

IMMUNOLOGY

In situ recruitment of regulatory T cells promotes donor-specific tolerance in vascularized composite allotransplantation

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Vascularized composite allotransplantation (VCA) encompasses face and limb transplantation, but as with organ transplantation, it requires lifelong regimens of immunosuppressive drugs to prevent rejection. To achieve donor-specific immune tolerance and reduce the need for systemic immunosuppression, we developed a synthetic drug delivery system that mimics a strategy our bodies naturally use to recruit regulatory T cells (T_{reg}) to suppress inflammation. Specifically, a microparticle-based system engineered to release the T_{reg} -recruiting chemokine CCL22 was used in a rodent hindlimb VCA model. These "Recruitment-MP" prolonged hindlimb allograft survival indefinitely (>200 days) and promoted donor-specific tolerance. Recruitment-MP treatment enriched T_{reg} populations in allograft skin and draining lymph nodes and enhanced T_{reg} function without affecting the proliferative capacity of conventional T cells. With implications for clinical translation, synthetic human CCL22 induced preferential migration of human T_{reg} in vitro. Collectively, these results suggest that Recruitment-MP promote donor-specific immune tolerance via local enrichment of suppressive T_{reg} .

INTRODUCTION

Each year, millions of individuals sustain unsalvageable composite tissue loss secondary to various etiologies. Vascularized composite allotransplantation (VCA) is an option in select patients, where current reconstructive strategies are suboptimal or fail in terms of cosmetic or functional outcomes. Over the past decade, various types of VCA transplants have been performed, including hand and face transplants, for which systemic therapy with two or more immunosuppressive drugs is the standard of care (1–3). Despite their efficacy in preventing early graft loss, the overriding concern with conventional immunosuppression is the associated toxic sequela. While early graft survival, functional, and immunological outcomes following VCA are promising, graft rejection and the effects of lifelong immunosuppression continue to hamper wider clinical application (2, 3).

An alternative approach to achieving allograft acceptance without systemic immunosuppressive drugs would be to harness the body's inherent mechanisms of immune regulation. Cells of the immune system use a variety of mechanisms to maintain immunological homeostasis and promote tolerance. As a hallmark example, our bodies contain a subset of lymphocytes termed regulatory T cells (T_{reg}) that play a critical role in establishing and maintaining immuno-

logical homeostasis (4–7). The absence of these cells results in destructive autoimmune responses that underlie a number of diseases (4–11). On the other hand, certain tumors harness endogenous immunological regulatory mechanisms, including T_{reg} recruitment, to avoid immune-mediated destruction (12). More specifically, these tumors recruit circulating T_{reg} to the tumor milieu via the release of the chemokine CCL22, which signals through the corresponding chemokine receptor (CCR4) preferentially expressed by T_{reg} (12–15).

To safely mimic this immune evasion strategy, we developed a synthetic, degradable, controlled release microparticle system capable of generating and sustaining a gradient of CCL22 from the site of microparticle placement in vivo. We previously demonstrated in vivo T_{reg} recruitment toward these CCL22-releasing microparticles (referred to as recruitment-microparticles or Recruitment-MP) via in vivo bioluminescence imaging (16), immunohistochemistry (17), and solid tissue flow cytometry (18). Furthermore, Recruitment-MP resolve destructive inflammation in models of both periodontal and dry eye disease (17, 18). Here, we hypothesized that local administration of Recruitment-MP could promote recruitment of endogenous T_{reg} to the graft and thus prevent immune-mediated VCA rejection (in a stringent hindlimb VCA model) as a first step toward developing a strategy to reduce or even eliminate the need for lifelong immunosuppression.

We demonstrate that local (intra-graft) administration of Recruitment-MP prolongs graft survival indefinitely (>200 days). This strategy led to a significant decrease in the expression of proinflammatory cytokines in graft skin and draining lymph nodes. Furthermore, our data suggest that Recruitment-MP can even promote antigen-specific tolerance, as evidenced by ex vivo T cell proliferation assays and secondary skin graft challenge. Together, our results demonstrate the unique ability of Recruitment-MP to selectively reduce inflammation within the graft and promote immune homeostasis in a robust model of major histocompatibility complex (MHC)-mismatched VCA (19).

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RESULTS

Characterization of Recruitment-MP

Recruitment-MP were formulated to produce CCL22 release kinetics such that a physiological gradient of CCL22 could be established for effective T_{reg} recruitment in vivo. These microparticles were made out of a biocompatible, biodegradable poly(lactic-co-glycolic acid) (PLGA) polymer using a well-established double emulsion solvent evaporation technique (16). Scanning electron micrographs of MP indicate that they are spherical and slightly porous (fig. S1A, inset). The surface of Recruitment-MP was formulated to be porous to allow continuous release (without periods of lag) of chemokine. Further, particles were designed to be large enough to avoid uptake by phagocytic cells (i.e., $>10 \mu\text{m}$) and to prohibit their movement across the vascular endothelium, with consequent immobilization at the site of placement (fig. S1B). Last, Recruitment-MP released CCL22 in a linear manner over a period of 40 days (fig. S1A).

Recruitment-MP prevents rejection and promotes long-term survival of hindlimb allografts

To test the ability of Recruitment-MP to prevent graft rejection in a clinically relevant model of VCA, vascularized hindlimbs were transplanted from Brown Norway (BN) rat donors to Lewis (LEW) recipients (complete MHC mismatch). Animals receiving only the baseline immunosuppression protocol of FK506/ALS (anti-rat lymphocyte serum) (timing presented in fig. S1C) served as controls. These animals consistently rejected grafts 2 to 3 weeks after FK506 was discontinued at postoperative day (POD) 21 (Fig. 1A). Three doses of Recruitment-MP (9, 50, and 100 mg) were tested to determine an effective gradient necessary for recruiting T_{reg} and prolonging graft survival. Minutes after hindlimb transplants were completed, particles were administered by a single subcutaneous injection on the lateral aspect of the hindlimb (to avoid the vascular anastomosis medially) after blunt dissection of the subcutaneous plane with an 18-gauge needle (to allow for better distribution of the particles). MP were administered similarly 21 days after transplant. Animals receiving 9 and 100 mg of Recruitment-MP had a graft mean survival time (MST) of 41.5 and 44.0 days, respectively (Fig. 1A). Treatment with 50 mg of Recruitment-MP significantly prolonged graft survival indefinitely, with long-term survival >200 days in six of eight animals (75%). Furthermore, animals treated with 50 mg of blank MP (MST, 39 days), soluble CCL22 (injected subcutaneously in the allograft) (MST, 37.5 days), and 50 mg of Recruitment-MP injected in the contralateral (nontransplanted) limb (MST, 36.0 days) did not exhibit prolonged graft survival compared to animals receiving only the baseline immunosuppression protocol.

