Published in final edited form as:

Adv Mater. 2012 September 11; 24(35): 4735–4738. doi:10.1002/adma.201202513.

Bioinspired Controlled Release of CCL22 Recruits Regulatory T Cells In Vivo

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Deficiency of regulatory T cells (Treg), a type of lymphocyte that promotes immunological homeostasis in the healthy steady state,^[1–3] is a causative factor for destructive inflammation and autoimmune disease.^[2,4] Accordingly, increasing the presence of Treg at the site of autoimmunity has been suggested as a potential method to treat these types of disorders.^[3,4] However, to our knowledge, techniques to increase the local presence of specific immune cell types in vivo do not yet exist. Herein, we develop and test a microscale controlled-release system for the recruitment of Treg that draws inspiration from a specific biological system: tumors. Specifically, a wide variety of tumors release the chemokine CCL22,^[5,6] which is responsible for tumor-directed migration of Treg and corresponding tumor-specific immune evasion. Our hypothesis was that if a steady CCL22 release could be maintained, by tuning the material properties of a controlled-release system, Treg may be preferentially recruited to a local site in vivo.

The first step towards testing this hypothesis was to design a release vehicle suitable for the steady release of CCL22. Although polymeric-microparticle-based controlled-release systems for proteins (including chemokines) have previously been developed,^[7,8] the release of hydrophilic proteins from these particles typically follows a triphasic release profile.^[9,10] Our goal was to achieve release of CCL22 without any periods of lag in order to produce a corresponding steady gradient of chemokine originating from a point source. To this end, new mechanistic descriptions of how controlled release from degradable materials^[9,11] suggest that one of the major factors affecting the rate of release is the erosion of a dense polymer matrix into a porous macrostructure that allows for protein egress. The timing of this process and, in turn, the period of lag is determined by the type of polymer, its molecular weight (inherent viscosity) and the degree of porosity in the matrix. Accordingly, we hypothesized that addition of contiguous pores to the microparticles during fabrication would pre-establish pathways for diffusive protein egress and should bypass the need for erosion as a requisite for release. Thus, we used the polymer poly(lactic-co-glycolic acid) (PLGA) of a specific inherent viscosity (0.16–0.24 dl/g in 0.1% chloroform; in order to achieve release within a 3–8 week period) and modified the surface porosity using an osmotic gradient between the inner aqueous emulsion and the outer bulk aqueous phase (Supporting Information, Figure S1). We observed that porous microparticles prepared using a specific osmotic gradient (corresponding to 15×10^{-3} M NaCl in inner aqueous emulsion), in contrast to unmodified degradable particles (Figure 1a), did not display the standard lag phase that would be expected with release of proteins and released CCL22 continuously over a 4-week period (Figure 1b). As an additional design parameter, the particles were made to be large enough to avoid uptake by phagocytic cells and to prohibit their movement across vascular endothelium (Supporting Information, Figure S2). As a consequence, particles would remain immobilized at the site of injection.

In order to test the ability of rationally designed CCL22MP to attract Treg, an in vivo adoptive transfer model coupled with non-invasive live-animal imaging was used. Specifically, fluorescently labeled CCL22MP were injected into the *triceps surae* of normal FVB mice followed by intravenous (i.v.) infusion of ex vivo-alloactivated Treg^[12]

