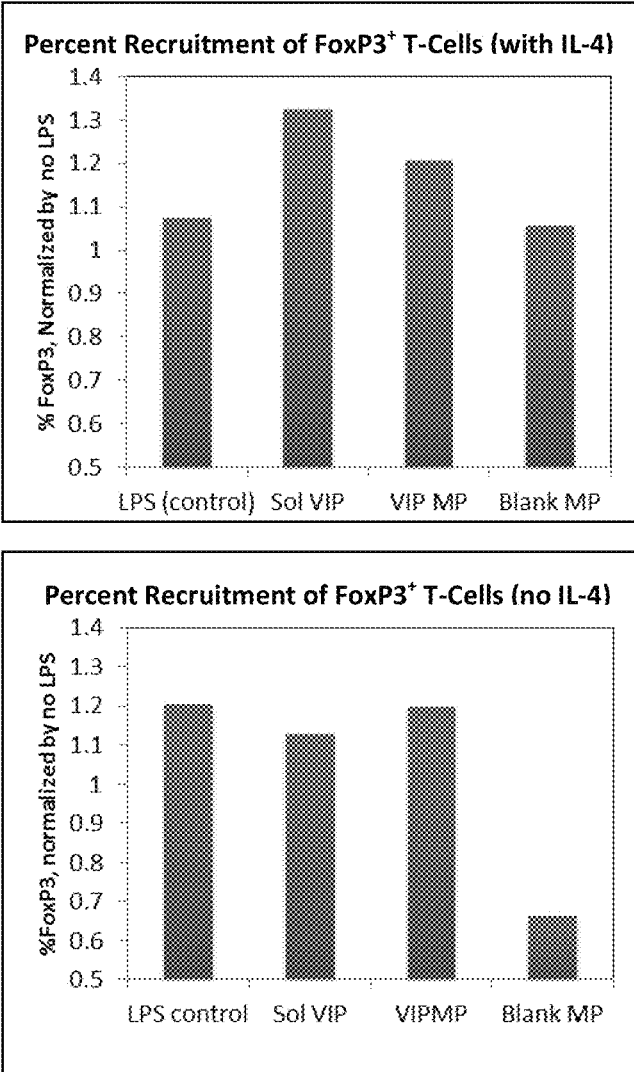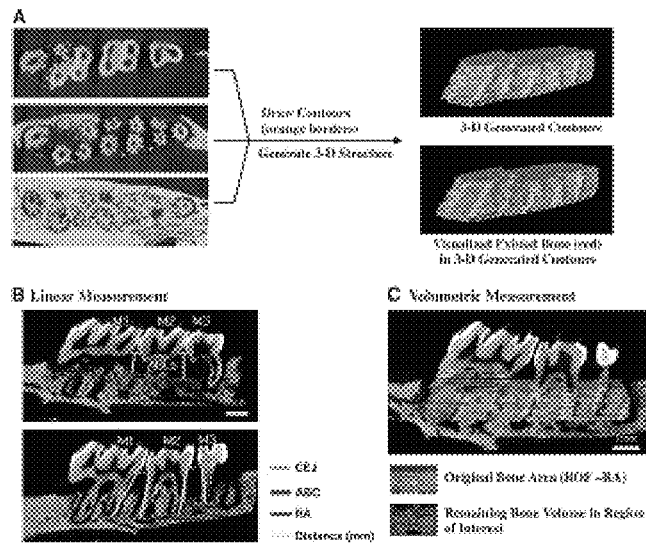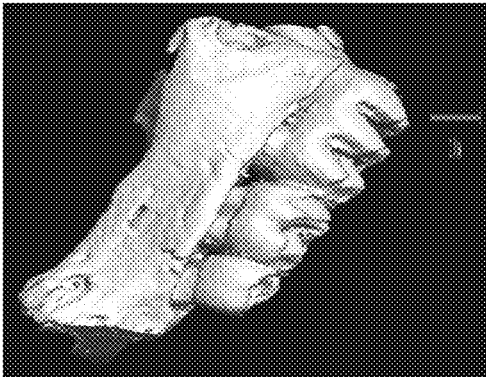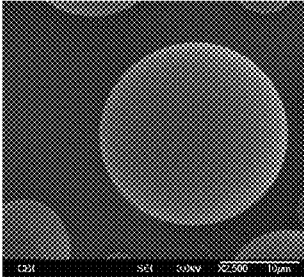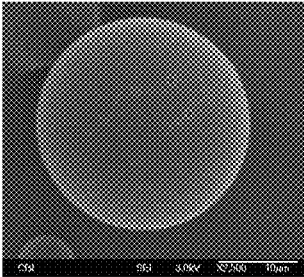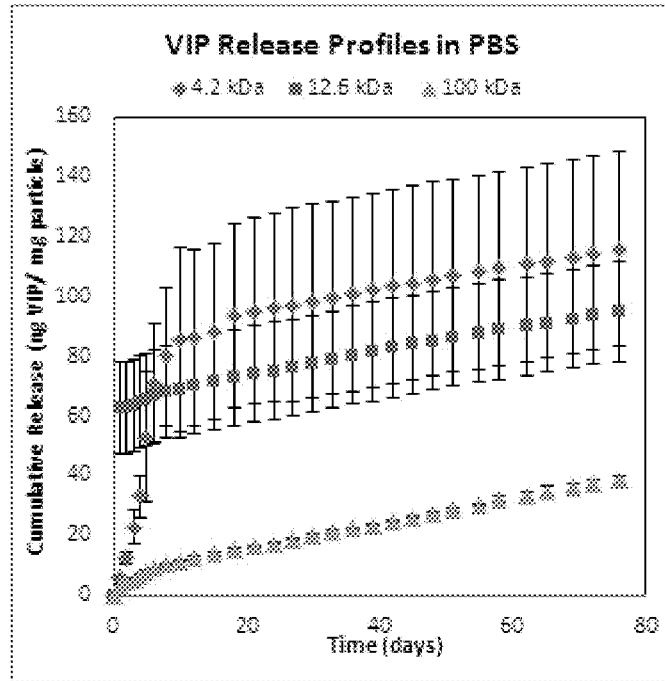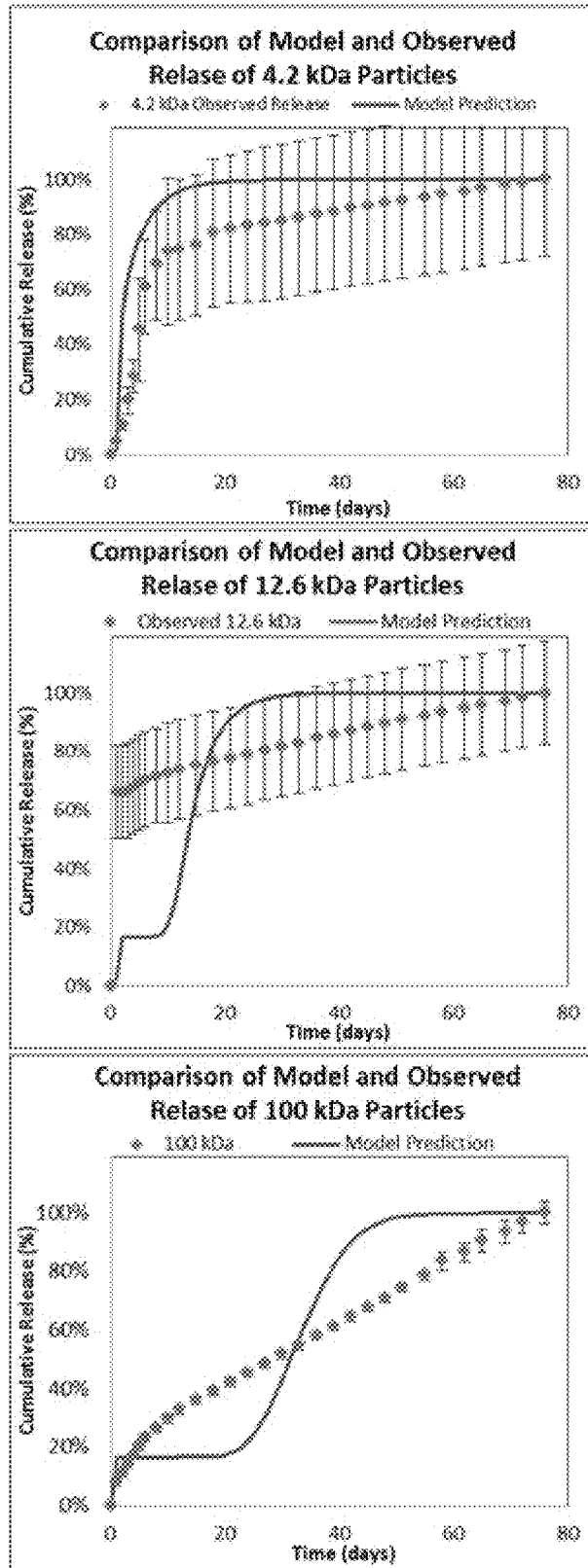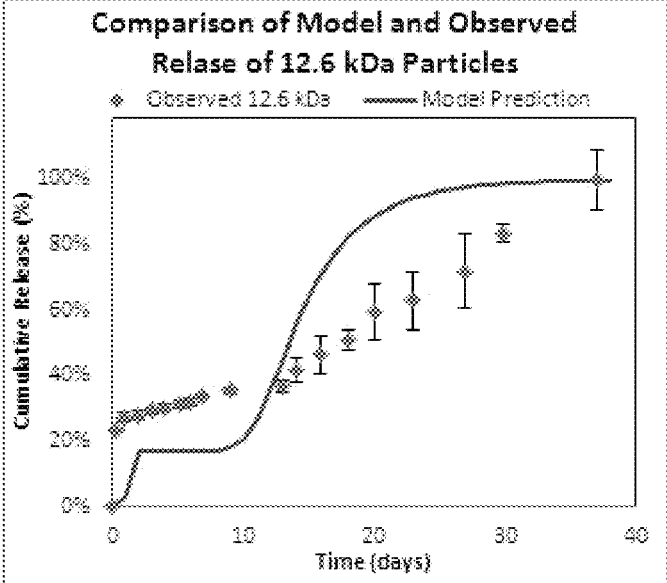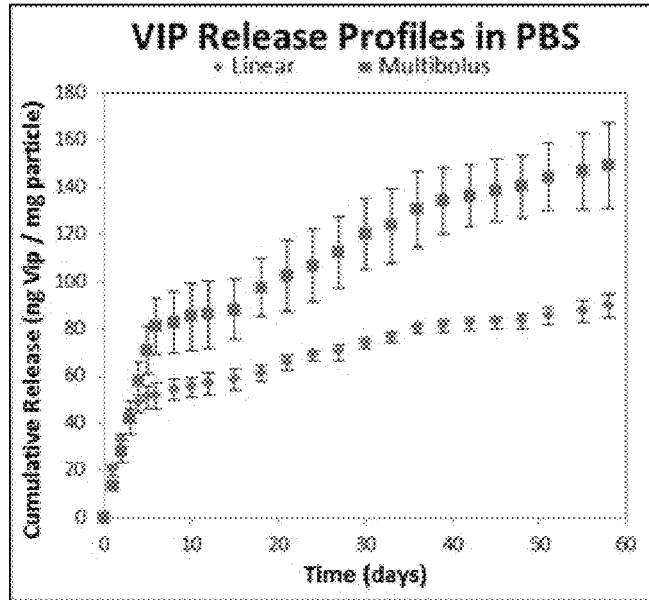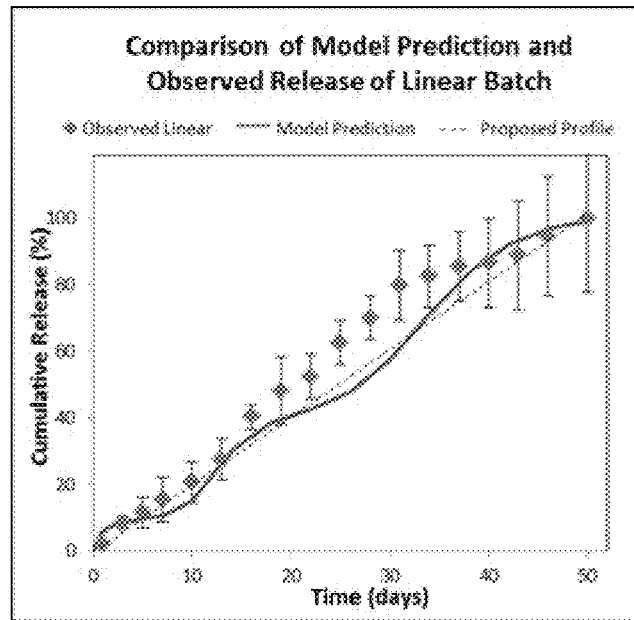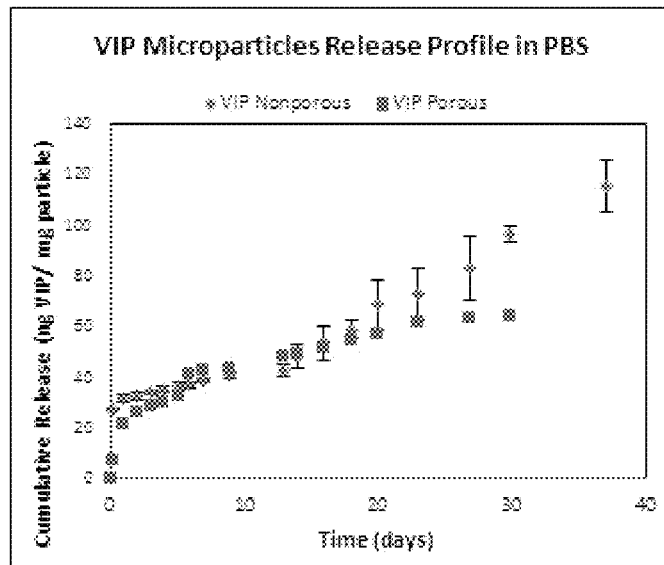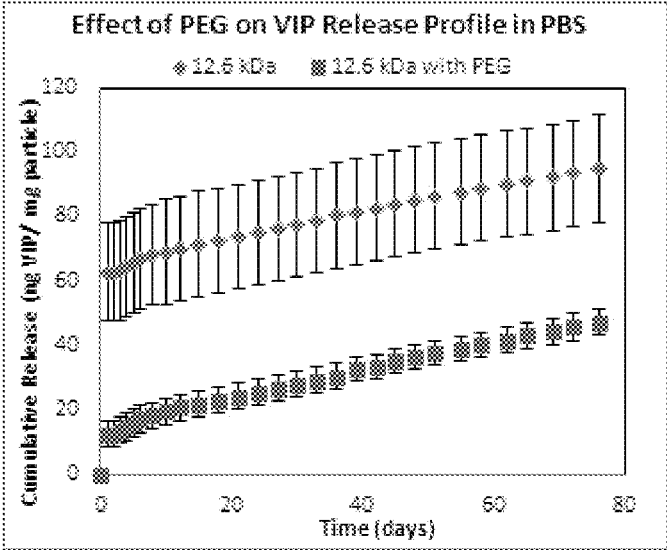
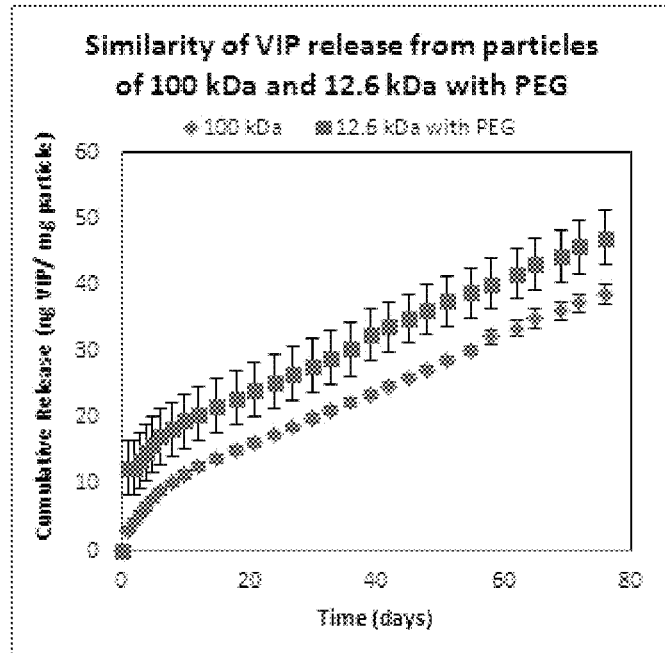Figure 34

A

B

Figure 35

Figure 36

Figure 37

Figure 38
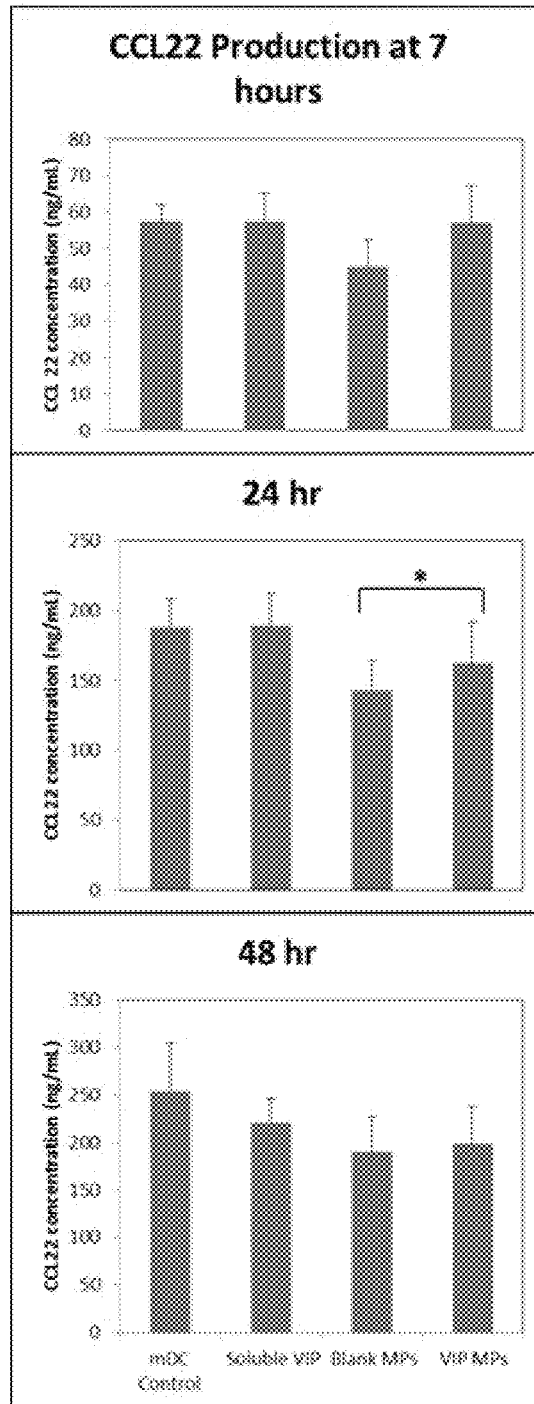
Figure 39

A

B
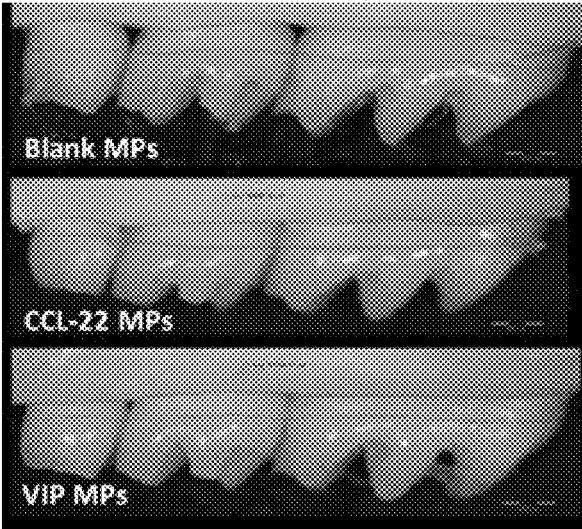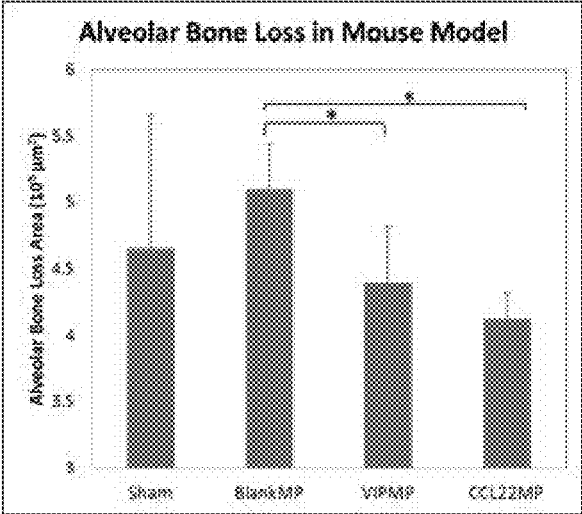
Figure 40

VASOACTIVE INTESTINAL PEPTIDE RELEASE FROM MICROPARTICLES

STATEMENT OF GOVERNMENT INTEREST

This invention was made with government support under grant numbers RR024154, DE021058, and A1067541 awarded by the National Institutes of Health. The government has certain rights in the invention.

FIELD OF THE INVENTION

The present invention is related to the treatment of immunological disorders, immunological diseases and/or transplantation rejection reactions. For example, certain embodiments of the invention result in the regulation of blood cells including, but not limited, to naïve blood cells and/or white blood cells (e.g., T cells and/or B cells) controlled by vasoactive intestinal peptide (VIP). In particular, the invention contemplates methods of creating VIP-induced regulatory T cells by controlled VIP release from custom designed microparticles.

BACKGROUND

Periodontal disease, also known as gingivitis or gum disease, involves the inflammation and destruction of the tissues that support the teeth. It is the leading cause of tooth loss and affects over 78 million Americans. Current treatments focus on removing bacteria associated with the onset of the disease through clinical procedures known as scaling and root planing. However, recent studies have demonstrated that while the bacteria initiates periodontal disease, an imbalance of the immune system propagates the damage, specifically through an absence of a subset of lymphocytes called regulatory T cells (Tregs). Cardoso et al., (2004) "Characterization of CD4+CD25+ natural regulatory T cells in the inflammatory infiltrate of human chronic periodontitis" *Journal of Leukocyte Biology* 84(1):311-318. Garlet et al. found that CD4+CD25+ and CD4+FoxP3+ regulatory T cells attenuate the severity of periodontitis in mice. Garlet also found that an inhibition of these cells was characterized by an increase in alveolar bone loss and disease progression, suggesting that restoration of regulatory T cells in the periodontium may alleviate symptoms and restore immunological balance. Garlet et al., (2010) "Regulatory T cells attenuate experimental periodontitis progression in mice" *Journal of Clinical Periodontology,* 37:591-600.

The science of transplantation, now half of a century old, has dramatically increased and improved the life of many individuals, including many children, with end stage diseases. Recent advancements in immunosuppressive agents have substantially decreased rejection of allografts over the past decade and a half in the United States. Hariharan et al., *N Engl J Med* 342, 605-612 (2000); and First, M. R., *Transplant Proc* 34:1369-1371 (2002). However, to avoid both episodes of acute rejection and the initiation of chronic rejection following transplantation, immunosuppressive drugs must be administered over the entire life of the organ recipient.

Consequences of long-term immunosuppressive drug administration are profound, including undesirable side effects, increasing the risk of infection, autoimmunity, heart disease, diabetes, and cancer. The chronic administration of these immunosuppressive drugs (especially when given systemically) lead to toxicity and significant side effects, thereby leaving the patient vulnerable to a variety of dis-

eases and systemic organ failure. The most desirable alternative to this extended state of vulnerability would be to render the patient's immune system to effectively suppress immune activation without systemic immunosuppression. In this case, no further immunosuppressant drug treatment would be necessary. Furthermore, the recipient's immune system would otherwise function normally, being capable of combating pathogens and malignant tumor cells.

What is needed in the art are compositions and methods to specifically induced regulatory T cells to treat tissues affected by immunological diseases including, but not limited to, those related to inflammatory conditions and/or tissue transplant rejection.

SUMMARY OF THE INVENTION

The present invention is related to the treatment of immunological disorders, immunological diseases and/or transplantation rejection reactions. For example, certain embodiments of the invention result in the regulation of blood cells including but not limited to naïve blood cells and/or white blood cells (e.g., T cells and/or B cells) controlled by vasoactive intestinal peptide (VIP). In particular, the invention contemplates methods of creating VIP-induced regulatory T cells by controlled VIP release from custom designed microparticles.

In one embodiment, the present invention contemplates a microparticle comprising at least three polymers, wherein each of said three polymers has a different molecular weight. In one embodiment, said at least three polymers have a molecular weight ranging between approximately 4-70 kDa 50:50 PLGA. In one embodiment, one of said at least three polymers ranges between approximately 7-17 kDa 50:50 PLGA (e.g., for example, 12.6 kDa RG502H). In one embodiment, one of said at least three polymers ranges between approximately 54-69 kDa 50:50 PLGA (e.g., for example, 100 kDa RG505).

In one embodiment, the present invention contemplates a method, comprising: a) providing; i) a patient comprising a target tissue and a blood cell population, wherein said target tissue exhibits at least one symptom of a disease; and ii) a microparticle population comprising a vasoactive intestinal peptide; b) administering said microparticle population to said patient under conditions such that said blood cell population is induced; and c) contacting said induced blood cell population with said target tissue such that said at least one symptom of said disease is reduced. In one embodiment, the disease comprises an immunological disease. In one embodiment, the blood cell is a white blood cell. In one embodiment, the white blood cell is a T cell. In one embodiment, the T cell is a regulatory T cell. In one embodiment, the white blood cell is a B cell. In one embodiment, the microparticle population comprises a plurality of polymers in a ratio predicted by a mathematical algorithm. In one embodiment, the plurality of polymers comprise a 4.2 kDa polymer, a 12.6 kDa polymer and a 55 kDa polymer. In one embodiment, the plurality of polymers comprise a 4.2 kDa polymer, a 12.6 kDa polymer and a 100 kDa polymer. In one embodiment, said ratio is 1:3:5.4. In one embodiment, said ratio is 1:1:1.

In one embodiment, the present invention contemplates, a microparticle comprising a 4.2 kDa polymer, a 12.6 kDa polymer and a 100 kDa polymer and a vasoactive intestinal peptide. In one embodiment, said 4.2 kDa polymer comprises 10.6% of said microparticle. In one embodiment, said 12.6 kDa polymer comprises 31.9% of said microparticle. In one embodiment, said 100 kDa polymer comprises 57.5% of

said microparticle. In one embodiment, the microparticle further comprises an aqueous inner phase. In one embodiment, the aqueous inner phase comprises polyethylene glycol. In one embodiment, the aqueous inner phase comprises sodium chloride. In one embodiment, said 4.2 kDa polymer includes, but is not limited to, a polylactide-co-glycolide (PLGA) polymer, an RG502H polymer and an RG505 polymer. In one embodiment, said 12.6 kDa polymer includes, but is not limited to, a polylactide-co-glycolide (PLGA) polymer, an RG502H polymer and an RG505 polymer. In one embodiment, said 100 kDa polymer includes, but is not limited to, a polylactide-co-glycolide (PLGA) polymer, an RG502H polymer and an RG505 polymer.

In one embodiment, the present invention contemplates a microparticle comprising a 4.2 kDa polymer, a 12.6 kDa polymer and a 55 kDa polymer and vasoactive intestinal peptide. In one embodiment, said 4.2 kDa polymer comprises 33.3% of said microparticle. In one embodiment, said 12.6 kDa polymer comprises 33.3% of said microparticle. In one embodiment, said 55 kDa polymer comprises 33.3% of said microparticle. In one embodiment, the 12.6 kDa polymer is an RG502H polymer. In one embodiment, the 55 kDA polymer is an RG505 polymer. In one embodiment, the microparticle further comprises an aqueous inner phase. In one embodiment, the aqueous inner phase comprises polyethylene glycol. In one embodiment, the aqueous inner phase comprises sodium chloride.

In one embodiment, the present invention contemplates a kit comprising: a) a first container comprising a composition comprising a microparticle population encapsulating a vasoactive intestinal peptide; b) a second container comprising a pharmaceutically acceptable vehicle for administration of said composition; and c) instructions for administering said composition to a patient comprising a target tissue exhibiting at least one symptom of a disease. In one embodiment, the microparticle population comprises a ratio of polymers predicted by a mathematical algorithm. In one embodiment, the ratio of polymers comprise a 4.2 kDa polymer, a 12.6 kDa polymer and a 55 kDa polymer. In one embodiment, the ratio of polymers comprise a 4.2 kDa polymer, a 12.6 kDa polymer and a 100 kDa polymer. In one embodiment, said ratio is 1:3:5.4. In one embodiment, said ratio is 1:1:1. In one embodiment, the polymer includes, but is not limited to, a polylactide-co-glycolide (PLGA) polymer, an RG502H polymer and/or an RG505 polymer. In one embodiment, the microparticle further comprises an aqueous inner phase. In one embodiment, the aqueous inner phase comprises polyethylene glycol. In one embodiment, the aqueous inner phase comprises sodium chloride.

The present invention is related to the field of inducing immunological tolerance by specifically manipulating immune-related cells. Such immunological tolerance may be induced by providing biomimetic artificial cells that bypass native immunological cells. For example, biomimetic artificial cell compositions are contemplated which comprise soluble factors that activate specific immune-related blood cells, including, but not limited to, macrophages and/or monocytes. Such factors may comprise chemoattractant factors. These biomimetic artificial particles may further present a specific biomimetic surface pattern that results in a targeted cell response such that the particles represent artificial presenting cells.

In one embodiment, the present invention contemplates an artificial particle comprising a plurality of soluble and surface bound regulatory T-cell factors. In one embodiment, the artificial particle comprises an artificial presenting cell.

In one embodiment, the factors comprise regulatory T cell stimulatory factors. In one embodiment, the factors comprise regulatory T cell inducing factors. In one embodiment, the factors comprise regulatory T cell chemoattractant factors. In one embodiment, the soluble factors undergo controlled released. In one embodiment, the controlled release is a short term release. In one embodiment, the short term release is between 30 minutes-1 hour. In one embodiment, the short term release is between 1 hour-3 hours. In one embodiment, the short term release is between 3 hours-10 hours. In one embodiment, the short term release is between 10 hours-24 hours. In one embodiment, the controlled release is a long term release. In one embodiment, the long term release is between 24 hours-36 hours. In one embodiment, the long term release is between 3 days-7 days. In one embodiment, the long term release is between 7 days-1 month. In one embodiment, the long term release is between 1 month-6 months. In one embodiment, the long term release is between 6 months-1 year. In one embodiment, the long term release is at least one year. In one embodiment, the surface bound factors comprises a biomimetic surface pattern. In one embodiment, the biomimetic surface pattern comprises an immunological synapse. In one embodiment, the artificial presenting cell take a shape selected from the group consisting of a sphere, a square, a rectangle, a triangle, a trapezoid, a hexagon, an octagon, or a tetrahedron.

In one embodiment, the present invention contemplates an artificial particle comprising at least one soluble T_{reg} cell factor and at least one surface bound T_{reg} cell factor, wherein said surface bound factor is displayed in a non-random pattern. In one embodiment, the artificial particle comprises an artificial presenting cell. In one embodiment, the at least one soluble or surface bound Treg cell factor comprises a regulatory T cell stimulatory factor. In one embodiment, the at least one soluble or surface bound Treg cell factor comprises a regulatory T cell inducing factor. In one embodiment, the at least one soluble or surface bound Treg cell factor comprises a regulatory T cell chemoattractant factor. In one embodiment, the artificial presenting cell further comprises a functionalized polymer, wherein said polymer encapsulates the soluble factor. In one embodiment, the artificial presenting cell further comprises compartments, wherein said compartments comprise the at least one soluble factor. In one embodiment, the artificial presenting cell is biodegradable such that the soluble factor undergoes controlled release. In one embodiment, the soluble factor comprises CCL22. In one embodiment, the soluble factor comprises IL2. In one embodiment, the soluble factor comprises TGF-β. In one embodiment, the polymer comprises poly-lactic acid-co-glycolic acid (PLGA). In one embodiment, the functionalized polymer non-covalently bonds the soluble factor. In one embodiment, the at least one surface bound T_{reg} stimulating factor forms a biomimetic surface pattern. In one embodiment, the biomimetic surface pattern comprises an immunological synapse. In one embodiment, the synapse comprises a cSMAC region, displayed on an apical portion of the artificial presenting cell. In one embodiment, the synapse comprises a pSMAC region displayed on a tropical region of the artificial presenting cell. In one embodiment, the synapse comprises a dSMAC region displayed on an equatorial portion of the artificial presenting cell. In one embodiment, the cSMAC region comprises CD3 antibodies and/or CD28 antibodies. In one embodiment, the pSMAC region comprises at least one adhesion molecule. In one embodiment, the dSMAC region comprises a CD45

monoclonal antibody. In one embodiment, the functionalized polymer covalently bonds the surface bound regulatory T cell stimulatory factor.

In one embodiment, the present invention contemplates a method, comprising: a) providing, i) an emulsified microparticle comprising at least one soluble T_{reg} cell factor and a functionalized polymer; and ii) at least one surface bound T_{reg} cell factor, wherein the surface bound factor is capable of attaching to the polymer; b) attaching a first surface bound factor to said polymer, wherein an apical region is created; c) attaching a second surface bound factor to said polymer, wherein a tropical region is created; and d) attaching a third surface bound factor to said polymer, wherein an equatorial region is created. In one embodiment, the microparticle comprises an artificial presenting cell. In one embodiment, the at least one soluble or surface bound Treg cell factor comprises a regulatory T cell stimulatory factor. In one embodiment, the at least one soluble or surface bound Treg cell factor comprises a regulatory T cell inducing factor. In one embodiment, the at least one soluble or surface bound Treg cell factor comprises a regulatory T cell chemoattractant factor. In one embodiment, the microparticle is biodegradable such that the soluble factor undergoes controlled release. In one embodiment, the soluble factor comprises CCL22. In one embodiment, the soluble factor comprises IL2. In one embodiment, the soluble factor comprises TGF-13. In one embodiment, the apical, tropical, and equatorial regions comprise a biomimetic pattern. In one embodiment, the biomimetic pattern comprises an immunological synapse. In one embodiment, the first surface bound factor is selected from the group consisting of a CD3 antibody and a CD28 antibody. In one embodiment, the second surface bound factor comprises an adhesion protein. In one embodiment, the third surface bound factor comprises a CD45 antibody. Alternatively, the microparticle may further comprise a CD3 ligand (i.e., MHC) and/or a CD28 ligand.

In one embodiment, the present invention contemplates a method, comprising: a) providing, i) a polymer capable of undergoing emulsification, ii) at least one soluble T_{reg} cell factor, and iii) at least one surface bound T_{reg} cell factor; and b) emulsifying the polymer and the soluble factor such that a biodegradable microparticle is created, such that the soluble factor undergoes controlled release. In one embodiment, the microparticle comprises an artificial presenting cell. In one embodiment, the at least one soluble or surface bound Treg cell factor comprises a regulatory T cell stimulatory factor. In one embodiment, the at least one soluble or surface bound Treg cell factor comprises a regulatory T cell inducing factor. In one embodiment, the at least one soluble or surface bound Treg cell factor comprises a regulatory T cell chemoattractant factor. In one embodiment, the emulsifying may be selected from the group including, but not limited to, precipitation, emulsions, melt casting, spray drying, crystallization, shearing, or milling. In one embodiment, the microparticle is porous. In one embodiment, the microparticle comprises compartment. In one embodiment, the method further comprises step (c) immobilizing a first surface bound stimulatory factor in an apical region of the microparticle. In one embodiment, the method further comprises step (d) immobilizing a second surface bound stimulatory factor in a tropical region of the microparticle. In one embodiment, the method further comprises step (e) immobilizing a third surface stimulatory factor in an equatorial region of the microparticle. In one embodiment, the polymer comprises poly-lactic acid-co-glycolic acid (PLGA). In one embodiment, the soluble stimulatory factor is selected from the group consisting of CCL22, IL2, and TGF-β. In one

embodiment, the first surface bound factor is selected from the group consisting of CD3 antibody and CD28 antibody. In one embodiment, the second surface bound factor comprises an adhesion molecule. In one embodiment, the third surface bound factor comprises a CD45 antibody. In one embodiment, the at least one surface bound signaling factor is randomly displayed on the microparticle.