Recruitment-MP treatment leads to long-term surviving allografts with normal tissue architecture and enhances T_{reg} proportions in allografts shortly after transplantation

With Recruitment-MP promoting allograft survival, as observed macroscopically, we next examined allograft tissues histologically. Rejecting grafts exhibited sloughing of the epidermis and substantial mononuclear cell infiltration in the dermis and perivascular regions (Fig. 2A). Rejecting grafts also exhibited substantial myositis as evidenced by mononuclear cell infiltration in muscle tissue and disruption of tissue architecture (Fig. 2B). Conversely, biopsies from Recruitment-MP-treated (50 mg) animals with long-term surviving allografts (>200 days) showed minimal cellular infiltration and intact tissue architecture, similar to muscle and skin biopsies from normal

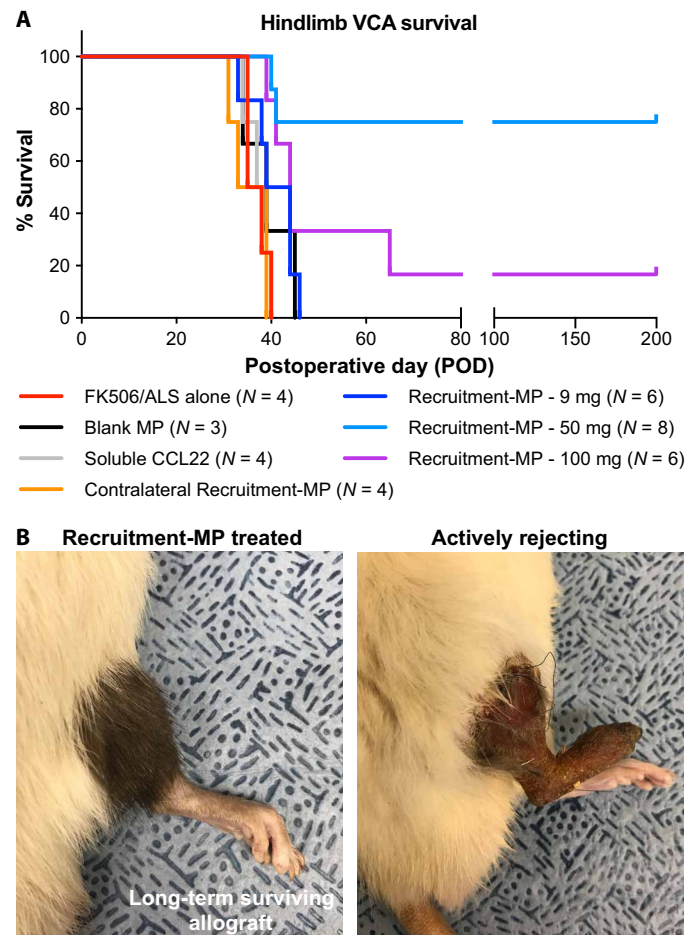


Fig. 1. Treatment with Recruitment-MP (50 mg) prolongs allograft survival indefinitely. (A) Hindlimb allograft survival curve showing indefinite survival (>200 days) in six of eight rats treated with Recruitment-MP (50 mg). These results are statistically significant ($P < 0.05$) when compared to all other groups using a log-rank test. (B) Macroscopically, treatment with 50 mg of Recruitment-MP results in acceptance of the graft with normal hair grown and gross skin appearance, as opposed to controls that exhibit hair loss and skin necrosis. Photo credit: James D. Fisher, University of Pittsburgh.

animals (Fig. 2, A and B). At earlier time points when allografts rejected in control animals, Recruitment-MP-treated hindlimbs exhibited noticeable mononuclear cell infiltrates in the dermis (albeit less than in rejecting limbs) and low to moderate epidermal hyperplasia (Fig. 2A); however, the epidermis remained intact, and there was substantially less evidence of cellular infiltrates in muscle tissue (Fig. 2B).

To characterize cellular infiltrates, skin from Recruitment-MP-treated and rejecting control allografts, as well as autologous skin from contralateral hindlimbs, was dissociated into single-cell suspensions and analyzed by flow cytometry. Rejecting allograft skin contained slightly more total leukocytes (live $CD45^+$ cells) and $CD8^+$ cytotoxic T cells than skin from Recruitment-MP-treated allografts, although the differences were not significant. $CD4^+$ $FoxP3^-$ effector T cells were significantly reduced in Recruitment-MP-treated grafts, while $CD4^+$ $FoxP3^+$ T_{reg} were found at comparable levels. Autologous skin had significantly fewer total leukocytes and T cells compared to rejecting and Recruitment-MP-treated allografts. The proportion of T_{reg} (% $FoxP3^+$ of total $CD4^+$ or $CD8^+$ T cells) in skin from

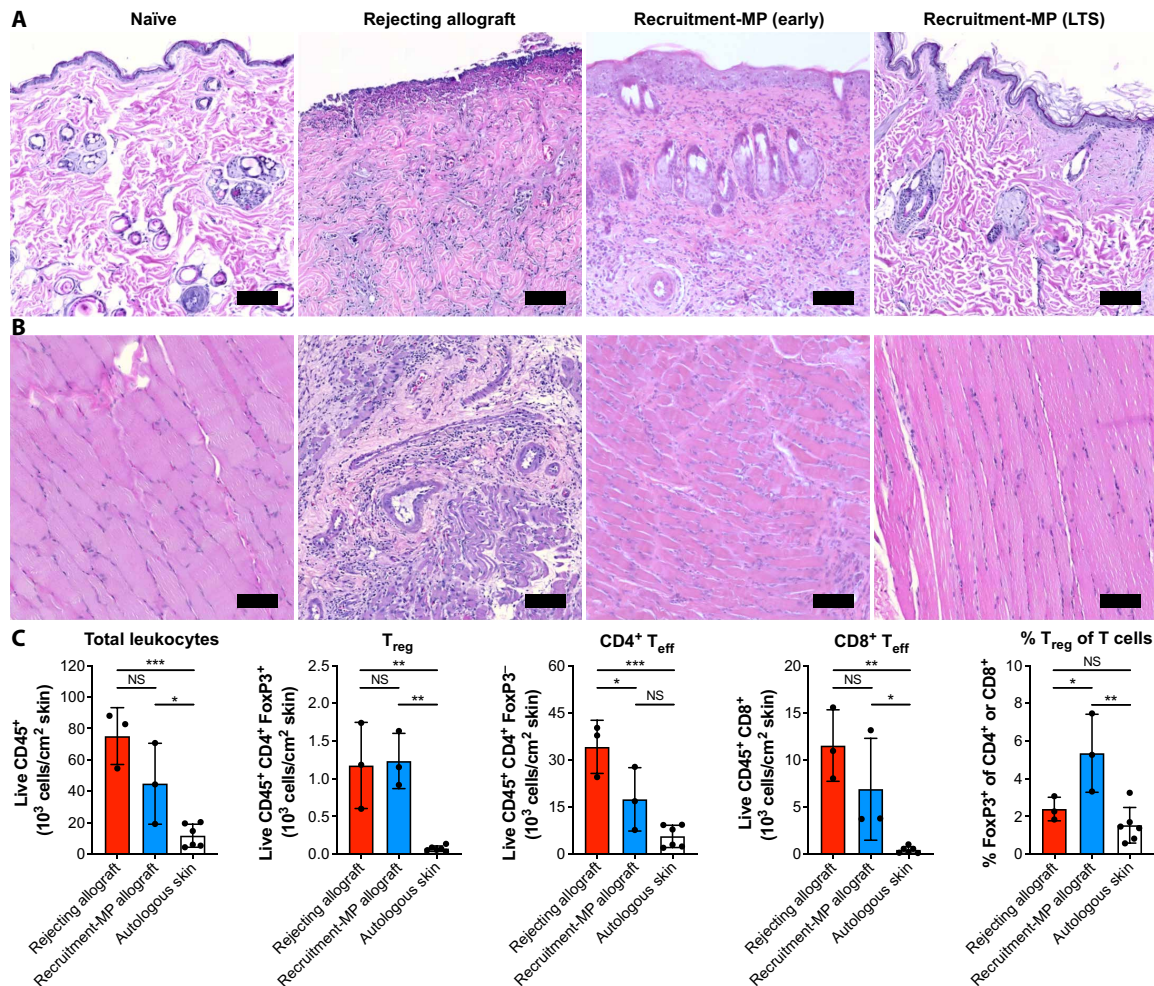


Fig. 2. Recruitment-MP preserves the architectural integrity of intragraft tissues and enhances cutaneous T_{reg} frequency. Representative histology from (A) skin and (B) muscle samples from naïve rats, rejecting allografts, and Recruitment-MP–treated allografts at early (POD 33) or late time points [POD > 200; long-term survivor (LTS)]. Tissue was stained with H&E. Scale bars, 100 μ m. (C) Immune cell populations in skin of rejecting allografts, Recruitment-MP–treated allografts, and autologous skin from contralateral hindlimbs (POD 29 to 43), as determined by flow cytometry on dissociated skin tissue. The flow cytometry gating strategy is presented in fig. S2. Each dot represents the mean of two 1-cm² skin biopsies from a single animal ($N = 3$ for allografts and $N = 6$ for autologous skin). T_{eff}, effector T cells; NS, not significant. Groups were compared by ANOVA, followed by Tukey's post hoc tests, and significant differences are indicated by * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Recruitment-MP–treated allografts was significantly increased compared to that in both rejecting allografts and autologous skin (Fig. 2C).