(AATreg) that constitutively expresses the luciferase gene. The hind limb muscles were chosen as the site for MP injections as these distal sites are not expected to produce large quantities of CCL22. The migration pattern of these bioluminescent AATreg was studied following the injection of non-labeled mature allogeneic dendritic cells (DC), which provide an activation stimulus (Supporting Information, Figure S3). Soon after DC stimulation, a significantly greater number of AATreg was recruited to the site of CCL22MP injection compared to an internal control of microparticles lacking CCL22 (Figure 2a and Supporting Information Figure S4). The adoptively transferred Treg are expected to translocate to sites in the body (e.g., lungs, gut etc.) that secrete CCL22, as observed in Figure S4, but not sites such as the hind limb (as confirmed by our preliminary experiments (data not shown)). However, a significant number of Treg do migrate towards the hind limb muscles upon injection of CCL22MP but not upon addition of blank particles ($p < 0.05$), suggesting that the migration must be due to the release of CCL22 from these particles. Importantly, several reports suggest that small changes in Treg numbers at local sites (leading to a change in the ratio of Treg to effector T cells) is sufficient to dramatically alter local immune responses.^[13] In line with these reports, and as a test for the ability of CCL22MP to replace the function of CCL22 secreting cells in vivo (e.g., site-specific recruitment of regulatory cells), we also demonstrate that CCL22MP are effectively able to delay the rejection of transplanted allogeneic cells. Specifically, allogeneic luciferase-expressing Lewis lung carcinoma cells (which do not endogenously produce CCL22) were implanted subcutaneously into mice at the site of CCL22MP injections (or for comparison BlankMP or Bolus CCL22 as controls), and the time to rejection was recorded using non-invasive live imaging (Figure S3). We observed that the rejection rates were significantly slower in the CCL22MP group when compared to both bolus CCL22 and BlankMP controls (Figure 2b and Figure S5), supporting the hypothesis that establishing a CCL22 gradient in vivo using synthetic systems can help modulate local immune responses.

Potential therapeutic implications of such a bioinspired degradable controlled-release formulation capable of recruiting Treg in vivo are manifold. One obvious application is the use of CCL22MP in combination with an infusion of Treg expanded ex vivo. Current pre-clinical data suggest that freshly-isolated or ex vivo-expanded Treg infusion can prevent organ transplant rejection or suppress autoimmune diseases.^[2,14,15] However, challenges such as obtaining adequate numbers and highly-purified populations of Treg have hindered progress into clinical trials.^[14,15] Using formulations that release CCL22, it may be possible to lower the numbers of injected Treg, or potentially use populations with lower purity. Another use of CCL22MP would be to attract endogenous Treg populations, wherein these formulations have the potential to function as “off-the-shelf” therapeutics for the treatment of a wide variety of disorders associated with unrestrained immune reactivity.

A potential drawback of using a CCL22 sustained release vehicle is that the receptor for this chemokine (CCR4) is expressed on both activated Treg and activated effector T cells,^[16] suggesting that both these cell populations would be attracted towards CCL22MP. Yet in vivo studies show that CCL22 production associated with tumors^[5,17] or long-surviving allografts^[18] result in preferential recruitment of Treg leading to local immunosuppression. One possible explanation for these results is that Treg could express significantly more CCR4 than effector T cells^[17] (Supporting Information, Figure S6). Further, it has been suggested that an optimal ratio of Treg to effector T cells, and not a complete absence of effector T cells, is necessary for effective local suppression of immunity.^[14,19] Regardless, if local effector T cells abrogate a suppressive environment, CCL22MP can easily be modified to simultaneously release immunosuppressive agents (such as rapamycin)^[20] to inhibit these effector T cells in situ, thereby assisting Treg to control adverse immune responses.

In conclusion, we demonstrate that site-specific attraction of Treg leading to local immunomodulation can be achieved in vivo using CCL22MP. These bioinspired controlled-release formulations are particularly attractive as modular platforms for therapeutic development, as well as tools to study Treg-dependent modulation of immune responses in situ.

Experimental Section

Mice

Six–eight week old female BALB/c and C57Bl/6 mice were purchased from Charles River Laboratories (Wilmington, MA); FVB mice were obtained from Taconic (Hudson, NY) and luciferase-expressing FVB (FVB.luc⁺) mice were from Xenogen (Caliper LifeSciences, Hopkinton, MA).^[21] All of the animals were maintained under specific pathogen-free conditions at the University of Pittsburgh. Experiments were conducted in accordance with the National Institutes of Health Guide for Care and Use for Laboratory Animals and under Institutional Animal Care and Use Committee approved protocols.