In one embodiment, the present invention contemplates a method, comprising: a) providing, i) a patient having received a tissue transplant, wherein said tissue is capable of immunolgical tolerance; ii) an artificial antigen presenting cell comprising at least one soluble T_{reg} cell factor and at least one surface bound T_{reg} cell factor; and b) administering the artificial antigen presenting cell to the patient under conditions such that an immunological tolerant state is induced in the tissue. In one embodiment, the at least one soluble or surface bound Treg cell factor comprises a regulatory T cell stimulatory factor. In one embodiment, the at least one soluble or surface bound Treg cell factor comprises a regulatory T cell inducing factor. In one embodiment, the at least one soluble or surface bound Treg cell factor comprises a regulatory T cell chemoattractant factor. In one embodiment, the artificial antigen presenting cell is porous. In one embodiment, the immunosuppression is not antigen-specific. In one embodiment, the artificial antigen presenting cell comprises compartments. In one embodiment, the compartments comprise the soluble factor. In one embodiment, the artificial antigen presenting cell is biodegradable, wherein the soluble factor undergoes controlled release. In one embodiment, the controlled release is a short term release. In one embodiment, the short term release is between 30 minutes-1 hour. In one embodiment, the short term release is between 1 hour-3 hours. In one embodiment, the short term release is between 3 hours-10 hours. In one embodiment, the short term release is between 10 hours-24 hours. In one embodiment, the controlled release is a long term release. In one embodiment, the long term release is between 24 hours-36 hours. In one embodiment, the long term release is between 3 days-7 days. In one embodiment, the long term release is between 7 days-1 month. In one embodiment, the long term release is between 1 month-6 months. In one embodiment, the long term release is between 6 months-1 year. In one embodiment, the long term release is at least one year. In one embodiment, the method further comprises step (c) inducing a tissue tolerance by the immunosuppressive state. In one embodiment, the tissue tolerance is permanent. In one embodiment, the soluble stimulatory factor is selected from the group consisting of CCL22, IL2, and TGF-β. In one embodiment, the surface bound factors are selected from the group consisting of CD3 antibody, CD28 antibody, an adhesion molecule, and a CD45 antibody. Alternatively, the microparticle may further comprise a CD3 ligand (i.e., MHC) and/or a CD28 ligand.

In one embodiment, the present invention contemplates a kit comprising a container comprising at least one artificial particle suspended in a pharmaceutically acceptable vehicle. In one embodiment, the artificial particle comprises an artificial presenting cell. In one embodiment, the kit further comprises instructions for using the artificial particle to induce immunosuppression in a patient. In one embodiment, the artificial particle comprises at least one encapsulated soluble T_{reg} factor. In one embodiment, the at least one soluble or surface bound Treg cell factor comprises a regulatory T cell stimulatory factor. In one embodiment, the at least one soluble or surface bound Treg cell factor comprises a regulatory T cell inducing factor. In one

embodiment, the at least one soluble or surface bound Treg cell factor comprises a regulatory T cell chemoattractant factor. In one embodiment, the soluble factor is selected from the group consisting of CCL22, IL2 or TGF-β.

In one embodiment, the present invention contemplates an artificial osteoblast cell comprising a plurality of soluble and surface bound osteoclast signaling factors. In one embodiment, the soluble signaling factors are controlled released. In one embodiment, the soluble signaling factor comprises MCSF. In one embodiment, the surface bound signaling factor comprises RANK-L, OSCAR-L, and/or ODF. In one embodiment, the controlled release is a short term release. In one embodiment, the short term release is between 30 minutes-1 hour. In one embodiment, the short term release is between 1 hour-3 hours. In one embodiment, the short term release is between 3 hours-10 hours. In one embodiment, the short term release is between 10 hours-24 hours. In one embodiment, the controlled release is a long term release. In one embodiment, the long term release is between 24 hours-36 hours. In one embodiment, the long term release is between 3 days-7 days. In one embodiment, the long term release is between 7 days-1 month. In one embodiment, the long term release is between 1 month-6 months. In one embodiment, the long term release is between 6 months-1 year. In one embodiment, the long term release is at least one year. In one embodiment, the surface bound signaling factors comprises a biomimetic surface pattern. In one embodiment, the biomimetic surface pattern comprises an osteological synapse. In one embodiment, the artificial osteoblast cell take a shape selected from the group consisting of a sphere, a square, a rectangle, a triangle, a trapezoid, a hexagon, an octagon, or a tetrahedron.

In one embodiment, the present invention contemplates an artificial osteoblast cell comprising at least one soluble osteoclast cell signaling factor and at least one surface bound osteoclast cell signaling factor. In one embodiment, the artificial osteoblast cell further comprises a functionalized polymer, wherein said polymer encapsulates the soluble factor. In one embodiment, the artificial osteoblast cell further comprises compartments, wherein said compartments comprise the soluble signaling factor. In one embodiment, the artificial osteoblast cell is biodegradable such that the soluble factor undergoes controlled release. In one embodiment, the soluble factor comprises MCSF. In one embodiment, the polymer comprises poly-lactic acid-co-glycolic acid (PLGA). In one embodiment, the functionalized polymer non-covalently bonds the soluble factor. In one embodiment, the at least one surface bound osteoclast signaling factor forms a biomimetic surface pattern. In one embodiment, the biomimetic surface pattern comprises an osteological synapse. In one embodiment, the surface bound osteoclast signaling factor comprises RANK-L, OSCAR-L, and/or ODF. In one embodiment, the functionalized polymer covalently bonds the surface bound osteoclast cell stimulatory factor.

In one embodiment, the present invention contemplates a method, comprising: a) providing, i) an emulsified microparticle comprising at least one soluble osteoclast cell signaling factor and a functionalized polymer; and ii) at least one surface bound osteoclast cell signaling factor, wherein the surface bound signaling factor is capable of attaching (i.e., for example, by conjugating and/or adsorbing) to the polymer; b) attaching a first surface bound factor to said polymer, wherein a first region is created; c) attaching a second surface bound factor to said polymer, wherein a second region is created; and d) attaching a third surface bound factor to said polymer, wherein a third region is created. In

one embodiment, the microparticle is biodegradable such that the soluble factor undergoes controlled release. In one embodiment, the at least one soluble signaling factor comprises MSCF. In one embodiment, the first, second, and third regions comprise a biomimetic pattern. In one embodiment, the biomimetic pattern comprises an osteological synapse. In one embodiment, the first surface bound signaling factor comprises RANK-L, OSCAR-L, and/or ODF.

In one embodiment, the present invention contemplates a method, comprising: a) providing, i) a polymer capable of undergoing emulsification, ii) at least one soluble osteoclast cell signaling factor, and iii) at least one surface bound osteoclast cell signaling factor; and b) emulsifying the polymer and the soluble signaling factor such that a biodegradable microparticle is created, such that the soluble factor undergoes controlled release. In one embodiment, the microparticle is porous. In one embodiment, the microparticle comprises compartments. In one embodiment, the emulsifying may be selected from the group including, but not limited to, precipitation, emulsions, melt casting, spray drying, crystallization, shearing, or milling. In one embodiment, the method further comprises step (c) immobilizing a first surface bound signaling factor in an apical region of the microparticle. In one embodiment, the method further comprises step (d) immobilizing a second surface bound signaling factor in a tropical region of the microparticle. In one embodiment, the method further comprises step (e) immobilizing a third surface signaling factor in an equatorial region of the microparticle. In one embodiment, the polymer comprises poly-lactic acid-co-glycolic acid (PLGA). In one embodiment, the soluble signaling factor comprises MCSF. In one embodiment, the at least one surface bound signaling factor comprises RANK-L, OSCAR-L, and/or ODF. In one embodiment, the at least one surface bound signaling factor is randomly displayed on the microparticle.

In one embodiment, the present invention contemplates a method, comprising: a) providing, i) a patient, wherein said patient is in need of bone healing; ii) an artificial osteoblast cell comprising at least one soluble osteoclast cell signaling factor and at least one surface bound osteoclast cell signaling factor; and b) administering the artificial osteoblast cell to the patient under conditions such that the bone healing is induced in the patient. In one embodiment, the patient has under gone bone surgery. In one embodiment, the patient comprises a bone injury. In one embodiment, the patient comprises a bone disease. In one embodiment, the artificial osteoblast cell is porous. In one embodiment, the artificial osteoblast cell comprises compartments. In one embodiment, the compartments comprise the soluble signaling factor. In one embodiment, the artificial osteoblast cell is biodegradable, wherein the soluble factor undergoes controlled release. In one embodiment, the controlled release is a short term release. In one embodiment, the short term release is between 30 minutes-1 hour. In one embodiment, the short term release is between 1 hour-3 hours. In one embodiment, the short term release is between 3 hours-10 hours. In one embodiment, the short term release is between 10 hours-24 hours. In one embodiment, the controlled release is a long term release. In one embodiment, the long term release is between 24 hours-36 hours. In one embodiment, the long term release is between 3 days-7 days. In one embodiment, the long term release is between 7 days-1 month. In one embodiment, the long term release is between 1 month-6 months. In one embodiment, the long term release is between 6 months-1 year. In one embodiment, the long term release is at least one year. In one embodiment, the bone surgery comprises bone replacement including, but not

limited to, hip replacement or knee replacement. In one embodiment, the bone surgery comprises bone reconstruction. In one embodiment, the bone surgery comprises bone fracture repair. In one embodiment, the soluble signaling factor comprises MCSF. In one embodiment, the surface bound signaling factor comprises RANK-L, OSCAR-L, and/or ODF.

In one embodiment, the present invention contemplates a kit comprising a container comprising at least one artificial osteoblast cell suspended in a pharmaceutically acceptable vehicle. In one embodiment, the kit further comprises instructions for using the artificial osteoblast cell to induce bone healing in a patient. In one embodiment, the artificial osteoblast cell comprises at least one encapsulated soluble osteoclast signaling factor. In one embodiment, the soluble signaling factor comprises MCSF.

In one embodiment, the present invention contemplates an artificial osteoclast cell comprising a plurality of soluble and surface bound osteoblast signaling factors. In one embodiment, the soluble signaling factors are controllably released. In one embodiment, the soluble signaling factor comprises MIM1. In one embodiment, the surface bound signaling factor comprises EphrinB2 and/or TGF-β. In one embodiment, the controlled release is a short term release. In one embodiment, the short term release is between 30 minutes-1 hour. In one embodiment, the short term release is between 1 hour-3 hours. In one embodiment, the short term release is between 3 hours-10 hours. In one embodiment, the short term release is between 10 hours-24 hours. In one embodiment, the controlled release is a long term release. In one embodiment, the long term release is between 24 hours-36 hours. In one embodiment, the long term release is between 3 days-7 days. In one embodiment, the long term release is between 7 days-1 month. In one embodiment, the long term release is between 1 month-6 months. In one embodiment, the long term release is between 6 months-1 year. In one embodiment, the long term release is at least one year. In one embodiment, the surface bound signaling factors comprises a biomimetic surface pattern. In one embodiment, the biomimetic surface pattern comprises an osteological synapse. In one embodiment, the artificial osteoblast cell take a shape selected from the group consisting of a sphere, a square, a rectangle, a triangle, a trapezoid, a hexagon, an octagon, or a tetrahedron.

In one embodiment, the present invention contemplates an artificial osteoclast cell comprising at least one soluble osteoblast cell signaling factor and at least one surface bound osteoblast cell signaling factor. In one embodiment, the artificial osteoclast cell further comprises a functionalized polymer, wherein said polymer encapsulates the soluble factor. In one embodiment, the functionalized polymer comprises a surface polymer. In one embodiment, the artificial osteoclast cell further comprises compartments, wherein said compartments comprise the soluble signaling factor. In one embodiment, the artificial osteoclast cell is biodegradable such that the soluble factor undergoes controlled release. In one embodiment, the soluble factor comprises MIM1. In one embodiment, the polymer comprises poly-lactic acid-co-glycolic acid (PLGA). In one embodiment, the functionalized polymer non-covalently bonds the soluble factor. In one embodiment, the at least one surface bound osteoblast signaling factor forms a biomimetic surface pattern. In one embodiment, the biomimetic surface pattern comprises an osteological synapse. In one embodiment, the surface bound osteoblast signaling factor comprises Eph-

rinB2 and/or TGF-β. In one embodiment, the functionalized polymer covalently bonds the surface bound osteoclast cell stimulatory factor.

In one embodiment, the present invention contemplates a method, comprising: a) providing, i) an emulsified microparticle comprising at least one soluble osteoblast cell signaling factor and a functionalized polymer; and ii) at least one surface bound osteoblast cell signaling factor, wherein the surface bound signaling factor is capable of attaching to the polymer; b) attaching a first surface bound factor to said polymer, wherein an apical region is created; c) attaching a second surface bound factor to said polymer, wherein a tropical region is created; and d) attaching a third surface bound factor to said polymer, wherein an equatorial region is created. In one embodiment, the microparticle is biodegradable such that the soluble factor undergoes controlled release. In one embodiment, the at least one soluble signaling factor comprises MIM1. In one embodiment, the apical, tropical, and equatorial regions comprise a biomimetic pattern. In one embodiment, the biomimetic pattern comprises an osteological synapse. In one embodiment, the first surface bound signaling factor comprises EphrinB2 and/or TGF-β.

In one embodiment, the present invention contemplates a method, comprising: a) providing, i) a polymer capable of undergoing emulsification, ii) at least one soluble osteoblast cell signaling factor, and iii) at least one surface bound osteoblast cell signaling factor; and b) emulsifying the polymer and the soluble signaling factor such that a porous biodegradable microparticle is created, such that the soluble factor undergoes controlled release. In one embodiment, the emulsifying may be selected from the group including, but not limited to, precipitation, emulsions, melt casting, spray drying, crystallization, shearing, or milling. In one embodiment, the method further comprises step (c) immobilizing a first surface bound signaling factor in an apical region of the microparticle. In one embodiment, the method further comprises step (d) immobilizing a second surface bound signaling factor in a tropical region of the microparticle. In one embodiment, the method further comprises step (e) immobilizing a third surface signaling factor in an equatorial region of the microparticle. In one embodiment, the polymer comprises poly-lactic acid-co-glycolic acid (PLGA). In one embodiment, the soluble signaling factor comprises MINE. In one embodiment, the at least one surface bound signaling factor comprises EphrinB2 and/or TGF-β.

In one embodiment, the present invention contemplates a method, comprising: a) providing, i) a patient having undergone bone surgery, wherein said patient is in need of bone healing; ii) a porous artificial osteoclast cell comprising at least one soluble osteoblast cell signaling factor and at least one surface bound osteoblast cell signaling factor; and b) administering the artificial osteoclast cell to the patient under conditions such that a bone healing is induced in the patient. In one embodiment, the porous artificial osteoclast cell comprises compartments. In one embodiment, the compartments comprise the soluble signaling factor. In one embodiment, the artificial osteoclast cell is biodegradable, wherein the soluble factor undergoes controlled release. In one embodiment, the controlled release is a short term release. In one embodiment, the short term release is between 30 minutes-1 hour. In one embodiment, the short term release is between 1 hour-3 hours. In one embodiment, the short term release is between 3 hours-10 hours. In one embodiment, the short term release is between 10 hours-24 hours. In one embodiment, the controlled release is a long term release. In one embodiment, the long term release is between 24 hours-36 hours. In one embodiment, the long

term release is between 3 days-7 days. In one embodiment, the long term release is between 7 days-1 month. In one embodiment, the long term release is between 1 month-6 months. In one embodiment, the long temp release is between 6 months-1 year. In one embodiment, the long term release is at least one year. In one embodiment, the bone surgery comprises bone replacement including, but not limited to, hip replacement or knee replacement. In one embodiment, the bone surgery comprises bone reconstruction. In one embodiment, the bone surgery comprises bone fracture repair. In one embodiment, the soluble signaling factor comprises MIM1. In one embodiment, the surface bound signaling factor comprises EphrinB2 and/or TGF-β.

In one embodiment, the present invention contemplates a kit comprising a container comprising at least one artificial osteoclast cell suspended in a pharmaceutically acceptable vehicle. In one embodiment, the kit further comprises instructions for using the artificial osteoclast cell to induce bone healing in a patient. In one embodiment, the artificial osteoclast cell comprises at least one encapsulated soluble osteoblast signaling factor. In one embodiment, the soluble signaling factor comprises MIM1.

In one embodiment, the present invention contemplates a method, comprising: a) providing, i) a patient, wherein said patient comprises at least one inflammatory symptom; ii) a microparticle comprising at least one soluble Treg factor; and b) administering the microparticle to the patient under conditions such that the at least one inflammatory symptom is reduced. In one embodiment, the at least one soluble or surface bound Treg factor comprises a regulatory T cell stimulatory factor. In one embodiment, the at least one soluble or surface bound Treg cell factor comprises a regulatory T cell inducing factor. In one embodiment, the at least one soluble or surface bound Treg cell factor comprises a regulatory T cell chemoattractant factor. In one embodiment, the patient has under gone periodontal surgery. In one embodiment, the patient further comprises a tissue injury. In one embodiment, the patient further comprises a periodontal disease. In one embodiment, the patient comprises a cancer disease. In one embodiment, the microparticle is porous. In one embodiment, the microparticle is biodegradable, wherein the Treg chemoattractant factor undergoes controlled release. In one embodiment, the controlled release is a short term release. In one embodiment, the short term release is between 30 minutes-1 hour. In one embodiment, the short term release is between 1 hour-3 hours. In one embodiment, the short term release is between 3 hours-10 hours. In one embodiment, the short term release is between 10 hours-24 hours. In one embodiment, the controlled release is a long term release. In one embodiment, the long term release is between 24 hours-36 hours. In one embodiment, the long term release is between 3 days-7 days. In one embodiment, the long term release is between 7 days-1 month. In one embodiment, the long term release is between 1 month-6 months. In one embodiment, the long term release is between 6 months-1 year. In one embodiment, the long term release is at least one year. In one embodiment, the Treg chemoattractant factor comprises CCL22 and/or vasoactive intestinal peptide.

Definitions

The term "target tissue" as used herein, refers to any bodily tissue that may be affected by a medical condition and/or disorder (e.g., an immunological disease or a transplanted tissue/organ) to which a population of regulatory T cells may be directed to by induction with an individual and/or a combination of T cell inducing factors released from microparticles.

The term "blood cell" as used herein, refers to any biological cell, either nucleated or enucleated, found circulating in the blood stream.

The term "white blood cell" as used herein, refers to any blood cell that is colorless, lacks hemoglobin, contains a nucleus, and may include, but is not limited to, lymphocytes, monocytes, neutrophils, eosinophils, and basophils—called also leukocytes, white blood corpuscles, white cells, and/or white corpuscles.

The term "T cell" as used herein, refers to any of several lymphocyte types (e.g., helper T cell or regulatory T cell) that differentiate in the thymus, possess highly specific cell-surface antigen receptors, that may control the initiation and/or suppression of cell-mediated and humoral immunity (as by the regulation of T and B cell maturation and proliferation) and others that lyse antigen-bearing cells—also referred to as a T lymphocyte.

The term "B cell" as used herein, refers to any of the several lymphocytes that have antigen-binding antibody molecules on their membrane surface, that comprise the antibody-secreting plasma cells when mature, and that in mammals differentiate in the bone marrow—also referred to as a B lymphocyte.

The term "microparticle population" as used herein, refers to a collection of microparticles and/or microspheres having similar properties and composition.

The term "polymer" as used herein, refers to any unit-based chain of molecules. For example, such molecules may include, but are not limited to, gelatin, collagen, cellulose esters, dextran sulfate, pentosan polysulfate, chitin, saccharides, albumin, synthetic polyvinyl pyrrolidone, polyethylene oxide, polypropylene oxide, block polymers of polyethylene oxide and polypropylene oxide, polyethylene glycol, acrylates, acrylamides, methacrylates including, but not limited to, 2-hydroxyethyl methacrylate, poly(ortho esters), cyanoacrylates, gelatin-resorcin-aldehyde type bioadhesives, polyacrylic acid and copolymers and block copolymers thereof. Alternatively, polymers may include, but are not limited to, poly(lactide-co-glycolide) polymer (e.g., PLGA), RG505, and/or RG502H. For example, one polymer may be comprised of at least three polymers ranging from 4-70 kDa 50:50 PLGA. Another polymer may be comprised of 7-17 kDa 50:50 PLGA (e.g., for example, 12.6 kDa RG502H). Another polymer may be comprised of 54-69 kDa 50:50 PLGA (e.g., for example, 100 kDa RG505).

The term "microparticle" as used herein, refers to any microscopic carrier to which a compound or drug may be attached and/or encapsulated. Preferably, microparticles contemplated by this invention are capable of formulations having controlled release properties.

The term "attached" as used herein, refers to any interaction between a medium (or carrier) and a drug. Attachment may be reversible or irreversible. Such attachment includes, but is not limited to, covalent bonding, ionic bonding, Van der Waals forces or friction, and the like. A drug is attached to a medium (or carrier) if it is impregnated, incorporated, coated, in suspension with, in solution with, mixed with, etc.

The term "encapsulated" as used herein, refers to any composition where a secondary compound (e.g., a drug, protein, peptide, nucleic acid etc.) is trapped within the composition and/or attached throughout the composition including the surface of the composition. Usually, encapsulation occurs when the secondary compound is present

during the formation of the composition, as opposed to being contacted with the composition after the formation of the composition.

The term "PLGA" as used herein, refers to mixtures of polymers or copolymers of lactic acid and glycolic acid. As used herein, lactide polymers are chemically equivalent to lactic acid polymer and glycolide polymers are chemically equivalent to glycolic acid polymers. In one embodiment, PLGA contemplates an alternating mixture of lactide and glycolide polymers, and is referred to as a poly(lactide-co-glycolide) polymer.

The term "at risk for" as used herein, refers to a medical condition or set of medical conditions exhibited by a patient which may predispose the patient to a particular disease or affliction. For example, these conditions may result from influences that include, but are not limited to, behavioral, emotional, chemical, biochemical, or environmental influences.

The term "effective amount" as used herein, refers to a particular amount of a pharmaceutical composition comprising a therapeutic agent that achieves a clinically beneficial result (i.e., for example, a reduction of symptoms). Toxicity and therapeutic efficacy of such compositions can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index, and it can be expressed as the ratio LD_{50}/ED_{50}. Compounds that exhibit large therapeutic indices are preferred. The data obtained from these cell culture assays and additional animal studies can be used in formulating a range of dosage for human use. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage varies within this range depending upon the dosage form employed, sensitivity of the patient, and the route of administration.

The term "symptom" as used herein, refers to any subjective or objective evidence of disease or physical disturbance observed by the patient. For example, subjective evidence is usually based upon patient self-reporting and may include, but is not limited to, pain, headache, visual disturbances, nausea and/or vomiting. Alternatively, objective evidence is usually a result of medical testing including, but not limited to, body temperature, complete blood count, lipid panels, thyroid panels, blood pressure, heart rate, electrocardiogram, tissue and/or body imaging scans.

The term "transplant rejection reaction" or "graft versus host disease" as used herein, refers to any activation of the immune system subsequent to the implantation of an exogenous tissue and/or organ into a patient that may result in damage and/or destruction of the transplanted tissue. Generally, transplant rejections are believed to be an adaptive immune response via cellular immunity (i.e., for example, mediated by killer T cells inducing apoptosis of target cells) as well as humoral immunity (mediated by activated B cells secreting antibody molecules), though the action is joined by components of innate immune response (phagocytes and soluble immune proteins).

The term "disease" as used herein, refers to any impairment of the normal state of the living animal or plant body or one of its parts that interrupts or modifies the performance of the vital functions. Typically manifested by distinguishing signs and symptoms, it is usually a response to: i) environmental factors (as malnutrition, industrial hazards, or climate); ii) specific infective agents (as worms, bacteria, or

viruses); iii) inherent defects of the organism (as genetic anomalies); and/or iv) combinations of these factors

The terms "reduce," "inhibit," "diminish," "suppress," "decrease," "prevent" and grammatical equivalents thereof (including, but not limited to, "lower," "smaller," etc.) when used in reference to the expression of any symptom in an untreated subject relative to a treated subject, mean that the quantity and/or magnitude of the symptoms in the treated subject is lower than in the untreated subject by any amount that is recognized as clinically relevant by any medically trained personnel. In one embodiment, the quantity and/or magnitude of the symptoms in the treated subject is at least 10% lower than, at least 25% lower than, at least 50% lower than, at least 75% lower than, and/or at least 90% lower than the quantity and/or magnitude of the symptoms in the untreated subject.

The term "migration" or "migrate" or "migrating" as used herein, refers to any movement of a cell (e.g., a T cell) in the direction of a compromised target tissue. Such migration may be accompanied by a stimulation from a gradient of chemoattractant and/or chemotactic factors (i.e., for example, lysophosphatidic acid) released by white blood cells.

The term "chemoattractant factor" as used herein, refers to any compound and/or molecule that induces movement of chemotactic cells in the direction of its highest concentration. For example, a chemoattractant factor may include, but is not limited to, CCL22 and/or vasoactive intestinal peptide (VIP).

The term "chemotactic cells" as used herein, refers to any biological cell exhibiting chemotaxis, wherein the chemotactic cells direct their movements according to certain chemicals in their environment.

The term "drug" or "compound" as used herein, refers to any pharmacologically active substance capable of being administered which achieves a desired effect. Drugs or compounds can be synthetic or naturally occurring, non-peptide, proteins or peptides, oligonucleotides or nucleotides, polysaccharides or sugars.

The term "administered" or "administering" as used herein, refers to any method of providing a composition to a patient such that the composition has its intended effect on the patient. An exemplary method of administering is by a direct mechanism such as, local tissue administration (i.e., for example, extravascular placement), oral ingestion, transdermal patch, topical, inhalation, suppository etc.

The term "patient" as used herein, is a human or animal and need not be hospitalized. For example, out-patients, persons in nursing homes are "patients." A patient may comprise any age of a human or non-human animal and therefore includes both adult and juveniles (i.e., children). It is not intended that the term "patient" connote a need for medical treatment, therefore, a patient may voluntarily or involuntarily be part of experimentation whether clinical or in support of basic science studies.

The term "protein" as used herein, refers to any of numerous naturally occurring extremely complex substances (as an enzyme or antibody) that comprise a polymer of amino acid residues joined by peptide bonds, containing the elements carbon, hydrogen, nitrogen, oxygen, and usually sulfur. In general, a protein comprises amino acids having an order of magnitude within the hundreds.

The term "peptide" as used herein, refers to any of various amides that are derived from two or more amino acids by combination of the amino group of one acid with the carboxyl group of another and are usually obtained by

partial hydrolysis of proteins. In general, a peptide comprises amino acids having an order of magnitude with the tens.

The term "pharmaceutically" or "pharmacologically acceptable" as used herein, refer to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when administered to an animal or a human.

The term, "pharmaceutically acceptable carrier", as used herein, includes any and all solvents, or a dispersion medium including, but not limited to, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils, coatings, isotonic and absorption delaying agents, liposome, commercially available cleansers, and the like. Supplementary bioactive ingredients also can be incorporated into such carriers.

The term, "purified" or "isolated" as used herein, may refer to a peptide composition that has been subjected to treatment (i.e., for example, fractionation) to remove various other components, and which composition substantially retains its expressed biological activity. Where the term "substantially purified" is used, this designation will refer to a composition in which the protein or peptide forms the major component of the composition, such as constituting about 50%, about 60%, about 70%, about 80%, about 90%, about 95% or more of the composition (i.e., for example, weight/weight and/or weight/volume). The term "purified to homogeneity" is used to include compositions that have been purified to 'apparent homogeneity" such that there is single protein species (i.e., for example, based upon SDS-PAGE or HPLC analysis). A purified composition is not intended to mean that some trace impurities may remain.

The term "substantially purified" as used herein, refers to molecules, either nucleic or amino acid sequences, that are removed from their natural environment, isolated or separated, and are at least 60% free, preferably 75% free, and more preferably 90% free from other components with which they are naturally associated. An "isolated polynucleotide" is therefore a substantially purified polynucleotide.

The term "small organic molecule" as used herein, refers to any molecule of a size comparable to those organic molecules generally used in pharmaceuticals. The term excludes biological macromolecules (e.g., proteins, nucleic acids, etc.). Preferred small organic molecules range in size from approximately 10 Da up to about 5000 Da, more preferably up to 2000 Da, and most preferably up to about 1000 Da.

The term "immunologically active" defines the capability of a natural, recombinant or synthetic peptide, or any oligopeptide thereof, to induce a specific immune response in appropriate animals or cells and/or to bind with specific antibodies.

The term "artificial particle" or "artificial construct" as used herein, refers to any fabricated degradable polymer construct that is capable of emulsification to encapsulate soluble factors, wherein the polymer may be functionalized to attached surface bound factors. The constructs may then manipulate native regulatory cells to induce immunological tolerance.

The term "artificial presenting cell" as used herein, refers to any artificial particle or artificial construct that further comprises an immunological synapse'.

The term "immunological synapse' as used herein, refers to any specific, non-random, arrangement of biological compounds (i.e., for example, antibodies or soluble proteins) on a biological antigen presenting cell that is complementary

to a contacting region on a target cell (i.e., for example, a lymphocyte). For example, an antigen presenting cell may comprise an immunological synapse that resembles a "bull's eye" and has a complementary contact region on a regulatory T cell (T_{reg} cell).

The term "artificial dendritic cell" or "artificial APC" as used herein, refers to any fabricated degradable, artificial presenting cell using microparticle-based controlled release technology capable of interacting with a regulatory T cell. For example, cell-sized PLGA microparticles may be used to encapsulate soluble factors that are capable of inducing regulatory T cells to induce immunosuppression. For investigational studies, the soluble factors may be labeled with monoclonal antibodies (i.e., for example, fluorescent labels).

The term "artificial osteoblast cell" as used herein, refers to any fabricated degradable microparticle having controlled release technology capable of interacting with an osteoclast cell. For example, cell-sized PLGA microparticles may be used to encapsulate soluble factors that are capable of inducing osteoclasts to initiate bone healing. For investigational studies, the soluble factors may be labeled with monoclonal antibodies (i.e., for example, fluorescent labels).

The term "artificial osteoclast cell" as used herein, refers to any fabricated degradable microparticle having controlled release technology capable of interacting with an osteoblast cell. For example, cell-sized PLGA microparticles may be used to encapsulate soluble factors that are capable of inducing osteoblasts to modulate bone healing. For investigational studies, the soluble factors may be labeled with monoclonal antibodies (i.e., for example, fluorescent labels).

The term "soluble regulatory T cell stimulating factors" as used herein, refers to any biological agent (i.e., for example, a protein, hormone, drug etc.) capable of interacting with T_{reg} cells that may become encapsulated within a polymer-based microparticle (i.e., for example, an artificial antigen presenting cell) and undergo controlled release during the degradation of the microparticle. Such soluble factors may be, for example, CCL22, IL2, or TGFβ.

The term "soluble osteoclast signaling factor" as used herein, refers to any biological agent (i.e., for example, a protein, hormone, drug etc.) capable of interacting with osteoclast cells that may become encapsulated within a polymer-based microparticle (i.e., for example, an artificial osteoblast cell) an undergo controlled release during the degradation of the microparticle. Such soluble factors may be for example, MIM-1.

The term, "surface bound regulatory T cell stimulating factors" as used herein, refers to any biological agent (i.e., for example, a protein, hormone, drug etc.) capable of interacting with T_{reg} cells, that are attached to the surface of a polymer-based microparticle (i.e., for example, an artificial antigen presenting cell) and are not released during the degradation of the microparticle. Such surface factors may be, for example, CD3 antibody, CD28 antibody, or adhesion molecules.

The term, "surface bound osteoclast signaling factors" as used herein, refers to any biological agent (i.e., for example, a protein, hormone, drug etc.) capable of interacting with osteoclast cells that are attached to the surface of a polymer-based microparticle (i.e., for example, an artificial antigen presenting cell) and are not released during the degradation of the microparticle. Such surface factors may be, for example, RANK-L.

The term "biomimetic" as used herein, refers to any artificial or synthetic construct that has a similar biological effect as a native biological compound. Such similar effects may be due to similarities is size, shape, or specific (i.e.,

non-random) display of receptors, antibodies, or other active biological compounds. Such shapes include, but are not limited to, a circle, a square, a rectangle, a triangle, a trapezoid, a hexagon, an octagon, or a tetrahedron.

The term "biocompatible" as used herein, refers to any material does not elicit a substantial detrimental response in the host. There is always concern, when a foreign object is introduced into a living body, that the object will induce an immune reaction, such as an inflammatory response that will have negative effects on the host. In the context of this invention, biocompatibility is evaluated according to the application for which it was designed: for example; a bandage is regarded a biocompatible with the skin, whereas an implanted medical device is regarded as biocompatible with the internal tissues of the body. Preferably, biocompatible materials include, but are not limited to, biodegradable and biostable materials.

The term "biodegradable" as used herein, refers to any material that can be acted upon biochemically by living cells or organisms, or processes thereof, including water, and broken down into lower molecular weight products such that the molecular structure has been altered.

The term "controlled release" as used herein, refers to the escape of any attached or encapsulated factor at a predetermined rate. For example, a controlled release of a factor may occur resulting from the predictable biodegradation of a polymer particle (i.e., for example, an artificial antigen presenting cell, a microparticle and/or a microsphere). The rate of biodegradation may be predetermined by altering the polymer composition and/or ratio's comprising the particle. Consequently, the controlled release may be short term or the controlled release may be long term. In one embodiment, the short term release is between 30 minutes-1 hour. In one embodiment, the short term release is between 1 hour-3 hours. In one embodiment, the short term release is between 3 hours-10 hours. In one embodiment, the short term release is between 10 hours-24 hours. In one embodiment, the long term release is between 24 hours-36 hours. In one embodiment, the long term release is between 3 days 7 days. In one embodiment, the long term release is between 7 days-1 month. In one embodiment, the long term release is between 1 month-6 months. In one embodiment, the long term release is between 6 months-1 year. In one embodiment, the long term release is at least one year.

The term "emulsification" as used herein, refers to any process that mixes together at least two compounds that are not naturally miscible together. For example, an emulsification process may utilize agents (i.e., emulsifiers) that facilitate the co-solubility of immiscible compounds. For example, emulsification may include methods that use techniques including, but not limited to, precipitation, emulsions, melt casting, spray drying, crystallization, shearing, or milling.

The term "functionalized polymer" as used herein, refers to any polymer where the terminal moiety has been chemically altered to facilitate the attachment of other chemicals and or compounds (i.e., for example, proteins, antibodies, hormones, drugs, etc.). For example, the terminal moiety may include, but is not limited to, a carboxylic acid, an amine, a sulfide, a hydroxyl etc.

The term "immobilization" as used herein, refers a state where a biological compound is fixed in position as a complex and is unable to move without the movement of the complex.

The term "covalent bond" as used herein, refers to a close association between at least two atoms, wherein the atoms share electrons in specific atomic orbital paths.

The term "non-covalent bond" as used herein, refers to a close association between at least two atoms, wherein the atoms do not share electrons.

The term "tissue transplant" as used herein, refers to any replacement of a tissue and/or organ within an individual with a similar tissue and/or organ from a different individual. In some cases, the individuals are from the same species. In other cases, the individuals are from different species.

The term "immunological tolerance" as used herein, refers to any modification of the immune system wherein specific antibodies may not be produced, but the immune system remains responsive to other antigens. For example, specific immune related cells including, but not limited to, T_{reg} cells, osteoclasts, and/or osteoblasts may be stimulated to induce immunosuppression. Such "immunological tolerance" may also be capable of controlling autoimmune diseases including, but not limited to, arthritis, Type I diabetes. Such "immunological tolerance" may also be capable of controlling inflammatory diseases including, but not limited to, periodontal disease such as periodontitis.

The term, "supramolecular activation clusters (SMACs)" as used herein, refer to a topologically complex, and discretely organized, arrangement of antigen presenting cell surface proteins and/or soluble proteins. SMACs may be comprised of a plurality of regions having localized density of specific surface proteins and/or soluble proteins (i.e., for example, central SMAC (cSMAC), peripheral SMAC (pSMAC), or distal SMAC (dSMAC). In general, the surface proteins comprise cell surface antibodies and/or receptors, while the soluble proteins comprise ligands directed to activating certain lymphocytes (i.e., for example, a regulatory T cell).

The term "apical region" as used herein, refers to the top of a sphere and spreading circumferentially from between approximately 1 degree to 30 degrees from a polar axis. In one embodiment, the apical region may spread circumferentially from between approximately 5 degrees to 20 degrees from a polar axis. In one embodiment, the apical region may spread circumferentially from between approximately 10 degrees to 15 degrees from a polar axis.

The term "tropical region" as used herein, refers to a circumferential region of a sphere located between approximately 30-70 degrees from a polar axis. In one embodiment, the tropical region may spread circumferentially from between approximately 35 degrees to 60 degrees from a polar axis. In one embodiment, the tropical region may spread circumferentially from between approximately 40 degrees to 50 degrees from a polar axis.

The term "equatorial region" as used herein, refers to a circumferential region of a sphere located between approximately 70-90 degrees from a polar axis. In one embodiment, the equatorial region may spread circumferentially from between approximately 75 degrees to 85 degrees from a polar axis. In one embodiment, the equatorial region may spread circumferentially from between approximately 87 degrees to 93 degrees from a polar axis.