Recruitment-MP reduces expression of proinflammatory mediators in skin and draining lymph nodes of hindlimb transplant recipients

To determine whether treatment with Recruitment-MP could suppress inflammation locally in the context of VCA, intragraft full-thickness skin samples and draining lymph nodes were harvested from actively rejecting animals (grade III to IV rejection) and Recruitment-MP–treated VCA recipients (experimental end point; POD > 200). We then measured expression of proinflammatory genes: *TNF- α* , *IFN- γ* , *IL-17A*, *Perforin-1*, and *Serglycin*. Expression of all five genes (normalized to expression in normal tissue) was decreased significantly in skin biopsies from Recruitment-MP–treated VCA recipients when compared to corresponding tissue samples from actively rejecting grafts (Fig. 3A). In draining lymph nodes of Recruitment-MP–treated VCA recipients, expression of *TNF- α* ,

IL-17A, *Perforin-1*, and *Serglycin* was also significantly decreased compared to actively rejecting animals (Fig. 3B).

Recruitment-MP increase the incidence of CD4⁺CD25^{hi}FoxP3⁺ T_{reg} in draining lymph nodes of long-term surviving allografts and tip the local immune balance

To assess potential mechanisms underlying the enhanced allograft survival associated with Recruitment-MP, local phenotypic changes in the draining lymph nodes of animals with long-term surviving grafts were examined. At the experimental end point (grade III rejection or POD > 200 for long-term survivors), draining and nondraining inguinal lymph nodes were harvested, and the local CD4⁺ T helper cell phenotype was analyzed. Allograft draining and nondraining lymph nodes from Recruitment-MP–treated animals exhibited a decreased incidence of inflammatory CD4⁺IFN- γ ⁺ cells (normalized to percentages from naïve animals) when compared to draining lymph nodes from actively rejecting animals. Further, draining lymph nodes from actively rejecting animals demonstrated a higher

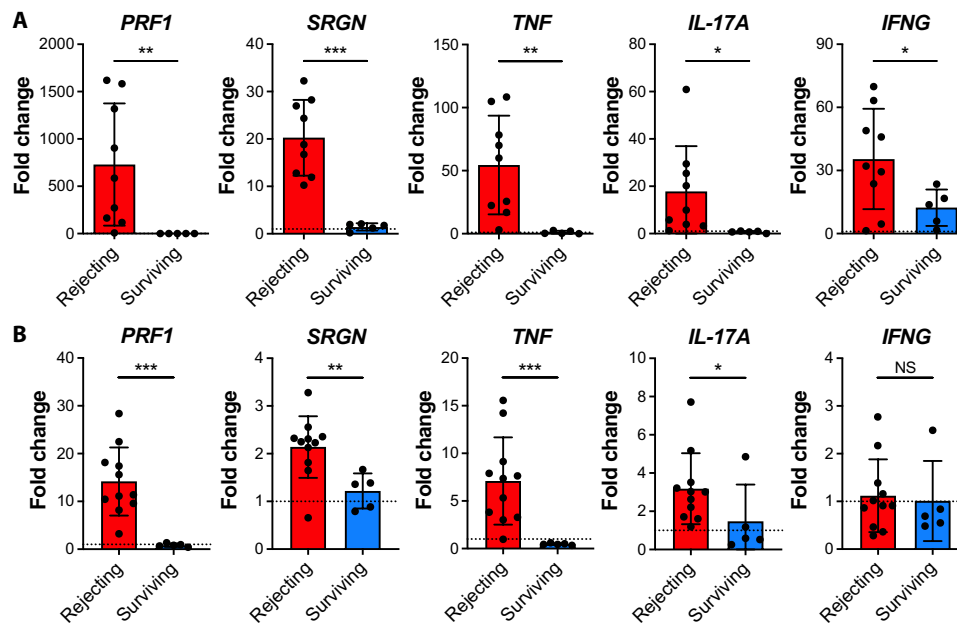


Fig. 3. Expression of proinflammatory mediators in skin and draining lymph nodes is reduced in Recruitment-MP-treated long-term surviving allografts. Relative mRNA expression in (A) skin samples and (B) draining lymph nodes from rejecting hindlimb allografts ($N=9$ for skin and $N=11$ for draining lymph nodes) or Recruitment-MP-treated long-term surviving allografts ($N=5$ for skin and draining lymph nodes). Expression levels are presented as fold changes ($2^{-\Delta\Delta C_t}$) relative to naïve skin ($N \geq 6$) or nondraining lymph nodes ($N=7$ to 9). Bars represent means \pm SD, and dots represent values from individual rats. Groups were compared by Welch's t test or Mann-Whitney U test, as appropriate, and significant differences are indicated by $*P < 0.05$, $**P < 0.01$, or $***P < 0.001$.

percentage of $CD4^+IFN-\gamma^+$ cells than from nondraining lymph nodes of actively rejecting animals (Fig. 4B). Allograft draining lymph nodes from Recruitment-MP-treated animals exhibited an increased incidence of $CD4^+CD25^{hi}FoxP3^+$ cells (normalized to percentages from naïve animals) when compared to both nondraining lymph nodes from Recruitment-MP-treated animals and draining lymph nodes from actively rejecting animals (Fig. 4A).

T_{reg} from recruitment-MP-treated animals exhibit superior suppressive ability compared to T_{reg} from naïve animals

The suppressive and proliferative capacity of T_{reg} and conventional T cells (T_{conv}) isolated from Recruitment-MP-treated VCA recipients and naïve LEW rats were measured in mixed leukocyte reaction (MLR). Specifically, splenocytes from both Recruitment-MP-treated VCA recipients and naïve animals were flow-sorted into two groups: $CD4^+CD25^{hi}$ (T_{reg}) or $CD4^+CD25^-$ (T_{conv}). T_{conv} from both sets of animals were then cultured with irradiated BN (donor) stimulator splenocytes. There was no observed immune hyporesponsiveness with T_{conv} isolated from Recruitment-MP-treated animals when compared to normal controls (Fig. 4C). To assess the suppressive function of T_{reg} from Recruitment-MP-treated animals, T_{reg} from naïve or Recruitment-MP-treated animals were cocultured with T_{conv} from naïve LEW (syngeneic) rats and irradiated BN (donor) splenocytes. T_{reg} isolated from Recruitment-MP-treated animals were more effective than naïve T_{reg} at inhibiting proliferation of naïve T_{conv} stimulated with BN splenocytes (Fig. 4D).

T_{reg} from Recruitment-MP-treated animals exhibit donor-specific suppression of T_{conv} proliferation

To test the donor antigen specificity of $CD4^+CD25^{hi}$ T_{reg} isolated from Recruitment-MP-treated VCA recipients, additional MLRs were

performed. Naïve LEW $CD4^+CD25^-$ T_{conv} were cocultured with $CD4^+CD25^{hi}$ T_{reg} from Recruitment-MP-treated VCA recipients and stimulated with irradiated BN (donor) or Wistar Furth (WF; third party, complete MHC mismatch) splenocytes. T_{reg} from long-term surviving grafts showed enhanced suppressive function ($P < 0.05$) against BN stimulation compared to WF stimulation on a per-cell basis (Fig. 5A).