Microparticle Preparation

Microparticles (MPs) containing rmCCL22 (R&D Systems, Minneapolis, MN) were prepared as described.^[8] Briefly, the nonporous (unmodified) particles were prepared by mixing 200 μ l of an aqueous solution containing CCL22 (5 μ g) and bovine serum albumin (BSA) (2 mg) with PLGA (200 mg; RG502H, Boehringer Ingelheim, Petersburg, VA) dissolved in dichloromethane (4 ml). This mixture was sonicated for 10 s (to form the first emulsion of water-in-oil) before being poured into 2% poly(vinyl alcohol) (PVA) solution (60 ml; M.W. $\approx$ 25 000 g mol⁻¹, 98 mol% Hydrolyzed, PolySciences, Warrington, PA) being homogenized (Silverson L4RT-A) at 3000 rpm. Following homogenization for 1 min (to form the second emulsion), the solution was mixed with 1% PVA and the dichloromethane was allowed to evaporate. After 3 h, the freshly formed MPs were centrifuged and washed ($\times$ 4) in deionized (DI) water. The MPs were then re-suspended in DI water (5 ml), frozen with dry ice and lyophilized (Virtis Benchtop K freeze dryer, Gardiner, NY; operating at 100 mTorr). For the porous CCL22MP, the procedure described above was used with the following modifications to the first step; 200 μ l NaCl solution (varying concentrations) containing CCL22 (5 μ g) and BSA (2 mg) was mixed with PLGA (200 mg; RG502H, Boehringer Ingelheim, Petersburg, VA) dissolved in dichloromethane (4 ml). Fluorescently labeled porous CCL22MP (CCL22MP-680) was prepared by adding 50 μ g of the dextran, Alexa Fluor 680 (M.W. 10 000 g mol⁻¹, Invitrogen, Carlsbad, CA) to the 200 μ l aqueous solution containing CCL22, BSA, and NaCl.

Microparticle Characterization

The surface morphology of CCL22MP was characterized using a scanning electron microscope (SEM) (JEOL JSM-6330F, Peabody, MA) and the CCL22MP size distribution was measured using volume impedance measurements on a Beckman Coulter Counter (Multisizer-3, Beckman Coulter, Fullerton, CA). The in vitro release characteristics were measured by suspending CCL22MP ($\approx$ 7 mg) in phosphate buffered saline (PBS) (1 ml) on an end-to-end rotator at 37 °C. At different time intervals, the suspensions were centrifuged, the supernatant collected, and the CCL22MP resuspended in PBS (1 ml). The amount of CCL22 in the supernatant was determined by enzyme-linked immunosorbent assay (ELISA). As one indication of the amount of CCL22 encapsulated in CCL22MP, the total amount of CCL22 released could be used along with the number of particles used in the release experiment. This loading amount was calculated to be 3.3 ± 0.1 ng per mg of particles. As an additional source of verification of CCL22 encapsulation, this same amount of loaded CCL22 was further verified by degrading CCL22MP (5 mg) in 0.05 N NaOH +

0.5% sodium dodecyl sulfate (SDS) solution (10 ml) for 2 h, followed by quantifying the amount of chemokine in the supernatant using a CCL22-specific ELISA kit.

Alloactivated Regulatory T Cell (AATreg) Cultures

[12] CD4⁺ CD25⁺ regulatory T cells (Treg) were isolated from the spleens and lymph nodes of FVB.luc⁺ mice using the following procedure. CD4⁺ T cells were enriched using the Dynal mouse CD4⁺ negative selection kit (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions. CD25⁺ T cells were isolated from the CD4⁺ cell pool by labeling the cells with anti-CD25-PE, followed by addition of anti-PE beads (Miltenyi) and separating the bead-labeled cells using a MACS column (Miltenyi, Auburn, CA). CD4⁺ CD25⁺ (enriched Treg) were then cultured for 10 d with immature allogeneic-DC (propagated from the bone marrow of BALB/c mice [22]) in complete media (containing 10% FBS) and recombinant human IL-2 (1000 Units). At the end of the culture period, anti-CD11c-labeled beads (Miltenyi) were added to the cells and DC removed using an AutoMACS separator (Miltenyi). The isolated, alloactivated Treg were then prepared for in vivo injections.