The term "random" as used herein, refers to a stochastic arrangement of objects formed without definite aim, purpose, method, or adherence to a prior arrangement; or in a haphazard way.

The term "non-random" as used herein, refers to a non-stochastic arrangement of objects with a definite aim, purpose, method and adheres to a prior conceived pattern.

BRIEF DESCRIPTION OF THE FIGURES

The file of this patent contains at least one drawing executed in color. Copies of this patent with color drawings

will be provided by the Patent and Trademark Office upon request and payment of the necessary fee.

FIG. **1** provides exemplary data showing a T_{reg} proliferative dose response induced by degradable particles with randomly attached CD3 antibodies and CD28 antibodies (ratio=1:1 or 1:4 cells/particle). Proliferation was measured by tritiated thymidine incorporation. Controls are equivalent amounts of soluble antibody (control). Inset: FITC labeled CD3 and CD28 antibodies for visualization),

FIG. **2** presents an illustrative embodiment showing how regulatory T cells (T_{regs}) are attracted to: (A) a tumor; or (B) an artificial presenting cell (i.e., for example, a synthetic dendritic cell) near the surface of an allograft.

FIG. **3** shows a fluorescent image of a representative "bull's eye" immunological synapse orientation from a biological dendritic cell. Grakoui et al., *Science* 285:221 (1999).

FIG. **4A** illustrates one embodiment of a method for fabricating artificial presenting cell construct patterns that mimic a cellular immunological synapse (IS).

FIG. **4B** presents an exemplary fluorescent photomicrograph image of a synthetic dendritic cell comprising a central supramolecular activation complex (cSMAC) region (green/yellow) and a peripheral supramolecular activation complex (pSMAC) region. See, Top Image arrows, respectively. Scanning Electron Microscopic backscatter imaging shows a bright, and intentionally over-labeled, cSMAC region (See, Bottom Image, white arrow=gold-bead labeled protein).

FIG. **5A** presents an illustration of one embodiment for the fabrication of polymer microparticles via a double-emulsion/solvent evaporation technique.

FIG. **5B** presents an exemplary freeze fracture scanning electron micrograph of a polymer microparticle created by the technique shown in FIG. **5A** demonstrating an inner aqueous phase.

FIG. **6** illustrates standard carbodiimide chemistry to create an amine reactive site on the surface of PLGA microparticles starting with a carboxylic acid generated from the degradation of ester bonds.

FIG. **7** presents one embodiment to label functionalized PLGA polymers using a novel compound (i.e., for example, 2-pyridyldithioethylamine) to convert a carboxylic acid group into a thiol reactive group (See, middle panel). A subsequent cleavage reaction liberates a detectable product upon conjugation which can be used to determine the extent of surface labeling (see, right panel).

FIG. **8** illustrates one embodiment of an in vitro chemotaxis assay. Microparticles containing chemokines are placed in the bottom of a transwell filter plate with T_{reg} cells isolated from a transgenic B6/GFP+mouse on top of the opaque filter (left). After a time, chemokine is released and T_{reg} cells migrate through the filter and up the concentration gradient of the chemokine (right). Fluorescence readings are taken with a top and bottom detector plate reader to determine the extent of Treg cell chemotaxis.

FIG. **9A** presents exemplary data showing a release profile of CCL22 from porous microparticles showing an approximate linear release for over twenty (20) days.

FIG. **9B** presents exemplary data showing the successful creation of microparticles encapsulating CCL22 as used in FIG. **9A**. The top portion shows a representative image of an intact microparticle, indicating the particle's structural integrity and confirming the size. The bottom portion shows a representative image of a fractured particle confirming the porous nature of the particle created by the double emulsion encapsulation process.

FIG. **10** presents exemplary data showing CCL22 labeling (red) using the microparticles in accordance with FIG. **9**.

FIG. **11** presents an illustration of one embodiment comparing healthy gum tissue (left side) with gum tissue affected by periodontal disease (right side).

FIGS. **12A** and **12B** present exemplary data showing the effect of artificial antigen presenting cells (e.g., a polymer microparticle) encapsulating CCL-22 on periodontal disease in a mouse model. AA: infected, untreated mice. LP: infected, aAPC-CCL22 treated mice; CP: infected, aAPC-only mice.

FIG. **13A** presents an illustration representing a hypothesized coupling interaction for osteoclast stimulation. For example, osteoclasts may be recruited by MCSF and activated by surface-bound RANK-L and/or OSCAR-L.

FIG. **13B** presents an illustration of an artificial osteoblast cell that mimics the coupling interactions shown in FIG. **13A**.

FIG. **14A** presents an illustration representing a hypothesized coupling interaction for osteoblast stimulation. For example, osteoblasts are recruited by MIM1 and activated by surface-bound TGF-beta and EphrinB2 (BMP2).

FIG. **14B** presents an illustration of an artificial osteoclast cell that mimics the coupling interactions shown in FIG. **14A**.

FIG. **15** presents representative scanning micrographs of CCL22-containing polymer microparticles.

FIG. **15A** shows the external porous structure of a polymer microparticle.

FIG. **15B** shows the internal structure of a polymer microparticle displaying an inner aqueous phase created by a water-in-oil emulsion.

FIG. **16** presents exemplary data showing release kinetics of CCL22 from polymer microparticles as measured in physiological buffered saline (PBS). Error Bars=Standard Deviations; n=3.

FIG. **17** presents exemplary data showing a representative volume of an averaged sized distribution of polymer microparticles comprising CCL22 determined by average volume diameter measurements. Error Bars=Standard Error of the Mean; n=6.

FIG. **18** presents exemplary data showing CCR4 expression and in vitro transwell migration assay.

FIG. **18A**: Histograms depicting the absence of CCR4 expression on naïve CD4+ T cells but its presence on activated CD4+ T cells, as determined by flow cytometry.

FIG. **18B**: Transwell migration assays show significantly greater migration of activated regulatory and effector CD4+ T cells when compared to respective populations of naïve cells at different concentrations of CCL22. (* p<0.05, one-tailed Students T test)

FIG. **18C**: Expression of CCL22 is enhanced in CD4+ cells in the presence FoxP3 in comparison to the absence of FoxP3.

FIG. **19** presents exemplary data showing an in vivo migration of Tregs towards polymer microparticles comprising CCL22.

FIG. **19A** shows a representative schematic describing one experimental design for live animal imaging Luc+ Treg indicates a Treg population isolated from transgenic mice constitutively expressing the luciferase gene.

FIG. **19B** presents exemplary data showing representative fluorescence and luminescence images obtained at day 7 post injection, fluorescence images were used to outline areas of particle localization (i.e., for example, with Pro Living Image® 2.60.1) and these outlines were superimposed onto luminescence images taken with the mouse in

the exact same position to demonstrate co-localization of Tregs and CCL22 microparticles.

FIG. 19C presents exemplary data showing kinetics of Treg migration towards the injection sites; average radiance measurements obtained from the luminescence images are displayed as a ratio of CCL22 microparticles (CCL22MP) to Blank microparticles (BlankMP). Error Bars=Standard Deviation; n=3.

FIG. 20 presents exemplary data of non-invasive live animal imaging. The left limb was injected with BlankMP-680 and the right limb was injected with CCL22MP-680. Representative fluorescence (left) and luminescence (right) images taken from a single mouse at different time points following injection. Days 4 & 7: Significant co-localization of Treg with CCL22 microparticles. Days 2 and 9: Very little or no co-localization of Treg with CCL22 microparticles. Values represent the total flux (photons/sec) of fluorescence or luminescence intensities. ROI (regions of interest) were drawn automatically using Igor Pro Living Image® 2.60.1. Influx of Treg only towards CCL22MP was observed over multiple experimental cycles (n=6). Over time it was observed that Treg localized in mucosal tissues such as the gut and the lung, which secrete CCL22 endogenously. Endogenous Treg migration to the hind limbs was negligible and the cells that migrated to these sites were specifically towards CCL22MP.

FIG. 21 presents one method of preparing polymer microparticles comprising a double emulsion and evaporation.

FIG. 22 presents exemplary data showing mRNA expression of markers in periodontal tissues resected from diseased mice:

FIG. 22A: FoxP3: a marker for Tregs.

FIG. 22B: Anti-inflammatory, pro-regenerative cytokine IL-10.

FIG. 22C: Bone resorbing cell (osteoclast) activation factor RANKL.

FIG. 22D: Endogenous CCL-22 (Treg recruiting) chemokine.

FIG. 23 presents exemplary data showing abrogation of alveolar bone resorption in a mouse model of periodontitis. Dissecting microscope images of resected maxilla were mechanically de-fleshed and soaked in dispase overnight. Dotted-red boxes outline large differences in alveolar bone levels between the internal control and CCL-22 MP treated.

FIG. 23A: Representative image from mice that received BlankMPs A RIGHT in the right maxilla and no treatment (internal control) A LEFT in the left maxilla.

FIG. 23B: Representative image from a mouse that received CCL-22 MPs B RIGHT in the right maxilla and no treatment (internal control) B LEFT in the left maxilla.

FIG. 23C: Area measurements (CEJ-ABC) for the right maxilla of mice from each treatment and control group. *p-value<0.05

FIG. 24 presents exemplary data showing tumor regression after induction of Treg cells. Transgenic luciferase expressing lewis lung carcinoma cells (derived from C57B1/6 mice) were injected into FVB mice 4 days post particle injections. Quantitative live animal imaging was performed subsequently at regular time intervals showing qualitative data obtained at 2 different time points, suggesting that the CCL22 microparticles are able to slow down rejection of cellular allo-transplants.

FIG. 25 presents tumor regression kinetic data from the experiment demonstrated in FIG. 24. * Indicates p<0.05; Wilcoxon rank-sum test for null hypothesis that normalized luminescence for CCL22MP is same as that of BlankMP or

bolus CCL22.# p≤0.05 (comparison between CCL22MP and BlankMP only). n=11 BlankMP and CCL22MP (n=5 for bolus CCL22).

FIG. 26 presents a representative schematic of a proposed mechanism for vasoactive intestinal peptide (VIP) induction of regulatory T cells. For example, by a direct activation of CD4+CD25− naïve T cells to inducible Tregs. Alternatively by an activation of tolerogenic dendritic cells which then promote inducible Treg generation through activation of CD8+CD25− naïve T cells.

FIG. 27 presents exemplary data showing VIP release from VIPMPs having release profiles predicted by a computer algorithm presented herein.

FIG. 27A: VIP release profiles from predicted linear release VIPMPs (diamonds) and predicted multi-bolus release VIPMPs (squares). Standard deviations are represented by bars at each data point.

FIG. 27B. Comparison of the algorithmic model release prediction (red line), observed VIP release (blue diamonds) from the predicted linear release VIPMPs to the desired profile model input (dashed line). The anomalous initial release burst was ignored for this comparison.

FIG. 28 presents exemplary data showing the percent of FoxP3+ CD4+ T-cells which were recruited through the transwells by DCs, following treatment with LPS and addition of soluble VIP (2.5×10^{-8}M), blank microparticles releasates, or VIP microparticle releasates (estimated to be at 2.5×10^{-8} M from previous release profiles). Percents were normalized by the group which did not receive LPS. Upper Graph: DC precursors treated with IL-4 and GM-CSF. Lower Graph: DC precursors cultured with 5×GM-CSF.

FIG. 29 presents exemplary data showing linear and volumetric measurements of alveolar bone loss using micro-CT imaging as shown in Park et al., *J Periodontol.* 78(2): 273-281 (2007).

FIG. 30 presents an exemplary micro-CT analysis performed on mice having periodontitis after receiving VIPMP treatment.

FIG. 31 presents exemplary scanning electron microscope images of VIP microparticles comprising a 12.6 kDa PLGA polymer. Nonporous (A) and porous (B) VIP microparticles had average diameters of 16.7 μm and 17.7 14.3 μm, respectively.

FIG. 32 presents exemplary VIP release profiles from PLGA VIP microparticles in PBS consisting of either: a 4.2 kDa polymer (blue diamond); a 12.6 kDa polymer (red square); or a 100 kDa polymer (green triangle). Standard deviations are represented by bars at each data point.

FIG. 33 presents an illustrative comparison of VIP release between a computer modeling prediction (red line) with actual observed VIP release data (blue diamonds) for individual PLGA microparticles consisting of 4.2 kDa polymers (upper panel), 12.6 kDa polymers (middle panel), or 100 kDa polymers (lower panel).

FIG. 34 presents exemplary data of a VIP release profile from microparticles consisting of 12.6 kDa PLGA polymers showing a smaller magnitude of initial burst than the data shown in FIG. 33.

FIG. 35 presents exemplary data comparing actual VIP release profiles in PBS from predicted linear and/or multi-bolus VIPMPs.

FIG. 35A: VIP release profiles from either predicted extended linear VIPMPs (blue diamonds) or predicted multi-bolus VIPMPs (red squares). Standard deviations are represented by bars at each data point.

FIG. 35B: Comparison of computer algorithm model prediction (red line), observed VIP release from predicted

linear VIPMPs (blue diamonds) and the desired profile computer model input (dashed line). The anomalous initial burst release was ignored for this comparison.

FIG. **36** presents exemplary data showing VIP release profiles from nonporous VIP 12.6 kDa polymer MPs (blue diamonds) and porous VIP 12.6 kDa polymer MPs (red squares) in PBS. Standard deviations are represented by bars at each data point.

FIG. **37** presents exemplary data showing cumulative VIP release profiles from 12.6 kDa polymer VIPMPs either with PEG (red squares) or without PEG (blue diamonds) in PBS. Standard deviations are represented by bars at each data point.

FIG. **38** presents an illustrative comparison of VIP release profiles from VIP 100 kDa PLGA polymer MPs (blue diamonds) and VIP 12.6 kDa PLGA polymer MPs with PEG (red squares) in the inner aqueous phase. PEG appears to minimize the VIP 12.6 kDa polymer MP initial burst as seen above, and provide a near-linear release of VIP similar to that of VIP 100 kDa polymer MPs.

FIG. **39** presents exemplary data of CCL-22 production by DCs measured at 7 hours (upper panel), 24 hours (middle panel), and 48 hours (lower panel) following treatment. mDC Control group: no LPS or drug. Soluble VIP group: 10^{-6} M soluble VIP. Blank MPs: 0.0556 mg/mL of nonporous empty MPs; and VIP MPs 0.0556 mg/mL of nonporous VIPMPs.

FIG. **40** presents exemplary data showing a reduction of alveolar bone loss in mice infected with periodontal disease that were treated with VIPMPs.

FIG. **40A**: Dissecting microscope images of bone loss between the alveolar bone crest and cementoenamel junction for mice treated with BlankMPs, CCL22MPs, or VIPMPs.

FIG. **40B**: Quantitative bone loss area for sham, BlankMPs, VIPMPs, and CCL22MPs. Mice treated with VIPMPs or CCL22MPs showed significantly less bone loss than the BlankMPs. $p<0.021$ and $p<0.002$, respectively.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is related to the treatment of immunological disorders, immunological diseases and/or transplantation rejection reactions. For example, certain embodiments of the invention result in the regulation of blood cells including, but not limited to, naïve blood cells and/or white blood cells (e.g., T cells and/or B cells) controlled by vasoactive intestinal peptide (VIP). In particular, the invention contemplates methods of creating VIP-induced regulatory T cells by controlled VIP release from custom designed microparticles.

The absence of regulatory T cells (Treg) may underlie immunological disorders including, but not limited to, autoimmunity, dermatitis, periodontitis and/or transplant rejection Enhancing local numbers of Treg through in situ Treg expansion or induction is contemplated herein as a treatment option for these disorders. Current methods for in vivo Treg expansion are not Treg-cell specific and are associated with many adverse side-effects. The data presented herein provides in vitro testing of a Treg-inducing microparticle populations providing a predictable controlled release of vasoactive intestinal peptide (VIP). These controlled release microparticles are also capable of inducing FoxP3+ Treg in human cells in vitro suggesting that these microparticles may be clinically useful for in vivo Treg induction and expansion therapy.

Current techniques for regulatory T cell induction can only be used in vitro as they rely on the use of soluble mediators. In some embodiments, the present invention contemplates compositions and methods to deliver T cell induction soluble factors in vivo in a sustained, predictable and controlled manner.

In one embodiment, the present invention contemplates methods for evaluating the therapeutic benefits of vasoactive intestinal peptide (VIP) in treating periodontitis. Although it is not necessary to understand the mechanism of an invention, custom microparticle compositions having specific VIP release profiles were predicted by computer modeling and validated by formulating microparticles having specific ratios of polymers having different molecular weights. For example, in one embodiment, custom microparticles were formulated using 4.2 kDa PLGA, 12.6 kDa PLGA, and 100 kDa PLGA. In other embodiments, the custom microparticle further comprises an inner aqueous phase comprising sodium chloride (NaCl) and/or polyethylene glycol (PEG). The data presented herein demonstrates that the released VIP from custom microparticles was bioactive and caused dendritic cells (DCs) to produce more CCL22 than DCs treated with blank particles after 7 and 24 hours of stimulation ($p<0.05$). The data further shows that DCs treated with VIP microparticle releasates recruited higher percentages of FoxP3+ T-cells in in vitro chemotaxis studies. In vivo mouse model testing indicated that VIP microparticles have significant therapeutic potential to treat periodontal disease by reducing the bone loss in infected mice relative to Blank microparticles given to the control group ($p<0.02$).

The present invention is also related to the field of inducing immunological tolerance by specifically manipulating immune-related cells. Such immunological tolerance may be induced by providing biomimetic artificial microparticles that bypass native immunological cells. For example, biomimetic artificial particle compositions are contemplated which comprise soluble factors that activate specific immune-related blood cells, including, but not limited to, macrophages and/or monocytes. Such factors may comprise chemoattractant factors. These biomimetic artificial microparticles may further present a specific biomimetic surface pattern that results in a targeted cell response such that the particles represent artificial presenting cells.

The present invention contemplates artificial microparticles such that a synthetic construct may be capable of presenting the same released/secreted and/or chemically-bound/plasma membrane-bound compounds (i.e., for example, soluble proteins, antibodies, receptors, hormones etc.) as actual living cells, and is not limited to modulating the immune system (i.e., for example, modulation of bone healing, tissue inflammation and/or tumor regression). The present invention contemplates various embodiments wherein these artificial microparticles replace biological cell-cell interactions within the processes of many biological functions including, but not limited to, wound healing, restoration from a diseased state, or enhancement in normal function of cellular processes.

In one embodiment, the present invention contemplates an investigational platform capable of testing the in vivo roles and efficiencies of both soluble secreted and surface bound T_{reg} cell factors. Such Treg cell factors may include, but are not limited to, stimulatory factors, inducing factors and/or chemoattractant factors. In one embodiment, the investigational platform comprises an in vivo testing platform (i.e., for example, an in vivo mouse model). In one embodiment, the investigational platform comprises an in vitro testing platform (i.e., for example, a primary cell

culture). These investigational platforms are capable of evaluating various combinations of membrane surface factors and/or soluble releasable factors that are attached to and/or encapsulated within, a biodegradable microparticle population that stimulates T_{res} cells and induces allograft tolerance.

Although it is not necessary to understand the mechanism of an invention, it is believed that these biodegradable microparticle populations may act as "artificial antigen presenting cells" that comprise a biomimetic immunological synapse that encapsulate and controllably release soluble factors for T_{reg} cell stimulation, T_{reg} cell proliferation, and T_{reg} cell chemotaxis. Hoffmann et al., *Blood* 104, 895-903 (2004); Green et al., *Proc Natl Acad Sci USA* 100: 10878-10883 (2003); Curiel et al., *Nat Med* 10:942-949 (2004); and Belghith et al., *Nat Med* 9:1202-1208 (2003). Further, these microparticle populations are capable of presenting surface-bound monoclonal antibodies (mAb) specific for T_{reg} cell surface activation factors.

I. Vasoactive Intestinal Peptide

Vasoactive intestinal peptide (VIP) is a neuropeptide of twenty-eight (28) amino acids in length that was originally isolated from the intestine that has numerous biological and regulatory functions. For example, VIP down-regulates a variety of pro-inflammatory responses and up-regulates anti-inflammatory responses. Reports have suggested that VIP may be useful in the treatment of experimental collagen-induced arthritis, sepsis, Crohn's disease, and experimental autoimmune encephalomyelitis (EAE) in mice. Delgado et al., (2001) "Vasoactive intestinal peptide prevents experimental arthritis by downregulating both autoimmune and inflammatory components of the disease" Nat. Med. 7:563-568; Fernandez-Martin et al., (2006) "Vasoactive intestinal peptide induces regulatory T cells during experimental autoimmune encephalomyelitis" *Eur. J. Immunol.* 36:318-326. Additionally, VIP may shift the Th1-Th2 balance in the favor of Th2 by promoting Th2-type cytokine production and, at the same time, inhibit Th1 development and responses. Gonzalez-Rey et al., (2007) "Vasoactive intestinal peptide and regulatory T-cell induction: a new mechanism and therapeutic potential for immune homeostasis" *Trends in Molecular Medicine* 13:241-251.

More recently, new mechanisms have been proposed that identify a potential role for VIP in inducing and recruiting regulatory T (Treg) cells for maintaining immune homeostasis. FIG. **26**. Both natural and inducible regulatory T cells may be involved in immune tolerance and regulation. Natural Treg cells can develop in the thymus as CD4+CD25+ FoxP3+ Tregs, and expand into the periphery. So-called inducible Treg cells can be generated from CD4+CD25- and CD8+CD25- naïve T cells under certain stimulation patterns. Treg induction by VIP is believed to occur in two primary pathways. First, VIP may directly activate naïve CD4+CD25- T cells into a regulatory phenotype. Secondly, VIP may steer immature dendritic cells (DCs) into a tolerogenic phenotype. Such tolerogenic DCs can then induce CD4+ and CD8+ naïve T cells toward a regulatory phenotype (inducible Tregs). Finally, VIP can induce tolerogenic dendritic cells to produce CCL22. CCL22 is a chemokine reported to be involved in the recruitment of regulatory T cells. Pozo et al., (2007) "Tuning immune tolerance with vasoactive intestinal peptide: A new therapeutic approach for immune disorders" *Peptides* 28:1833-1846; and Delgado et al., (2004) "VIP/PACAP preferentially attract Th2 effectors through differential regulation of chemokine production by dendritic cells" *FASEB J* 18:1453-1455.

One difficulty in VIP-based therapies is the short half-life of VIP in the body. One approach to address this challenge is to develop VIP analogs which are bioactive but have improved metabolic stability. Misaka et al., "Inhalable powder formulation of a stabilized vasoactive intestinal peptide (VIP) derivative: Anti-inflammatory effect in experimental asthmatic rats" *Peptides* (2010) 31:72-78. Another approach is to protect the VIP by encapsulation into sterically stabled liposomes. However, the reports show limitations to these approaches. For example, VIP analogues only extend the half-life to 4 hours and the liposomes offer only short-term release, releasing 77-87% of encapsulated VIP within the first two hours in vitro. Wernig et al., (2008) "Depot formulation of vasoactive intestinal peptide by protamine-based biodegradable nanoparticles" *J of Controlled Release* 130:192-198; and Misaka et al., (2010) "Inhalable powder formulation of a stabilized vasoactive intestinal peptide (VIP) derivative: Anti-inflammatory effect in experimental asthmatic rats" *Peptides* 31:72-78. As contemplated herein, microparticles which deliver an extended release profile solves the above problems of conventional VIP-based therapy platforms.

The data presented herein describes an alternative method of quantifying VIP release using microparticles composed of 12.6 kDa PLGA. The released VIP was detected using a commercially available VIP Enzyme Immunoassay kit (Phoenix Pharmaceuticals). Dendritic cell (DC) cultures were tested to determine the bioactivity of VIP released from these microparticles. The results suggest that VIP microparticles have a significant therapeutic potential as a treatment for periodontitis.

In one embodiment, the present invention contemplates a composition comprising a VIPMP formulation having a polymer composition predicted by mathematical analysis to provide a pre-determined release profile.

II. Regulatory T Cells

A. Immunological Role And Function

Over the past two decades, regulatory T cells (Treg) have been identified as one component of the mammalian immune system. Sakaguchi et al., "Immunologic tolerance maintained by CD25+ CD4+ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance" *Immunol Rev.* (2001) 182:18-32; Sakaguchi et al., "Regulatory T cells and immune tolerance" *Cell* (2008) 133:775-787; Campbell et al., "Phenotypical and functional specialization of FOXP3+ regulatory T cells" *Nat Rev Immunol* (2011) 11:119-130; and Bour-Jordan et al., "Regulating the regulators: costimulatory signals control the homeostasis and function of regulatory T cells" (2009) 229:41-66. One commonly described, widely studied, and abundant regulatory T cells in the body are those that express CD4, CD25, and/or FoxP3. These CD4+ CD25+ FoxP3+ cells (Treg) are believed to play a role in suppressing the activity of self-reactive immune cells and in re-establishing homeostasis following infection. Sakaguchi et al., "Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases" *J Immunol.* (1995) 155:1151-1164; Hori et al., "Control of regulatory T cell development by the transcription factor Foxp3" *Science* (2003) 299:1057-1061; Fontenot et al., "Foxp3 programs the development and function of CD4+ CD25+ regulatory T cells" *Nat. Immunol.* (2003) 4:330-336; and Khattri et al., "An essential role for Scurfin in CD4+CD25+ T regulatory cells" *Nat. Immunol.* (2003) 4:337-342.

B. Clinical Applications

Treg proliferation has been reported to suppress diverse inflammatory diseases such as: i) autoimmunity (de Kleer et al., "CD4+CD25 bright regulatory T cells actively regulate inflammation in the joints of patients with the remitting form of juvenile idiopathic arthritis" *J Immunol.* (2004) 172: 6435-6443: ii) transplant rejection (Raimondi et al., "Mammalian target of rapamycin inhibition and alloantigen-specific regulatory T cells synergize to promote long-term graft survival in immunocompetent recipients" *J Immunol* (2010) 184:624-636; and Lee et al., "Recruitment of Foxp3+ T regulatory cells mediating allograft tolerance depends on the CCR4 chemokine receptor" *J Exp Med* (2005) 201:1037-1044; iii) dermatitis (Robinson et al., "Tregs and allergic disease" *J Clin Invest.* (2004) 114:1389-1397; iv) psoriasis (Wang et al., "TGF-beta-dependent suppressive function of Tregs requires wild-type levels of CD18 in a mouse model of psoriasis" *J Clin Invest.* (2008) 118:2629-2639; Bovenschen et al., "Foxp3+ Regulatory T Cells of Psoriasis Patients Easily Differentiate into IL-17A-Producing Cells and Are Found in Lesional Skin" *J Invest Dermatol.* (2011) 131:1853-1860; and v) periodontitis Garlet et al., "*Actinobacillus* actinomycetemcomitans-induced periodontal disease in mice: patterns of cytokine, chemokine, and chemokine receptor expression and leukocyte migration" *Microbes Infect.* (2005) 7:738-747; Garlet et al., "Regulatory T cells attenuate experimental periodontitis progression in mice" *J Clin Periodontol.* (2010) 37:591-600.

Proliferation of Treg cell populations at local tissue sites has been reported by various methods such as: i) ex vivo expansion of Treg cells followed by a local administration or systemic re-infusion; and ii) in vivo manipulation of immune cells that increases the Treg/Teff ratio. Riley et al., "Human T regulatory cell therapy: take a billion or so and call me in the morning" *Immunity* (2009) 30:656-665; Safinia et al., "Adoptive regulatory T cell therapy: challenges in clinical transplantation" *Curr Opin Organ Transplant* (2010) 15:427-434; and Wieckiewicz et al., "T regulatory cells and the control of alloimmunity: from characterisation to clinical application" *Curr Opin Immunol.* (2010) 22:662-668. Selective enhancement of Treg cell populations in vivo has been reported using biologic therapies. For example, i) anti-IL-2 monoclonal antibody (Webster et al., "In vivo expansion of Treg cells with IL-2-mAb complexes: induction of resistance to EAE and long-term acceptance of islet allografts without immunosuppression" *J Exp Med.* (2009) 206:751-760; ii) superagonistic anti-CD28 monoclonal antibody (Beyersdorf et al., "Selective targeting of regulatory T cells with CD28 superagonists allows effective therapy of experimental autoimmune encephalomyelitis" *J Exp Med.* (2005) 202:445-455; and iii) agonistic anti-CD4 monoclonal antibody (Anonymous, "Deal watch: Boosting Tregs to target autoimmune disease" *Nat. Rev. Drug Discovery* (2011) 10:566. These approaches have specific disadvantages including, but not limited to, a limited understanding of the underlying mechanism of action and human safety for clinical administration. In fact, phase I clinical trials of the superagonistic anti-CD28 monoclonal antibody (TGN1412) resulted in severe negative reactions (cytokine 'storm') in all 6 human subjects who received the monoclonal antibody. Suntharalingam et al., "Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412" *N Engl J Med.* (2006) 355:1018-1028. Establishment of a local immunosuppressive environment that selectively favors Treg expansion has also been shown to increase Treg cell population numbers. An environment rich in IL-2, transforming growth factor-β1 (TGF-β) and

rapamycin (an inhibitor of the serine-threonine kinase mammalian target of rapamycin; mTOR) has been shown to favor Treg development, even under inflammatory conditions. Haxhinasto et al., "The AKT-mTOR axis regulates de novo differentiation of CD4+Foxp3+ cells" *J Exp Med.* (2008) 205:565-574; Kopf et al., "Rapamycin inhibits differentiation of Th17 cells and promotes generation of FoxP3+ T regulatory cells" *Int Immunopharmacol.* (2007) 7:1819-1824; and Cobbold et al., "Infectious tolerance via the consumption of essential amino acids and mTOR signaling" *Proc Natl Acad Sci USA* (2009) 106:12055-12060.

However, formulations providing a predictable continuous release of these factors in vivo, has proven difficult. This problem is solved as described herein by microparticle compositions and methods showing controlled release profiles of Treg cell inducing compounds, such as vasoactive intestinal peptide can be mathematically predicted by adjustment of polymeric ratios comprising the microparticles. These predicted microparticle compositions are expected to have a better therapeutic efficacy and safety than current antibody Treg induction models, thereby having a superior clinical application to treat medical disorders and disease.

Therapies that enhance Treg numbers and function may have the potential to suppress transplant rejection and autoimmunity. Clinical trials are currently underway to test cellular therapies involving Treg cells as potential therapeutics for treating graft versus host disease. Hippen et al., "Generation and large-scale expansion of human inducible regulatory T cells that suppress graft-versus-host disease" *Am J. Transplant.* (2011) 11:1148-1157; and Bronstein et al., "Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics" *Blood* (2011) 117:1061-1070.

However, Treg-based cellular therapies face many challenges, which include, but are not limited to: i) difficulties in isolating pure and homogenous populations and large quantities of Treg from the blood; ii) inconsistent maintenance of the Treg phenotype and suppressive function post-proliferation; and iii) the need for GMP facilities. Riley et al., "Human T regulatory cell therapy: take a billion or so and call me in the morning" *Immunity* (2009) 30:656-665; Safinia et al., "Adoptive regulatory T cell therapy: challenges in clinical transplantation" *Curr Opin Organ Transplant.* (2010) 15:427-434; and Wieckiewicz et al., "T regulatory cells and the control of alloimmunity: from characterisation to clinical application" *Curr Opin Immunol.* (2010) 22:662-668. Hence, acellular therapies that can increase numbers and/or the suppressive potency of Treg without the need for ex vivo culture represent an answer to a long unsolved problem in the art.

III. Induction of Regulatory T Cell Phenotypes

Treg cells can be induced under a variety of in vitro and in vivo conditions. Treg cells may be induced under different conditions to have distinct characteristics that distinguish them from each other and from naturally-occurring Treg cells. Identification of these distinctive characteristics provides insight into their potential use in treating inflammatory disorders (such as autoimmunity and allergy) and transplant rejection, or in preventing tumor growth and metastasis. For example, it has been demonstrated that RA can help to convert naïve T cells to induced Treg cells (iTreg). These RA-iTreg are stable under inflammatory conditions and have the potential to prevent inflammation in the gut due primarily to their ability to specifically migrate to the gut. However, such tissue-specific migration could potentially be a hindrance to using these types of iTreg cells to treat autoimmunity or transplant rejection at other peripheral sites.

Regulatory T cells (Treg) may be involved in maintaining immune homeostasis. Consequently, it is believed that Treg cell therapy might be useful to treat medical conditions including, but not limited to, a variety of immune mediated disorders. Current Treg cell-based clinical therapies have disadvantages including, but not limited to, obtaining insufficient numbers of cells from peripheral blood for expansion and re-infusion. One alternative method to induce the formation of Treg cells from non-Treg cells has been reported that contacts non-Treg cells with a soluble cytokine (e.g., TGF-β). However, this approach has disadvantages including, but not limited to, the fact that these methods do not induce stable Treg cells in sufficient number to be useful. Although it is not necessary to understand the mechanism of an invention, it is believed that all-trans retinoic acid (RA) and/or rapamycin (RAPA) may aid in achieving a stable induced Treg (iTreg) phenotype. Even so, such iTreg phenotypes have not been characterized as to phenotype, function and/or migratory characteristics

In one embodiment, the present invention contemplates a method to induce a specific and stable phenotype and/or function of iTreg cells. In one embodiment, the iTreg cells may be produced by contacting non-Treg cells with a compound such as VIP.

Regulatory T (Treg) cell-based therapies are widely regarded as promising treatment options for immunological diseases including, but not limited to, autoimmunity and/or transplant rejection. Currently, several therapies involving the use of ex vivo expanded Treg cells are being tested in clinical trials. However, there are significant barriers to ex vivo Treg cell-based therapies, such as difficulty in isolating pure populations of these rare cells and expanding them to sufficiently large numbers while maintaining their phenotype and function. Wieckiewicz et al., (2010) "T regulatory cells and the control of alloimmunity: From characterisation to clinical application" *Curr Opin Immunol* 22: 662-668; Riley et al., (2009) "Human T Regulatory Cell Therapy: Take a Billion or So and Call Me in the Morning" *Immunity* 30:656-665; Brusko et al., (2008) "Human regulatory T cells: Role in autoimmune disease and therapeutic opportunities" *Immunol Rev* 223: 371-390; Trzonkowski et al., (2009) "First-in-man clinical results of the treatment of patients with graft versus host disease with human ex vivo expanded CD4+CD25+CD127− T regulatory cells" *Clin Immunol* 133: 22-26; Brunstein et al., "Alternative donor transplantation after reduced intensity conditioning: results of parallel phase 2 trials using partially HLA-mismatched related bone marrow or unrelated double umbilical cord blood grafts" *Blood* 118: 282-288; and Safinia et al., (2010) "Adoptive regulatory T cell therapy: Challenges in clinical transplantation" *Curr Opin Organ Transplant* 15: 427-434.

One possible alternative to circumvent these issues is to generate adaptive or induced Treg (iTreg) from the patient's own naïve T cells either ex vivo or in vivo. Past reports have demonstrated that IL-2 and transforming growth factor β1 (TGF-β) can induce a Treg cell phenotype having functional characteristics from naïve T cells upon in vitro stimulation. Fu et al. (2004) "TGF-beta induces Foxp3+ T-regulatory cells from CD4+CD25− precursors" *American Journal of Transplantation* 4: 1614-1627; and Chen et al., (2003) "Conversion of Peripheral CD4+CD25− Naive T Cells to CD4+CD25+ Regulatory T Cells by TGF-beta Induction of Transcription Factor Foxp3" *Journal of Experimental Medicine* 198:1875-1886. However, TGF-β-induced Treg cells (TGFβ-iTreg cells) have been shown to be unstable in long term in vitro cultures and upon antigenic re-stimulation. Floess et al., (2007) "Epigenetic control of the foxp3 locus in regulatory T cells" *PLoS Biology* 5: 0169-0178. Additionally, the presence of inflammatory cytokines such as IL-6 can antagonize TGF-β-mediated induction of Treg cells, making the presence of such inflammatory mediators a potential impediment to inducing Treg cells in vivo at the site of a diseased tissue. Veldhoen et al., (2006) "TGF-beta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells" *Immunity* 24: 179-189; and Bettelli et al., (2006) "Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells" *Nature* 441: 235-238.

Numerous reports suggest that these problems might be overcome through the use of small molecules that work in concert with TGF-β to induce Treg cells. For example, all-trans retinoic acid (RA) is known to potently synergize with IL-2 and TGF-β to induce FoxP3 expression in naïve T cells and allows for induction of Treg cells even in the presence of inflammatory cytokines. Thorough characterization of the phenotype and function of RA-induced Treg (RA-iTreg) cells demonstrates suppressor activity and are more stable than TGFβ-iTreg cells. Nevertheless, RA-iTreg cells have a specific disadvantage in that they migrate primarily to the mucosal tissues in the gut, which might limit their use. Mucida et al., (2007) "Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid" *Science* 317: 256-260; and Lu et al., (2011) "Characterization of Protective Human CD4+CD25+ FOXP3+ Regulatory T Cells Generated with IL-2, TGF-β and Retinoic Acid" *PLoS ONE* 5: 1-12; and Benson et al., (2007) "All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation" *Journal of Experimental Medicine* 204: 1765-1774.