Recruitment-MP treatment confers systemic donor-specific tolerance to hindlimb recipients

To test whether Recruitment-MP could impart donor antigen-specific dominant tolerance, animals with long-term surviving allografts (>200 days) were challenged with nonvascularized skin allografts from LEW (syngeneic), BN (donor), and WF (third party). These animals received no further immunosuppression or microparticle treatment beyond POD 21. All three animals acutely rejected skin grafts from WF animals, as evidenced by a lack of graft take, characterized by necrosis, wound contraction, and scarring. However, animals accepted both LEW and BN grafts, with minimal wound contracture and eventual hair regrowth (Fig. 6, B and C).

Synthetic and recombinant CCL22 induce selective migration of human T_{reg}

Given promising results with CCL22 delivery in the rat hindlimb VCA model, we sought to determine whether chemically synthesized CCL22 (with potential advantages for clinical translation) could serve as an alternative to recombinant CCL22 to selectively enrich human T_{reg} . Migration of human $CD3^+$ T cells from peripheral blood toward human CCL22 was evaluated using in vitro transwell migration assays. Both recombinant and synthetic CCL22

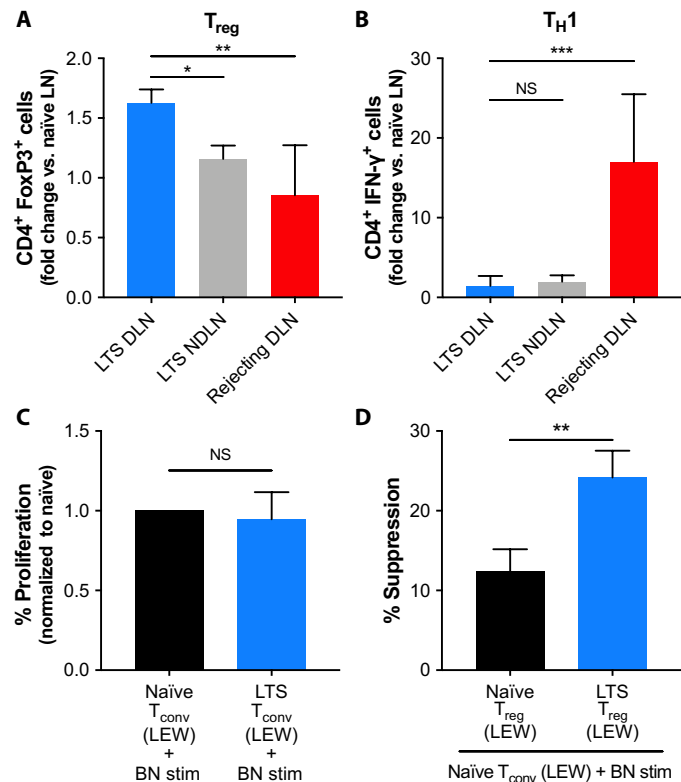


Fig. 4. Functional and phenotypic analysis of CD4⁺ T cells from animals with long-term surviving hindlimb allografts. Flow cytometric analysis of (A) CD4⁺ FoxP3⁺ cells (T_{reg}) and (B) CD4⁺ IFN-γ⁺ cells (T_{H1}) isolated from allograft draining lymph nodes (DLN) and contralateral nondraining lymph nodes (NDLN) of long-term surviving (LTS) rats treated with 50 mg of Recruitment-MP, and allograft draining lymph nodes from actively rejecting controls (N = 5). Percent T_{reg} and T_{H1} were normalized to naive controls. (C) Proliferative capacity of CD4⁺CD25⁻ T_{conv} from long-term surviving Recruitment-MP-treated LEW rats with BN allografts, relative to T_{conv} from naive LEW rats (N = 3). T_{conv} were stimulated with BN splenocytes, and percent proliferation normalized to that of naive LEW T_{conv} with BN stimulation. (D) CD4⁺CD25^{hi} T_{reg} isolated from Recruitment-MP-treated animals (N = 4) were more effective on a per-cell basis at suppressing BN-induced proliferation of naive T_{conv} than CD4⁺CD25^{hi} T_{reg} isolated from naive LEW rats (N = 3). Groups were compared by ANOVA, followed by Tukey's post hoc tests (A and B), one-sample t test (C), or Student's t test (D), and significant differences are indicated by *P < 0.05, **P < 0.01, or ***P < 0.001.

promoted specific migration of human CD4⁺ CD25⁺ CD127^{low} T cells from a diverse pool of total CD3⁺ T cells in a dose-dependent manner (Fig. 6), and selective FoxP3 expression by these cells confirmed their identity as T_{reg} (Fig. 6C). Compared to CD4⁺ CD25^{+/-} CD127⁺ helper T cells and CD3⁺ CD4⁻ T cells, which include CD8⁺ T cells and a smaller population of γδ T cells, T_{reg} expressed higher levels of CCR4 (Fig. 6C). This differential expression of CCR4 resulted in enrichment of T_{reg} in the migrating fraction (Fig. 6A), with T_{reg} frequencies among migrating T cells enhanced by as much as 1.6-fold compared to starting populations of total CD3⁺ T cells. While the greatest T_{reg} enrichment corresponded to the greatest CCL22 concentration (1000 ng/ml) (Fig. 6A), T_{reg} migration indices, which represent CCL22-induced T_{reg} migration, appear to peak at a lower concentration of 100 ng/ml (Fig. 6B). Human T_{reg} migrated comparably toward both recombinant and synthetic CCL22.

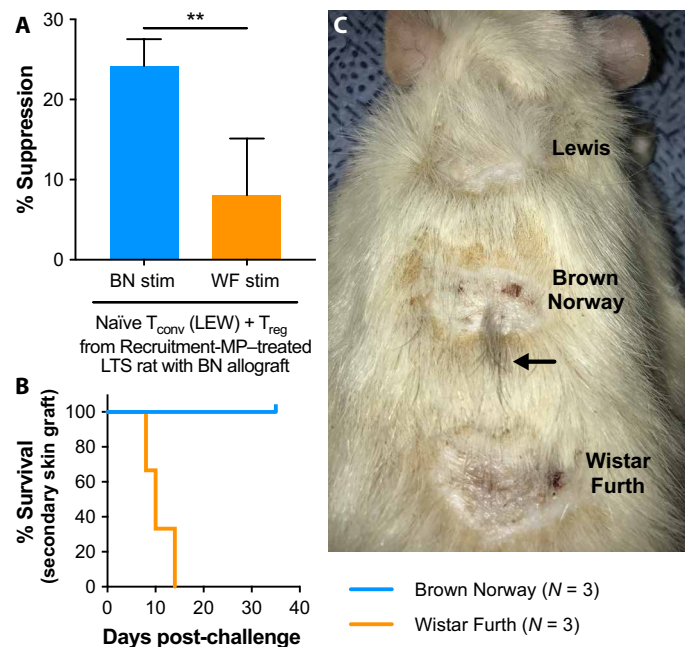


Fig. 5. Recruitment-MP confers donor antigen-specific tolerance to animals with long-term surviving grafts. (A) CD4⁺ CD25^{hi} T_{reg} from Recruitment-MP-treated animals (N = 4) were cocultured with CD4⁺ CD25⁻ T_{conv} from naive LEW rats and subject to stimulation with either BN or WF (third-party) splenocytes. T_{reg} isolated from Recruitment-MP-treated animals were more effective at suppressing BN-mediated proliferation than WF-mediated proliferation. Groups were compared by Student's t test, and a significant difference is indicated by **P < 0.01. (B and C) To test for donor antigen-specific tolerance in vivo, Recruitment-MP-treated animals with long-term surviving BN allografts were challenged with full-thickness nonvascularized skin grafts from LEW (syngeneic control), BN (allogeneic), and WF (allogeneic, third-party) donors. Recruitment-MP-treated animals accepted BN grafts, as evidenced by wound healing and hair growth (C, arrow), but failed to accept third-party WF grafts, as evidenced by contracture and graft necrosis. Additional skin graft images are presented in fig. S3. Photo credit: James D. Fisher, University of Pittsburgh.