In Vivo Treg Migration

CCL22MP-680 (2.5 µg suspended in 50 µl of PBS) was injected into the triceps surae of FVB mice on day -3 or day -6 (Figure 2a or Figure S3a schematic). BlankMP-680 injections in the contra lateral limb served as an internal control. AATreg was injected i.v. on day 0 (2×10^6 cells in 200 µl of PBS), followed by mature allogeneic DC injection (1×10^6 cells in 200 µl of PBS). Non-invasive, live animal bioluminescence imaging was performed at defined time intervals using Xenogen Vivovision (IVIS 200, Caliper LifeSciences, Hopkinton, MA). Luc⁺ Treg were detected by injecting luciferin (200 µl; Caliper LifeSciences) 10 min prior to imaging. The position of each mouse remained unchanged, while both fluorescence and luminescence imaging were performed. Images acquired from the live-animal imaging apparatus were analyzed using Igor Pro Living Image 2.60.1 (Caliper LifeSciences). Contours of the equivalent fluorescence signals from each leg were automatically drawn by the software, and then applied to the luminescence images to determine colocalization of AATreg with the particle depots. Statistical analysis was performed using the Student's 't' test on average luminescence measurements obtained from the BlankMP-680 and CCL2MP-680 groups.

Allogeneic-Cell-Transplant Studies

Either CCL22MP-680 or BlankMP-680 was injected subcutaneously into FVB mice 4 d prior to the implantation of the allogeneic cells. Transgenic luciferase expressing lewis lung carcinoma cells (1×10^6 ; originally derived from C57Bl/6 mice) were injected at the sites of particle injection (the area of particle localization was determined using fluorescence imaging) in PBS (100 µl). At defined time intervals bioluminescence imaging was performed as described previously. For analysis of acquired images, equivalent circles enveloping the area of luminescence signal were drawn and average luminescence (photons $s^{-1} cm^{-2} Sr^{-1}$) measured (average luminescence measurements negate any differences in signal that may arise due to variations in the selection of the area of luminescence). The data are reported as normalized average luminescence, where the luminescence intensity measured from any given mouse on any given day was divided by the luminescence intensity measured for the the same mouse on day 1. Statistical analysis was performed on the normalized data using the Mann-Whitney U test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Sam Rothstein for his help with the mathematical models for controlled release from polymeric matrices, and Rachel Sikorski for her assistance with live-animal imaging. This publication was made possible by Grant KL2 RR024154 and R01 DE021058-01 (to S.R.L.), R01 AI67541 (to AWT) and by the Arnold and Mabel Beckman Foundation (to S.R.L.). G.R. is in receipt of an American Heart Association Grant-in-Aid, the Starzl Transplantation Institute Joseph Patrick Fellowship and the American Diabetes Association Junior Faculty Award. A.J.G. is in receipt of NIH F31 DE021297-01 award. S.J., G.R., A.W.T. and S.R.L. designed the experiments and wrote the manuscript. S.J. performed all the experiments. A.J.G., S.J.H., and D.H.M. assisted with fabrication of the controlled-release formulations and associated data analysis. G.R. generated AATreg populations and S.H.T. helped with the in vivo Treg migration experiments and associated data analysis.

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Figure 1. CCL22MP Characteristics. a) Scanning electron micrographs of nonporous (left; top and bottom) and porous (right; top and bottom) CCL22 microparticles (CCL22MP). The top images were taken at 500 $\times$ magnification, while the bottom images were taken at 2000 $\times$ magnification (representative images of at least 6 independent particle formulations). b) Release kinetics of nonporous and porous CCL22MP, as measured in PBS. The porous particles stopped releasing by day 35, while the nonporous particles continued to release (after a period of lag until day 30) till approximately day 75; the errors bars represent cumulative SEM based on $n = 3$ experiments.

a Colocalization of Treg with CCL22MP

b CCL22MP Delays Rejection of Allo-transplants

Figure 2. CCL22MP *in vivo*. a) Representative fluorescence (red-gold) and luminescence images (blue-yellow) showing localization of particles and Treg, respectively; fluorescence images were used to outline (red-line) areas of particle localization (Igor Pro Living Image 2.60.1) and these outlines were super-imposed on luminescence images taken with the mouse in identical position to demonstrate colocalization of Treg and CCL22MP. b) Representative luminescence images of mice injected with luciferase-expressing allogeneic lewis lung carcinoma (LLC) cells showing that cells survive for longer when injected with CCL22MP when compared with injections with BlankMP.