Further, other evidence suggests additional disadvantages to RA-iTreg cells in that, depending on the immunological microenvironment, RA can induce inflammation rather than tolerance. DePaolo et al., (2011) "Co-adjuvant effects of retinoic acid and IL-15 induce inflammatory immunity to dietary antigens" *Nature* 471: 220-224. Also, RA has been shown to induce hypervitaminosis-A upon local administration, and hence it would be difficult to use this combination (cytokines+RA) to induce Treg cells in vivo. Jones D H, (1989) "The role and mechanism of action of 13-cis-retinoic acid in the treatment of severe (nodulocystic) acne" *Pharmacol Ther* 40: 91-106; and Barua et al., (1996) "Percutaneous absorption, excretion and metabolism of all-trans-retinoyl beta-glucuronide and of all-trans-retinoic acid in the rat" *Skin Pharmacol* 9: 17-26.

Another small molecule that synergizes with IL-2 and TGF-β to induce FoxP3 expression in naïve T cells is the serine/threonine protein kinase inhibitor rapamycin (RAPA). It has been demonstrated that, like RA, RAPA can induce Treg cells even in the presence of IL-6. However, the phenotype and function of RAPA-induced Treg (RAPA-iTreg) cells has yet to be characterized. Kopf et al., (2007) "Rapamycin inhibits differentiation of Th17 cells and promotes generation of FoxP3+ T regulatory cells" *Int Immunopharmacol* 7: 1819-1824; Haxhinasto et al., (2008) "The AKT-mTOR axis regulates de novo differentiation of CD4+ Foxp3+ cells" *J Exp Med* 205: 565-574; and Cobbold et al., (2009) "Infectious tolerance via the consumption of essential amino acids and mTOR signaling" *Proc Natl Acad Sci USA* 106: 12055-12060.

IV. Conventional Controlled Release Formulations

Several drug delivery systems are known that provide for a roughly uniform and controllable rate of release. A variety

of different media are described below that are useful in creating drug delivery systems.

Microparticles generally refer to compositions including, but not limited to, nanoparticles, microspheres, nanospheres, microcapsules, and nanocapsules. Preferably, some microparticles contemplated by the present invention comprise poly(lactide-co-glycolide), aliphatic polyesters including, but not limited to, poly-glycolic acid and poly-lactic acid, hyaluronic acid, modified polysacchrides, chitosan, cellulose, dextran, polyurethanes, polyacrylic acids, psuedo-poly(amino acids), polyhydroxybutrate-related copolymers, polyanhydrides, polymethylmethacrylate, poly(ethylene oxide), lecithin and phospholipids. Microparticles are generally distinguished from liposomes, as microparticles are usually made of organic polymers, while liposomes are usually made of lipids, such as phospholipids.

Microspheres and microcapsules are useful due to their ability to maintain a generally uniform distribution, provide stable controlled compound release and are economical to produce and dispense. Microspheres are obtainable commercially (Prolease®, Alkerme's: Cambridge, Mass.). For example, a freeze dried medium comprising at least one therapeutic agent is homogenized in a suitable solvent and sprayed to manufacture microspheres in the range of 20 to 90 μm. Techniques are then followed that maintain sustained release integrity during phases of purification, encapsulation and storage. Scott et al., "Improving Protein Therapeutics With Sustained Release Formulations" *Nature Biotechnology*, Volume 16:153-157 (1998).

Modification of a microsphere composition by the use of biodegradable polymers can provide an ability to control the rate of therapeutic agent release. Miller et al., "Degradation Rates of Oral Resorbable Implants. Polylactates and Polyglycolates: Rate Modification and Changes in PLA/PGA Copolymer Ratios" *J. Biomed. Mater. Res.*, Vol. II:711-719 (1977).

Alternatively, a sustained or controlled release microsphere preparation is prepared using an in-water drying method, where an organic solvent solution of a biodegradable polymer metal salt is first prepared. Subsequently, a dissolved or dispersed medium of a therapeutic agent is added to the biodegradable polymer metal salt solution. The weight ratio of a therapeutic agent to the biodegradable polymer metal salt may for example be about 1:100000 to about 1:1, preferably about 1:20000 to about 1:500 and more preferably about 1:10000 to about 1:500. Next, the organic solvent solution containing the biodegradable polymer metal salt and therapeutic agent is poured into an aqueous phase to prepare an oil/water emulsion. The solvent in the oil phase is then evaporated off to provide microspheres. Finally, these microspheres are then recovered, washed and lyophilized. Thereafter, the microspheres may be heated under reduced pressure to remove the residual water and organic solvent.

Other methods useful in producing microspheres that are compatible with a biodegradable polymer metal salt and therapeutic agent mixture are: i) phase separation during a gradual addition of a coacervating agent; ii) an in-water drying method or phase separation method, where an anti-flocculant is added to prevent particle agglomeration and iii) by a spray-drying method.

Controlled release microcapsules may be produced by using known encapsulation techniques such as centrifugal extrusion, pan coating and air suspension. Such microspheres and/or microcapsules can be engineered to achieve desired release rates. For example, Oliosphere® (Mac-romed) is a controlled release microsphere system. These particular microsphere's are available in uniform sizes rang-

ing between 5-500 μm and composed of biocompatible and biodegradable polymers. Specific polymer compositions of a microsphere can control the therapeutic agent release rate such that custom-designed microspheres are possible, including effective management of the burst effect. Pro-Maxx® (Epic Therapeutics, Inc.) is a protein-matrix delivery system. The system is aqueous in nature and is adaptable to standard pharmaceutical delivery models. In particular, ProMaxx® are bioerodible protein microspheres that deliver both small and macromolecular drugs, and may be customized regarding both microsphere size and desired release characteristics.

A microsphere or microparticle may comprise a pH sensitive encapsulation material that is stable at a pH less than the pH of the internal mesentery. The typical range in the internal mesentery is pH 7.6 to pH 7.2. Consequently, the microcapsules should be maintained at a pH of less than 7. However, if pH variability is expected, the pH sensitive material can be selected based on the different pH criteria needed for the dissolution of the microcapsules. The encapsulated compound, therefore, will be selected for the pH environment in which dissolution is desired and stored in a pH preselected to maintain stability. Examples of pH sensitive material useful as encapsulants are Eudragit® L-100 or S-100 (Rohm GMBH), hydroxypropyl methylcellulose phthalate, hydroxypropyl methylcellulose acetate succinate, polyvinyl acetate phthalate, cellulose acetate phthalate, and cellulose acetate trimellitate. In one embodiment, lipids comprise the inner coating of the microcapsules. In these compositions, these lipids may be, but are not limited to, partial esters of fatty acids and hexitiol anhydrides, and edible fats such as triglycerides. Lew C. W., "Controlled-Release pH Sensitive Capsule And Adhesive System And Method" U.S. Pat. No. 5,364,634 (herein incorporated by reference).

Microparticles may also comprise a gelatin, or other polymeric cation having a similar charge density to gelatin (i.e., poly-L-lysine) and is used as a complex to form a primary microparticle. A primary microparticle is produced as a mixture of the following composition: i) Gelatin (60 bloom, type A from porcine skin), ii) chondroitin 4-sulfate (0.005%-0.1%), iii) glutaraldehyde (25%, grade 1), and iv) 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC hydrochloride), and ultra-pure sucrose (Sigma Chemical Co., St. Louis, Mo.). The source of gelatin is not thought to be critical; it can be from bovine, porcine, human, or other animal source. Typically, the polymeric cation is between 19,000-30,000 daltons. Chondroitin sulfate is then added to the complex with sodium sulfate, or ethanol as a coacervation agent.

V. Custom-Tailored Controlled Release Profiles

In some embodiments, the present invention contemplates compositions and methods that encapsulate and/or attach VIP into and/or onto custom-tailored poly(lactic-co-glycolic acid) (PLGA) microspheres (MPs). For example, the custom-tailored microsphere result from a procedure that predictably controls VIP release of microparticles as a function of time. Such predictable controlled release of VIP was achieved by using custom-tailored microspheres having different polymer molecular weights. Alternatively, predictable controlled release of VIP was achieved by using custom-tailored micro spheres having polyethylene glycol (PEG) and/or sodium chloride (NaCl) in an inner aqueous phase of the microparticle.

In some embodiments, the present invention contemplates compositions providing a predictable controlled release of compounds to induce a Treg cell phenotype (e.g., deter-

mined by the expression of canonical Treg markers and migratory surface markers). In one embodiment, the induced Treg phenotype includes, but is not limited to, VIP-iTreg.

In one embodiment, the present invention contemplates compositions and methods for the development and testing of controlled release formulations for Treg cell induction compounds (e.g., VIP) having a predictable release profile. In other words, a predicted VIP release profile is achieved with a custom-tailored predetermined kinetic and temporal pattern. Rothstein et al., "A simple model framework for the prediction of controlled release from bulk eroding polymer matrices" *J Mater Chem* (2008) 18:1873-1880; and Rothstein et al., "A unified mathematical model for the prediction of controlled release from surface and bulk eroding polymer matrices" *Biomaterials* (2009) 30:1657-1664. As is shown herein, a specific composition of a microparticle can be determined, in advance, that results in the predicted release profile. The data presented herein show that VIP microparticles (VIPMPs) are capable of Treg cell population induction in vitro using either mouse or human cells.

The absence of regulatory T (Treg) cells may be involved in a wide variety of disorders including, but not limited to, autoimmunity, dermatitis, periodontitis and/or transplant rejection. Logically, one potential treatment option for these disorders would be to increase local Treg cell numbers. For example, enhancing local numbers of Treg cells might be achieved through in situ Treg cell expansion or induction. Current methods for in vivo Treg cell expansion rely on biologic therapies, which are not Treg cell-specific and are associated with many adverse side-effects.

A. Controlled Release Microparticle Characterization The data presented herein use a VIPMP comprising a polymer (e.g., RG502H) having a viscosity of 0.16-0.24 dl/g. Such microparticles are spherical and have average diameter of approximately 15-30 µm. Additionally, the microparticles may comprise a slightly porous exterior surface. These particles were specifically formulated to be porous by altering osmotic pressures between the inner emulsion and the outside aqueous phase during microparticle preparation. It was predicted that this composition would comprise a high initial VIP release burst followed by a continuous release.

VIP microparticles fabricated using a 12.6 kDa RG502H polymer had mean diameters of 16.7 µm and 17.7 µm for nonporous and porous particles, respectively. See, FIG. 31.

B. VIP Release From VIPMPs Having Pre-Determined Release Profiles

Various tactics can be employed to alter the release of encapsulated VIP from PLGA microspheres including, but not limited to: a) VIP release may be decreased as polymer molecular weight increases as evident in the present data showing that release of VIP from 100 kDa polymer particles had a very small initial burst followed by a region of near linear release; b) addition of NaCl or PEG to the inner aqueous phase influences VIP release; c) porous microparticles fabricated with NaCl showed a diminished initial burst possibly due to electrostatic interactions; d) electrostatic interactions between VIP with the PLGA polymer may delay VIP release in that VIP has an exceptionally high isoelectric point of 11.8, meaning that at pH of 7, the peptide has a net positive charge and since the polymer is negatively charged, the VIP could aggregate with the polymer, which may be enhanced when NaCl is present.

Controlled release of VIP from VIPMPs made in accordance with Example Da was measured over a 60 day period and evaluated for both a predicted linear release profile and a predicted multi-bolus release profile.

VIP release profiles from VIP MPs were evaluated using ELISA plates. The VIP MPs contained particles of pure polymer weight: 4.2 kDa, 12.6 kDa with and without PEG, and 100 kDa. Predictive model calculations generated proposed ratios of microparticles with different polymer molecular weights (10.6% of the 4.2 kDa, 31.9% of the 12.6 kDa without PEG, and 57.5% of the 100 kDa polymer) that predicted a linear release for a period of approximately 50 days. Rothstein et al., "A simple model framework for the prediction of controlled release from bulk eroding polymer matrices" *J of Mater. Chem.* (2007) 18:1873-1880. This algorithmic model further suggested that when 4.2 kDa, 12.6 kDa with PEG, and 55 kDa polymers were mixed in equal amounts (e.g., a ratio of 1:1:1, or 33.3% of each polymer), the resultant microparticle would be expected to have an extended multi-bolus release profile.

A VIP release profile for a linear VIPMPs showed an initial burst followed by an extended release over a period of 60 days. See. FIG. 27A. As would be expected from the algorithm prediction, the multi-bolus data showed a secondary burst around day 15 and a possible start of a tertiary burst at day 45. Further, the multi-bolus group released about twice as much VIP as compared to the linear VIPMP release profile. The linear VIPMPs released much slower than the multi-bolus VIPMPs and at day 5, release of VIP from the linear VIPMPs began releasing in a near-linear fashion. See. FIG. 27A. A comparison of the observed release and the model prediction of the linear VIPMPs is presented that ignores the initial burst occurring during the first five days of release profile. See, FIG. 27B.

With the exception of the initial burst, the computer model successfully predicted the polymer ratio necessary to achieve a near-linear release of VIP for a period of 50 days. Although it is not necessary to understand the mechanism of an invention, it is believed that that the release of VIP may be delayed due to ionic interactions of VIP with the PLGA polymer. VIP has an exceptionally high isoelectric point of 11.8, meaning that at pH of 7, the peptide has a net positive charge. Since the polymer is negatively charged, the VIP could aggregate with the polymer. It appeared that the VIP did not finish releasing from both the linear and multi-bolus groups, although the model predicted complete release by day 50.

The above described controlled release of VIP over an extended period was performed using particles formulated with mixtures of 4.2 kDa, 12.6 kDa, and 100 kDa polymers. Release assays were performed in triplicate for each batch of VIP microparticles generating cumulative release profiles for each polymer molecular weight. FIG. 32. Particles of the lowest polymer molecular weight released most quickly as predicted. The 100 kDa particles released markedly slower than both the 4.2 kDa and 12.6 kDa particles in an almost linear fashion. The 12.6 kDa particle set showed an unexpected (e.g., anomalous) large initial burst on the first day.

Each release profile in FIG. 32 was compared to a release profile predicted by a computer model. FIG. 33. The model was most accurate in predicting release from the 4.2 kDa polymer particles. The large magnitude of the initial burst of the 12.6 kDa batch was not predicted by the model. In fact, a second VIP release profile from 12.6 kDa polymer particles conducted over a shorter time period where the initial burst was much smaller in magnitude. See, FIG. 34. The 100 kDa particles began releasing VIP earlier than the profile predicted by the model, which did not capture the near-linear release from the 100 kDa particles.

The predictive computer modeling described herein was also used to generate custom-tailored microparticles having

a mixture of a specific ratio of different polymer molecular weights (e.g., for example, 4.2 kDa (10.6%), 12.6 kDa (31.9%), and 100 kDa polymer (57.5%)). An extended multi-bolus release profile was predicted by computer modeling using a mixture of 4.2 kDa polymers (33.3%), 12.6 kDa polymers+PEG (33.3%), and 100 kDa polymers (33.3%). Cumulative release profiles of these linear and multi-bolus groups are shown. FIG. 35A. A comparison was then made of the observed VIP release and the model prediction of the linear group while ignoring the initial burst that occurred during the first five days of release. FIG. 35B. Both the linear and multi-bolus groups showed initial bursts followed by extended release over the period of 60 days. The multi-bolus group released about two times as much VIP and showed a secondary burst around day 15 and a possible start of a tertiary burst at day 45. The linear batch released much slower and at day 5, release of VIP from the linear batch began releasing in a near-linear fashion.

The effects of NaCl or poly(ethylene glycol) (PEG) in the inner aqueous phase on release dynamics were also examined. Cumulative VIP release profiles were generated using nonporous 12.6 kDa PLGA VIPMPs and porous 12.6 kDa PLGA VIPMPs (made with 7.5 mm NaCl inner aqueous phase). FIG. 36. The nonporous VIP microparticles experienced a slight initial burst and a secondary burst around Day 14. The porous VIP particles experienced less of an initial burst and slower release than the nonporous particles.

Release profiles from 12.6 kDa polymer microparticles with or without a 4×10 mM PEG inner aqueous phase were also generated. FIG. 37. The initial burst and release rate was reduced dramatically when PEG was added to the inner aqueous phase. When PEG was added to the inner aqueous phase of 12.6 kDa polymer particles, the initial burst was greatly reduced. Interestingly, VIP release from the 12.6 kDa polymer particles with PEG greatly resembled the release from 100 kDa polymer particles. See FIG. 40. Although it is not necessary to understand the mechanism of an invention, it is believed that PEG may minimize hydrophobic interactions with the polymer.

C. VIP Bioactivity Assays

In some examples, the data presented herein was collected in cultured dendritic cells to ascertain if VIP released from microparticles promotes CCL22 production by steering DCs towards a tolerogenic phenotype. DCs treated with VIP microparticles were also used in chemotaxis studies to measure the recruitment of FoxP3+ CD4+ T-cells.

To show that VIP microparticles released bioactive VIP, dendritic cell (DC) cultures and chemotaxis studies were performed. Three separate DC cultures with 24 individual wells were completed in accordance with Example IVX. Both GM-CSF and IL-4 were used to culture the cells in the first two DC cell cultures, but for the third DC culture, the IL-4 was omitted to reduce its possible interference in steering immune cells toward a tolerogenic phenotype. Chemotaxis studies of CD4+ T-cells were performed and the percent of FoxP3+ T-cells which flowed through the transwells for each group was evaluated. The data is presented as a normalized percent of FoxP3+ cells which were recruited by the DCs for the first DC when both IL-4 and GM-CSF were used to culture the DC precursors and when GM-CSF was used alone in the last DC culture. The DCs treated with soluble VIP or releasates from VIP microparticles recruited a higher percentage of FoxP3+ cells than DCs that only received LPS or releasates from blank particles. See, FIG. 28.

DCs cultured in the absence of IL-4 showed similar trends in the recruitment of regulatory T cells, suggesting that VIP itself (not IL-4) is responsible for CCL22 induction. When the DC culture was repeated with the absence of IL-4 and addition of 5×GM-CSF, flow cytometry indicated slightly different trends. The DCs treated with LPS alone, soluble VIP, and releasates from VIP microparticles recruited the same percentage of FoxP3+ cells. The DCs treated with releasates from the blank microparticle groups recruited less FoxP3+ cells than the other groups. The distinction between the VIP microparticle group and the blank microparticle group was increased when no IL-4 was used.

In the in vitro DC cultures, a higher percentage of FoxP3+ regulatory T-cells after chemotaxis indicated that the DCs produced more CCL22, a chemokine known to be involved in regulatory T cell recruitment. The released VIP appeared to be bioactive since the DCs treated with releasates from VIP microparticles showed higher percentage recruitment of FoxP3+ cells than those treated with blank releasates for all three experiments. Studies have shown that CCL22 production of DC cells does not increase as significantly after treatment with soluble VIP. Delgado et al., "VIP/PACAP preferentially attract Th2 effectors through differential regulation of chemokine production by dendritic cells" (2004) *FASEB J* 18:1453-1455. As IL-4 has been suggested to skew DC differentiation, IL-4 was omitted from the fourth DC culture in attempt to yield greater distinction between groups in percent FoxP3+ cell recruitment. Robinson et al., "Chapter 17. Generation of Murine Bone-Marrow-Derive Dendritic Cells" (2001) *Dendritic Cell Protocols* 191-98. While there was a greater difference between the blank microparticles and VIPMPs of the DC culture without IL-4, the LPS control, soluble VIP, and VIP microparticle groups all appeared to show similar trends.

CCL-22 production was also determined from samples collected at 0, 7, 24 and 48 hours from a mature DC control group and a soluble mature DC group. CCL-22 production was similar between the mature DC control group and the soluble mature DC groups. CCL-22 concentration rapidly decreased as microparticle concentration was increased in all microparticle groups. Therefore, CCL22 production by DCs treated with a low concentration of nonporous microparticles relative to the control and soluble VIP group was investigated further. CCL22 concentrations produced by dendritic cells was determined in the presence or absence of VIP, both in soluble form and in microparticles, at 7, 24, and 48 hours. FIG. 39. The VIP microparticles induced more CCL22 production than the blank microparticles at each time point, gaining statistical significance at 24 hours with p<0.05.

Released VIP from microparticles appeared bioactive in the in vitro DC cultures. The observed CCL22 production of DCs treated with microparticles was significantly higher than that of the respective blank microparticles and showed a significant increase at 24 hours in the nonporous VIP group. This trend was most evident at 7 and 24 hours. At 48 hours following treatment with drugs and LPS, the concentration of CCL22 began to equilibrate between the microparticle groups. The effects of the presence of LPS for an extended time period have not been determined but are thought to be detrimental for the cells. Published protocols recommend that cells should be exposed to LPS for a maximum of 18 hours, but the protocol of previous studies involving VIP treatments to DCs did not imply washing of the LPS in a 72 hour period. Delgado et al., (2004) "VIP/PACAP preferentially attract Th2 effectors through differential regulation of chemokine production by dendritic cells" *FASEB J* 18:1453-1455. Additionally, the microparticles themselves may negatively impact the cells and cause

lower CCL22 production by DCs relative to the control and soluble VIP groups. The particle degradation products, such as PLGA fragments or lactic or glycolic acid, may disrupt the cells and may cause some cells to die.

Additionally, it was expected that the DCs cultured with soluble VIP would show a more significant increase in CCL22 production relative to the mature DC control group, as was shown in previous studies. Delgado et al., (2004) "VIP/PACAP preferentially attract Th2 effectors through differential regulation of chemokine production by dendritic cells" *FASEB J* 18:1453-1455. IL-4 has been proposed to skew DC differentiation to exhibit a regulatory phenotype and might therefore diminish the difference in CCL22 production between the groups. Rothstein et al., (2007) "A simple model framework for the prediction of controlled release from bulk eroding polymer matrices" *J of Mater. Chem.* 18:1873-1880. In subsequent chemotaxis studies that measured FoxP3+ T-cell recruitment by DCs treated with VIP microparticle releasate, omission of IL-4 during cell culture increased the distinction between FoxP3+ T cell recruitment. This suggested that released VIP itself (not IL-4) is responsible for CCL22 induction and shows some degree of bioactivity in vitro.

D. In Vivo Murine Model of Periodontitis

After VIP microparticles demonstrated bioactivity in vitro, it was shown that they also promote therapeutic effects in vivo. In some examples, the data presented herein evaluates VIP microparticles as a treatment in an in vivo murine model of periodontitis.

Micro-computerized tomographic analysis images of mouse alveolar bone were used to determine bone loss in mice following administration of blank MPs, CCL22MPs, and VIPMPs. The data distinguishes the cementoenamal junction (CEJ) as a focal point on which to quantitate bone loss data using either a linear measurement or a volumetric measurement technique. FIG. **29**. Park et al., "Three Dimensional Micro-Computed Tomographic Imaging of Alveolar Bone in Experimental Bone Loss or Repair" (2007) *J. Periodontol.* (2007) 78:273-281.

Micro-CT analysis has not yet been reported as a technique to evaluate alveolar bone loss in mice having periodontitis treated with VIP microparticles. However, micro-CT analysis has been performed on mice having periodontitis which received CCL22MP treatment. FIG. **30**.

Dissecting microscope images show alveolar bone loss in infected mice treated with blank, CCL22, and VIP microparticles. FIG. **40**A. VIP microparticles were shown to reduce alveolar bone loss as indicated by decreased distances between the ABC and CEJ with area quantifications confirming these results. FIG. **40**B. Mice infected with periodontal disease and treated with VIP microparticles showed significantly less bone loss than infected mice that received blank microparticles (with p<0.021). As shown previously, CCL22 microparticles also produce a similar effect.

VIP microparticles significantly reduced bone loss in mice infected with periodontal disease relative to mice treated with blank microparticles. However, there was not a significant decrease in bone loss relative to untreated mice, possibly because the sample size of the untreated was reduced to due to accidental fractures of the maxillae during testing. The brittleness of the untreated maxillae relative to those treated with VIP microparticles, may be a testament to the therapeutic potential of VIP.

VI. Predicting Microparticle Formulation Release Profiles

In some embodiments, the present invention contemplates improving upon methods comprising increasing a Treg cell:Teff cell ratio. For example, is has been reported that a combination of soluble T cell inducing factors such as IL-2, TGF-β and rapamycin establish an environment that favors an increase in this ratio. Haxhinasto et al., "The AKT-mTOR axis regulates de novo differentiation of CD4+Foxp3+ cells" *J Exp Med* (2008) 205565-574; Kopf et al., "Rapamycin inhibits differentiation of Th17 cells and promotes generation of FoxP3+ T regulatory cells" *Int Immunopharmacol* (2007) 7:1819-1824; Cobbold et al., "Infectious tolerance via the consumption of essential amino acids and mTOR signaling" *Proc Natl Acad Sci USA* (2009) 106:12055-12060; and Thomson et al., "Immunoregulatory functions of mTOR inhibition" *Nat Rev Immunol.* (2009) 9:324-337. In one embodiment, the present invention contemplates a method to create an improved immunosuppressive, Treg cell-inducing environment by providing VIPMP formulations that provide a predictable and sustained release of T cell inducing factors at a local site. In one embodiment, the VIPMP formulation comprises a polymer (e.g., PLGA). Although it is not necessary to understand the mechanism of an invention, it is believed that these formulations are prepared in accordance with a pre-fabrication mathematical analysis that creates a unique microparticle composition that results in a pre-determined release profile tailored for the induction and proliferation of specific Treg cell populations.

In one embodiment, the present invention contemplates a composition comprising a VIPMP formulation. Although it is not necessary to understand the mechanism of an invention, it is believed that such VIPMP formulations are as effective as soluble factors that induce Treg from naïve T cells. In one embodiment, the VIPMP iTreg cells provide a robust Treg cell proliferation, express canonical Treg surface markers, and suppress naïve T cell proliferation. Further, it was observed that Treg induction and proliferation occurred even when the cells were in contact with microparticles, suggesting that the microparticles do not have adverse on these cells. In one embodiment, VIPMP formulations induce human T cells into an iTreg cell population.

In one embodiment, the present invention contemplates method comprising administering VIPMP populations in vivo for treating medical conditions such as transplant rejection and/or autoimmunity mediated by a local Treg cell population induction.

VII. Mathematical Prediction of Microparticle Release Profiles

In some embodiments, the present invention contemplates using a broadly applicable model for predicting controlled release that eliminates the need for exploratory, in vitro experiments during the design of new biodegradable matrix-based therapeutics. For example, a simple mathematical model can predict the release of many different types of agents from bulk eroding polymer matrices without regression. Such models comprise deterministically calculating the magnitude of the initial burst and the duration of the lag phase (time before Fickian release) by making predictions based upon easily measured or commonly known parameters. This model describes the release of water-soluble agents that are discretely encapsulated in bulk eroding, polymer matrices and that dissolve rapidly, relative to the time scale of release. In addition to using specific equations, the model includes two correlations that enable predictions with knowledge of just five parameters, all commonly known or easily measured prior to release. These parameters are microsphere radius (R_p), occlusion radius (R_{occ}), polymer degradation rate (kC_w), polymer initial molecular weight (M_{wo}), and agent molecular weight (M_{wA}). A regression to a desired dosing schedule generates a set of matrix

design parameters to guide the fabrication of a matching controlled release therapeutic.

Briefly, the operation of the predictive algorithm can be described as follows. Consider an initially uniform matrix of known geometry comprised of a biodegradable polymer, such as a polyester or polyanhydride, and with randomly distributed entrapped release agent (e.g. drug of concentration C_{Ao}), loaded below its percolation threshold (such that agent remains discrete) to ensure matrix mediated release. This agent can either be dispersed as crystals (such as in the case of uniformly loaded systems, e.g. single emulsion-based particulates) or housed as a solution in occlusions (e.g. double emulsion-based particulates). At time zero, an aqueous reservoir begins to hydrate the matrix, a process which happens quickly for the bulk eroding polymers matrices considered herein. As the matrix hydrates, encapsulated agent adjacent to the matrix surface (with a direct pathway for egress) diffuses into the reservoir in a phase typically dubbed "the initial burst". The relative size of the occlusion (R_{occ}) occupied by the encapsulated agent is proportional to the magnitude of the initial burst. As the initial burst release commences, degradation of the polymer begins, increasing chain mobility and effectively leading to the formation of pores in the polymer matrix. Although a number of mechanisms have been proposed for this heterogeneous degradation profile, one hypothesis, which has been reinforced by experimental data, is based upon regions of varying amorphicity and crystallinity. It is believed that (as shown using scanning electron microscopy). These pores appear to be essential for subsequent release.

With the cumulative growth and coalescence of these pores, agents are able to diffuse towards the surface of a polymer matrix that would otherwise be too dense to allow their passage. Thus, a pore is defined as a region of polymer matrix with an average molecular weight low enough to allow the release of encapsulated agent. (This is in contrast to the occlusion, which is defined as a region occupied by dissolved or solid agent, marked by the absence of polymer matrix). Further, the molecular weight associated with release may vary for each encapsulated agent type (small molecule, peptide, protein, etc.), leading to a size-dependent restriction for agent egress.

With a size-dependent restriction on egress established, the degradation controlled release of any encapsulated agent can only occur when the following four conditions are satisfied. 1) The release agent must be present in the polymer matrix. 2) A pore must encompass the release agent. 3) That release agent must be able to diffuse through the encompassing pore. 4) The pore must grow and coalesce with others to create a pathway for diffusion to the surface.

Agent concentration within a matrix (such a microsphere, rod, or thin film) can be calculated from Fick's second law (Equation 1) for any point in time (t) or space (r), provided that the agent is not generated or consumed in any reactions while within the matrix.

$$\frac{\partial C_A}{\partial t} = \nabla(D_{eff} \nabla C_A) \tag{1}$$

where D_{eff} is an effective diffusivity term. Integrating C_A/C_{ao} over the entire matrix volume yields the cumulative fraction of agent retained in the matrix (P(t)) (Equation 2).

$$P(t)=V^{-1}\int C_A/C_{Ao}dV \tag{2}$$

In turn, the cumulative fraction of agent released (R(t)), a metric commonly used to document formulation performance, is simply (Equation 3):

$$R(t)=1-P(t) \tag{3}$$

At the center point, line, or plane of the matrix (r=0) symmetry conditions are defined such that $dC_A/dr=0$. At the matrix surface ($r=R_p$) perfect sink conditions are specified. A boundary also exists at a depth of R_{occ} in from the matrix surface ($r=R_p-R_{occ}$) where continuity conditions are defined. In the subdomain from R_p to R_p-R_{occ} (terminating one occlusion radius in from the particle surface), agent is subject to the initial release, such that D_{eff} is simply a constant (D), reflecting the movement of agent through the hydrated occlusions abutting the matrix surface. In the subdomain from 0 to R_p-R_{occ}, agent is subject to pore-dependent release, such that $D_{eff}=D\epsilon$ where D is the diffusivity of the agent through the porous matrix and ϵ is the matrix porosity.

For a system of like matrices, such as microspheres or sections in a thin film, that degrade randomly and heterogeneously, the accessible matrix porosity is simply a function of time as a discrete pore has, on average, an equal probability of forming at any position in the polymer matrix. Hence, the time until pore formation can be calculated from the degradation of the polymer matrix, as any differential volume containing a pore would have a lower average molecular weight than its initial value. Assuming that the polymer degradation rate is normally distributed, the induction time for pore formation will also follow a normal distribution. As this pore formation is cumulative, the time dependent matrix porosity (3(t)) can be described with a cumulative normal distribution function (Equation 4).

$$\varepsilon(t) = \frac{1}{2}\left[\text{erf}\left(\frac{t-\bar{\tau}}{\sqrt{2\sigma^2}}\right)+1\right] \tag{4}$$

In this equation, τ is the mean time for pore formation and σ^2 is the variance in time required to form pores.

Calculating the cumulative normal induction time distribution (ϵ(t)) requires values for τ and σ^2. For polymers that obey a first order degradation rate expression, the mean time for pore formation (τ) can be determined as follows:

$$\bar{\tau} = \frac{-1}{kC_w}\ln\left|\frac{M_{wr}}{M_{wo}}\right| \tag{5}$$

where kC_w is the average pseudo-first order degradation rate constant for the given polymer type, M_{wo} is the initial molecular weight of the polymer, and we define M_{wr} as the average polymer molecular weight in a differential volume of matrix that permits the diffusion of the encapsulated agent. For blended polymer matrices, the value for τ was calculated by averaging the results obtained from Equation 5 for each component.

It is reasonable to believe that the matrix molecular weight at release (M_{wr}), which dictates how much degradation is required before release can occur, would vary depending on the size of the encapsulated agent. Macromolecules or larger agents can only diffuse through a section of matrix if it is almost entirely free of insoluble polymer chains. Hence the M_{wr} for such agents is considered the polymer solubility molecular weight (e.g., 668 Da for 50:50 PLGA). As agent size decreases (as indicated by M_{wA}), however,

egress can occur through more intact sections of polymer matrix (higher M_{wr}), as less free space is needed to allow their passage.

The distribution of polymer degradation rates ($kC_w(n)$) attributed to matrix crystallinity is needed to calculate the variance (σ^2) in the induction time distribution for pore formation ($\epsilon(t)$). To determine $kC_w(n)$, the first order degradation rate equation $M_w = M_{wo}e^{-kC_w t}$ was linearly fitted at three different time periods to published degradation data for the desired hydrolysable polymer. Fitting the initial slope of the degradation profile provides the degradation rate constant of amorphous polymer as degradation occurs faster in amorphous regions of the matrix. Fitting data from the final weeks of degradation produces a rate constant for the crystalline material, as amorphous regions are largely eroded by this point. Finally, a fit of the entire degradation profile yielded a rate constant indicative of the overall morphology.

With values for $kC_w(n)$ defined, a distribution of induction times ($t(n)$) was calculated using equation 5. For blended polymer matrices this $T(n)$ includes values calculated at all component $kC_w(n)$ and M_{wo}. The standard deviation was taken for $\tau(n)$, then divided by a crystallinity-based factor and squared, yielding an adjusted variance (σ^2), which conforms with lamellar size data.

This crystallinity-based factor adjusts the probability of finding pores formed from the fastest degradation rate in $kC_w(n)$ to match the probability of finding a differential volume of matrix containing purely amorphous polymer. For all modeled cases, this differential volume is defined as a region large enough to allow the passage of a small virus or protein complex (20 nm diameter). As multiple lamellar stacks can fit into this differential volume, the probability that such a volume is purely amorphous can be calculated from of the number of stacks per differential volume and the average crystallinity of the matrix. From crystallinity data on 50:50 PLGA matrices, the probability of finding a purely amorphous differential volume is calculated as 0.05%. Thus, to ensure that the probability of finding a pore formed from the fastest degradation rate in $kC_w(n)$ also equals 0.05%, the standard deviation in the induction time distribution for pore formation was adjusted by a factor of 5. Similarly, factors of 4 and 2 were calculated from crystallinity data for 75:25 PLGA and polyanhydride matrices, respectively.

With values for τ and σ^2 selected (defining $\epsilon(t)$), a finite element solution to Equation 1 may be calculated using a commercially available software program (e.g., Comsol®, v3.3) for the given matrix geometry, using default solver settings. To decrease computation time, the matrix geometry can be simplified to one dimension based on symmetry, for a sphere, or high aspect ratio, for a thin film. The resulting concentration profiles are numerically integrated to calculate the cumulative fraction of agent released (Equations 2 and 3). For validation, the numerical solutions of the model can be fit to experimental data sets by varying M_{wr} and D. It should be noted that data points charting the kinetics of the initial burst can be omitted from these regressions, as the model only predicts the magnitude of this phase. Each fit may be optimized using a commercially available software program (e.g., Matlab®, R2007a) based on a minimized sum-squared error (SSE) or weighted sum-squared error (wSSE) when error bars are provided.

The above described computer model was most accurate in predicting release of VIP from 4.2 kDa polymer. The large magnitude of the initial burst observed from the 12.6 kDa polymer microparticles was not predicted by the program. However, in other release assays of VIP from microparticles

made of the 12.6 kDa polymer, the initial burst was not as dramatic. For these other studies, the model was more accurate in predicting the release from the 12.6 kDa polymer microparticles, although the observed release appeared to last longer than predicted. The model failed to predict the near linear release from the 100 kDa polymer microparticles and again underestimated the duration of release, suggesting that drug-polymer interactions between VIP and the PLGA polymer may be delaying release. While the model struggled to capture the dynamics of the microparticles consisting of either 12.6 kDa polymers or 100 kDa polymers, it was fairly accurate in predicting that a mixture of a specific ratio of polymers would generate a specific linear release profile if the initial burst was disregarded. The initial observed release from the mixture of 4.2 kDa, 12.6 kDa, and 100 kDa polymers resembled release from the pure 4.2 kDa polymer but was not captured by the model. The extended near linear release was most likely produced by the high percentage of 100 kDa polymer particles in the mixture.