DISCUSSION

The largest issue facing the field of transplant immunology is graft loss due to immune-mediated rejection that necessitates the need for lifelong anti-rejection drug treatment. These problems are exacerbated in the emerging field of VCA, as grafts are not viewed as life-saving interventions, raising ethical issues concerning the risk/reward ratio of such procedures. As such, to allow for the routine and widespread use of VCA, it is of paramount importance to develop alternative strategies to limit or even eliminate the need for chronic, systemic immunosuppression.

Over the past decade, mounting evidence has accumulated, implicating a pivotal role for T_{reg} in the prevention of allograft rejection and the induction of tolerance (5, 6, 20, 21). Given the substantial challenges with ex vivo-expanded T_{reg} therapies (22), it is desirable to develop an alternative strategy that is capable of harnessing the highly sophisticated immunoregulatory potential of a patient's endogenous T_{reg}. Some malignant cells reportedly secrete the T_{reg}-recruiting chemokine CCL22 to create a proregulatory environment in the tumor milieu (12). Previous attempts to mimic this phenomenon have involved transduction of pancreatic islet cells with adenovirus expressing CCL22, as an experimental treatment

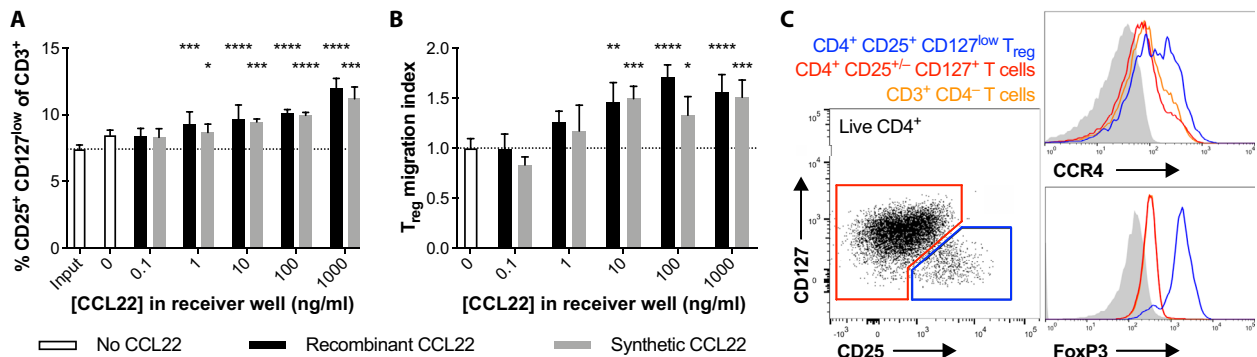


Fig. 6. Recombinant or synthetic CCL22 induces preferential migration of human T_{reg} to enrich T_{reg} frequency. Total $CD3^+$ T cells were isolated from human PBMCs and allowed to migrate through transwell membranes toward different concentrations of recombinant or synthetic CCL22. After 2 hours, migrating cells were collected in the receiver wells and analyzed by flow cytometry ($N=4$ wells per group; means \pm SD). (A) Frequency of $CD4^+ CD25^+ CD127^{low}$ T_{reg} among total migrating T cells. Dotted line represents the average T_{reg} frequency among the total $CD3^+$ T cell population before migration (“input”). Groups were compared by two-way independent ANOVA, followed by Dunnett’s test of dose effect. Significant differences compared to the starting population (input) are indicated by * $P < 0.05$, *** $P < 0.001$, or **** $P < 0.0001$. (B) T_{reg} migration index represents the number of $CD4^+ CD25^+ CD127^{low}$ T_{reg} that migrated toward CCL22, normalized to the average number of T_{reg} that spontaneously migrated in the absence of chemokine. Groups were compared by two-way independent ANOVA, followed by Dunnett’s test of dose effect. Significant differences compared to the no CCL22 control group are indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, or **** $P < 0.0001$. (C) Representative flow cytometry plots confirming specific FoxP3 expression by $CD4^+ CD25^+ CD127^{low}$ T_{reg} (blue) and showing greater CCR4 expression by T_{reg} (blue) than conventional $CD4^+$ T cells (red) and $CD3^+ CD4^-$ T cells (orange).

tested in murine models of autoimmune diabetes and islet allotransplantation (23, 24). In the present study, we used synthetic, biodegradable Recruitment-MP to recruit endogenous T_{reg} to VCA tissues to promote allograft tolerance. While recruitment of CCR4-expressing T_{reg} is a key mechanism through which CCL22 suppresses inflammation, other complementary functions of CCL22 have been elucidated more recently and may also contribute to allograft tolerance. In particular, CCL22 can also recruit invariant natural killer T cells and plasmacytoid dendritic cells (DCs), as well as enhance expression of T_{reg} factors, such as CTLA-4 (cytotoxic T lymphocyte antigen-4), which, in turn, induce production of suppressive indoleamine dioxygenase by DCs (25, 26).

Here, a rat hindlimb model of VCA was used, using BN rats as donors and LEW rats as recipients. All animals received the same short-course baseline immunosuppression protocol consisting of two doses of ALS induction therapy and 21 days of FK506 maintenance therapy (fig. S1C). Our rationale for including a short course of immunosuppression was twofold: (i) to ensure that Recruitment-MP were given sufficient time to establish an adequate gradient of CCL22 and (ii) to evaluate whether the Recruitment-MP could be used together with an immunosuppression protocol resembling that used clinically (2). While FK506 suppresses IL-2 production, resulting in suppression of conventional T cell activity, there is a paucity of information regarding the effect of FK506 on regulatory populations (27). Some studies even report favorable effects of FK506 on T_{reg} induction and function (28, 29). Last, while effective at depleting host lymphocytes, ALS reportedly spares regulatory cells (30) and could serve to compliment the effect of Recruitment-MP.

Because the effectiveness of Recruitment-MP hinges upon the generation of a gradient of CCL22 chemokine to recruit T_{reg} , three doses of Recruitment-MP (9, 50, and 100 mg) were tested to determine an effective formulation for sufficient directional migration of T_{reg} to the transplanted limb. If the concentration of CCL22 is too high, chemokine receptor saturation and/or internalization can inhibit T_{reg} chemotaxis (31, 32). Conversely, if the concentration of CCL22 is too low, then it may not be possible to generate a gradient

capable of affecting and sustaining the migration of T_{reg} distant from the site of Recruitment-MP placement (33). Our data show that treatment with only two doses of 50 mg of Recruitment-MP (at POD 0 and 21) can prolong hindlimb allograft survival when compared to the 9- and 100-mg doses (Fig. 1A). Furthermore, a formulation capable of sustaining release of CCL22 was required, as a subcutaneous bolus injection of CCL22 did not prolong graft survival (Fig. 1A). Given the lack of long-term survival with the high and low doses of Recruitment-MP or with a bolus of CCL22, we did not quantify in vivo T_{reg} recruitment to the allograft for those treatments; however, such studies could provide insight into how Recruitment-MP dose affects in vivo T_{reg} recruitment, as well as what degree of local T_{reg} enrichment is necessary for allograft survival. Last, it should be noted that the tolerogenic effects seen with Recruitment-MP were due to local release of CCL22 on the order of nanogram per kilogram per day, as opposed to conventional immunosuppression that requires up to milligrams of drug per day, administered systemically.

Flow cytometric analysis of lymph nodes from long-term surviving Recruitment-MP VCA recipients demonstrated a significantly higher incidence of $CD4^+ FoxP3^+$ T_{reg} in allograft draining lymph nodes than in nondraining lymph nodes and draining lymph nodes of rejecting animals (Fig. 4A). Recruitment-MP also increased the proportion of T_{reg} among total T cells in allograft skin around the time that allografts rejected in control animals (Fig. 2C). These findings further support our hypothesis that Recruitment-MP can promote VCA tolerance via local enrichment of T_{reg} via chemotactic recruitment. Furthermore, increased percentages of functional T_{reg} were correlated with a concomitant decrease in the incidence of $CD4^+ IFN-\gamma^+$ T helper 1 (T_H1) effector T cells in draining lymph nodes of long-term surviving allografts (Fig. 4B). Not unsurprisingly, there was an increase in the incidence of T_H1 cells in draining lymph nodes of rejecting animals (Fig. 4B). In addition to reducing inflammation at the cellular level, Recruitment-MP substantially reduced inflammation at the RNA level in both intragraft tissue and in draining lymph nodes. Expression of $TNF-\alpha$, Perforin-1, Serglycin,

and IL-17 was decreased in draining lymph nodes and intragraft skin tissue of long-term survivors when compared to actively rejecting animals (Fig. 3). This result is not surprising, as T_{reg} are known to inhibit T_H1/T_H17 T cells and their production of proinflammatory cytokines (6, 9, 34, 35). Collectively, results at the macroscopic, microscopic, and cellular level indicate that Recruitment-MP can achieve anti-donor hyporesponsiveness via enrichment of suppressive T_{reg} .

T_{reg} were further implicated as an essential component of VCA tolerance by examining the function of T_{conv} and T_{reg} ex vivo. Recruitment-MP did not alter the intrinsic proliferative capacity and alloreactivity of T_{conv} , as $CD4^+CD25^- T_{conv}$ from long-term survivors showed no immune hyporesponsiveness toward donor stimulation when compared to $CD4^+CD25^- T_{conv}$ from naïve rats (Fig. 4C). Furthermore, not only were T_{reg} populations enriched in skin and draining lymph nodes from Recruitment-MP-treated allografts, but also T_{reg} isolated from long-term survivors were also more effective at suppressing donor antigen-induced T_{conv} proliferation than T_{reg} from naïve animals (Fig. 4D). This suggests that Recruitment-MP impart donor-specific suppressive function to VCA recipients with long-term surviving grafts. We also show that T_{reg} from long-term survivors are more effective at suppressing BN (donor)-induced proliferation than WF (third party)-induced proliferation, suggesting an antigen-specific inhibitory function of the T_{reg} isolated from Recruitment-MP-treated recipients (Fig. 5A). Collectively, these results suggest that allograft tolerance induced by Recruitment-MP is mediated by T_{reg} .

As a final step to ascertain donor specificity, full-thickness skin grafts were grafted from both BN (donor) and WF (third party) to Recruitment-MP-treated rats with long-surviving grafts. These rats accepted skin grafts from BN donors, as evidenced by growth of brown fur from previously shaved skin grafts, which would not occur in nonviable grafts (Fig. 5C and fig. S3A). Furthermore, third-party (WF) grafts failed to take status 14 days after skin grafting, as evidenced by graft necrosis (fig. S3B). The WF grafts eventually sloughed off as eschars, leaving a healing wound bed (Fig. 5C). This in vivo test of antigen specificity mirrored the in vitro results, with all animals accepting donor (BN) skin grafts but failing to accept third-party (WF) grafts (Fig. 5, B and C).

While the data presented here suggest that Recruitment-MP can establish donor-specific tolerance in rodents receiving hindlimb transplants by enriching T_{reg} locally, there are a few limitations worth noting. Rats were used instead of mice because of their comparatively larger vasculature, which reduces the technical challenges associated with microsurgical anastomoses of blood vessels; however, biochemical and genetic tools (e.g., knockout or reporter strains) for more extensive mechanistic investigation are less readily available for rats. For example, without specific transgenic animal models (e.g., $FoxP3^{cre} \times CCR4^{fllox}$ mice, in which $CCR4$ would be selectively deleted only in T_{reg} , or $CCR4^{-/-}$ mice with targeted $CCR4$ transgene expression in $FoxP3^+$ cells), we cannot rule out the possibility that some other $CCR4^+$ cells might also be recruited to the allograft and contribute to allograft tolerance. Tracking T_{reg} in allografts throughout the course of transplantation, Recruitment-MP treatment, and long-term survival (or allograft rejection) also remains a challenge in the rat hindlimb VCA model and would require either costly serial sacrifice and tissue harvesting or serial in vivo imaging of animals with fluorescent or luminescent reporter T_{reg} (available for mice but not rats).

As the next step toward translation, we are beginning to test Recruitment-MP in a well-established large-animal VCA flap model in outbred swine. These preclinical studies will provide critical information with respect to dose scaling of Recruitment-MP for human trials. Swine are also a suitable species for translational studies as they share anatomic, physiologic, and immunologic similarities with humans. We previously demonstrated that micro-particles releasing recombinant human CCL22 locally recruit T_{reg} in vivo in a large animal (canine) model, providing a basis for scaling and efficacy in larger animal models (17). The probability of favorable safety data in humans is also dramatically increased by the extremely low doses to be released locally (nanogram per kilogram per day).

Although preferential chemotaxis of human T_{reg} toward recombinant CCL22 has been reported previously (13), we have now confirmed that synthetic human CCL22 exhibits comparable T_{reg} -specific chemotactic potency (Fig. 6), suggesting that either could be used for human Recruitment-MP. From a translational perspective, synthetic chemokines produced by solid-phase peptide synthesis (36) offer several advantages over recombinant chemokines produced by engineered microbial, mammalian, or plant cells. Cell-free solid-phase synthesis using automated peptide synthesizer equipment can substantially accelerate the production of cGMP (current good manufacturing practice)-grade chemokines with lower costs than recombinant chemokines. Furthermore, for regulatory purposes, recombinant CCL22 would be treated as a biologic or new molecular entity, while synthetic CCL22 would more likely be treated as a drug or new chemical entity. As such, synthetic CCL22 would be subject to regulatory provisions of the Federal Food, Drug, and Cosmetic Act but not additional regulations of the Public Health Service (PHS) Act, which also applies to biologics. The more complex manufacturing and regulatory pathway for recombinant CCL22, however, could be at least partially offset by an extended 12-year market exclusivity for biologics following U.S. Food and Drug Administration approval [PHS Act, Section 351(a)] compared to 5-year exclusivity (21 CFR 314.108) for a new chemical entity (e.g., synthetic CCL22). Thus, considerations for clinical translation and commercialization of protein therapeutics, like Recruitment-MP, should weigh the benefits of faster and more cost-effective development of a synthetic protein against the seven additional years of market exclusivity for a recombinant protein.