VIII. VIPMP Kits

In another embodiment, the present invention contemplates kits for the practice of the methods of this invention. The kits preferably include one or more containers containing a VIPMP composition to perform methods of this invention. The kit can optionally include a pharmaceutically acceptable excipient and/or a delivery vehicle. The reagents may be provided suspended in the excipient and/or delivery vehicle or may be provided as a separate component which can be later combined with the excipient and/or delivery vehicle. The kit may optionally contain additional therapeutics to be co-administered with the microparticle compositions.

The kits may also optionally include appropriate systems (e.g. opaque containers) or stabilizers (e.g. antioxidants) to prevent degradation of the reagents by light or other adverse conditions.

The kits may optionally include instructional materials containing directions (i.e., protocols) providing for the use of the reagents in the performance of the methods described herein. In particular the disease can include any one or more of the disorders described herein. While the instructional materials typically comprise written or printed materials they are not limited to such. Any medium capable of storing such instructions and communicating them to an end user is contemplated by this invention. Such media include, but are not limited to electronic storage media (e.g., magnetic discs, tapes, cartridges, chips), optical media (e.g., CD ROM), and the like. Such media may include addresses to internet sites that provide such instructional materials.

In one embodiment, the present invention contemplates a kit comprising VIPMP populations to provide an 'off-the-shelf' therapeutic for creating a local immunosuppressive environment and increasing the presence of Treg cells at sites of inflammation. In one embodiment, the kits may be used to treat various medical conditions including, but not limited to, contact dermatitis, periodontitis and diseases where immune homeostasis is lost and needs to be restored, skin disorders, composite tissue transplantation, prevention of graft rejection. Although it is not necessary to understand the mechanism of an invention, it is believed that VIPMP formulations can be administered to treat diseases and disorders while the systemic immune system integrity such that the immune system can continue to fight infections and inhibit malignancies.

IX. Immunological Tolerance

In developing a strategy to replace and/or decrease post-transplant immunosuppressant therapy, "reprogramming"

the immune system to selectively accept a foreign antigen without an systemic immune down-regulation is seen as a desirable option. This state may be referred to as a permanent immunological tolerance. One modulator of immunological tolerance is suspected to be regulatory T-cells (T_{reg}). T_{reg} cells have been shown to be capable of antigen-independent immune suppression and can be activated by immature or tumor-manipulated dendritic cells, thereby inducing immunosuppression. In vitro, T_{reg} cells respond to several known soluble factors and even latex beads coated with monoclonal antibodies specific for several T_{reg} cell surface receptors. Tumors actively secrete compounds to modulate regulatory T-cells so as to evade immune recognition. Although not intuitively obvious, it is believed that the induction of permanent allograft tolerance may be achieved by mimicking tumor-induced immunosuppression.

CD4+CD25+ regulatory T-cells are believed to express the CCR4 receptor which binds to the chemokine CCL22. CCL22 was reported to be involved in the migration of regulatory T-cells to tumor sites because tumors also actively secrete CCL22. Curiel et al., *Nat Med* 10:942-949 (2004). This represents just one of the methods that tumors have for evading immune recognition. Evasion of an immune response provides for tumor survival given that many of its basic biological features such as genetic instability, invasive growth, and tissue disruption are inherently pro-inflammatory. Besides attracting regulatory T-cells to its vicinity, tumors are believed to also have the ability to influence biological antigen presenting cells (i.e., for example, biological dendritic cells) to down-regulate processing and presentation of tumor associated antigens, inhibit co-stimulatory expression (i.e. retain immature phenotype), and alter their cytokine secretion profile towards Immune tolerance. Pardoll, D., *Annual Review of Immunology* 21:807-839 (2003). It has been reported that tumors also secrete TGF-β, which may influence regulatory T-cell mediated tolerance. Chen et al., *Proc Natl Acad Sci USA* 102:419-424 (2005). Although it is not necessary to understand the mechanism of an invention, it is believed that these other mechanisms similar to those of tumor immune evasion and survival may also be useful in inducing allograft tolerance.

Control over the induction of immunological tolerance would afford permanent allograft acceptance, bypassing a lifelong regimen of immunosuppressive agents. Although it is not necessary to understand the mechanism of an invention, it is believed that this tolerance can be mediated by activating suppressive regulatory lymphocytes (i.e., for example, regulatory T cells).

In one embodiment, the present invention contemplates a therapeutically-relevant, modular platform to deliver multiple soluble factors and membrane surface-bound factors. In one embodiment, these factors are delivered in vivo by artificial particles into the vicinity of regulatory T cells (T_{reg} cells). In one embodiment, the delivered factors modulate T_{reg} cell proliferation. In one embodiment, the delivered factors modulate T_{reg} cell immunosuppressive capacity. In one embodiment, the delivered factors induce Treg cell chemotaxis. In one embodiment, the platform is delivered in vivo. In one embodiment, the platform comprises a biocompatible and/or biodegradable synthetic construct, such as an artificial antigen presenting cell (aAPC). In one embodiment, the aAPC is approximately the size of a biological cell. In one embodiment, the aAPC comprises covalently-bound monoclonal antibodies and/or encapsulated soluble cytokines/chemokines. In one embodiment, the antibodies and cytokine/chemokines stimulate lymphocyte proliferation. In one embodiment, the lymphocyte stimulation is

performed in vitro. In one embodiment, the lymphocyte stimulation is performed in vivo.

In one embodiment, the present invention contemplates a construct comprising a pattern of membrane surface factors such that the pattern mimics a biological dendritic cell/regulatory T-cell immunological synapse (IS). In one embodiment, the pattern increases the stimulatory capacity of the construct. In one embodiment, the synapse comprises a "bull's-eye" pattern. In one embodiment, the "bull's-eye" pattern comprises an apical region and an equatorial region. In one embodiment, the pattern further comprises a region between the apical and equatorial regions.

In one embodiment, the present invention contemplates a method for creating an artificial biomimetic immunological synapse. In one embodiment, the synapse is discrete and topologically complex. In one embodiment, the synapse comprises at least two immunosuppressive factors. In one embodiment, the synapse is located on the surface of an artificial presenting cell. In one embodiment, the synapse comprises a "bull's-eye" pattern. In one embodiment, the "bull's-eye" pattern comprises an apical region and an equatorial region. In one embodiment, the pattern further comprises a region between the apical and equatorial regions.

A. Tolerogenic Cells

The biological processes whereby tolerance is induced involve the suppression or deletion of alloreactive T-cells which would otherwise destroy an allograft. This process is thought to be mediated by a subset of regulatory T-cells which represent 5-10% of all peripheral CD4+ lymphocytes. Mahnke et al., *Blood* 101:4862-4869 (2003).

Suppressive lymphocyte involvement in immune response was first suggested in the early 1970s. Gershon et al., *Immunology* 21:903-914 (1971). Recently, however, a suppressive regulatory T-cell (T_{reg}) has been implicated in the development of immune tolerance. For example, a population of these lymphocytes that express high levels of CD25, the a subunit of the IL2 receptor, have been reported as an immune suppressor. Sakaguchi et al., *J Immunol* 155:1151-1164 (1995).

T_{reg} cells also express a unique transcription factor called FoxP3, which is thought to be required for the development of T_{reg} suppressive capacity and is a recognized marker for T_{reg} cells. Hori et al., *Science* 299:1057-1061 (2003); and Fontenot et al., *Nat Immunol* 4:330-336 (2003). Furthermore, it is believed that the extent of expression of this transcription factor can correlate with the suppressive capacity and activation state of a T_{reg} cell. It has been shown that transfer of the gene for FoxP3 into cells which are CD4+ CD25– confers regulatory capacity which is otherwise absent. Fontenot et al., *Nat Immunol* 4:330-336 (2003). T_{reg} cells may also express high levels of CTLA-4 (i.e., for example, CD152) which may also be involved in their regulatory capacity as this molecule can bind to the B7 class of co-stimulatory molecules in place of CD28 thereby resulting in the production of transforming growth factor-β, or TGF-β. Perez et al., *Immunity* 6:411-417 (1997). TGF-β, along with engagement of the T-cell receptor, has been demonstrated to differentiate naïve, peripheral CD4+ CD25– T-cells into CD4+CD25+ cells with suppressive capacity, suggesting that this factor may be important for the in vivo generation and maintenance of T_{reg} cells. Walker et al., *J Clin Invest* 112:1437-1443 (2003). Regulatory T-cells are also believed to express chemokine receptors including, but not limited to, CCR4 and CCR8, rendering them fully capable of migration (i.e., for example, by chemotaxis) to a

site of inflammation or to the lymph nodes upon appropriate signaling. Iellem et al., *J Exp Med* 194:847-853 (2001).

Mechanisms of action used by regulatory T-cells in promoting tolerance in vivo has been a topic of dispute. Some believe that cell-to-cell contact between T_{reg} cells and the alloreactive lymphocytes provide the activation stimulus that results in immunosuppression. Others believe that specific stimulatory factors (i.e., for example, soluble factors and/or membrane surface bound factors) which have been shown to be secreted from regulatory T-cells may be responsible for contact-independent suppression (i.e., for example, TGF-β, IL-10, CTLA-4, and IL-4). For example, studies which support the latter hypothesis demonstrate that: i) T_{reg} activation by TNF-β may be blocked by monoclonal antibodies (Belghith et al., *Nat Med* 9:1202-1208 (2003); and ii) T_{reg} activation is diminished in TGF-β receptor knock-out mice (Green et al., *Proc Natl Acad Sci USA* 100:10878-10883 (2003)). However, it has been reported that suppression by T_{reg} cells in vitro is not inhibited by blocking TGF-β and suggest a cellular contact dependent mechanism. Piccirillo et al., *J Exp Med* 196:237-246 (2002).

Nevertheless, one interesting notion regarding the method of T_{reg} cell suppression which is gaining popular acceptance is an antigen independent suppression mechanism, known as "bystander regulation". Several new reports are providing evidence for "bystander regulation" which opposes a previous hypothesis that regulatory T-cells preferentially mediate immunosuppression in an antigen specific manner Graca et al., *Proc Natl Acad Sci USA* 101:10122-10126 (2004); and Karim et al., *Blood* 105:4871-4877 (2005). In particular, Karim et al. reported that CD25+CD4+ T_{reg} cells generated by nominal antigens under cover of anti-CD4 monoclonal antibody treatments were fully capable of tolerizing mice to cardiac allografts comprised of completely different antigens. Therefore, although most studies show that T-cell receptor stimulation seems to be involved in tolerization, antigen specificity may or may not be limiting in the overall effects of immunosuppression induced by regulatory T-cells. The data presented herein suggest that T_{reg} cell immunosuppression may be meditated through a combination of contact dependent and independent mechanisms.

1. Antigen Presenting Cells

In one embodiment, the present invention contemplates methods that would reconcile the apparently conflicting in vitro and in vivo data regarding cytokine-dependent suppressive capacity. In one embodiment, the method comprises using cell types that are not present in the in vitro experimental models (i.e., for example, a co-mediator cell). Although it is not necessary to understand the mechanism of an invention, it is believed that these co-mediator cells may secrete their own immunomodulating cytokines or act in other suppressive capacities at the same time as regulatory T-cells. For example, a co-mediator cell may comprise an antigen presenting cells (APCs). Antigen presenting cells are also referred to as dendritic cells and have been shown to have a tolerogenic capacity. Matzinger et al., *Nature* 338:74-76 (1989); Steinman et al., *Proc Natl Acad Sci USA* 99:351-358 (2002); Brocker et al., *J Exp Med* 185:541-550 (1997); and Volkmann et al., *J Immunol* 158:693-706 (1997).

APCs and lymphocytes may interact at a contact point in accordance with a "two signal" model of stimulation. For example, the presence of T-cell receptor (TCR)/Major Histocompatibility Complex (MHC) engagement (i.e., for example, Signal 1) without the presence of a proper co-stimulatory molecule engagement (i.e., for example, Signal 2) may lead to lymphocyte progression toward a state of anergy or deletion through apoptosis. In support of this hypothesis, it has been demonstrated that engagement of the T-cell receptor using monoclonal antibodies specific for CD3 (i.e., for example, T-cell receptor subunit believed responsible for signal transduction), in the absence of co-stimulation, led to long lasting proliferative unresponsiveness in murine T-cell clones. Jenkins et al., *J Immunol* 144:16-22 (1990).

Dendritic cells (i.e., for example, APCs) in an immature state (i.e. that have not been stimulated by inflammatory factors) have been observed to possess a low level expression of co-stimulatory molecules while still maintaining expression of MHC Class I and II. These immature dendritic cells have a high capacity to take up material by receptor-mediated endocytosis, pinocytosis, and phagocytosis and are thought to survey the periphery and constantly process and present self-antigen. These observations suggest that an immunological tolerant state is maintained in the absence of co-stimulatory molecules even when self-antigen is persistently presented. Consequently, it would be reasonable to believe that the presence of co-stimulatory molecules represent danger signals. Matzinger, P. *Science* 296:301-305 (2002). Although it is not necessary to understand the mechanism of an invention, it is believed that this would explain why autoimmunity develops when the normal, perpetual tolerant state is somehow disrupted.

In light of the above, it is most likely not a coincidence that persistent expression of antigen in the periphery is also thought to be sufficient for generation of regulatory T-cells. Taams et al., *Eur J Immunol* 32:1621-1630 (2002). Furthermore, soluble factors are secreted by T_{reg} cells that generate and maintain APCs in an immature state including, but not limited to: i) IL10 (De Smedt et al., *Eur J Immunol* 27:1229-1235 (1997)); ii) TGF-β (Geissmann et al., *J Immunol* 162:4567-4575 (1999)); and IL4 (Inaba et al., Journal of Experimental Medicine 176:1693-1702 (1992)). On the other hand, others have shown that immature/tolerogenic dendritic cells also have the ability to mediate contact-dependent stimulation of T_{reg} cells. These secreted soluble factors are also suggested to generate and maintain T_{reg} cells thereby creating the possibility for feedback regulation. Steinman et al., *Annu Rev Immunol* 21:685-711 (2003). This feedback mechanism might even extend to the apoptosis of dendritic cells which are already activated or mature by regulatory T-cells. Frasca et al., *J Immunol* 168:1060-1068 (2002). These data suggest that APCs and T_{reg} cells interact to modulate immunotolerance.

The current understanding of biological processes whereby regulatory T-cells (T_{reg}) promote tolerance is presented in Table 1.

TABLE 1

Summary Of T$_{reg}$ Observations

Observation	Significance	Reference
Tolerogenic T$_{reg}$ cells express high surface levels of CD4 & CD25.	These markers can aid in the isolation of T$_{reg}$ cells	Sakaguchi et al., *J Immunol* 155: 1151 (1995).
The transcription factor Foxp3 is highly expressed in activated T$_{reg}$.	Activation state of T$_{reg}$ can be determined by measuring Foxp3.	Fontenot et al., *Nat Immunol* 4: 330 (2003).
TGF-β1 can generate T$_{reg}$ while inhibiting other T-cells.	TGF-β1 is important to preferential development of T$_{reg}$.	Belghith et al., *Nat Med* 9: 1202 (2003); and Green et al., *PNAS* 100: 10878 (2003).
T$_{reg}$ express the chemokine receptors CCR4 and CCR8 and are attracted by their ligands.	Chemokines such as CCL22 may be used to preferentially recruit T$_{reg}$.	Chen et al., *American Journal of Transplantation* 6: 1518 (2006); and Curiel et al., *Nat Med* 10: 942 (2004).
Tolerogenic dendritic cells have been associated with, and have the capacity to stimulate, T$_{reg}$.	Contact-mediated and soluble factor stimulation provided by these APCs can generate T$_{reg}$	Steinman et al., *Annu Rev Immunol* 21: 685 (2003).
T$_{reg}$ stimulated by a particular antigen can mediate immune suppression in the presence of an allograft without this antigen.	T$_{reg}$ are capable of promoting allograft tolerance regardless of the antigen/TCR-stimulus used to generate them.	Karim et al., *Blood* 105: 4871 (2005).
Beads coated with mAb specific to CD3 and CD28 in the presence of IL2 were capable of stimulating and proliferating T$_{reg}$ in vitro.	A similar strategy could be utilized in vivo by including soluble factor(s) in a biodegradable format.	Hoffmann et al., *Blood* 104: 895 (2004).

Although it is not necessary to understand the mechanism of an invention, it is believed that the stimulation, and behavior of, T$_{reg}$ cells can generate immune tolerance. Professional antigen presenting cells (APCs) (i.e., for example, dendritic cells: DCs) have also been shown to have tolerogenic capacity, although the precise mechanism of their suppressive function is unclear. Matzinger et al., *Nature* 338:74 (1989); and Steinman et al., *Proc Natl Acad Sci USA*, 99:351 (2002). A relationship between DCs and T$_{reg}$ cells has been observed where a persistent presentation of antigen by tolerogenic DCs generates T$_{reg}$ cells through both contact-mediated and secreted soluble signal-mediated stimulation. Steinman et al., *Annu Rev Immunol* 21:685 (2003). Thus, APCs might mediate immunosuppression by stimulating T$_{reg}$ cells through a dual pronged mechanism.

Although it is not necessary to understand the mechanism of an invention, it is believed that an artificial particles comprising Treg cell factors should modulate T$_{reg}$ cells in a manner similar to biological antigen presenting cells. For example, a membrane surface presentation (i.e., for example, an immunological synapse) of stimulating protein factors on a biological dendritic cell is not displayed in a random pattern. In one embodiment, the present invention contemplates an artificial antigen presenting cell comprising soluble and/or membrane bound stimulating factors displayed in non-random patterns.

In particular, biological antigen presenting cells have been reported to develop a mature immunological synapse (IS) and are composed of discretely defined spatial regions of receptors. Monks et al., *Nature* 395:82 (1998). Some in the art have termed these regions as supramolecular activation clusters, or SMACs. Grakoui et al., *Science* 285:221 (1999). A central SMAC (cSMAC) region may comprise areas providing factors for TCR stimulation and/or soluble factors for co-stimulation. The cSMAC may be surrounded by at least one peripheral SMAC (pSMAC) region that are usually rich in adhesion molecules (i.e., for example, ICAM). Further, pSMACs may be surrounded by a distal SMAC (dS-MAC) region that may have, for example, a high CD45 density. Using a dual probe fluorescence imaging technique, the SMAC spatial organization resembles a "bulls-eye". See, FIG. **3**.

2. In Vitro T$_{reg}$ Stimulation

In vitro testing with primary, regulatory T-cells provides a way to determine if the soluble factors encapsulated within an artificial particle are biologically active. Preferred assays are directly related to the biological activity of the encapsulated factors with respect to their modulatory effects on T$_{reg}$ cells. In vitro primary T$_{reg}$ cells also provide a testing platform for the optimization of artificial particles in preparation for therapeutic in vivo administration.

A method for large scale in vitro expansion of T$_{reg}$ cells has been reported. Hoffmann et al., *Blood* 104:895 (2004). In this study, the proliferation and suppressive capacity of cultured T$_{reg}$ cells was increased using latex beads randomly coated with soluble IL2 and CD3/CD289 monoclonal antibodies. It was unreported if the T$_{reg}$ cell populations produced by Hoffmann et al. are functionally the same as those produced in vivo by biological antigen presenting cells. Sufficient discrepancies are observed between in vitro and in vivo suppressive mechanisms to indicate that they are not functionally the same. Belghith et al., *Nat Med* 9:1202 (2003); and Green et al., *PNAS* 100:10878 (2003). Such a problem in the art identifies a need to study in vivo T$_{reg}$ cell activation. A significant disadvantage of the Hoffmann et al. technique, is that the random distribution stimulatory factors on the latex beads did not present the T$_{reg}$ cells with an SMAC IS complex.

Nonetheless, in vitro data is provided herein demonstrating the stimulatory capacity of synthetic dendritic cells by several subsets of T-cells. For example, primary lympho-

cytes were harvested and incubated with synthetic dendritic cells and the resulting level of activation were measured.

Commercially available cell isolation kits (i.e., for example, MACS) may be used to harvest CD4+CD25+ T$_{reg}$ and CD4+CD25− helper T-cells from lymph nodes of B6 mice. Once incubated with artificial antigen presenting cells, regulatory T-cells can be examined for proliferative capacity using techniques including, but not limited to, tritiated thymidine incorporation, CSFE dilution, or Flow Cytometry. Cytokine secretion profiles (i.e., for example, IL-2 or IFN-gamma) may be determined using ELISPOT. To assess regulatory T-cell activation the presence of the intracellular transcription factor, FoxP3, may also be measured in permeabilized cells using Flow Cytometry.

To verify the exclusivity of T$_{reg}$ cell stimulation, artificial antigen presenting cells can also be incubated with other lymphocyte populations (CD4+CD25−, and CD8+) which are then tested for known activation markers. Although it is not necessary to understand the mechanism of an invention, it is believed that because of the release of TGF-β1 by aAPCs these non-suppressor cells will not be stimulated and may actually even gain suppressive capacity. Fu et al., *Am J Transplant* 4:1614 (2004). It is further believed that the removal of CD28 co-stimulation may result in anergy of helper T-cells. Ragazzo et al., *PNAS* 98:241 (2001). Thus, it is not presumed that a cSMAC region must contain both membrane bound TCR stimulation factors and soluble co-stimulation factors simultaneously to achieve preferential regulatory T-cell activation.

Nonetheless, T$_{reg}$ cells may be studied in vitro using artificial APCs comprising multiple membrane surface-bound stimulatory factors and soluble secretable stimulatory factors. For example, the activity of encapsulated soluble stimulatory factors may be tested using primary T$_{reg}$ cells in vitro by assays measuring effects on cell differentiation, cell proliferation, and/or chemotaxis. In some embodiments, a plurality of aAPC formulations may be used wherein each aAPC formulation comprises a different amount of both surface bound factors and soluble releasable factors. In one embodiment, the present invention contemplates a method comprising identifying an optimized formulation.

3. In Vivo Effects of Artificial Particles on Regulatory T Cells (T$_{regs}$)

In one embodiment, the present invention contemplates a method comprising implanting artificial particles in vivo wherein T$_{reg}$ cells are recruited and/or activated. In one embodiment, the T$_{reg}$ cell recruitment and/or activation induces biological graft tolerance. Although it is not necessary to understand the mechanism of an invention, it is believed that the biological graft tolerance is a result of T$_{reg}$ cell induced immunosuppression.

Before T$_{reg}$ cells can be directly manipulated by an artificial particle, however, they need to be attracted to an artificial particle. In one embodiment, the present invention contemplates an artificial particle comprising an encapsulated chemotactic factor (i.e., for example, CCL22). Alternatively, the T$_{reg}$ cell population may be increased by performing a T$_{reg}$ cell adoptive transfer. This procedure is especially useful during investigational and/or technique verification studies wherein the transferred T$_{reg}$ cells are labeled with green fluorescent protein (i.e., for example, collected from transgenic GFP+ B6 mice). The resulting enriched T$_{reg}$ population in vivo facilitates histological and flow cytometry analyses. Further, an optimization of the chemotactic factor is most likely necessary because of inherent differences between in vivo and in vitro techniques, as discussed above.

The embodiments of the present invention contemplate many transplantation models for use as testing models. In some embodiment, testing models include, but are not limited to, skin allograft or heterotopic heart transplants, and are used to compare high and low amounts of donor derived antigen presenting cells. These donor biological particles may interfere or enhance the function of the artificial particles. However, it should be theoretically feasible to drastically outnumber the biological antigen presenting cells through administration of multiple depot injections near the transplantation site and/or coating the allograft with artificial particles. In one embodiment, a skin allograft testing model is chosen due to the simplicity of the procedure and the ability to use a control syngenic graft on the same animal. In one embodiment, a heterotopic heart transplant testing model is chosen, not only due to its relatively simple procedure and method of data collection, but also because of the consistency in the rejection profile as opposed to liver and kidney transplants.

In one embodiment, the present invention contemplates a method comprising measuring regulatory T cell migration and activation following exposure to synthetic, biodegradable constructs. In one embodiment, the method comprises an in vivo murine model. This model provides unexpected and unpredictable advantages over traditional in vitro models, because, as discussed elsewhere, the behavior of T$_{reg}$ cells in vitro is not believed to be equivalent to that observed in vivo. M. Belghith et al., *Nat Med* 9:1202 (September, 2003); Green et al., *PNAS* 100:10878 (2003).

In one embodiment, the present invention contemplates a testing method comprising co-implanting a tissue transplant graft and a plurality of artificial particles in vivo, wherein the artificial particles recruit and activate the T$_{reg}$ cells to induce a permanent graft tolerance. For example, artificial particles may be tested for the ability to recruit and stimulate regulatory T-cells by injecting artificial particles into mice which have been adoptively transferred with GFP+ T$_{reg}$ cells. Imaging studies can then easily identify in vivo localizations of the GFP+ T$_{reg}$ cell following artificial particle injection. Further, various artificial particle formulations may be co-administered at the time of tissue transplantation (i.e., for example, skin allograft or heterotopic heart transplantation) to examine their therapeutic ability to induce allograft tolerance.

In one embodiment, the present invention contemplates an in vivo method comprising manipulating T$_{reg}$ cells using synthetic dendritic cells. In one embodiment, the synthetic dendritic cells stimulate T$_{reg}$, thereby resulting in immunosuppression. Although it is not necessary to understand the mechanism of an invention, it is believed that that synthetic dendritic cells which are approximately 10 μm do not move from the site of implantation given that they are too large to cross the endothelial barrier or redistribute via the reticuloendothelial system. In one embodiment, the method further comprises administering synthetic dendritic cells with encapsulated soluble factors and/or surface factors into a first leg flank of a B6 mouse. In one embodiment, the administering comprises injection. In one embodiment, the injection comprises a subcutaneous injection. In one embodiment, the injection comprises an intramuscular injection. In one embodiment, a second leg flank is injected with a synthetic dendritic cell without encapsulated soluble and/or surface factors (i.e., for example, as a negative control). At 1 Day or 3 Days after the injection(s), mice will be euthanized and skin or muscle will be resected for histological analysis (i.e., for example, staining for T$_{reg}$ cells and Foxp3). Wang et al., *Am J Transplant* 6:1297 (June, 2006).

The sensitivity of this protocol can be enhanced by adoptively transferring fluorescent T_{reg} isolated from GFP+B6 mice into normal B6 mice to increase the number of detectable cells available for chemotaxis.

In one embodiment, the present invention contemplates a method comprising prolonging the survival of a skin allograft using a biomimetic immunosuppressive synthetic dendritic cell.

4. T_{reg} Cells And Transplant Tolerance

The generation of T_{reg} suppressor cells has been attempted in vivo prior to transplantation with the goal of inhibiting allograft rejection. Most of these methods involve the administration of an alloantigen, with the final result being an increase in the number and activation state of regulatory T-cells. In mice, it has been shown that naïve regulatory T-cells still have suppressive capacity, but 10 fold greater quantities are required to promote graft acceptance when compared to populations that have been stimulated prior to transplantation with alloantigen. Graca et al., *J Immunol* 168:5558-5565 (2002). Furthermore, administration of this alloantigen is usually accompanied by CD4 monoclonal antibodies to avoid eliciting a hostile immune response. Kingsley et al., *J Immunol* 168:1080-1086 (2002).

Other monoclonal antibodies which block co-stimulation pathways may also be used to generate regulatory T-cells including, but not limited to, monoclonal antibodies that block CD154-CD40 engagement. van Maurik, A., Herber, M., Wood, K. J. & Jones, N. D. (2002) J Immunol 169, 5401-4; Taylor et al., *Blood* 99:4601-4609 (2002). Also, CD-3 monoclonal antibodies were used to generate regulatory T-cells via stimulation through the T-cell receptor (TCR) and was reported to control the progression of diabetes. Herold et al., *N Engl J Med* 346:1692-1698 (2002). Native, immature dendritic cells may be capable of generating CD4+CD25+ T_{reg} cells in vivo if alloantigen is specifically targeted to them by an antibody which binds to DEC205; a receptor involved in the recycling of antigen through late endosomal compartments. Mahnke et al., *Blood* 101:4862-4869 (2003).

Ex vivo expansion and manipulation of tolerogenic cells holds much promise for therapeutic application given their relatively low frequency in peripheral tissues. For this potential to be realized, this expansion would have to maintain an appropriate regulatory phenotype, including the ability to migrate to the correct sites of inflammation and the lymphoid compartments. Attempts at generating regulatory T-cells in vitro have involved stimulation with alloantigen with co-stimulants, such as: i) vitamin D3 and dexamethasone (Barrat et al., *J Exp Med* 195:603-616 (2002)); ii) IL10 (Levings et al., *J Exp Med* 193:1295-302 (2001)); iii) TGF-β (Yamagiwa et al., *J Immunol* 166:7282-7289 (2001)); and iv) IL2 (Taylor et al. *Blood* 99:3493-3499 (2002)). Transfection of T-cell subsets with the gene for FoxP3 in vitro has also endowed regulatory capacity. Fontenot et al., *Nat Immunol* 4:330-336 (2003).

X. Biomimetic Artificial Antigen Presenting Cells

In one embodiment, the present invention contemplates a method comprising fabricating cell-sized, degradable particles (i.e., for example, a synthetic dendritic cell or artificial antigen presenting cell). In one embodiment, the particles release soluble factors. In one embodiment, the particles display surface bound factors in a non-random pattern. In one embodiment, the particles display surface bound factors in a random pattern. In one embodiment, the non-random pattern simulates an immunological synapse. In one embodiment, the synapse comprises a supramolecular activation complex.

In one embodiment, the artificial particle comprises a biodegradable construct thereby providing a controlled release of incorporated and/or attached compounds (i.e., for example, therapeutic agents, antibodies, cytokines, or chemokines). In one embodiment, the particle comprises a degradable polyester including, but not limited to, poly (lactic-co-glycolic) acid (PLGA). PLGA has been used in FDA-approved grafts, sutures, and/or drug delivery microparticulates such as Lupron Depot®. Degradable PLGA polymer microparticles are superior to conventional latex or polystyrene "artificial APCs" because PLGA confers biodegradability. Further, unlike latex and polystyrene polymer particles that only allow surface attachment of proteins, PLGA polymer particles. allow encapsulation of protein cell factors (i.e., for example, IL2, TGF-β, and CCL22) through a double emulsion/solvent evaporation procedure. Odonnell et al., *Advanced Drug Delivery Reviews* 28:25-42 (1997). Further, a controlled release of soluble cell factors from PLGA polymers can be engineered to create an appropriate local concentration of these cell factors, which would be accompanied by cell-to-cell contact with immobilized molecules on the particle surface. Such immobilized molecules (i.e., for example, a monoclonal antibody) can be easily bound to the particle through a streptavidin-biotin linkage using several established chemical techniques. Further, the controlled release of encapsulated one such soluble protein factor for twenty days from microparticles has been demonstrated (i.e., for example, CCL22). See, FIG. 9A. Scanning electron microscopy confirmed the porous nature of the microparticles responsible for the controlled release characteristics. See, FIG. 9B.

Artificial stimulation using randomly coated micron-sized spherical constructs to promote cytotoxic T-cell receptor engagement along with co-stimulation has been used to gain insight into the role of IL2 as a soluble stimulation factor. Mescher et al., *J Immunol* 149:2402-2405 (1992); and Curtsinger et al., *J Immunol Methods* 209:47-57 (1997). When applied to regulatory T-cells, stimulation by randomly coated constructs provided substantial increases in total cell count, but also enhanced suppressive capabilities over non-stimulated naïve CD4+CD25+ cells. Others have shown that randomly coated particles produce expanded and activated regulatory T-cells were polyclonal, and maintained expression of appropriate chemokine receptors. Hoffmann et al., *Blood* 104:895-903 (2004).

Nevertheless, these references using randomly coated particles do not contemplate the specific advantages of creating and using non-randomly patterned artificial presenting cells that specifically mimic biological cell surface protein patterns (i.e., for example, a supramolecular activation complex). In one embodiment, the present invention contemplates fabricating an artificial tolerogenic dendritic cell designed to stimulate a regulatory T-cell. In one embodiment, these cell sized constructs present protein factors capable of both cooperating in TCR engagement and releasing T_{reg} cell co-stimulatory moieties. For example, the constructs are able to contain and controllably release soluble chemokines and proliferation promoting factors. These constructs highly flexible, being capable of modification for both the type, and level, of surface bound and/or soluble releasable stimulating factors. In some embodiments, the use of biodegradable materials will provide uses of these "artificial dendritic cells" for in vivo diagnostics to identify the role of individual T_{reg} cell stimulating factors, and therapeutically to promote allograft tolerance.

A. The Particle Core

In one embodiment, the present invention contemplates a particle comprising CD3 antibodies and CD28 antibodies non-randomly attached to the surface of a poly-lactic-co-glycolic acid (PLGA) particle. In one embodiment, the antibodies are attached by absorption. In one embodiment, the antibodies are attached covalently. In one embodiment, the particles are covalently surface modified with streptavidin. In one embodiment, the particles comprise biotinylated mAb, wherein the mAbs stimulate T_{reg} cells. In one embodiment, the attached antibodies are in a biologically active state. See, FIG. 1.

These biodegradable particles have numerous advantages over T_{reg} cell modulators known in the art, for example: a) particle biodegradability and biocompatibility allow for in vivo investigational and therapeutic use for controlling T_{reg} cells in immune suppression; or 2) such particles are capable of controllably releasing soluble proteins such as cytokines and chemokines from the particle interior while also presenting immobilized protein factors on their surface; and 3) surface-mediated stimulation and release of soluble factors from the same artificial construct closely mimics T_{reg} cell stimulation by biological dendritic cells.

In one embodiment, the particles comprise releasable soluble factors that preferentially activate different T-cell subsets. In one embodiment, the releasable factors are encapsulated within the particles. Although it is not necessary to understand the mechanism of an invention, it is believed that the releasable factors are controllably released subsequent to particle degradation. In one embodiment, the factor comprises a suppressive cytokine (i.e., for example, TGF-β12). In one embodiment, the factor comprises a T_{reg}-preferential cytokine (i.e., for example, CCL-226). These particles behave similarly to tumor tissues that are believed to actively suppress immune responses by selectively secreting TGF-β12 and/or CCL-226 to attract T_{reg}s to their vicinity. Otherwise, a body's normal immune response might otherwise destroy the malignancy. Curiel et al., *Nat Med* 10:942 (2004); and Chen et al., *Proc Natl Acad Sci USA* 102:419 (2005). Although it is not necessary to understand the mechanism of an invention, it is believed that the particles contemplated by the present invention are capable of both delivering the immobilize surface stimulation factors and the secreted soluble stimulation factors to act in a similar manner as tolerogenic biological dendritic cells (i.e., for example, APCs) to influence T_{reg} mediated immunoresponsivity. See, FIG. 2.

B. Artificial Antigen Presenting Cell Surface Protein Patterns

Recent studies have attempted to study IS orientations in vitro using microfabricated, patterned "dots" of CD3 antibody (i.e., providing TCR stimulation) in a field of ICAM adhesion molecules. Doh et al., *Proc Natl Acad Sci USA* 103:5700 (2006). In vitro models have distinct disadvantages, however, because T_{reg} cell stimulation is more pronounced when using a spherical structure as opposed to a flat surface, such as a cell culture dish. Curtsinger et al., *J Immunol Methods* 209: 47 (1997). Further, the in vitro approach provided in Curtsinger et al. does not allow simulation for the study of all three (3) SMAC regions in a mature IS. Especially lacking in Curtsinger et al. is the ability to study biomimetic SMAC regions. Although recent data demonstrate patterns having protein orientations using two (2) factors on a particle surface, a method of precisely orienting a pattern of >2 factors on a particle has yet to be discovered. Chen et al., *Proc Natl Acad Sci USA* 104:11173 (2007).

In one embodiment, the present invention contemplates a method comprising creating a synthetic dendritic cell surface comprising a biomimetic IS pattern. See, FIG. 4. In one embodiment, the IS pattern comprises a cSMAC region. In one embodiment, the IS pattern comprises a pSMAC region. In one embodiment, the IS pattern comprises a dSMAC region. Although it is not necessary to understand the mechanism of an invention, it is believed that this method is analogous to draining water out of a tub filled with bowling balls. After only a fraction of the liquid has drained, the top portion of the ball (having a circular shape, if viewed from above) will have been exposed (thereby making the surface available for a first modification) while the remaining portion is still covered by the water (not available for a first modification). After further draining, an annular ring comprising the first modification and the remaining portion is exposed, thereby making the remaining portion available for labeling with a second factor, thereby creating a second annular ring. This process may be repeated as many times as desired limited only by the size of the ball and the width of the multiple annular rings.

A method for patterning a prototype particle with multiple factor layers was demonstrated using poly-dimethylsiloxane (PDMS). As is known from photolithography techniques, PDMS can be etched by the application of a solvent. This is analogous to partial draining of water from a bathtub full of bowling balls. Using PDMS, a prototype synthetic dendritic cell comprising a "bull's eye" IS was made by surface etching an artificial cSMAC region surrounded by artificial pSMAC region. See. FIG. 4. In one embodiment, the present invention contemplates a method for stimulating T_{reg} cells using "bull's eye" patterned IS-mimetic particles, wherein the stimulation is qualitatively and quantitatively superior to randomly-labeled synthetic dendritic cells.

The above construction of prototype synthetic dendritic cells using dual labels definitively shows preferential labeling in different particle regions. Nonetheless, some factor overlap is present indicated by overlapping fluorescent signals (i.e., a yellow central region indicating the presence of both labels) and some gold-bead labeling in the SEM images outside of the intended central region. These observations may be a result of uneven PDMS etching which may be improved by using techniques that are more easily controllable with respects to speed, distance, and residual matter. For example, a sodium silicate ("liquid-glass") system can provide a more easily controlled etching process by using<5% hydrofluoric acid (HF) evenly applied at a controllable rate of approximately 100 nm per minute resolution (data not shown).

C. Functional Protein Patterns on Synthetic Dendritic Cell Prototypes

The present invention contemplates compositions comprising a fabricated cell-sized, biodegradable particle exhibiting secretable soluble factors and immobilized surface factors displayed in a biomimetic pattern.

In one embodiment, the present invention contemplates a synthetic dendritic cell comprising PLGA microparticles. In one embodiment, the present invention contemplates a method of producing a synthetic dendritic cell comprising encapsulating soluble and/or surface factors into the PLGA microparticles using one of many emulsification and/or encapsulation procedures (i.e., for example, double emulsion). In one embodiment, the synthetic dendritic cells comprise microparticle diameters of approximately 1-1000 μm. In one embodiment, the microparticle diameters are approximately 5-500 μm. In one embodiment, the synthetic dendritic cells comprise microparticle diameters of approxi-

mately 10-50 μm. Lu et al., *J Biomed Mater Res* 50:440 (2000). Encapsulation methods are commonly used to load agents into polymer matrices that are capable of releasing factors by modifying parameters including, but not limited to, copolymer ratios and/or molecular weight. Odonnell et al., *Advanced Drug Delivery Reviews* 28:25 (1997).

Encapsulation loading of synthetic dendritic cells can be easily verified by dissolution techniques using sodium hydroxide and/or dimethylsulfoxide followed by protein detection assays such as bicinchoninic acid (BCA) or enzyme linked immunosorbent assay (ELISA) to detect total protein. Fluorescent surface factor labeling determines which construction method (PDMS vs. "liquid glass") produces the most consistent results. For example, three separate labels can be applied using a bioconjugate strategy, for example: i) a first exposed region may be protected with small-molecule biotinylation; ii) a second exposed region may be protected with an asymmetric disulfide (for subsequent thiol exchange) 20; and iii) a third exposed region may be protected with standard N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC) chemistry.

In one embodiment, the present invention contemplates a method for evaluating three (3) exposed particle regions by fabricating the particles using different formulations. In one embodiment, a formulation comprises cSMAC (CD3 mAb)+pSMAC (ICAM) (2 labels/2 regions) for comparison to formulations created on a flat surface (i.e., for example, an in vitro cell culture dish). In one embodiment, a formulation comprises cSMAC (CD3+CD28 mAbs)+pSMAC (ICAM) (3 labels/2 regions) for comparison with randomly displayed CD3 mAbs and CD28 mAbs particles. In one embodiment, a formulation comprises cSMAC (CD3/CD28)+pSMAC (ICAM)+dSMAC (CD45 mAb) for comparison with a complete synthetic immunological synapse comprising at least three (3) SMACs.

XI. Controlled Release of Compounds from Artificial Particles

Controlled release of compounds from microparticles (i.e., for example, PLGA microparticles) fabricated from the double emulsion procedure provide many advantages as an artificial construct for releasing encapsulated compounds as opposed to more traditional compounds such as latex or styrene based particles. PLGA microparticle advantages include, but are not limited to: i) the double emulsion procedure allows for encapsulation of any number of water soluble activation factors; ii) particle size can be easily adjusted based on known fabrication parameters; iii) PLGA is non-toxic, biodegradable, and has properties which allow it to be tuned to adjust release of the encapsulated agents; and 4) the surface of PLGA particles can be easily labeled using EDC (carbodiimide) chemistry.

In one embodiment, the present invention contemplates a method comprising encapsulating and controllably releasing soluble factors while maintaining relatively stable surface presentation of monoclonal antibody. Although it is not necessary to understand the mechanism of an invention, it is believed that T$_{reg}$ cells are attracted towards an artificial particle, engage surface protein stimulation factors, and receive the proper proliferation and maturation signals from secreted soluble proteins at the same time. In one embodiment, the encapsulating comprises at least one soluble secretable factors, including but not limited to, IL2, TGF-β, and CCL22. These factors may be encapsulated individually or in any combination. In one embodiment, the releasing is controlled by using different molecular weight PLGA or through other fabrication parameters including, but not limited to, drug distribution, occlusion radius, amorphicity/

crytallinity of the polymer, excipients etc. Rothstein et al., *J Materials Chem* 18:1873-1880 (2008). An empirical process determines the final amounts of factors to be encapsulated given that the appropriate quantity of these factors for optimal stimulation of regulatory T-cells in vivo is yet still unknown.

In one embodiment, the particle surface is covalently labeled to determine the number of functionalized sites capable of attaching the immobilized protein factors. In one embodiment, the labeling comprises 2-pyridyldithioethylamine. In covalent labeling of the particle, it would be desirable to allow for positioning of the surface mediated regulatory T-cell factor(s) (i.e., for example, monoclonal antibodies for CD3 or CD28) in a way which does not covalently link the active site. To achieve this, some embodiments conjugate streptavidin to the particle surface. Although it is not necessary to understand the mechanism of an invention, it is believed that at least one of streptavidin's four active binding sites for biotin will be exposed. Consequently, an artificial particle may be labeled using commercially available biotinylated antibodies specific for many commercially available surface molecules. Alternatively, streptavidin may be thiolated (i.e., for example, by using readily available products such as NHS esters of S-acetyl-thioacetic acid or Trauts reagent) and integrated into the 2-pyridyldithioethylamine labeling embodiment described herein.

In some embodiments, biodegradable polymers pre-labeled with biotin may be used in fabrication of artificial particles. For example, poly(lactic acid) (PLA) copolymers with poly(ethylene glycol) (PEG) are reported to be conjugated with biotin. Cannizzaro et al., *Biotechnol Bioeng* 58:529-535 (1998). Microparticles have been made from these polymers have been reported to produce microparticles having surface binding sites using emulsion/solvent-evaporation techniques. Further, the microparticle surface was successfully labeled using an excess of neutravidin followed by biotinylated monoclonal antibodies and proteins. Sakhalkar et al., *Proc Natl Acad Sci USA* 100:15895-15900 (2003).

XII. Release Characteristics of CCL22

The data presented herein demonstrates that a sustained release of CCL22 was achieved by loading the chemokine into degradable poly(lactic-co-glycolic) acid (PLGA)-based microparticles (CCL22MP) using a water-oil-water double emulsion-evaporation technique. Zhao et al., *Biomaterials* 26:5048-5063 (2005). Scanning electron micrographs (SEM) of intact microparticles indicates that these microparticles are spherical and slightly porous. FIG. **15A**. SEM particle cross-sections also show a plurality of internal structures formed during creation in a water-in-oil primary emulsion. FIG. **15B**. The surface of a microparticle comprising CCL22 (CCL22MP) was specifically formulated to be porous, to allow continuous release of chemokine (i.e., for example, release occurring without intermittent periods of lag). Rothstein et al., *J. Mater. Chem.* 18:1873-1880 (2008); Rothstein et al., *Biomaterials* 30:1657-1664 (2009). For example, release experiments performed in phosphate-buffered saline demonstrate a constant release of CCL22 for over 3 weeks. See, FIG. **16**. Further, the microparticles were designed to be large enough (i.e., for example, >10 μm) to avoid their uptake by phagocytic cells and to prohibit their movement through capillaries, with consequent immobilization at the site of injection. FIG. **17**.

Simultaneously, the ability of CCL22 to attract murine T cells was examined. Expression of chemokine receptors for CCL22 (i.e., for example, the CCR4 receptor) are known to

control the ability of cells to respond to CCL22 gradients. Moreover, there is evidence that only activated T cells express the CCR4 receptor. Baatar et al., *Journal of Immunology* 178: 4891-4900 (2007); Lim et al., *Journal of Immunology* 177:840-851 (2006); and Lee et al., *Journal of Experimental Medicine* 201:1037-1044 (2005); and FIG. **18**A. The data presented herein show that activated T cells (both regulatory and effector cell populations), but not naïve T cells, migrated towards a CCL22 gradient in vitro using a transwell chamber-based assay, a response that is consistent with their surface receptor expression patterns. FIG. **18**B.

Having confirmed that activated Tregs migrate toward a CCL22 gradient and that controlled release formulations could be used to sustain the release of CCL22, the capability of CCL22MPs to attract Tregs in vivo was tested. For example, CCL22-releasing polymeric particles were injected into the triceps surae of FVB mice followed by i.v. infusion of ex vivo activated, alloantigen-specific Tregs (AATregs) that constitutively expressed the luciferase gene. Following an activation stimulus (i.e., for example, an allogeneic-dendritic cell injection) the migration pattern of AATregs could be monitored by non-invasive live animal imaging. FIG. **19**A. The data demonstrate that immediately after allogeneic-dendritic cell stimulation (i.e., for example, approximately 4-7 days after injection) a significantly greater number of AATregs were recruited to the site of the CCL22MP injection when compared to an internal control of microparticles lacking CCL22 (BlankMP). FIG. **19**B and FIG. **20**. Further, upon analyzing the kinetics of migration, it was determined that cellular localization was transient and that within 7 days of the stimulation event, no significant numbers of AATreg persisted at the site of particle injections. See, FIG. **19**C.

One explanation of the data is that the decline in AATreg numbers may be due to the absence of stimulatory/inflammatory signals at the site of CCL22MP injection at that time. Presumably, the presence of Treg can be extended in a stimulatory microenvironment, in combination with other survival factors. The potential therapeutic implications of a degradable controlled release formulation capable of recruiting Tregs are manifold. For example, CCL22MP may be used in combination with an infusion of cells expanded ex-vivo. Current pre-clinical data suggest that infusion of freshly-isolated or ex vivo-expanded Tregs can be used to prevent rejection of organ transplants and to suppress autoimmune diseases, but challenges such as obtaining adequate numbers and highly purified populations of Tregs have hindered progress into clinical trials. Brusko et al., *Immunological Reviews* 223:371-390 (2008); and Riley et al., *Immunity* 30:656-665 (2009). Given that the present data suggest that Tregs can be attracted to a local site using CCL22, it may be possible to lower the numbers of injected cells, or potentially, to use populations with lower purity. In one embodiment, the present invention contemplates a method comprising a microparticle population encapsulating CCL22, wherein a controlled release of CCL22 from the microparticle improves tissue transplantation success (i.e., for example, murine pancreatic islet cell transplantation). In one embodiment, the released CCL22 attracts Tregs to the pancreatic islet cells Zhang et al., *Immunity* 30:458-469 (2009).

Another use of CCL22-containing polymeric microparticles would be to attract naturally occurring Treg populations. If the numbers of recruited Tregs prove sufficient to control adverse immune responses at the site of particle injection, then therapeutic effects could be realized without infusion of ex vivo-expanded Tregs. This hypothesis has been reported within the context of a murine model of periodontitis, a disease that is associated with dissipation of Tregs from the gingival tissues and loss of immune homeostasis. Garlet et al., *Microbes and Infection* 7:738-747 (2005). The data presented herein indicates that administration of CCL22MP leads to local recruitment of FoxP3+ cells to the gingival tissues and corresponding reversal of adverse outcomes associated with periodontitis (infra). Conventional methods of CCL22-based sustained release have disadvantages in that this particular chemokine is known to attract both activated Tregs and activated effector T cells. FIG. **18**; and Bromley et al., *Nature Immunology* 9:970-980 (2008); Thus, in some cases it is quite possible that the numbers of recruited Tregs may be insufficient to control the immune response.

However, CCL22-based release formulations can easily be modified to simultaneously release immunosuppressive agents that can suppress the functions of activated effector T cells in situ, thereby assisting the Tregs in controlling adverse immune responses. Additionally, although CCL22 can attract both activated Tregs and effector T cells in vitro, studies in vivo suggest that CCL22 production and release associated with tumors or long-surviving allografts results in local immunosuppression. Curiel et al., *Nature Medicine* 10:942-949 (2004); and Lee et al., *Journal of Experimental Medicine* 201:1037-1044 (2005), respectively. Although it is not necessary to understand the mechanism of an invention, it is believed that this CCL22-associated effect may be due to an enriched population of recruitable Tregs in the periphery when compared to activated effector T cells, or where equivalent numbers of both populations are recruited and the suppressive effect of Tregs is dominant.

In conclusion, the local attraction of Tregs in vivo using chemokine-loaded sustained delivery vehicles has been demonstrated. Controlled release microparticle formulations as contemplated herein are particularly useful as a modular platform for therapeutic development as well as a tool to study Treg-dependent modulation of immune responses in situ.

XIII. CCL22 Microparticle Therapy

To verify that Tregs are recruited toward microparticles that sustain release of CCL-22, formulations were administered into the periodontium of diseased mice. Briefly, mice were orally exposed to the periodontal pathogen *A. actinomycetemcomitans* (Aar) on day 0, and also received CCL-22 microparticles (CCL-22 MPs) or blank (empty) microparticles (BlankMPs) in their periodontal pockets. Specifically, mice received microparticle injections at day –1, 10 and 20, mice treated with VIP injections received systemic injections at days –1, 7, 14, 21, 28. Untreated mice served as negative controls. At day 30, all mice were sacrificed and maxilla's were resected. Real-time polymerase chain reaction (PCR) analysis provided strong evidence that our formulations recruit Tregs to a greater extent than other groups as detected by greater expression of FoxP3. FIG. **22**A. This same treatment group also showed statistically greater expression of the pro-regenerative cytokine IL-10. Figure BIB. Further, a decreased expression of bone resorbing cell activator RANKL was observed. FIG. **22**C. Interestingly, systemic VIP injections induced endogenous production of CCL-22 presumably leading to Treg recruitment, and the disease symptom attenuation as demonstrated below.

To confirm that Treg recruiting formulations attenuate periodontal disease symptoms, levels of alveolar bone resorption was examined in experimental mouse periodontitis. G. P. Garlet et al., Clin Exp Immunol 147, 128 (January, 2007); D. T. Graves, D. Fine, Y. T. Tang, T. E. Van Dyke, G.

Hajishengallis, J Clin Periodontol 35, 89 (February, 2008); and J. J. Yu et al., Blood 109, 3794 (2007). To this end, B6 mice (n=6) were infected orally with the periodontal pathogen *A. actinomycetemcomitans* (Aa) and treated with microparticles containing CCL-22 (CCL-22 MPs) or empty microparticles (BlankMPs) according to the schedule outlined above. Additionally vasoactive intestinal peptide (VIP) was administered intraperitoneally. Mice receiving only PBS and the thickener carboxymethyl cellulose served as controls (Untreated). Experimental treatments were administered in the periodontal pocket of the right maxilla while left maxillae were used as internal negative controls in each mouse. Alveolar bone loss was quantified as the area between the cementoenamel junction (CEJ) and the alveolar bone crest (ABC) FIG. **23**, CEJ and FIG. **23A**, ABC). Alveolar bone levels are shown on the left (internal control, FIG. **23A** LEFT) and right (BlankMP treated, FIG. **23A** RIGHT) maxilla. Both the internal control and BlankMP treated maxilla (FIG. **23A**, both) exhibited significant alveolar bone loss. However, FIG. **23** RIGHT depicts a maxilla treated with CCL-22 MPs, revealing significantly reduced alveolar bone loss, both compared to the internal control maxilla (FIG. **28** LEFT) and the BlankMP treated maxilla (FIG. **23A** RIGHT). Quantification of total bone loss (area in mm2) reveals that CCL-22 MP as well as VIP (systemic IP injections) treatments led to significantly less alveolar bone resorption than BlankMPs. FIG. **23C**.

IVX. Clinical Applications

A. Immunosuppressant Therapy Substitution for Tissue/Graft Transplants

The development of new immunosuppressive agents has been the general direction in efforts to improve the transplantation success rate. For example, the use of agents which inhibit the production of lymphocyte proliferation factors have extended the half life of pediatric heart transplants. Pietra et al., *Prog Pediatr Cardiol* 11:115-129 (2000). Presently, research is underway in efforts to further increase pediatric heart half-lives using agents which inhibit the de novo synthesis of nucleotides and block co-stimulation of T-cells.

Presently available immunosuppressant drugs act to abrogate acute rejection of tissue transplants. Tissue transplant rejections are usually characterized by recognition of the foreign donor antigen by the adaptive immune system and the subsequent expansion of lymphocytes which attack the transplanted tissue. Symptoms which accompany acute rejection (including, but not limited to, pain at the transplantation site or fever-like symptoms) are more frequent and severe immediately following transplantation, but commonly recur 6-12 months subsequent to the procedure. Persistent episodes of acute rejection are thought to lead to a state of chronic rejection coupled with a failure of many immunosuppressant drugs. The most viable option following the onset of chronic tissue rejection is re-transplantation, which is undesirable from both the standpoint of allograft availability and the increased difficulty of the repeated operation.

A major disadvantage of immunosuppressant therapy is that in order to avoid both episodes of acute rejection and the initiation of chronic rejection, immunosuppressant drugs must be administered over the entire life of the transplant recipient. A further disadvantage of immunosuppressant therapy is the frequent incidence of side effects. For example, in the case of cyclosporine treatment (a commonly used agent which decreases the production of interleukin 2, or IL2), patients have been known to experience tremors, headaches, blurry vision, high blood pressure, and inhibited

kidney function. Other commonly used immunosuppressants also cause frequent side effects, which range from growth retardation to behavioral instability to neurotoxicity. Besides these drug specific side effects, the consequences of long term immunosuppression in general can be profound including, but not limited to, increases in the risk of infection, heart disease, diabetes, and cancer.

While a combined administration of the immunosuppressant mycophenolate mofetil along with Vitamin D3 has been reported to generate dendritic cells with a tolerogenic phenotype resulting in increased numbers of regulatory T-cells in mice, mycophenolate mofetil suffers from the same adverse side effects as the immunosuppressants mentioned above and has also been implicated in causing birth defects in animals. Gregori et al., *J Immunol* 167:1945-1953 (2001). Furthermore, immunosuppressants can cause inhibition of regulatory T-cells as well as the T-cells these therapies intend to impede. Li et al., *Nat Med* 5:1298-1302 (1999). Due to the potential of these tolerogenic cells, it is important that a greater understanding of the factors involved in the expansion and employment of T_{reg} cells in vivo is developed.

The present invention contemplates alternatives to extended immune system down-regulation provided by immunosuppressant therapy. Such alternatives are expected to provide a dramatic improvement over the state-of-the-art in the clinic. For example, the most desirable of these alternatives would render the patient's immune system in a state of complete, permanent tolerance to a specific foreign body (i.e., for example, a single antigen and/or antigen complex). Once this complete and permanent tolerance is attained, no further treatment is necessary to prevent transplant-related adverse reactions. Furthermore, the recipient's immune system would otherwise function normally, being fully capable of fighting pathogens and/or stopping the progression of tumor cells. Although it is not necessary to understand the mechanism of an invention, it is believed that infants up to fourteen (14) months old innately have tissue tolerance due to the lack of soluble and/or surface rejection factors which mediate immediate rejection of the transplant. As the infant's immune system matures, however, this innate tissue tolerance is lost. Fan et al., *Nat Med* 10, 1227-1233 (2004).

B. Implant Tolerance

Numerous polymeric biomaterials and metal materials are implanted each year in human bodies. Among them, drug delivery devices provide local therapeutic effect for diseases which lack efficient treatments. Controlled release systems are in direct and sustained contact with the tissues, and some of them degrade in situ. Thus, both the material itself and its degradation products must be devoid of toxicity. The knowledge and understanding of the criteria and mechanisms determining the biocompatibility of biomaterials are therefore of great importance.

The classical tissue response to a foreign material leads to the encapsulation of the implant, which may impair the drug diffusion in the surrounding tissue and/or cause implant failure. This tissue response depends on different factors, especially on the implantation site. Indeed, several organs possess a particular immunological status, which may reduce the inflammatory and immune reactions. Among them, the central nervous system is of particular interest, since many pathologies still need curative treatments.

In one embodiment, the present invention contemplates a method of inducing implant tolerance by the administration of artificial antigen presenting cells as described herein.

C. Tissue Healing

In one embodiment, the present invention contemplates using artificial microparticles comprising soluble and surface bound factors to induce tissue healing states including, but not limited to bone healing, wound healing, and disease-induced injury tissue restoration.

1. Bone Healing

Over the past several decades, those having ordinary skill in the art have been attempting to understand the interactions between the cells that mediate the process of bone remodeling and healing. It is believed that the cells that mediate bone formation (i.e., for example, osteoblasts) may be capable of stimulating osteoclast cells. Osteoclast cells have been reported to resorb and remodel bone. Mundy G. R., *Bone* 24(5 Suppl):35S-38S (1999). Osteoclasts, derived from a hemopoietic monocyte cell lineage (e.g. in the same cell lineage as dendritic cells and macrophages), resorb bone using proton and enzymatic secretion. Teitelbaum S. L., *Science* 289; 1504-1508. It is generally believed that approximately 10-15% of human bone is perpetually undergoing remodeling in order for our mineralized tissue to grow and adapt to stress. However, the current evidence suggests that when the osteoblast-osteoclast communication pathway breaks down, osteosclerotic symptoms may occur including, but not limited to, fractures, severe infections, blindness, deafness, deformities, or stroke.

Signaling factors (i.e., for example, soluble signaling factors and surface-bound signaling factors) have been consistently implicated as intermediaries between osteoblasts and osteoclasts. Mundy G. R., *Bone* 24(5 Suppl):35S-38S (1999). See, FIG. **13**A. In one embodiment, the present invention contemplates a composition comprising an antibody-ligated OSCAR, wherein the antibody/OSCAR composition serves as a co-stimulus with the signaling factors. Merck et al., *Journal of Immunology* 176(5):3149-3156 (2006). Although it is not necessary to understand the mechanism of an invention, it is believed that the antibody/OSCAR composition may be necessary but not sufficient for osteoclast activation alongside other surface-bound signals (i.e., for example, RANK-L) and soluble signaling factor MCSF27.

Conversely, it is commonly known that secretion of osteoprotegrin (OPN) (a molecule that inhibits RANK-L signaling) can diminish osteoclastic activity. Udagawa et al., *Endocrinology* 141:3478-3484 (2000); and Itoh et al., *Endocrinology* 142:3656-3662 (2001). As dendritic cells may undergo suppression via apoptosis/anergy (supra), a similar mechanism downregulating the osteoblast-osteoclast interaction could be application to diseases where the degree of osteoclast resorption exceeds that of osteoblast healing including, but not limited to, osteoporosis or osteopenia.

Finally, has been reported that a balance between bone resorption and healing may be mediated not only by communication of osteoblasts to osteoclasts, but also by communication of osteoclasts to osteoblasts. Martin et al., *Trends in Molecular Medicine* 11: 76-81 (2005). This crosstalk is commonly referred to as "osteo-coupling". Although bone resorption processes are believed to release soluble factors embedded in bone matrix that can recruit and differentiate osteoblastic precursors, some studies suggest that osteoclasts still strongly stimulate osteoblasts in the absence of resorption. Although it is not necessary to understand the mechanism of an invention, it is believed that several soluble signaling factors and several surface-bound signaling factors are likely to be involved.

In one embodiment, the present invention contemplates an artificial biodegradable osteoblast cell that directly and

indirectly interacts with an osteoclast cell. In one embodiment, the artificial osteoblast cell mimics osteo-coupling signaling that results in the modulation of bone healing. In one embodiment, the artificial osteoblast cell acts as a biomimetic compound by providing surface bound RANK-L signaling factors, anti-OSCAR antibodies, and a soluble signaling factor (i.e., for example, MIM-1 and/or MCSF-27). Although it is not necessary to understand the mechanism of an invention, it is believed that the artificial osteoblast cell is capable of stimulating osteoclast cells to a greater degree than the "unorganized" forms because of a patterned organization of the signaling factors. In other embodiments, a surface-bound signaling factor comprises osteoclastic differentiation factor (ODF). ODF has been reported to act as a co-stimulator with RANK-L. Suda et al., Endocrinology Rev. 13:66-80 (1992). In other embodiments, OPN is used to suppress osteoclasts. Udagawa et al., *Endocrinology* 141:3478-3484 (2000).

In some embodiments, osteo coupling may involve a variety of signaling factors. In one embodiment, a soluble signaling factor comprises MIM1, a chemokine specific to osteoblast precursors. Falany et al., *Biochemical and Biophysical Research Communications* 281(1):180-1805 (2001). In one embodiment, a surface-bound signaling factor comprises EphrinB2, that appears to stimulate osteogeneic differentiation. Zhao, et al., *Cell Metabolism* 4:111-121 (2006). In one embodiment, a surface-bound signaling factor comprises TGF-13, believed to stimulate osteoblasts. Pfeilschifter et al., *Proceedings of the National Academy of Sciences* 84:2024-2028 (1987). Although it is not necessary to understand the mechanism of an invention, it is believed that cell-sized biodegradable artificial osteoblast cells displaying surface bound signaling factors (i.e., for example, RANK-L, ODF, EphrinB2 and/or TGF-β) on a polymer surface and controllably releasing a soluble signaling factor (i.e., for example, MIM1 and/or MSCF-27) might act, in combination, as "a synthetic bone surface/synthetic osteoclast" thereby stimulating osteoblasts and/or osteoprecursors to a greater degree than the "unorganized" forms of these factors (i.e., for example, by independent local and/or systemic administration). See, FIGS. **14**A and **14**B.

In one embodiment, the present invention contemplates a method of making a functional artificial osteoblast. In one embodiment, the present invention contemplates a method of making a function artificial osteoclast. In one embodiment, the method comprises emulsifying a polymer with a soluble signaling factor to produce a controlled release microparticle. In one embodiment, the controlled release microparticle comprises surface bound signaling factors. In one embodiment, an artificial osteoblast microparticle comprises a soluble MSCF and surface bound factors comprising RANK-L, αOSCAR, and/or EPF. See, FIGS. **14**A and **14**B. In one embodiment, an artificial osteoclast microparticle comprises a soluble MIM1 and surface bound factors EphB4 and/or TGF-β. See, FIGS. **15**A and **15**B. Although it is not necessary to understand the mechanism of an invention, it is believed that the encapsulation and release of these agents, and even the ability to precisely pre-program the profile of release may be measured and/or predicted using mathematical modeling of controlled delivery and variation of the microstructure and chemical structure of the particles.

In one embodiment, the present invention contemplates a method for examining combinations of soluble factors (i.e., for example secreted) and surface-bound factors (i.e., for example, immobilized) in vitro using known differentiation markers and functional assays for osteoblast and osteoclast stimulation. In one embodiment, the method measures the

cellular effects via standard co-culture assays. Takahashi et al., *Endocrinology* 123: 2600-2601 (1988); and Suda et al., *Endocrinology* 128:1792-1796 (1991). In one embodiment, the method uses assays for osteoblast activity and osteoprecursor differentiation including staining for alkaline phosphatase, Von-Kossa, and flow cytometric analysis. In one embodiment, the method uses assays for calcium phosphate pit-formation and TRAP staining.

In one embodiment, the present invention contemplates a method for in vivo osteo-coupling using artificial osteoblast and/or osteoclast cells. In one embodiment, the method utilizes a subcutaneous, mouse craniofacial model. In one embodiment, the model determines the effects of combinations of multiple soluble and surface-bound factors by intradermal and/or subcutaneous injections of artificial osteoblast and/or osteoclast cells can be made right under the skin, wherein the cells result in contacting a bone surface. Although it is not necessary to understand the mechanism of an invention, it is believed that these artificial cells can be made large enough as to not move from the site of injection. In one embodiment, the effect of these various artificial cell formulations in vivo are determined using software capable of quantifying bone density and lacunae (resorption) formation in histological sections of the craniofacial bone tissue (BioQuant, Osteoimage).

2. Tissue Healing

Periodontal disease, or periodontitis, is characterized by destructive inflammation of the periodontium (i.e., for example, gum tissue, supporting bone, and/or ligaments) and is considered the most pressing oral health concern today. Importantly, this disease affects not only tooth loss, but also the incidence of cardiovascular disease, kidney disease, respiratory diseases, diabetes, and even premature childbirth. Seymour, G. J., Ford, P. J., Cullinan, M. P., Leishman, S. & Yamazaki, K. Relationship between periodontal infections and systemic disease. Clin Microbiol Infect 13 Suppl 4, 3-10 (2007); Fisher, M. A. et al. Periodontal disease and other nontraditional risk factors for CKD. Am J Kidney Dis 51, 45-52 (2008); Boggess, K. A., Beck, J. D., Murtha, A. P., Moss, K. & Offenbacher, S. Maternal periodontal disease in early pregnancy and risk for a small-for-gestational-age infant. Am J Obstet Gynecol 194, 1316-1322 (2006); Backes, J. M., Howard, P. A. & Moriarty, P. M. Role of C-reactive protein in cardiovascular disease. Ann Pharmacother 38, 110-118 (2004); and Offenbacher, S. & Beck, J. D. A perspective on the potential cardioprotective benefits of periodontal therapy. Am Heart J 149, 950-954 (2005). Periodontal disease is strikingly prevalent in the United States, affecting 34% of individuals over the age of 30, or an estimated 78 million Americans. Garlet, G. P. et al. Regulatory T cells attenuate experimental periodontitis progression in mice. Journal of Clinical Periodontology 37, 591-600. It is the number-one cause of tooth loss according to the American Dental Association. Beyond the US, periodontal disease is estimated to affect up to 20% of the adult population worldwide. Furthermore, the periodontal biofilm hosts a wide variety of potentially hazardous bacterial species that can lead to systemic infections and inflammatory immune diseases. The most predominant periodontal pathogens, *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Tannerella forsythia* and *Fusobacterium nucleatum*, present virulence factors that have been associated with: 1) systemic infections and complications; 2) a 4-fold increase in premature births, 3) anorexia-cachexia syndrome, 4) atherosclerosis, and 5) myocardial infarction and ischemic stroke. As the correlations between periodontitis and the incidence of these other conditions

continue to be elucidated, reducing the prevalence of periodontal disease is a significant current medical problem and must be solved. The continuing prevalence of periodontal disease is likely perpetuated by a misunderstanding of the disease etiology consonant with current therapies that are aimed at removal of these remarkably common bacterial species. The current standard of care involves the debridement of calculus and plaque, often accompanied by local delivery of an antibiotic such as minocycline (Arestin®). These conventional treatments temporarily kill pathogens, but do not protect against inevitable future infections nor address the sensitivity that is observed in patients that are disposed to immune dysfunction. Although invasive bacterial species are protagonists of the disease, tissue destruction is mediated by an adverse host inflammatory immune response. Baker, P. J. The role of immune responses in bone loss during periodontal disease. Microbes and Infection 2, 1181-1192 (2000); Graves, D. T., Fine, D., Teng, Y. T., Van Dyke, T. E. & Hajishengallis, G. The use of rodent models to investigate host-bacteria interactions related to periodontal diseases. J Clin Periodontol 35, 89-105 (2008).

In some embodiments, the present invention contemplates a method for treating inflammation caused by periodontal disease. Although it is not necessary to understand the mechanism of an invention, it is believed that as the disease progresses, several populations of lymphocytes are recruited to the periodontium, guided by local gradients of specific lymphocyte-attracting chemokines. It is further believed that the overall cytokine milieu produced by these specific populations of lymphocytes that ultimately directs the expression of factors that promote hard and soft tissue destruction.

For example, in some embodiments an artificial antigen presenting cell provides a therapy for inflammation resulting from periodontal gum disease. Periodontal disease is strikingly prevalent in the United States (34% of individuals over age 30 or an estimated 78 million Americans). Eke et al., "CDC Periodontal Disease Surveillance Project: background, objectives, and progress report" *J Periodontol* 78:1366-1371 (2007). Periodontal disease is a cause of tooth loss according to the American Dental Association and is a current oral health concern. It is believed that periodontal disease not only affects tooth loss but also contributes to the incidence of cardiovascular disease, diabetes, and respiratory disease such as pneumonia. Furthermore, women who have periodontal disease are over 4 times more likely to give birth to a child prematurely. Boggess et al., "Maternal periodontal disease in early pregnancy and risk for a small-for-gestational-age infant" *Am J Obstet Gynecol* 194:1316-1322 (2006). Clearly, finding a solution to the problem of periodontal disease is needed in the art. In: Pennsylvania Department of Health report: "Status of Oral health in Pennsylvania" (2002).

The physiological events associated with periodontal disease are well characterized but the pathologic mechanisms of this disease are unknown. It is believed that periodontal disease affects the composition and integrity of periodontal structures at the dento-gingival junction, alveolar bone, cementum and periodontal ligament. Further it is believed that periodontal disease causes the destruction of connective tissue matrix and cells, loss of fibrous attachment, and resorption of alveolar bone. FIG. 11. Although it is not necessary to understand the mechanism of an invention, it is believed that tissue destruction in periodontal patients is thought to be a result of a complex inflammatory and immune response initiated and perpetuated by gram-negative anaerobic rods and spirochetes. Previous work has

suggested that B cells and T cells accumulate in large numbers in the periodontal tissues although much about their functions in the disease process is not clearly understood.

Current therapies for periodontal disease focus upon reducing or controlling bacterial infection in the gums. Traditional, mechanical debridement techniques temporarily remove the accumulated bacterial plaque responsible for inflammation. One treatment strategy provides a controlled release of the antibiotic minicycline from PLGA microspheres that are placed in the periodontal pocket (Arestin®, OraPharma). Although both of these techniques may temporarily reduce inflammation associated with bacteria, they do not intend to treat the susceptibility of the area to inflammation and the reformation of pockets that cultivate further infection leading to re-establishment of disease. Since periodontal tissue destruction is initiated and exacerbated by inflammatory host response, there has been an increased focus toward understanding the cellular immune responses in the periodontal space in order to treat the source of the physiological response.

Recently, several groups have discovered evidence that the cause of periodontal disease may related to the regulation of inflammation in the local tissues. Cardoso et al., "Characterization of CD4+CD25+ natural regulatory T cells in the inflammatory infiltrate of human chronic periodontitis" *J Leukoc Biol* (2008); and Ernst et al., "Diminished forkhead box P3/CD25 double-positive T regulatory cells are associated with the increased nuclear factor-kappaB ligand (RANK-L+) T cells in bone resorption lesion of periodontal disease" *Clin Exp Immunol* 148:271-280 (2007). It was reported that cytokines which may inhibit and/or regulate inflammation (i.e., for example, IL10) are substantially diminished in tissues with periodontal disease. Although it is not necessary to understand the mechanism of an invention, it is believed that the absence of these anti-inflammatory cytokines not only result in increased inflammation, but also produces a cascade leading to the production of RANK-L. Although it is not necessary to understand the mechanism of an invention, it is believed that RANK-L comprises a factor that differentiates monocyte precursors (i.e., for example, osteoblasts) into bone-resorbing cells called osteoclasts.

The regulation of anti-inflammatory cytokines and more generally, the regulation of the resultant harmful autoimmune responses, may be mediated by immunosuppressive T-cells (i.e., for example, T_{reg} cells). Tang et al., "The Foxp3+ regulatory T cell: a jack of all trades, master of regulation" *Nat Immunol* 9:239-244 (2008). For example, it has been reported that while markers for T_{reg} cells were present in healthy tissues, they were absent in diseased periodontal tissues. Further, it has been reported that patients with rheumatoid arthritis (predisposition for autoimmune regulation breakdown) have 8-fold increased odds of developing periodontal disease. Pischon et al., "Association among rheumatoid arthritis, oral hygiene, and periodontitis" *J Periodontol* 79:979-986 (2008).

As discussed above, periodontal disease is currently treated with strategies focused only on the removal of invasive bacterial species. Specifically, the clinical procedure called scaling and root planing involves the mechanical removal of plaque and bacteria from beneath the gingiva (i.e., for example, debridement), and is typically performed by a periodontal specialist. In severe cases, antibiotic treatments in the form of controlled release microparticles may be injected into the periodontal pocket (i.e., for example, Arrestin®, PLGA microparticles encapsulating and control-

lably releasing the antibiotic minocycline for 21 days). Williams, R. C. et al. Treatment of periodontitis by local administration of minocycline microspheres: A controlled trial. Journal of Periodontology 72, 1535-1544 (2001). This treatment approach is insufficient because, antibiotic treatments only temporarily removes the bacterial species and recurrent infections are common, requiring patients to repetitively undergo these expensive procedures. While such antibiotic treatments temporarily remove the bacterial insult, they do not address the sensitivity seen in patients that are susceptible to such immune-mediated tissue destruction. Furthermore, antibiotic treatment and/or scaling is completely ineffective in approximately 20% of the population and may induce antibiotic resistance, thereby limiting patient options for future treatment.