In summary, the current approach to inducing allograft tolerance by T_{reg} recruitment via local sustained release of a chemokine (CCL22) represents a marked departure from conventional immunosuppression regimens for VCA, as well as many recent experimental approaches involving sustained and/or local delivery of the same immunosuppressive agents currently administered systemically in the clinic (37, 38). By inducing dominant alloantigen-specific tolerance without suppressing systemic immune responses (as evidenced by functional conventional T cells in long-term surviving allograft recipients treated with two doses of Recruitment-MP), our novel therapeutic approach has the potential to prevent allograft rejection and avoid patient compliance and toxicity issues associated with current long-term immunosuppression regimens, which also leave patients immunocompromised and unable to fight infections and malignancies. Last, the translational implications of an immunosuppression-free tolerance inducing regimen extend beyond VCA. Recruitment-MP may also prevent rejection of allogeneic skin grafts used to treat third-degree burns or cover exposed bone,

hardware, or tendons, without native morbidity, or in cases of insufficient autologous donor sites. Ultimately, Recruitment-MP may have even broader relevance in acute or chronic inflammatory and autoimmune pathologies, such as rheumatoid arthritis, where available treatments are also limited by their global immunosuppressive or off-target toxic side effects.

METHODS

Animals

Six- to 8-week-old male LEW, BN, and WF rats (Charles River Laboratories, Wilmington, MA) were used. Animals were maintained under an Institutional Animal Care and Use Committee–approved protocol in a specific pathogen–free environment at the University of Pittsburgh.

Hindlimb transplantation

Using techniques developed in the University of Pittsburgh’s Department of Plastic Surgery (39), hindlimbs from donor BN rats were transplanted orthotopically to recipient LEW rats. Briefly, donor femoral vessels were anastomosed end-to-end to recipient femoral vessels using 10-0 nylon sutures, and femoral osteosynthesis was performed with an 18-gauge intramedullary rod. Attachment of muscles and skin closure were achieved with simple interrupted sutures. Surgical time was approximately 2 to 3 hours. Anesthesia was achieved perioperatively with inhaled isoflurane, and buprenorphine was used for analgesia.

Recruitment-MP fabrication and characterization

PLGA MPs containing recombinant mouse CCL22 (R&D Systems, Minneapolis, MN) were prepared using a standard water-oil-water double emulsion procedure, as described (16). Briefly, PLGA (RG502H; Boehringer Ingelheim, Petersburg, VA) MP were prepared by mixing 200 μ l of an aqueous solution containing 25 μ g of rmCCL22 and 2 mg of bovine serum albumin and 15 mmol NaCl with 200 mg of polymer dissolved in 4 ml of dichloromethane. The first water-in-oil emulsion was prepared by sonicating this solution for 10 s. The second oil-in-water emulsion was prepared by homogenizing (Silverson L4RT-A) this solution with 60 ml of an aqueous solution of 2% polyvinyl alcohol (molecular weight, ~25,000 Da; 98% hydrolyzed; PolySciences, Warrington, PA) for 60 s at 3000 rpm. This solution was then mixed with 1% polyvinyl alcohol and placed on a stir plate agitator for 3 hours to allow the dichloromethane to evaporate. The microparticles were then collected and washed four times in deionized (DI) water, to remove residual polyvinyl alcohol, before being resuspended in 5 ml of DI water, frozen, and lyophilized for 72 hours (VirTis BenchTop K freeze dryer, Gardiner, NY; operating at 100 mtorr).

Surface characterization of microparticles was conducted using scanning electron microscopy (JEOL JSM-6510LV/LGS), and microparticle size distribution was determined by volume impedance measurements on a Beckman Coulter counter (Multisizer 3, Beckman Coulter, Fullerton, CA). CCL22 release from microparticles was determined by suspending 7 to 10 mg of microparticles in 1 ml of phosphate-buffered saline placed on an end-to-end rotator at 37°C. CCL22 release sampling was conducted by centrifuging microparticles and removing the supernatant for CCL22 quantification using enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN) (16).

Study design and groups

All hindlimb recipients in all groups received the same baseline immunosuppression protocol consisting of 21 days of tacrolimus (LC Laboratories, Woburn, MA) at a dose of 0.5 mg/kg per day, injected intraperitoneally from the time of transplant. Rats also received two doses of rabbit ALS (Accurate Chemical, Westbury, NY) injected intraperitoneally on day –4 and POD 1 (fig. S1C). MP were injected via an 18-gauge needle after subcutaneously undermining dermal tissue to allow for even spreading of the particles. Further, MP were injected in the lateral aspect of the transplanted limb (unless otherwise noted) immediately following hindlimb transplantation and again on POD 21. Animals given transplants were allocated into groups consisting of the following treatments: (i) FK506/ALS baseline immunosuppression only ($N = 4$); (ii) 9 mg of Recruitment-MP (10 mg/ml; $N = 6$); (iii) 50 mg of Recruitment-MP (50 mg/ml; $N = 8$); (iv) 100 mg of Recruitment-MP (50 mg/ml; $N = 6$); (v) 50 mg of blank MP ($N = 3$); (vi) soluble CCL22 assuming 100% encapsulation efficiency of group 3 ($N = 4$); (vii) 50 mg of Recruitment-MP injected in the contralateral, native naïve (nontransplanted) limb ($N = 4$).

Hindlimb allograft monitoring

To assess rejection, hindlimbs were monitored daily and scored for rejection (appearance grading) based on physical examination (39). Limbs were given a daily score using the following scale: grade 0 (no rejection), grade I (edema), grade II (erythema and edema), grade III (epidermolysis), and grade IV (necrosis and “mummification”). Grafts were considered rejected when displaying signs of progressive grade III rejection.

Graft histology

Skin and muscle samples were obtained from the transplanted limbs at their experimental end point: progressive grade III rejection, early nonrejecting (POD 29 to 33), or long-term survival (>200 days). Samples were fixed in 10% neutral buffered formalin, paraffin-embedded, sectioned at 5 μ m, and stained with hematoxylin and eosin (H&E) for microscopic examination of tissue architecture and mononuclear cell infiltration.

Flow cytometric analysis

Draining and nondraining (contralateral) inguinal lymph nodes were harvested at the experimental end point (progressive grade III rejection or long-term survival >200 days). Lymph nodes were then processed to form single-cell suspensions. Cells were stained with fluorescently labeled antibodies for CD4 (clone OX-35), CD25 (OX-39), FoxP3 (FJK-16s), and IFN- γ (DB-1) (BD Biosciences, San Jose, CA; eBioscience, San Diego, CA). The FoxP3/Transcription Factor Staining Buffer Set (eBioscience) was used for intracellular staining. Before IFN- γ cytokine staining, cells were cultured overnight with Cell Stimulation Cocktail (plus protein transport inhibitor; eBioscience). Skin samples (1 cm \times 1 cm) were cut into thin strips for enzymatic digestion in culture medium supplemented with collagenase D (1 mg/ml) and deoxyribonuclease (DNase) (1 mg/ml). Tissue was incubated for 2 hours at 37°C and 100 rpm shaking and then mechanically dissociated through 70- μ m nylon filters. Cells were stained with antibodies for CD4 (OX-35), CD8a (OX-8), CD45 (OX-1), and FoxP3 (FJK-16s), as well as Fc block (anti-CD32, D34-485) and a fixable viability dye (eBioscience). Stained lymph node and skin cells were analyzed on a BD LSR Fortessa cytometer, and

CountBright Absolute Counting Beads (Thermo Fisher Scientific, Waltham, MA) were added to skin cell suspensions to determine total cell counts. Results were analyzed using FlowJo (Ashland, OR).