Recent periodontal disease research has investigated the inflammatory immune reaction itself. For example, one recent experimental treatment aimed at reducing the host inflammatory response involves the administration of the drug Resolvin®. Resolvin® blocks neutrophil-mediated inflammation and the associated pro-inflammatory cytokine milieu. Hasturk, H. et al. Resolvin E1 regulates inflammation at the cellular and tissue level and restores tissue homeostasis in vivo. J Immunol 179, 7021-7029 (2007). However, it has been shown that direct, long-term, inhibition of inflammatory cytokines by traditional blocking strategies (i.e., for example, anti-inflammatory compounds) can compromise periodontal tissue healing. Gurgel, B. C. et al. Selective COX-2 inhibitor reduces bone healing in bone defects. Brazilian oral research 19, 312-316 (2005); Ribeiro, F. V. et al. Selective cyclooxygenase-2 inhibitor may impair bone healing around titanium implants in rats. Journal of Periodontology 77, 1731-1735 (2006); Simon, A. M., Manigrasso, M. B. & O'Connor, J. P. Cyclo-oxygenase 2 function is essential for bone fracture healing. Journal of Bone and Mineral Research 17, 963-976 (2002); Vuolteenaho, K., Moilanen, T. & Moilanen, E. Non-steroidal anti-inflammatory drugs, cyclooxygenase-2 and the bone healing process. Basic and Clinical Pharmacology and Toxicology 102, 10-14 (2008); Zhang, X., Kohli, M., Zhou, Q., Graves, D. T. & Amar, S. Short- and long-term effects of IL-1 and TNF antagonists on periodontal wound healing. Journal of Immunology 173, 3514-3523 (2004). Further, it has also been shown that traditional blocking of the protective immune responses actually results in increased bacterial burden and acute systemic reactions. Garlet, G. P. et al. The essential role of IFN-gamma in the control of lethal *Aggregatibacter actinomycetemcomitans* infection in mice. Microbes Infect 10, 489-496 (2008); and Garlet, G. P. et al. The dual role of p55 tumour necrosis factor-α receptor in *Actinobacillus actinomycetemcomitans*-induced experimental periodontitis: Host protection and tissue destruction. Clinical and Experimental Immunology 147, 128-138 (2007). Consequently, an untested strategy toward disease resolution may not require complete blocking of the protective immune responses and would clearly be a more desirable approach.

Notably, harmful inflammatory responses are not regulated by blocking leukocyte infiltration, but rather by balancing inflammatory leukocyte recruitment with regulatory lymphocyte recruitment. Vignali, D. A. A., Collison, L. W. & Workman, C. J. How regulatory T cells work. Nature Reviews Immunology 8, 523-532 (2008); and Campanelli, A. P. et al. CD4+CD25+ T cells in skin lesions of patients with cutaneous leishmaniasis exhibit phenotypic and functional characteristics of natural regulatory T cells. J Infect Dis 193, 1313-1322 (2006). Regulatory T-cells (Treg) may exert their control over other lymphocytes both by several

mechanisms including but not limited to secreting factors and through direct cell-cell interactions, ultimately leading to targeted inflammatory-immune cell arrest. However, progress has been made toward utilizing Treg therapeutically for a wide variety of autoimmune and inflammatory diseases. Adoptive (Treg) cell transfer (i.e. cell infusion therapies) have seen the most pre-clinical success. Yet clinical translation of the complicated ex vivo cellular expansion protocols has proven difficult. Riley, J. L., June, C. H. & Blazar, B. R. Human T Regulatory Cell Therapy: Take a Billion or So and Call Me in the Morning. Immunity 30, 656-665 (2009). One may speculate that periodontal disease treatment would have the best clinical impact with an off-the-shelf therapeutic that improves the body's natural mechanisms for immune regulation (recruitment/activation of endogenous Treg) in attempt to restore local immune homeostasis and balance.

Naturally, immune cells are recruited to peripheral sites via chemokines secreted by tissue resident cells. Specifically, biological gradients of chemokines direct immune cells toward the origin of secretion (i.e., for example, at an infection site). For example, one way in which tumors appear to avoid immune surveillance and clearance is through the recruitment of regulatory T cells. Specifically, tumors produce and sustain a biological gradient of CCL22 (a Treg-associated chemokine) that directs Treg migration. Dutzan, N., Gamonal, J., Silva, A., Sanz, M. & Vernal, R. Over-expression of forkhead box P3 and its association with receptor activator of nuclear factor-s B ligand, interleukin (IL)-17, IL-10 and transforming growth factor-β during the progression of chronic periodontitis. Journal of Clinical Periodontology 36, 396-403 (2009); Sather, B. D. et al. Altering the distribution of Foxp3+ regulatory T cells results in tissue-specific inflammatory disease. Journal of Experimental Medicine 204, 1335-1347 (2007); Garlet, G. P., Avila-Campos, M. J., Milanezi, C. M., Ferreira, B. R. & Silva, J. S. *Actinobacillus* actinomycetemcomitans-induced periodontal disease in mice: patterns of cytokine, chemokine, and chemokine receptor expression and leukocyte migration. Microbes Infect 7, 738-747 (2005). Once co-localized, Treg suppress effector immune cells by secreting factors such as IL-10 and TGF-β, thereby establishing immunological homeostasis in an milieu that would otherwise present itself as highly inflammatory. Rabinovich, G. A., Gabrilovich, D. & Sotomayor, E. M. in Annual Review of Immunology, Vol. 25 267-296 (2007).

Although it is not necessary to understand the mechanism of an invention, it is believed that engineering principles may result in the production of controlled release systems that can produce a stable biological gradient of certain chemokines (i.e., for example, CCL22). These microparticles can be fabricated using an FDA-approved polyester and will degrade in a well characterized manner in vivo. Rothstein, S. N., Federspiel, W. J., Little, S. R. A unified mathematical model for the prediction of controlled release from surface and bulk eroding polymer matrices. Biomaterials (2009); Garlet, G. P. et al. The dual role of p55 tumour necrosis factor-alpha receptor in *Actinobacillus actinomycetemcomitans*-induced experimental periodontitis: host protection and tissue destruction. Clin Exp Immunol 147, 128-138 (2007). Theoretically, by mimicking the natural immune-evasion mechanisms of tumors using rationally-designed, CCL22-releasing, polymeric microparticles, Tregs may be recruited to a site of destructive inflammation (i.e., for example, a diseased periodontium).

In one embodiment, the present invention contemplates a method comprising a controlled delivery of Treg chemoat-

tractants (i.e., for example, CCL22 and/or vasoactive intestinal peptide, VIP) in the periodontium that can abrogate periodontal disease symptoms. In one embodiment, the method is clinically viable, biocompatible, and non-inflammatory. Rothstein, S. N., Federspiel, W. J., and Little, S. R. A simple model framework for the prediction of controlled release from bulk eroding polymer matrices. Journal of Materials Chemistry 18, 1873-1880 (2008); Rothstein, S. N., Federspiel, W. J., Little, S. R. A unified mathematical model for the prediction of controlled release from surface and bulk eroding polymer matrices. Biomaterials (2009); Delgado, M., Gonzalez-Rey, E. & Ganea, D. VIP/PACAP preferentially attract Th2 effectors through differential regulation of chemokine production by dendritic cells. FASEB Journal 18, 1453-1455 (2004); Little, S. R. et al. Poly-beta amino ester-containing microparticles enhance the activity of non-viral genetic vaccines. Proc Natl Acad Sci USA 101, 9534-9539 (2004). In one embodiment, the method comprises a controlled release formulation that is tunable and provides a long-lasting delivery of a drug. Although it is not necessary to understand the mechanism of an invention, it is believed that these embodiments provide a potential solution for immune imbalance and dysfunction associated with periodontal disease, while avoiding immune-blocking strategies. It is further believed that these embodiments are supported by preliminary data showing: 1) Tregs are attracted toward a preliminary formulation of CCL22 microparticles, in vitro and in vivo; 2) administration of CCL22 microparticles results in resolution of periodontal disease symptoms in in vivo animal models; 3) transformation/proliferation of CD4+ lymphocyte populations into FoxP3+ regulatory lymphocytes is possible using factors including, but not limited to, TGF-β, IL2, and/or rapamycin.

Inflammatory imbalances may cause periodontal tissue destruction wherein the inflammatory cascade is primarily initiated by bacterial recognition through innate immune cells bearing toll like receptors (TLRs). Garlet, G. P., Avila-Campos, M. J., Milanezi, C. M., Ferreira, B. R. & Silva, J. S. *Actinobacillus actinomycetemcomitans*-induced periodontal disease in mice: Patterns of cytokine, chemokine, and chemokine receptor expression and leukocyte migration. Microbes and Infection 7, 738-747 (2005); McGinity, J. W. & O'Donnell, P. B. Preparation of microspheres by the solvent evaporation technique. Adv Drug Deliv Rev 28, 25-42 (1997); Burns, E., Bachrach, G., Shapira, L. & Nussbaum, G. Cutting edge: TLR2 is required for the innate response to *Porphyromonas gingivalis*: Activation leads to bacterial persistence and TLR2 deficiency attenuates induced alveolar bone resorption. Journal of Immunology 177, 8296-8300 (2006); Kajita, K. et al. Quantitative messenger RNA expression of Toll-like receptors and interferon-alpha1 in gingivitis and periodontitis. Oral Microbiol Immunol 22, 398-402 (2007); Nakamura, H. et al. Lack of Toll-like receptor 4 decreases lipopolysaccharide-induced bone resorption in C3H/HeJ mice in vivo. Oral Microbiology and Immunology 23, 190-195 (2008). Triggering of these pathogen receptors may lead to the induction of pro-inflammatory mediators including, but not limited to, TNF-α, IL-1β and IFN-Y. Garlet, G. P., Martins Jr, W., Ferreira, B. R., Milanezi, C. M. & Silva, J. S. Patterns of chemokines and chemokine receptors expression in different forms of human periodontal disease. Journal of Periodontal Research 38, 210-217 (2003); Garlet, G. P. et al. Cytokine pattern determines the progression of experimental periodontal disease induced by *Actinobacillus actinomycetemcomitans* through the modulation of MMPs, RANKL, and their physiological inhibitors. Oral Microbiology and Immu-

nology 21, 12-20 (2006). This skewed, pro-inflammatory environment may upregulate soft tissue destroying matrix metalloproteinases (MMPs) and/or RANKL a receptor activator of nuclear factor κB ligand and a potent activator of bone resorbing osteoclasts. Garlet, G. P. et al. The essential role of IFN-gamma in the control of lethal *Aggregatibacter actinomycetemcomitans* infection in mice. Microbes Infect 10, 489-496 (2008); Garlet, G. P. et al. The dual role of p55 tumour necrosis factor-α receptor in *Actinobacillus actinomycetemcomitans*-induced experimental periodontitis: Host protection and tissue destruction. Clinical and Experimental Immunology 147, 128-138 (2007); Tang, Q. & Bluestone, J. A. The Foxp3+ regulatory T cell: A jack of all trades, master of regulation. Nature Immunology 9, 239-244 (2008); Normenmacher, C. et al. DNA from periodontopathogenic bacteria is immunostimulatory for mouse and human immune cells. Infection and Immunity 71, 850-856 (2003); Graves, D. T. & Cochran, D. The contribution of interleukin-1 and tumor necrosis factor to periodontal tissue destruction. J Periodontol 74, 391-401 (2003). Inflammatory mediators may result in tissue destruction in the periodontium and inhibit the production of anti-inflammatory factors conducive for disease amelioration and healing. Consequently, the present invention contemplates a method for solving the periodontal disease problem by controlling the inflammatory response associated with the known immunological imbalance associated with periodontitis.

Tregs have been shown to reestablish immune homeostasis through a wide variety of mechanisms, both at the site of inflammation (i.e., for example, the periodontium) and/or at the draining lymph nodes (i.e., for example, the cervical lymph nodes). Taubman, M. A., Valverde, P., Han, X. & Kawai, T. Immune response: the key to bone resorption in periodontal disease. J Periodontol 76, 2033-2041 (2005) Specifically, Tregs act to balance these pro-inflammatory mediators by secreting anti-inflammatory factors including, but not limited to, IL-10 and/or TGF-β. Vignali, D. A. A., Collison, L. W. & Workman, C. J. How regulatory T cells work. Nature Reviews Immunology 8, 523-532 (2008); Cardoso, C. R. et al. Characterization of CD4+CD25+ natural regulatory T cells in the inflammatory infiltrate of human chronic periodontitis. Journal of Leukocyte Biology 84, 311-318 (2008). Indeed, recent reports have shown that IL-10 levels are substantially diminished in patients with severe periodontitis. Zhang, N. et al. Regulatory T Cells Sequentially Migrate from Inflamed Tissues to Draining Lymph Nodes to Suppress the Alloimmune Response. Immunity 30, 458-469 (2009). IL-10 has been shown to play a major role in attenuation of periodontal disease by upregulating an extracellular RANKL inhibitor osteoprotegerin (OPG) and promoting increased levels of intra-inflammatory-cell 'suppressors of cytokine signaling' (SOCS). Claudino, M. et al. The broad effects of the functional IL-10 promoter-592 polymorphism: Modulation of IL-10, TIMP-3, and OPG expression and their association with periodontal disease outcome. Journal of Leukocyte Biology 84, 1565-1573 (2008); Garlet, G. P., Cardoso, C. R., Campanelli, A. P., Martins Jr, W. & Silva, J. S. Expression of suppressors of cytokine signaling in diseased periodontal tissues: A stop signal for disease progression? Journal of Periodontal Research 41, 580-584 (2006). Furthermore, IL-10 not only regulates the inflammatory immune response, but also plays a key role in bone anabolism, leading to maturation of bone forming osteoblasts. Abe, T. et al. Osteoblast differentiation is impaired in SOCS-1-deficient mice. J Bone Miner Metab 24, 283-290 (2006); Lorentzon, M., Greenhalgh, C. J., Mohan, S., Alexander, W. S. &

Ohlsson, C. Reduced bone mineral density in SOCS-2-deficient mice. Pediatr Res 57, 223-226 (2005); Ouyang, X. et al. SOCS-2 interferes with myotube formation and potentiates osteoblast differentiation through upregulation of JunB in C2C12 cells. J Cell Physiol 207, 428-436 (2006). Finally, TGF-β has been shown to play an important role in immune regulation and tissue healing, specifically through the recruitment and guidance of periodontal ligament cells. Ernst, C. W. O. et al. Diminished forkhead box P3/CD25 double-positive T regulatory cells are associated with the increased nuclear factor-kB ligand (RANKL+) T cells in bone resorption lesion of periodontal disease. Clinical and Experimental Immunology 148, 271-280 (2007).

In one embodiment, the present invention contemplates a method for restoring the immunosupressive regulatory balance in periodontal space, thereby reversing periodontal disease progression. In one embodiment, the population of Treg cells in the periodontal space is increased. In one embodiment, the amount of immunosupressive cytokines (i.e., for example, CCL-22) in the periodontal space is increased. Although it is not necessary to understand the mechanism of an invention, it is believed that immunosupressive cytokines activate T_{reg} cells that actually mediate the immunosuppression.

Treg populations have been identified in periodontal lesions. In one embodiment, the present invention contemplates a method providing a controlled release of soluble T_{reg} stimulatory factors (i.e., for example, CCL-22) from soluble biodegradable microparticles after injection into a periodontal space. The data presented herein demonstrates a sustained release of CCL-22 over a period of 1 month from microparticles composed of the FDA-approved polymer, PLGA. FIG. 9A. Although it is not necessary to understand the mechanism of an invention, it is believed that the released cytokine recruits T_{reg} cells back into the periodontal space, thereby restoring immune regulation and abrogating periodontal disease progression. See, Example XII.

In one embodiment, the present invention contemplates a method for the controlled delivery of Treg chemoattractants in the periodontium thereby promoting localization of endogenous Treg and abrogating periodontal disease symptoms. In one embodiment, the method further comprises recruiting endogenous Treg to the periodontium. In one embodiment, the periodontal disease symptoms are abrogated by reducing inflammation. Although it is not necessary to understand the mechanism of an invention, it is believed that the this method is supported by preliminary data demonstrating that: 1) controlled release of CCL22 leads to the recruitment of endogenous, Foxp3+ regulatory T-cells to the periodontium; 2) administration of CCL22 controlled release formulations leads to both higher numbers of Treg in draining lymph nodes and resolution of periodontal disease symptoms in in vivo animal models; and 3) beyond recruitment of Treg, a combination of specific factors have been identified that could induce the proliferation and/or transformation of local CD4+ lymphocytes toward enriched Treg populations.

In one embodiment, the present invention contemplates formulations capable of a long-lasting release of Treg-inducing factors. In one embodiment, the formulation is used for the treatment of periodontitis. In one embodiment, microparticles comprising CCL22 and/or vasoactive intestinal peptide (VIP), are constructed of biodegradable poly (lactic-co-glycolic acid) (PLGA) polymer. Rothstein, S. N., Federspiel, W. J., and Little, S. R. A simple model framework for the prediction of controlled release from bulk eroding polymer matrices. Journal of Materials Chemistry

18, 1873-1880 (2008). In one embodiment, the PLGA microparticles facilitate Treg recruitment to the periodontium. In one embodiment, the microparticle is a controlled release microparticle.

In one embodiment, the present invention contemplates a method comprising a microparticle formulation for treating periodontal disease, wherein the formulation promotes Treg recruitment, thereby reducing alveolar bone loss. Although it is not necessary to understand the mechanism of an invention, it is believed that local lymphocytes may expand toward an enriched population of Tregs through controlled release of a combination of several key molecules. Although it is not necessary to understand the mechanism of an invention, it is believed that recruited Tregs may play a role in periodontal disease abrogation and host response. For example, CCL22 microparticle formulation and systemic VIP effectively induce Treg migration to periodontal tissues and may abrogate disease symptoms. In one embodiment, the present invention contemplates formulations that induce Treg chemotaxis and therapeutic function in the periodontium by monitoring Treg residence and disease symptoms. For example, normal mice may be compared to mice deficient in receptors thought to play a role in chemotactic migration and immunological function. Further, gene expression levels of Treg markers, inflammatory mediators, soft and hard tissue destroying factors and biomolecules involved in bone growth to elucidate the mechanisms of Treg-mediated periodontal disease abrogation may also be assessed.

XV. Antibodies

The present invention provides for the use of antibodies (i.e., for example, polyclonal or monoclonal). In one embodiment, the present invention provides monoclonal antibodies that specifically bind to a polypeptide residing on a T_{reg} cell.

An antibody against a protein of the present invention may be any monoclonal or polyclonal antibody, as long as it can recognize the protein. Antibodies can be produced by using a protein of the present invention as the antigen according to a conventional antibody or antiserum preparation process.

The present invention contemplates the use of both monoclonal and polyclonal antibodies. Any suitable method may be used to generate the antibodies used in the methods and compositions of the present invention, including but not limited to, those disclosed herein. For example, for preparation of a monoclonal antibody, protein, as such, or together with a suitable carrier or diluent is administered to an animal (e.g., a mammal) under conditions that permit the production of antibodies. For enhancing the antibody production capability, complete or incomplete Freund's adjuvant may be administered. Normally, the protein is administered once every 2 weeks to 6 weeks, in total, about 2 times to about 10 times. Animals suitable for use in such methods include, but are not limited to, primates, rabbits, dogs, guinea pigs, mice, rats, sheep, goats, etc.

For preparing monoclonal antibody-producing cells, an individual animal whose antibody titer has been confirmed (e.g., a mouse) is selected, and 2 days to 5 days after the final immunization, its spleen or lymph node is harvested and antibody-producing cells contained therein are fused with myeloma cells to prepare the desired monoclonal antibody producer hybridoma. Measurement of the antibody titer in antiserum can be carried out, for example, by reacting the labeled protein, as described hereinafter and antiserum and then measuring the activity of the labeling agent bound to the antibody. The cell fusion can be carried out according to

known methods, for example, the method described by Koehler and Milstein (Nature 256:495 [1975]). As a fusion promoter, for example, polyethylene glycol (PEG) or Sendai virus (HVJ), preferably PEG is used.

Examples of myeloma cells include NS-1, P3U1, SP2/0, AP-1 and the like. The proportion of the number of antibody producer cells (spleen cells) and the number of myeloma cells to be used is preferably about 1:1 to about 20:1. PEG (preferably PEG 1000-PEG 6000) is preferably added in concentration of about 10% to about 80%. Cell fusion can be carried out efficiently by incubating a mixture of both cells at about 20° C. to about 40° C., preferably about 30° C. to about 37° C. for about 1 minute to 10 minutes.

Various methods may be used for screening for a hybridoma producing the antibody (e.g., against a tumor antigen or autoantibody of the present invention). For example, where a supernatant of the hybridoma is added to a solid phase (e.g., microplate) to which antibody is adsorbed directly or together with a carrier and then an anti-immunoglobulin antibody (if mouse cells are used in cell fusion, anti-mouse immunoglobulin antibody is used) or Protein A labeled with a radioactive substance or an enzyme is added to detect the monoclonal antibody against the protein bound to the solid phase. Alternately, a supernatant of the hybridoma is added to a solid phase to which an anti-immunoglobulin antibody or Protein A is adsorbed and then the protein labeled with a radioactive substance or an enzyme is added to detect the monoclonal antibody against the protein bound to the solid phase.

Selection of the monoclonal antibody can be carried out according to any known method or its modification. Normally, a medium for animal cells to which HAT (hypoxanthine, aminopterin, thymidine) are added is employed. Any selection and growth medium can be employed as long as the hybridoma can grow. For example, RPMI 1640 medium containing 1% to 20%, preferably 10% to 20% fetal bovine serum, GIT medium containing 1% to 10% fetal bovine serum, a serum free medium for cultivation of a hybridoma (SFM-101, Nissui Seiyaku) and the like can be used. Normally, the cultivation is carried out at 20° C. to 40° C., preferably 37° C. for about 5 days to 3 weeks, preferably 1 week to 2 weeks under about 5% CO2 gas. The antibody titer of the supernatant of a hybridoma culture can be measured according to the same manner as described above with respect to the antibody titer of the anti-protein in the antiserum.

Separation and purification of a monoclonal antibody (e.g., against a cancer marker of the present invention) can be carried out according to the same manner as those of conventional polyclonal antibodies such as separation and purification of immunoglobulins, for example, salting-out, alcoholic precipitation, isoelectric point precipitation, electrophoresis, adsorption and desorption with ion exchangers (e.g., DEAE), ultracentrifugation, gel filtration, or a specific purification method wherein only an antibody is collected with an active adsorbent such as an antigen-binding solid phase, Protein A or Protein G and dissociating the binding to obtain the antibody.

Polyclonal antibodies may be prepared by any known method or modifications of these methods including obtaining antibodies from patients. For example, a complex of an immunogen (an antigen against the protein) and a carrier protein is prepared and an animal is immunized by the complex according to the same manner as that described with respect to the above monoclonal antibody preparation.

A material containing the antibody against is recovered from the immunized animal and the antibody is separated and purified.

As to the complex of the immunogen and the carrier protein to be used for immunization of an animal, any carrier protein and any mixing proportion of the carrier and a hapten can be employed as long as an antibody against the hapten, which is crosslinked on the carrier and used for immunization, is produced efficiently. For example, bovine serum albumin, bovine cycloglobulin, keyhole limpet hemocyanin, etc. may be coupled to an hapten in a weight ratio of about 0.1 part to about 20 parts, preferably, about 1 part to about 5 parts per 1 part of the hapten.

In addition, various condensing agents can be used for coupling of a hapten and a carrier. For example, glutaraldehyde, carbodiimide, maleimide activated ester, activated ester reagents containing thiol group or dithiopyridyl group, and the like find use with the present invention. The condensation product as such or together with a suitable carrier or diluent is administered to a site of an animal that permits the antibody production. For enhancing the antibody production capability, complete or incomplete Freund's adjuvant may be administered. Normally, the protein is administered once every 2 weeks to 6 weeks, in total, about 3 times to about 10 times.

The polyclonal antibody is recovered from blood, ascites and the like, of an animal immunized by the above method. The antibody titer in the antiserum can be measured according to the same manner as that described above with respect to the supernatant of the hybridoma culture. Separation and purification of the antibody can be carried out according to the same separation and purification method of immunoglobulin as that described with respect to the above monoclonal antibody.

The protein used herein as the immunogen is not limited to any particular type of immunogen. For example, a protein expressed resulting from a virus infection (further including a gene having a nucleotide sequence partly altered) can be used as the immunogen. Further, fragments of the protein may be used. Fragments may be obtained by any methods including, but not limited to expressing a fragment of the gene, enzymatic processing of the protein, chemical synthesis, and the like.

XVI. Artificial Antigen Presenting Cell Kits

In another embodiment, the present invention contemplates kits for the practice of the methods of this invention. The kits preferably include one or more containers containing an artificial antigen presenting cell of this invention. The kit can optionally include a soluble T_{reg} stimulation protein. The kit can optionally include a surface T_{reg} stimulation protein. The kit can optionally include nucleic acids such as FoxP3. The kit can optionally include chemicals capable of functionalizing the polymer ends of an artificial antigen presenting cell. The kit can optionally include a pharmaceutically acceptable excipient and/or a delivery vehicle (e.g., a liposome). The reagents may be provided suspended in the excipient and/or delivery vehicle or may be provided as a separate component which can be later combined with the excipient and/or delivery vehicle. The kit may optionally contain additional therapeutics to be co-administered with an artificial antigen presenting cell.

The kits may also optionally include appropriate systems (e.g. opaque containers) or stabilizers (e.g. antioxidants) to prevent degradation of the reagents by light or other adverse conditions.

The kits may optionally include instructional materials containing directions (i.e., protocols) providing for the use of the reagents in the induction and maintenance of tissue tolerance. While the instructional materials typically comprise written or printed materials they are not limited to such. Any medium capable of storing such instructions and communicating them to an end user is contemplated by this invention. Such media include, but are not limited to electronic storage media (e.g., magnetic discs, tapes, cartridges, chips), optical media (e.g., CD ROM), and the like. Such media may include addresses to interne sites that provide such instructional materials.

XVII. Pharmaceutical Compositions

The present invention further provides pharmaceutical compositions (e.g., comprising the artificial antigen presenting cells described above). The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including vaginal and rectal delivery), pulmonary (e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration.

Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable.

Compositions and formulations for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets or tablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable.

Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions that may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, and liposome-containing formulations. These compositions may be generated from a variety of components that include, but are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids.

The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient (s). In general the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

The compositions of the present invention may be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions of the present invention may also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances that increase the viscosity of the

suspension including, for example, sodium carboxymethyl-cellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

In one embodiment of the present invention the pharmaceutical compositions may be formulated and used as foams. Pharmaceutical foams include formulations such as, but not limited to, emulsions, microemulsions, creams, jellies and liposomes. While basically similar in nature these formulations vary in the components and the consistency of the final product.

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions. Thus, for example, the compositions may contain additional, compatible, pharmaceutically-active materials such as, for example, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the compositions of the present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the present invention. The formulations can be sterilized and, if desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic substances and the like which do not deleteriously interact with the nucleic acid(s) of the formulation.

Dosing is dependent on type and amount of tissue that is transplanted. The course of treatment lasting from several days to several months, or in some cases, for the lifetime of the recipient. Optimal dosing schedules can be calculated from measurements of compound accumulation in the body of the patient. The administering physician can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC_{50s}, found to be effective in in vitro and in vivo animal models or based on the examples described herein. In general, dosage is from 0.01 µg to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly. The treating physician can estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues.

EXPERIMENTAL

Data presented herein is reported as means±standard deviation values (error bars) unless otherwise indicated. A paired or unpaired Student's 't' test was used for statistical comparison between any 2 given samples unless otherwise indicated.

Example I

Emulsification of Regulatory T-Cell Modulating Agents

Preparing emulsions of water soluble factors using biodegradable polymers is a commonly used strategy to deliver materials over extended periods of time and can make administration of factors with short half lives in vivo a reality. The use of the double-emulsion/solvent-evaporation technique is a commonly used strategy. Odonnell et al., *Advanced Drug Delivery Reviews* 28: 25-42 (1997), See,

FIG. **5**. A frequently used polymer in these formulations is poly-lactic-co-glycolic acid (PLGA), which is both biocompatible and FDA approved. PLGA is also attractive because its degradation rates can be controlled by the ratio of its monomers (lactide which is more hydrophobic, and glycolide which is more hydrophilic). This control over degradation behavior provides the ability to release factors over periods from days to months. Hanes et al., *Pharm Biotechnol* 6:389-412 (1995). Microparticles prepared in this manner can carry large payloads and encapsulate multiple agents including, but not limited to: i) cytokines (Thomas et al., *J Pharm Sci* 93: 1100-1109 (2004)); ii) small peptides (Haining et al., *J Immunol* 173:2578-2585 (2004)); iii) small molecule drugs; and iv) nucleic acids (Little et al., *Proc Natl Acad Sci USA* 101: 9534-9539 (2004)). The size can be controlled by parameters such as the concentration of the polymer solution, agitation speeds during fabrication, and amount of surfactant used in the outer aqueous phase during the emulsification procedure. These formulations can be administered in a variety of ways, including injection through a needle and syringe.

Any number of compounds may be emulsified using the above methodology. However, of most interest to the embodiments discussed herein, factors which are thought to influence the anti-inflammatory environment during tolerance induction are completely compatible with the system described here (i.e., for example, IL2, TGF-β, and CCL22). Further, the emulsification and release of IL2 in PLGA microparticles using a double and single emulsion systems has recently been reported. Thomas et al., *J Pharm Sci* 93: 1100-1109 (2004). Specifically, IL2 was encapsulated and released in an active state for periods of up to 4 months. Similarly, TGF-β has been encapsulated in microparticles using PLGA polymer blends with release demonstrated for a period of over 1 month. Kempen et al., *J Biomed Mater Res A* 70:293-302 (2004); and Lu et al., *J Biomed Mater Res* 50:440-51 (2000). CCL20, but not CCL22, has been encapsulated in PLGA microparticles which have been shown stimulate significant amounts of in vitro dendritic cell chemotaxis (i.e., for example, over 0.5 mm from their original position to the point of contact with the particle). Zhao et al., Biomaterials 26:5048-5063 (2005) Importantly, this chemotaxis was greater than that following bolus administration of the chemokine. CCL22 has been reported to induce regulatory T-cell chemotactic migration both in vitro and in vivo. Curiel et al., *Nat Med* 10:942-949 (2004).

Example II

Labeling Artificial Presenting Cell Surface Polymer Functionalization

This example provides a simple, rapid detection method to detect particle surface polymer modifications capable of attaching to soluble and membrane T_{reg} stimulation factors.

Particle surface labeling of biodegradable polymers has been accomplished using several established techniques. The most common strategy for PLGA is to utilize carboxylic acid groups, which are present at the end groups and are generated upon degradation of the ester bonds in its backbone. This can be accomplished using carbodiimide chemistry and can be facilitated using NHS esters. See, FIG. **6**.

Another approach has been to create more of these carboxylic acid groups on the surface using a surfactant which is rich in these chemical groups during fabrication of the particle. Keegan et al., *Macromolecules* 37:9779-9784

(2004). Also, short treatments with 0.1M NaOH can generate an abundance of these groups on the particle surface.

A new technique is presented in this example that quantifies surface labeling using a modification of the carbodiimide chemistry approach. In this technique, 2-pyridyldithioethylamine, reacts with activated esters and also contains a disulfide bond which is, in turn, readily reacted with thiol compounds. See, FIG. 7 middle panel. This reaction liberates a new chemical entity which absorbs light at 342 nm. See, FIG. 7 right panel.

Light absorption can be readily detected using any standard absorbance plate reader. The absorbance data, along with the total amount of particles and their average surface area, can determine an estimate of the number of labeling events per unit area on the particle surface. This technique has successfully labeled the surface of 10 micrometer PLGA microparticles wherein dithiothreitol was used to liberate the absorbent product (data not shown).

Alternatively, streptavidin may be used to facilitate the binding of commercially available biotin-labeled antibodies. For example, EDC/carbodiimide and NHS chemistry can take place in MES buffer in which microparticles are suspended. The reaction tube will need to be constantly rotated to keep particles evenly suspended for approximately 2 hours. Particles will then be washed and 2-pyridyldithioethylamine (PDA) will be added to the activated ester by 4 hour incubation under agitation.

After washing, these particles are labeled with thiolated streptavidin in excess. Particles will be spun down and supernatants removed for analysis at 343 nm.

Values obtained will be compared to a standard curve to determine the number of moles reacted/weight of particles. The average total available surface area for reaction can be determined by preparing an aqueous suspension of particles with a known concentration. This suspension will be analyzed by Coulter Counter to determine the number of particles per unit weight. The value, along with the average diameter of the particles and the number of moles of streptavidin reacted/weight particles leads to the number of streptavidin linkages per unit area on the microparticle surface. This value can be checked with the use of commercially available biotin conjugated to a fluorescent linker. Particles could be labeled with this material, washed, dissolved using NaOH/SDS, and this solution's fluorescence could be measured. This intensity, as compared to a standard curve, would indicate the amount of labeling/weight of particles. A pH insensitive fluorescent probe would need to be used in these experiments. These fluorescently labeled particles will also be incubated (n=3) in a similar fashion as described above for the controlled release assay. Stability of the surface linkage will be determined by the amount of fluorescence in the supernatant after centrifugation (label that has been liberated from the particle surface)

Example III

Emulsification of Soluble T_{reg} Cell Factors in PLGA Microparticles

This example describes the emulsification (i.e., for example, encapsulation) of soluble factors within PLGA microparticles using a modified double emulsion procedure. Odonnell et al., *Advanced Drug Delivery Reviews* 28:25-42 (1997).

Briefly, 20 µg of protein factor and 20 mg of bovine serum albumen will be dissolved in 200 µL of sterile PBS and added to a solution of 100 mg of 10, 18-30, or 40-78 kDa

PLGA with a lactide:glycolide monomer ratio of 50:50 in 2 ml of dichloromethane. This two phase system will be sonicated for 10 seconds using a probe sonicator to form the primary emulsion. The emulsion will immediately be transferred to 50 mL of a homogenizing (5000 rpm), aqueous solution containing 5% by weight poly(vinyl) alcohol (PVA) which serves as a surfactant. After 30 seconds of homogenization, the double emulsion is transferred to a stirring solution of 1% PVA (100 mL) to allow for evaporation of the dichloromethane over a period of 3 hours. The particles will then be centrifuged at <150 rcf in a refrigerated centrifuge and the supernatant containing PVA will be decanted. Particles will be resuspended, washed with cold water, and the process repeated 3×. Washed particles will then be suspended in a minimal amount of water and lyophilized for 3 days to allow sufficient time for removal of residual chloroform. Smaller particles can be prepared using homogenization at 7000 rpm (1 µm). Larger particles can be prepared using a lower concentration of surfactant in the outer aqueous phase of the emulsions (i.e. PVA=1% and 0.5% by weight) which will result in 10-20 µm particles.

Particle size will be determined by a Coulter Counter using volume impedance. Particle loading will be checked by exposing 5 mg of particles to a 0.5 M NaOH, 2% SDS solution to promote total degradation of the particles and dissolution of the protein. Concentrations can be determined using a BCA assay kit via the manufacturer's instructions and loading can be determined (n=3). It should be reasonable to assume that the relative amount of T_{reg} cell factor will remain the same with respect to BSA during encapsulation. However, this assumption can be checked using factor-specific ELISA in place of the BCA assay initially. A typical encapsulation efficiency using the double-emulsion/solvent-evaporation method for proteins is approximately 50%.

Example IV

Controlled Release of Soluble Factors from PLGA Microparticles

This example provides a method to determine the controlled release of soluble factors from microparticles made in accordance with Example III.

Approximately 10 mg of microparticles (n=3) will be weighed out into microcentrifuge tubes and suspended in 500 µL of PBS. Tubes will be sealed and placed on a shaker plate at 37° C. At 12 hour time intervals, tubes will be centrifuged, supernatants removed and stored at −70° C., and new buffer will be added to the particles. At the end of 2 weeks, supernatants will be analyzed for T_{reg} cell stimulating factor using a specific ELISA and release will be plotted over time with standard deviations. Values obtained for all particle formulations in these studies can be used for setting up the appropriate conditions for measuring the activity of these factors and determining their effects on T_{reg} cells in vitro.