Gene expression and PCR

Gene expression profiles of inflammatory markers were evaluated in the skin and lymph nodes of long-term graft survivors, actively rejecting animals, and naïve rats. Total RNA was extracted from samples using TRI Reagent according to the manufacturer's instructions and quantified using a NanoDrop 2000 spectrophotometer. For each reverse transcriptase assay, 4 µg of RNA was converted to complementary DNA using a QuantiTect Reverse Transcription kit. Quantitative real-time polymerase chain reaction (qRT-PCR) was then performed using VeriQuest Probe qPCR Mastermix, according to the manufacturer's instructions, with 5' nuclease PrimeTime qPCR assays specific for IFN-γ (Rn00594078_m1 Dye: VIC-MGB_PL), TNF-α (Rn99999017_m1 Dye: VIC-MGB_PL), Perforin-1 (Rn00569095_m1 Dye: VIC-MGB_PL), Serglycin (Rn00571605_m1 Dye: VIC-MGB_PL), IL-17 (Rn01757168_m1 Dye: VIC-MGB_PL), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; endogenous control, Rn99999916_s1). Duplex reactions (target gene + GAPDH) were run and analyzed on the StepOnePlus Real-Time PCR System. Relative fold changes of IFN-γ, TNF-α, Perforin-1, Serglycin, and IL-17 expression were calculated and normalized based on the $2^{-\Delta\Delta CT}$ method, and then further normalized to normal control. Skin biopsies from naïve animals or contralateral limbs served as untreated controls.

Cell proliferation and suppression assays

Spleens from rats with long-surviving hindlimb grafts and naïve LEW rats were processed into single-cell suspensions. Red blood cells (RBCs) were lysed using RBC lysis buffer (Thermo Fisher Scientific). CD4⁺ T cells were isolated using CD4 T cell enrichment columns according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). CD4⁺ enriched cells were then stained with anti-CD4 (OX-35) and anti-CD25 (OX-39). CD4⁺CD25⁻ (T_{conv}) and CD4⁺CD25^{hi} (T_{reg}) populations were sorted using a BD FACSAria cell sorter. To assess proliferative function, CD4⁺CD25⁻ (T_{conv}) from long-term surviving and naïve rats were stained with violet proliferation dye (VPD450; BD Biosciences) and each was cocultured/stimulated with irradiated splenocytes harvested from donor strain rats at 2:1 T_{conv}:stimulators. At the end of the 7-day MLR period, proliferation was measured via VPD450 dilution by flow cytometry. The proliferative capacity of T_{conv} from long-term surviving rats was normalized to that of naïve rats.

To quantify the suppressive cell function of CD4⁺CD25^{hi} (T_{reg}) isolated from long-term surviving and normal control rats, we tested their ability to suppress T_{conv} proliferation in MLR. CD4⁺CD25⁻ T_{conv} from naïve rats were stained with VPD450 and cocultured/stimulated with irradiated splenocytes from BN rats and CD4⁺CD25^{hi} T_{reg} from either long-term surviving or naïve rats at 2:1 T_{conv}:T_{reg}. At the end of the 7-day culture period, proliferation was measured via VPD450 dilution by flow cytometry. Percent suppression was calculated using the following formula: $\{1 - [(\% \text{ Proliferation of naïve T}_{\text{conv}} \text{ cultured with BN splenocytes and T}_{\text{reg}}) / (\% \text{ Proliferation of naïve T}_{\text{conv}} \text{ cultured with BN splenocytes})]\} \times 100\%$.

MLRs were also set up to test for antigen specificity of the CD4⁺CD25^{hi} T_{reg} isolated from long-term surviving rats. CD4⁺CD25⁻ T_{conv} from naïve rats were stained with VPD450 and stimulated

with either BN or WF (third party) irradiated splenocytes in the presence of CD4⁺CD25^{hi} T_{reg} isolated from long-term surviving rats. At the end of the 7-day culture period, proliferation was measured via VPD450 dilution using FlowJo (Ashland, OR). Percent suppression was calculated using the following formula: $\{1 - [(\% \text{ Proliferation of naïve T}_{\text{conv}} \text{ cultured with BN or WF splenocytes and T}_{\text{reg}}) / (\% \text{ Proliferation of naïve T}_{\text{conv}} \text{ cultured with BN or WF splenocytes})]\} \times 100\%$.

Full-thickness secondary skin grafting

Donor antigen-specific tolerance was assessed in vivo in long-surviving graft recipients (>200 days) from the 50-mg Recruitment-MP group via skin graft challenge. Skin allografts were harvested from normal donor strain (BN) or third-party strain rats (WF) and transplanted to the dorsal thoracic area of long-term survivors >200 days after VCA. Grafts were supported in place with xeroform and surgical gauze for 5 days and subsequently evaluated daily for signs of rejection. Rejection was defined as 80% necrosis of the skin graft.

Human CCL22 T cell migration and T_{reg} enrichment assay

Healthy donor human peripheral blood was obtained from the Central Blood Bank (Pittsburgh, PA), and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque Plus (1.077 g/ml; GE Healthcare, Chicago, IL) gradient centrifugation. Total CD3⁺ T cells were isolated from PBMCs by immunomagnetic negative selection with an EasySep Human T Cell Isolation kit (StemCell Technologies, Cambridge, MA). Chemotaxis assays were conducted in 24-well plates with 5-µm pore polycarbonate transwell filters (Corning Costar, Corning, NY), as previously reported (13). Serum-free AIM V medium (600 µl; Thermo Fisher Scientific) containing recombinant human CCL22 (0, 0.1, 1, 10, 100, or 1000 ng/ml) (R&D Systems), or synthetic human CCL22 (Almac, Souderton, PA), was added to the lower receiver wells. T cells suspended in serum-free AIM V medium were added to the top chambers of the transwells (100 µl, 5×10^5 cells per well). For total cell "input" controls, cells were placed directly in receiver wells. After incubating at 37°C with 5% CO₂ for 2 hours, migrating cells in the lower wells were recovered and transferred to fluorescence-activated cell sorting (FACS) tubes for staining. Cells were stained with fluorescently labeled antibodies for CD4 (RPA-T4; BD Biosciences), CD25 (2A3; BD Biosciences), CD127 (hIL-7R-M21; BD Biosciences), and a fixable viability dye (eBioscience). Some cells were also stained for CCR4 (1G1; BD Biosciences) and FoxP3 (236A/E7; eBioscience), or mouse IgG1,κ isotype controls, using the eBioscience FoxP3/Transcription Factor Staining Buffer Set. Immediately before analysis on a BD LSR II flow cytometer, CountBright Absolute Counting Beads were added to each tube. Frequencies of T_{reg} (CD4⁺ CD25⁺ CD127^{low}) in the migrating populations were calculated. To determine total numbers of migrating T cells, cell counts were normalized to total counting beads. T_{reg} chemotaxis migration indices were calculated by normalizing numbers of T_{reg} migrating in the presence of CCL22 to the average number of spontaneously migrating T_{reg} in the absence of CCL22.

Statistics

Statistical analyses were performed in GraphPad Prism v8. All data are expressed as means ± SD, and significant differences between experimental groups were determined by two-tailed Student's *t* test, Welch's *t* test, or Mann-Whitney *U* test for two independent samples,

or one-way analysis of variance (ANOVA) followed by Tukey's post hoc tests for comparison of multiple groups. For human T_{reg} migration assays with two types of CCL22 and multiple doses, results were analyzed by two-way ANOVA, followed by Dunnett's pairwise comparisons for dose effect. A one-sample *t* test was used to compare fold changes in an experimental group to a value of one, and the effects of various treatments on VCA survival were analyzed using a log-rank test. Differences were considered significant if *P* < 0.05.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <http://advances.sciencemag.org/cgi/content/full/6/11/eaax8429/DC1>

Fig. S1. Characterization of Recruitment-MP and experimental timeline for hindlimb transplantation.

Fig. S2. Skin flow cytometry gating strategy used for Fig. 2C.

Fig. S3. Representative images of a hindlimb VCA recipient challenged with LEW, BN, and WF nonvascularized skin grafts.

[View/request a protocol for this paper from Bio-protocol.](#)

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tissues for analyses. J.D.F., S.C.B., V.S.G., and S.R.L. wrote the manuscript, with additional discussion and contributions from H.R.T., A.W.T., and M.G.S. **Competing interests:** The authors declare that they have no competing interests. **Data and materials availability:** All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

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