Example V

Primary Regulatory T-Cell Isolation

Lymph nodes will be harvested from BALB/c mice and processed to achieve a single cell suspension. Magnetic cell sorting (MACS) will be employed to purify the population via a depletion step which enriches the CD4+ population of cells (mostly CD4+CD25−) and a positive selection step

involving CD25 (resulting in mostly CD4+CD25+). Cells will be cultured thereafter in RPMI 1640 media including 10% FCS, glutamine, HEPES, nonessential amino acids, penicillin/streptomycin, and 2-mercaptoethanol.

Example VI

Biological Activity of Encapsulated TGF-β and IL2

For the determination of TGF-β and IL2 activity, the effect of released factors are measured on a population of CD4+ cells prior to positive selection of CD25. This assay is takes advantage of the fact that TGF-β and IL2 can aid in the differentiation of peripheral CD4+ cells to increase their expression of FoxP3 and suppress alloreactive lymphocytes. Fu et al., *Am J Transplant* 4:1614-27 (2004). Both TGF-β and IL2 are present at the same time as TCR engagement thereby facilitating activation.

CD4+ CD25− cells are treated for 5 days with experimental groups, mAb specific to CD3 (1 µg/ml), and syngenic, lymphocyte-depleted and irradiated splenocytes. To examine biological activity of encapsulates, the following experimental groups (n=3) are used: 1) fresh, TGF-β and IL2 (1 ng/ml) as a positive control, 2) particles with no encapsulated factors and no external addition of IL2 or TGF-β, 3) IL2 particle incubation with the cells+(1 ng/ml fresh TGF-β), 4) TGF-β particle incubation with the cells+(1 ng/ml fresh IL2).

After treatment, cells will be analyzed by flow cytometry for the increased presence of CD25 and intracellular expression of FoxP3 (via Cytofix/Cytopenn kit and labeled FoxP3 mAb) which would indicate activation of the cells. The amount of particles to be used in these experiments will be based on the results of the controlled release assay (described above) to allow for equivalent amounts of administered growth factor. Groups 3 and 4 will be compared to Group 1 to determine the biological activity of the released IL2 and TGF-β, respectively.

Example VII

Measurement of T_{reg} Cell In Vitro Chemotaxis

This example provides one method for measuring the in vitro chemotaxis of T_{reg} cells in response to chemokine release (i.e., for example, CCL22) from particles. See, FIG. 8.

Regulatory T-cells will be isolated as described above using green fluorescent protein transgenic mice (GFP+−C57BL/6 mice). This provides fluorescently labeled cells which have not been treated by any external reagents. Microparticles containing CCL22 (n=3) will be placed below opaque, transwell filters (mean pore size=3 µm and 5 µm). Cells will be seeded on top of this membrane and allowed to incubate for 5 hours before measuring fluorescence. A top and bottom fluorescent reading format is used which allows for the determination of the number of fluorescent cells that have migrated through the membrane at any point. No checkerboard analysis is needed to differentiate between chemotaxis and chemokinesis given that the only factor we are using in our experiments is a well-known chemotactic agent.

Alternatively, a carboxyfluorescein diacetate succinimidyl ester, or CSFE (a factor used to track T-cell proliferation), may be used instead of the GFP transgenic mice to label the T_{reg} cells. A manual counting of cells both below and above the transwell filter (using unlabeled cells as a

control) should be performed to determine if CSFE has any effect on the chemotaxis of the regulatory T-cells.

Experimental controls include, but are not limited to: 1) wells with blank microparticles (as a negative control); and 2) wells containing soluble CCL22 at 0.5-20 ng/ml (the typical manufacturer's stated ED50 range for this material) as a positive control. The amount of microparticles used in this experiment are derived from the controlled release experiment (described above) to equalize the released CCL22 with the positive controls.

A t-test using two-tailed, unequal variance will be used to determine differences between groups with p<0.05 being deemed significant.

Example VIII

In Vitro Measurement of Soluble Released Factors

This example identifies one embodiment for measuring the effect of surface co-stimulatory and soluble released factors on T^{reg} cells in vitro.

Materials

Latex beads with streptavidin can be easily purchased in 1, 5, and 10 µm sizes and can be labeled in one step with biotinylated mAb specific to CD3 and CD28. Optionally, the surface bound moieties can be optimized

In addition to determining the optimal size for the surface labeled microparticles, label ratios are also varied to optimize the proliferation of primary T_{reg} cells (i.e., for example, 0% CD3/100% CD28; 25% CD3/75% CD28, 50% CD3/: 50% CD28; 75% CD3/25% CD28: 100% CD3/0% CD28).

Furthermore, saturating concentration of the stimulatory factors on the particle surface may or may not be optimal for T_{reg} cell proliferation. For example, stimulation of regulatory T-cells by CD28 requires an optimized dose. Fu et al., *Am J Transplant* 4:1614-27 (2004); Salomon et al., *Immunity* 12:431-40 (2000). To determine this dose, the total percentage of streptavidin sites accessible to mAb bound biotin are reduced (i.e., for example, by limiting the addition of free biotin during the labeling procedure). All samples (n=3) will be compared using a two-tailed t-test (unequal variance) with p<0.05 being deemed significant.

Methods

Primary T_{reg} cells will be isolated from C57BL/6 mice and expanded using helper cell-free in vitro conditions including the addition of fresh IL2. The latex bead system will serve as a positive control for comparison with labeled PLGA microparticles of different sizes. Negative controls will include untreated cells and cells treated with particles without surface label.

Soluble IL2 will be replenished, and the cells recounted bi-weekly, while new particles will be administered twice over a two week period. Besides measurement of total regulatory T-cell proliferation, samples will be taken from the culture flasks bi-weekly for flow cytometry analysis. Cells will be stained with fluorescently labeled mAbs which recognize CD25, and CD4. Intracellular staining for FoxP3 is used to determine the activation state of the regulatory T-cells.

Labeling both IL2 and IL2+TGF-β encapsulated particles are performed under the same conditions which produced the optimal surface labeled formulation. Also, this protocol will allow the determination if an artificial antigen presenting cell is capable of activating and inducing the proliferation of regulatory T-cells without the need for manual addition of soluble factors.

Example IX

In Vivo Injection of Chemokine Encapsulated
Particles

This example demonstrates the injection of chemokine encapsulated particles to determine recruitment capacity in vivo.

Particles will be prepared with the full amount of CCL22 (as described in the first experimental section), $\frac{1}{3}$ this amount, and $\frac{1}{10}$ of this amount. Primary cells (1×10^5) from transgenic GFP+–B6 mice will be adoptively transferred into normal B6 mice for in vivo chemotaxis experiments. At this time mice will be given an injection of the three types of CCL22 encapsulated microparticles in a solution of sterile PBS and 1% carboxymethylcellulose (to avoid particle settling and needle clogging with the high concentrations of particles used). This injection will either be on a shaved and sterilized belly of the animal (i.e., for example, intraperitoneally) (n=3) or in the tibealis anterior muscle (hind leg) (n=3). On the other side of the animal, blank microparticles will be injected S.C. or I.M. as a control. Another experimental group provides an S.C. or I.M. injection to mice using a soluble CCL22 in the same amount as the CCL22 encapsulated particles (full amount encapsulated) (n=3).

At t=1 day and 3 days, mice will be euthanized and skin or muscle at the site of an injection will be resected for histology sections. In these sections, the more GFP expressing cells near the site of the depot, the greater the success of the formulation. For a more quantitative analysis, we will form a single cell suspension with the resected tissue and analyze using flow cytometry (at 488 nm) to determine how many adoptively transferred regulatory T-cells were recruited compared to the blank microparticle-treated, negative control (deemed statistically significant by t-test, p<0.05).

Example X

In Vivo Injection of Artificial Antigen Presenting
Cells

This example demonstrates the injection of artificial antigen presenting cells to determine manipulative effect on T_{reg} cells in vivo.

Artificial antigen presenting cells will be prepared by encapsulating an optimized CCL22 formulation plus IL2 and TGF-β followed by an optimized surface labeling strategy. Experimental groups will be designed to explore the necessity of the included factors for stimulation of the recruited regulatory T-cells in vivo (i.e. for example, one formulation with each soluble and surface stimulating component being absent). Mice will be adoptively transferred with transgenic, GFP+ regulatory T-cells as described above and administered injections in the same manner, but this time with the artificial APC formulations instead of encapsulated CCL22 alone (n=3 for each group). Cells from resected skin and muscle will be intracellularly stained for the level of FoxP3 expression (given the strong correlation between its up-regulation and T_{reg} cell stimulatory capacity).

These experiments are repeated with the intent of sorting CD4+ cells instead of intracellular FoxP3 staining. The sorted cells will subsequently be used as suppressor cells (suppressor: responder ratios of 1:1, 2:1 and 5:1) with freshly isolated CD4+CD25– which have been pre-labeled with CSFE (15 μM) and stimulated for 3 days using monoclonal antibodies specific for CD3 (1 μg/ml) and syngenic,

irradiated splenocytes (depleted of lymphocytes). Cultures will be monitored using flow cytometry. Efficient suppression would result in a decreased dilution (or higher signal at 488 nm) of CSFE in the cellular population due to the fewer cell divisions of a suppressed lymphocyte. To determine the necessity of each of the individual factors, experimental groups will be compared via two tailed t-test assuming unequal variance with p<0.05 being considered significant.

Example XI

Induction of Allograft Tolerance Using Artificial
Antigen Presenting Cells

This example demonstrates therapeutic use of optimized artificial antigen presenting cells for induction of allograft tolerance.

The results of the in vivo suppressor assay (supra) provides optimized formulations and are used for the in vivo allograft tolerance assays. These optimized formulations will be co-administered using either a depot administration of, or by coating the transplant in, artificial antigen presenting cells (n=5). Controls will include mice with no treatment and mice administered (15 mg/kg day) cyclosporine S.C. as a positive control (n=5). Furthermore, an optional experimental group comprising an artificial APC group with an initial cyclosporine dose of 15 mg/kg day, and then reduced to examine the effect of immunosuppressant promotion or inhibition of immune tolerance induction (n=5). The experimental endpoint will be 100 days post operation.

For skin transplantation, mice will be shaven, sterilized, and anesthetized before preparation of two graft beds on the posterior chest wall. Rosenberg A. S. (1991), eds. Coligan, J. E., Kruisbeek, A. M., Margulies, D. H., Shevach, E. M. & Strober, W. (John Wiley and Sons, New York), pp. 4.4.1-4.4.12. These graft beds will engraft full thickness skin grafts from the tail of a donor and syngenic control. After one week of recovery, the transplant will be monitored daily for redness, hair growth, hemorrhaging, and status of graft borders. The percentage of viable skin will be recorded daily until only 10% remains, at which time the animal will be euthanized.

Heterotopic heart transplants will be performed as previously described. Zhang et al., *Transplantation* 62:1267-1272 (1996). Rejection will be monitored via direct abdominal palpitation using the following ranking system: A). Strongly beating, B) Noticeable decline from A, or C) Termination of pulsation at which time the animal will be euthanized.

Following termination of the animals in both transplant models, the allografts will be fixed in 10% formaldehyde and sent for embedding, staining, and evaluation of rejection. Percentage of surviving grafts in each group will be plotted against time for comparison.

A log rank test will be used to compare groups with transplantation rejection data as analyzed by any one of many available software packages.

Example XII

Restoring Immunological Regulation in Periodontal
Diseased Tissues

Results Overview

In this experiment, artificial antigen presenting cells providing a CCL-22 controlled release formulation were injected into palatal gingival tissue of right molars of mice (n=3) at the times of –1, 10 and 20 days of periodontal

infection. At 30 days, total RNA was extracted from periodontal tissues as previously described and analyzed for regulatory T-cell and periodontal disease factor. Garlet et al., "The essential role of IFN-gamma in the control of lethal *Aggregatibacter actinomycetemcomitans* infection in mice. In: *Microbes and infection/Institut Pasteur* (2008). Infected, untreated mice (AA) and non-loaded particle treated (CP) groups had low levels of FoxP3 (a regulatory T-cell marker) and IL10 (a suppressive cytokine secreted by T_{reg} cells), but elevated levels of RANK-L (an osteoclast differentiation factor). See, FIGS. **12**A & **12**B. Conversely, CCL-22 particle treatment groups (LP) had significantly higher levels of FoxP3 and IL10, and significantly lower levels of RANK-L. To determine if the particles had therapeutic effect, mice were given the aforementioned treatments and alveolar bone loss was measured at 30 days as described. Garlet et al., "*Actinobacillus actinomycetemcomitans*-induced periodontal disease in mice: patterns of cytokine, chemokine, and chemokine receptor expression and leukocyte migration". *Microbes and infection/Institut Pasteur* 7, 738-747 (2005). Mice treated with CCL-22 loaded particles (LP) exhibited significantly reduced bone loss compared to neg. controls. See, FIG. **12**B.

Methods

Fabrication and Characterization of Controlled Release Formulations: Encapsulation of recombinant murine CCL-22 into PLGA microparticles was performed using a double emulsion procedure to generate particles in the 10 μm range (supra). Loading was verified by dissolution (NaOH, DMSO) followed by protein detection assays such as ELISA to detect total protein encapsulated. Particle sizing and SEM analysis were performed for quality control purposes.

Example XIII

Therapeutic CCL-22 aAPCs in a Murine Mouse Model for Periodontal Disease

To induce periodontal disease in a mouse, we delivered 1×10^9 CFU of a diluted culture of *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans* JP2 (anaerobically grown in supplemented agar medium, TSBV) in 100 μl of PBS with 2% carboxymethylcellulose, placed in the oral cavity of mice with a micropipette in accordance with Example XII. This procedure will be repeated at 48 and 96 hours later to fully establish periodontal disease.

Experimental groups will comprise of eight-week-old C57B1/6 mice. CCL22 loaded particles will be injected in the palatal gingival tissue of right molars from the mesial of first molar until the distal face of the third molar, at the times of −1, 10 and 20 days of infection. Negative controls will include non-infected and sham-infected mice, which receive heat-killed bacteria in 2% carboxymethylcellulose solution, mice that receive PBS injection in left maxilla of the same LP group, and mice that receive the injection of control non-loaded particles. For a positive control, we will utilize mice that receive regular IP injections of VIP (vasoactive intestinal peptide) as this material induces systemic proliferation of regulatory T-cells. Chorny et al., "Vasoactive intestinal peptide induces regulatory dendritic cells that prevent acute graft-versus-host disease while maintaining the graft versus-tumor response" Blood 107:3787-3794 (2006). After 30 days of infection, mice will be euthanized and the samples collected for the following experimental analyses:

Alveolar Bone Loss/Healing Analysis. Evaluation of the extent of alveolar bone loss will be performed in accordance

with Example XII and FIG. **12** above. Further, quantitative histological methodology will be used to characterize bone healing. Briefly, the maxillae will be hemisected, exposed overnight in 3% hydrogen peroxide and mechanically defleshed. The palatal faces of the molars will be photographed at 20× magnification using a dissecting microscope (Leica, Wetzlar, Germany), and the images will be analyzed using ImageTool 2.0 software (University of Texas Health Science Center, San Antonio). Quantitative analysis will be used for the measurement of the area between the cement-enamel junction (CEJ) and the alveolar bone crest (ABC) in the 3 posterior teeth, in arbitrary units of area (AUA). In total, 5 animals will be analyzed, and for each animal, the alveolar bone loss will be defined as the average of CEJ-ABC between the right and left arches.

Histological Assessment for Bone Healing. At the specified times, animals will be euthanized and block sections removed for immunohistochemistry analyses. Tissue sections will be divided into 2 groups (every other section), one for histological/histomorphometry analysis and the other for immunohistochemistry to assess new bone formation by staining for the Dentin Matrix Protein 1 and Bone Sialoprotein.

Real-Time PCR Analysis. Extraction of total RNA from periodontal tissues (upper molars with whole surrounding buccal and palatal periodontal tissues) will be performed with Trizol reagent (Invitrogen, Rockville, Md.) and cDNA synthesis. RealTime-PCR quantitative mRNA analyses will be done in a MiniOpticon system (BioRad, Hercules, Calif.), using SybrGreen Master Mix (Invitrogen), 100 nM specific primers, and 2.5 ng of cDNA in each reaction. We will analyze tissues for the presence of CCL-22, FoxP3, IL-10, RANK-L, and TGF-b. For mRNA analysis, the relative level of gene expression is calculated in reference to beta-actin using the cycle threshold (Ct) method.

Quantification and Visualization of Regulatory T-cells. Tissue will be resected and either: 1) sectioned for staining specific for regulatory T-cells using reported methods (Raimondi et al J Immunol 176, 2808-16 (2006) or 2) digested and separated using Ficoll in order to isolate white blood cells for flow cytometric analysis. The latter will allow us to determine the degree of FoxP3 expression specifically in regulatory T-cells (directly correlating to suppressive activity and also the percentage total regulatory T-cells in the periodontal space in comparison to other lymphocyte populations, CD4+CD25−, CD8+.

Statistical analysis. Preliminary data (n=3 mice per group with an internal control) obtained statistical significance which was slightly less than the p=0.05 cutoff. Power analysis based upon standard deviations and response magnitudes in pilot data (assuming power=0.8) indicates that we require n=5 to obtain desired confidence in our experimental results. Statistical significance between the infected and control mice of both strains will be analyzed by ANOVA, followed by Bonferroni post test. Values of P<0.05 will be considered statistically significant. We will also test both 1 month and 2 month time-points to examine whether or not therapeutic efficacy can be maintained and whether or not healing occurs at these time-points. PCR and AUA based measurements of bone loss/healing can be performed on the same set of mice (n=5×2 time points×4 groups) and histological analysis and flow cytometry can be performed on another set of mice (n=5×2 time-points×4 groups) leading to a total of 80 mice. Experimental groups include: 1) sham

control, 2) blank particle control, 3) VIP positive control, and 4) CCL-22 particle experimental group.

Example XIV

Making Anisotropic Microspheres with Soft Protein Islets

Commercially available carboxylated phosphatidylserine (PS) microspheres (mean diameter 6.37 μm) were purchased from Bangs Laboratories Inc, USA. In preparation for the procedure, microspheres were washed twice in deionised water, then washed once in ethanol, centrifuged at 3500 rpm for 5 min, and re-suspended in water. Microbiology-grade glass cover slips were washed in soap solution, distilled water, and then ethanol (70%) under bath sonication for 15 min each. "Microwells" were developed by spotting 3 μl of microsphere suspension (10% w/v in water) onto a cover slip and dried sequentially at 25° C. for 1 hr 40° C. for 30 min and 60° C. for 10 min (Tg of PS bulk is 100° C. and reduces to 70° C. as the thickness reduces) 27 to avoid surface melting. The wells were further filled 4 times with concentrated microsphere suspension (30% w/v) in water with intermittent drying at 4-8° C. between additions. The colloidal crystals were further dried at room temperature for 3 hours. Prior to the addition of PDMS, cover slips with colloidal crystals were preheated to 60° C. for 5 min on a leveled hot plate (The PDMS ((PDMS/catalyst) (10:1) (w/w)) (Sylgard 184 silicone elastomer kit, Dowcorning Corp, Mich., USA) solution was added and allowed to saturate and stabilize the interstitial spaces for a minute. This mixture was then immediately heated to 90° C. for 15 min. After setting of the PDMS scaffold (16 hrs at 40° C.), the cover slip was carefully removed to prepare the PDMS scaffold/colloidal crystal for protein patterning.

For protein labeling, scaffolds were first immersed in 0.1% Tween solution, to reduce the non-specific adsorption of proteins. Biotin-PEO (EZ Link®, Pierce, USA) was immobilized onto the microspheres in the PDMS scaffold. Carboxylate groups on the surface of microspheres (except the region at PDMS mask) were activated with 0.1M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Acros, N.J., USA), and 0.2M N-hydroxysulfosuccinimide (Sulfo-NHS) (Thermo scientific, IL, USA) in 2000 in 0.2M MES (pH5.5) buffer for 2 hours. Following the reaction, microspheres were washed two times with MES buffer prior to addition of 50 mM biotin-PEO (Fisher scientific, USA) in 200 μl MES buffer. After 2 hrs, the solution was removed and microspheres were washed two times with MES buffer in the scaffold. For etching, Tetra Butyl Ammonium Flouride (TBAF) (1M solution in Tetrahydrofuran, sigma-Aldrich Inc) in 1-Methyl, 2-Pyrrolidininone (NMP) (Sigma-Aldrich Inc)/Deionised Water (1:6) (v/v) in 0.1% Tween 80 solution was used. After 1 hour incubation of microspheres in etchant solution, the scaffolds were washed two times with the MES buffer. The second protein was then immobilized to the newly exposed area using EDC-NHS chemistry. For that the succinimide derivatized particles 200 μl of 0.2 mM albumin rhodamine (Albumin from bovine serum, tetramethyl rhodamine conjugate, Invitrogen) in 0.1% Tween 80/MES buffer (pH 5.5) was added to label the patches. The reaction proceeded for 2 hrs in the dark followed by washing with 1% (w/v) Bovine Serum albumin (BSA) (Sigma-Aldrich Inc) in 200 μl MES (pH 5.5) buffer. For labeling of the remaining surface of the particle, 200 μl MES buffer (pH 5.5) with 0.1% Tween and 1% BSA containing 0.2 mM avidin flourescein (Immunopure®, Pierce, USA) was added to the washed

microspheres in the scaffolds. After 2 hours, the scaffolds were washed two times with MES buffer (pH5.5), and further washed one time in deionised water prior to storage at 4-8° C. The microspheres were scrapped out of the scaffold with a steel spatula. Optical microscopy images were taken with an Optical microscope (Caltex systems 3D Digital Video Inspection measurement system+signatone 1160 Probe Station). SEM studies were done with JSM 6330F. CLSM studies were performed using an Olympus FluoroView 1000 confocal microscope.

Example XV

Microparticle Fabrication

Rationally design of controlled release microparticles may be created using a predictive mathematical model. S. R. Little, S, N. Rothstein, W. J. Federspiel, Journal of Materials Chemistry 18, 1873 (2008); S, N. Rothstein, Federspiel, W. J., Little, S. R., Biomaterials, (2009). The model allows computationally design formulations with specific release profiles based on drug/polymer characteristics and the desired release kinetics. Computer generated fabrication parameters are then used to inform the production process. Specifically, emulsion technique can encapsulate drugs in microparticles. FIG. 21. This model specifies that formulations should have the following characteristics to produce a 20 day linear release profile: Particle Size=20 μm; Internal Drug Pocket Size=500 nm; Average Polymer Molecular Weight=15 kDa; Polydispersity=1.5. FIG. 16.

Furthermore, a VIP (vasoactive intestinal peptide) microparticle formulation will be designed to provide a constant supply of the peptide over 20 days using the following characteristics: Particle Size=20 μm; Internal Drug Pocket Size=300 nm; Average Polymer Molecular Weight=15 kDa; Polydispersity=1.5. If VIP release behavior deviates from the designed profile, we are able to easily reformulate the microparticles to appropriately address the deviant characteristic. Release of VIP from microparticles are expected to provide data similar to FIG. 16.

Example XVI

Preparation of iTreg Cells

This example describes one method of preparing iTreg cells following an in vitro administration of soluble vasoactive intestinal peptide.

Animals

Six-eight week-old C57BL/6 and B6.SJL-Ptprca/BoyAi-Tac (CD45.1) mice were purchased from Taconic and used within two months of delivery. B6(Cg)-Tyrc-2J/J (albino C57BL/6 mice) were purchased from The Jackson Laboratory. C57BL/6.Luc+ mice were a kind gift from Dr. Stephen Thorne (Dept. of Surgery, University of Pittsburgh). All animals were maintained under specific pathogen-free conditions.

Materials

Mouse CD4 negative isolation kit, αCD3/αCD28-labeled beads (aAPC Dynal®) and Vybrant CFDA-SE cell tracer kit were from Invitrogen Corporation (Carlsbad, Calif., USA). Recombinant mouse IL-2 (R&D systems, Minneapolis, Minn., USA), and vasoactive intestinal peptide.

T Cell Isolation

Spleen and lymph nodes are dissected from mice, and single cell suspensions are prepared using mechanical digestion. Following RBC lysis, CD4+ cell isolation are per-

formed using the CD4 negative isolation kit (Invitrogen) as per the manufacturer's instructions. To enrich for CD25– cells, CD4+ cells are incubated with anti-mouse CD25– PE antibody (eBioscience) followed by addition of anti-PE microbeads (Miltenyi). Bead-bound CD25+ cells are isolated by passing cells through a magnetic column. Unbound CD4+ CD25– cells are separated and used to induce Treg.

Treg Induction

Freshly-isolated naïve CD4+ CD25– cells are cultured with aAPC Dynal® beads at a 2:1 (dynal:cell) ratio in the presence of between approximately 3-50 ng/ml vasoactive intestinal peptide. To obtain effector T cells (Teff), CD4+ CD25– cells are cultured with aAPC Dynal® beads and 10 ng/ml IL-2 only. Cell cultures are maintained for 4 days, and cells separated subsequently from the magnetic Dynal® beads. To determine induction of Treg phenotype, FoxP3 staining and flow cytometry (BD-LSRII) are performed at the end of the 4-day culture period as per the manufacturer's instructions (eBioscience).

Example XVII

In Vitro Suppression Assay

Freshly-isolated naïve CD4+ CD45.1+ cells are stained with Carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen, as per the manufacturer's instructions) and co-cultured with autologous induced Treg (generated as described above) at different ratios in 96-well plates. The number of naïve CD4+ CD45.1+ cells is always 50,000 cells/well. For stimulation, 25,000 aAPC Dynal® beads per well are used (2:1, naïve cell:Dynal ratio). Co-cultures are maintained for 4 days, followed by staining for flow cytometry.

Example XVIII

In Vitro iTreg Stability Testing

iTreg cells generated under different conditions and effector T cells (Teff, generated by stimulating naïve T cells in the presence of IL-2 only) are obtained from 4-day cultures and rested in 10 ng/ml IL-2 for 2 days. Thereafter the cells are cultured along with aAPC Dynal® beads as stimulators: (i) for Dynal® only (no Teff and no factors) group, 100,000 iTreg were cultured with 200,000 Dynal® beads along with 10 ng/ml VIP; (ii) for Dynal®+Teff (no factors) group, 50,000 iTreg cells are cultured with 50,000 Teff cells and 100,000 Dynal® beads along with 10 ng/ml VIP; (iii) for Dynal®+factors (no Teff) group, 100,000 iTreg cells are cultured with 200,000 Dynal® beads and respective factors at the concentrations described above; (iv) for Dynal®+ Teff+factors group, 50,000 iTreg cells are cultured with 50,000 Teff cells and 100,000 Dynal® beads and respective factors. Re-stimulation experiments were carried out for 4 days and cells were then stained and analyzed by flow cytometry.

Example IXX

VIPMP Formulations

VIP microparticles (VIPMPs) were prepared following calculations using a model guided fabrication protocol. Rothstein et al., "A simple model framework for the prediction of controlled release from bulk eroding polymer matrices" *J of Mater. Chem.* (2008) 18:1873-1880. The

mathematical algorithm of the computer model suggests specific polymer ratios to achieve desired release kinetic profiles. Separate batches of microparticles were fabricated which could then be combined in model-specified ratios to obtain complex release behaviors.

Three batches were comprised of individual PLGA polymer molecular weights using 4.2 kDa polymer, RG502H PLGA polymer (12.6 kDa), and RG505 polymer (55 kDa). Additionally, a fourth batch was fabricated with the 12.6 kDa polymer which contained poly(ethylene glycol) (PEG) at approximately 4×10^4 mM in the inner aqueous phase. The inner aqueous phases of all VIPMPs comprises approximately 1250 µg/ml VIP. Unloaded (or "blank") sets of particles were also fabricated for each batch.

In a separate study, nonporous and porous (with an inner aqueous phase comprised of 7.5 mm NaCl) VIP microparticles were prepared using RG502H polymer (approximately 12.6 kDa). The inner aqueous phases of the nonporous and porous microparticles consisted of 1250 µg/ml VIP. Blank sets of nonporous and porous particles were also fabricated.

Example XX

VIPMP Release Assays

Release samples were collected for each of the four batches of VIPMPs made in accordance with Example XI. VIP release was also measured for two sets of VIPMPs with varying polymer ratios determined by a computer model that predicted a linear and multi-bolus release profile, respectively.

For the predicted linear release profile VIPMPs, the computer model suggested a microparticle composition comprising ratios of 10.6% of the 4.2 kDa polymer, 31.9% of the 12.6 kDa polymer (without PEG), and 57.5% of the 100 kDa polymer.

For multi-bolus release profile VIPMPs the computer model suggested a microparticle composition comprising ratios of 33.3% each of the 4.2 kDa, 12.6 kDa (with PEG), and 100 kDa polymers.

Release was also measured for the porous and nonporous microparticle groups. Additionally, eight sets of blank microparticles for each group were also collected at each time-point. Release assays in physiological buffered saline (PBS) conducted for each set of microparticles. Ten milligrams of blank microparticles or VIPMPs and 1 mL of either media or PBS were incubated at 37° C. Vials were centrifuged at 2000 rpm, and 800 µL of supernatant was removed and saved at –80° C., and replaced with fresh PBS for each time point.

VIP concentration was determined using a VIP EIA kit purchased from Phoenix Pharmaceuticals. Samples were diluted up to 10×.

Example XXI

VIP Bioactivity Assays

Dendritic cells were isolated from murine bone marrow and cultured following standard procedures.

In the first two experiments, both GM-CSF and IL-4 were added to DC media (modified RPMI 1640) of bone marrow cell cultures during media changes on Days 0, 3 and 6. In the third experiment, the IL-4 was purposely omitted and GM-CSF was added in 5× concentration. On Day 6, CD11c+ DC cells were isolated using MACS cell sorting beads and plated in a 24-well plate at 100,000 cells/well. Five different

groups of four wells each were analyzed and received treatment. An immature DC control group did not receive LPS. A mature DC control group received only LPS. Three different groups were matured with LPS and received different concentrations of either soluble VIP or releasates of VIP microparticles or blank microparticles (using the 12.6 kDa polymer).

Preliminary Protocol: Soluble VIP was added to the third group in concentration of 2.5×10^{-8} M. Ten milligrams of microparticles were first incubated for 4-6 hours in media and 250 μL of releasates were added to each well. After treatment, the DCs were incubated for approximately 18 hours. Prior to the CD4+ T cell chemotaxis study, the 24-well plate was centrifuge and the media was removed. The cells were then starved with 600 μl, PBS+1% BSA and incubated for one hour.

Dose Response Protocol: Soluble VIP was added in concentrations of 10^{-6} M, 10^{-8} M, and 10^{-9} M to both immature DC groups and DCs matured with LPS. Twelve different groups were matured with LPS and received different concentrations of either blank porous, blank nonporous, VIP porous, or VIP nonporous microparticles (of the 12.6 kDa polymer). Microparticles were added in concentrations of either 2.77 mg/mL, 1.39 mg/mL, and 0.277 mg/mL. In a second DC culture, the low concentration of microparticles was reduced to 0.0556 mg/mL. At timepoints of 7, 24, and 48 hours, 100 μl was removed from each well and frozen for ELISA analysis. 100 μl of fresh media was then added to each well after sample removal to maintain a total well volume of 600 μl.

For chemotaxis studies, in the first two experiments, both GM-CSF and IL-4 were added to DC media (modified RPMI 1640) of bone marrow cell cultures during media changes on Days 0, 3 and 6. In the third experiment, the IL-4 was purposely omitted and GM-CSF was added in 5× concentration. On Day 6, CD11c+ DC cells were isolated using MACS cell sorting beads and plated in a 24-well plate at 100,000 cells/well. Five different groups of four wells each were analyzed and received treatment. An immature DC control group did not receive LPS. A mature DC control group received only LPS. Three different groups were matured with LPS and received different concentrations of either soluble VIP or releasates of VIP microparticles or blank microparticles (using the 12.6 kDa polymer). Soluble VIP was added to the third group in concentration of 2.5×10^{-8} M. Ten milligrams of microparticles were first incubated for 4-6 hours in media and 250 μL of releasates were added to each well. After treatment, the DCs were incubated for approximately 18 hours. Prior to the CD4+ T cell chemotaxis study, the 24-well plate was centrifuged and the media was removed. The cells were then starved with 600 μL PBS+1% BSA and incubated for one hour prior to chemotaxis studies.

Example XXII

Microparticle Characterization

The size distribution of each set of microparticles was determined using a Coulter counter. Additionally, scanning electron microscopy was used to analyze microparticles.

Example XIII

ELISA Test for CCL22 Production

An ELISA test purchased from R&D Systems was used to measure CCL22 production by dendritic cells following

modified lab protocol. An alternate blocking buffer of 1 mL Tween dissolved in 50 mL PBS was used. Samples were diluted up to 500×.

Example IVXX

CD4+ Cell Isolation and Chemotaxis

CD4+ T-cells were isolated from the spleen and lymph nodes of a mouse using Dynal beads following the lab protocol. Transwells were added to the 24-well plate containing the DCs and 100 μL of PBS and 1% BSA containing 500,000 CD4+ cells were added to each transwell and incubated for two hours. T Cells that flowed through were analyzed for FoxP3+ expression using flow cytometry.

Example XXV

Murine Periodontitis Model

Wild Type C57BL/6 mice were infected with *Actinobacillus actinomycetemcomitans* three times during the first week of a 30 day test to initiate the periodontal disease. A 'sham' control group received no bacteria or microparticles and an 'untreated' group received only bacteria. A 'blank' control group will receive bacteria and injections of unloaded microparticles. All microparticles were administered at a concentration of 1.25 mg/50 μL in sterile PBS with 2% carboxymethylcellulose. Microparticles were administered on days −1, 10 and 20 into the periodontal pockets of right maxillary molars. VIP microparticles were injected into a fourth group of infected mice as a potential treatment. On Day 30, all mice were sacrificed and the maxillae were harvested for alveolar bone loss analysis.

Example XXVI

Evaluation of Bone Loss

Mice maxillae were incubated in 1× dispase overnight at 37° C. and then placed in hydrogen peroxide for two hours to remove any soft tissue. The area between the cementoenamal junction (CEJ) and the alveolar bone crest (ABC) on the buccal side of right maxilla was measured using a dissecting microscope and CellSans software.

Example XXVII

IL-4 Mediation of Chemotaxis

IL-4 will be omitted from an in vitro chemotaxis study to verify that VIP promotes tolerogenic phenotypes in DCs and not IL-4. It is expected that the omission of IL-4 will have no effect on chemotaxis. ELISAs will be used to measure CCL22 production of DCs cultured with VIP microparticle releasate in the absence of IL-4.

Example XXVIII

Induction of Naïve CD4+CD25+-Naïve T Cells

This example will determine if released VIP is able to directly induce naïve CD4+CD25− naïve T cells to exhibit regulatory phenotypes as shown in literature. Fernandez-Martin et al., (2006) "Vasoactive intestinal peptide induces

regulatory T cells during experimental autoimmune encephalomyelitis" *Eur. J. Immunol.* 36:318-326.

Example IXXX

Repetitive Measures on In Vivo Murine Model Periodontisis Treatment

This example will verify the above in vivo murine model experiments by increasing the sample size and improve statistical significance.

QCPR analysis and flow cytometry will also be conducted to measure the expression of FoxP3+ T cells (Tregs) in draining lymph nodes and in the periodontal pocket. A higher expression of FoxP3+ T cells (Tregs) in draining lymph nodes is expected to show that VIP microparticles induce the production of Tregs. Additionally, an increase in tolerogenic DCs in draining lymph nodes is expected and can be evaluated by observing a decrease in CD86 and CD80 and MHCII on the DCs using flow cytometry.

We claim:

1. A method for reducing periodontal disease symptoms in a patient in need thereof, wherein the patient comprises a white blood cell population and a periodontium that exhibits at least one symptom of a periodontal disease, the method comprising:
   a) providing a biodegradable microparticle population comprising a vasoactive intestinal peptide;
   b) administering said microparticle population to said periodontium under conditions such that said vasoac-

tive intestinal peptide is released from said biodegradable microparticle population;
   c) inducing said white blood cell population with said released vasoactive intestinal peptide to create a regulatory T cell population; and
   d) reducing said at least one symptom of said periodontal disease with said regulatory T cell population.

2. The method of claim **1**, wherein said periodontal disease is an immunological periodontal disease.

3. The method of claim **1**, wherein said white blood cell population comprises a T cell.

4. The method of claim **1**, wherein said white blood cell population comprises a B cell.

5. The method of claim **1**, wherein said periodontal disease is periodontitis.

6. The method of claim **1**, wherein said at least one symptom of said periodontal disease comprises inflammation.

7. The method of claim **1**, wherein said inflammatory immune cell arrest is caused by said regulatory T cell release of secretory factors or direct cell-cell interactions.

8. The method of claim **7**, wherein said secretory factors are selected from the group consisting of immunological homeostasis factors, CCL22, IL-10 and TGF-β.

9. The method of claim **1**, wherein said regulatory T cell population reduces said at least one symptom by inflammatory arrest.

* * * * *