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Brief communication

Differential promotion of hematopoietic chimerism and inhibition of alloreactive T cell proliferation by combinations of anti-CD40Ligand, anti-LFA-1, everolimus, and deoxyspergualin

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ABSTRACT

Allogeneic bone marrow (BM) engraftment for chimerism and transplantation tolerance may be promoted by combinations of costimulation blocking biologics and small molecular weight inhibitors. We showed previously in a mouse model that anti-CD40Ligand (anti-CD40L, CD154) combined with anti-LFA-1 or everolimus (40-*O*-(2-hydroxyethyl)-rapamycin) resulted in stable chimerism in almost all BM recipients, whereas anti-LFA-1 plus everolimus conferred ~50% chimerism stability. Here, we investigated whether this lower incidence could be increased with deoxyspergualin (DSG) in place of or in addition to everolimus. However, DSG and everolimus were similarly synergistic with costimulation blockade for stable hematopoietic chimerism. This correlated with allospecific T cell depletion and inhibition of acute but not chronic skin allograft rejection. Different treatments were also compared for their inhibition of alloreactive T cell proliferation *in vivo*. While anti-CD40L did not impair T cell proliferation, anti-LFA-1 reduced both CD4 and CD8 T cell proliferation, and combining anti-LFA-1 with everolimus or DSG had an additive inhibitory effect on CD4 T cell proliferation. Thus, despite their strong inhibition of alloreactive T cell proliferation, combinations of anti-LFA-1 with everolimus or DSG did not reach the unique potency of anti-CD40L-based combinations to support stable hematopoietic chimerism in this system.

Thymic deletion of alloreactive T cells in hematopoietic chimeras offers the most stable form of tolerance to transplantation antigens represented by cells of hematopoietic origin. CD40L blocking antibodies have been essential components of combination treatments that permitted allogeneic bone marrow (BM) engraftment and hematopoietic chimerism in animal models [1]. Thromboembolic complications following treatment with anti-CD40L antibodies in monkeys and in human clinical trials, however, precluded the clinical use of these reagents [2]. The multi-facetted roles of lymphocyte function-associated antigen-1 (LFA-1) in T cell trafficking, T cell-antigen presenting cell adhesion, natural killer cell–target cell interaction and as a costimulatory molecule have made this integrin an attractive target in autoimmunity and transplantation (Tx), including clinical and experimental BMTx [3–6]. A 50% incidence of stable chimerism following allogeneic BMTx under cover of combined LFA-1 blockade with the mTOR inhibitor everolimus suggested a potential for an anti-CD40L-free anti-LFA-1-based regimen, if a combination with improved synergism could be identified [5]. In the light of several reports suggesting that LFA-1 blockade and mTOR inhibition impair CD8 T cells more strongly than CD4 T cells, a potent CD4 T cell inhibitor might be a more effective combination partner for anti-LFA-1 [7–10]. Deoxyspergualin (DSG) was shown to suppress CD4 T cell expansion and ameliorate CD4 T cell-dependent autoimmunity [11]. Furthermore, DSG and its analogue LF15-0195 impaired the maturation and activation potential of antigen presenting cells, thereby further inhibiting T cells indirectly and inducing allograft tolerance in some studies [12–14]. We therefore compared the synergistic capacities of everolimus and DSG for promoting stable mixed hematopoietic chimerism in the same fully allo-mismatched BALB/c to C57BL/6 (B6) BMTx model as used before [5]. All experimental procedures for BMTx, recipient treatments and chimerism analysis by flow cytometry were performed as described previously and as depicted in Fig. 1 [5]. Lyophilized DSG (Novartis, Basel) was reconstituted in water. The percentage of chimerism among granulocytes/monocytes, B cells, and T cells is the percentage of H-2D^d-positive cells among PBL gated for a specific marker, CD11b, B220, and CD3, respectively.

To assess transplantation tolerance, full-thickness tail skin of ~1 cm^2 was transplanted on the dorsolateral thorax of B6 recipients ~3 months after BMTx, as described [5]. Endpoints for acute skin graft rejection were defined as 100% graft necrosis. Because endpoints for the slow

Abbreviations: BM, bone marrow; CD40L, CD40Ligand; CFSE, carboxyfluorescein succinimidyl ester; DC, dendritic cell; DSG, deoxyspergualin; ICAM, intercellular adhesion molecule; LFA-1, lymphocyte function-associated antigen-1; mTOR, molecular target of rapamycin; TEC, thymic epithelial cell; Tx, transplantation.

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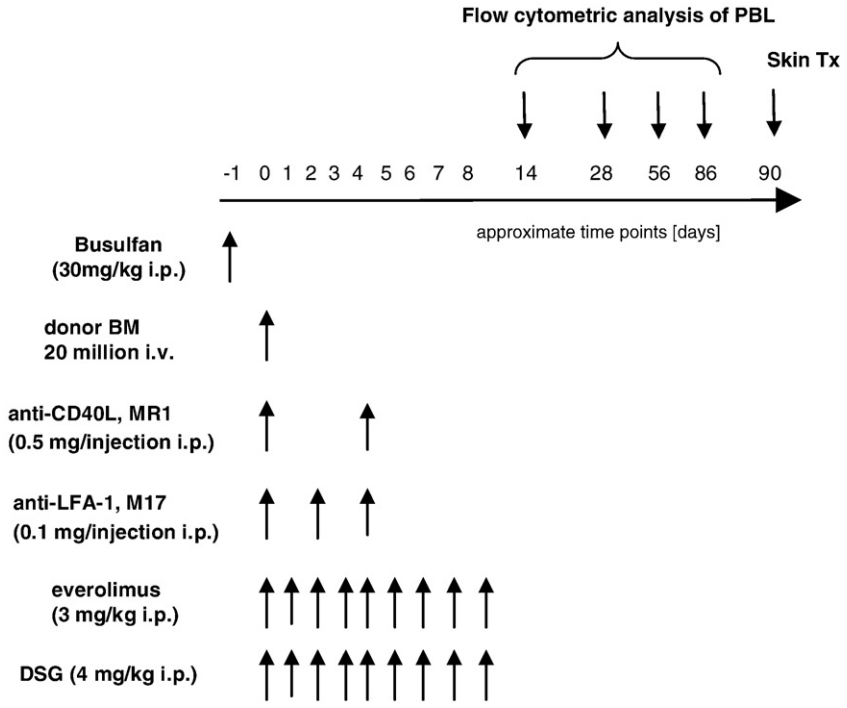

Fig. 1. Experimental design. Arrows show the time points of treatments with the indicated reagents or BM cells, dosed as depicted for each item. Arrows above the time scale indicate approximate time points of chimerism analysis and skin Tx.

process of chronic rejection were less clear, this is indicated as the first day of noticeable changes such as dryness and flakiness. For histological analysis, excised skin grafts were fixed in 10% buffered formalin for at least 48 hours and processed and embedded in paraffin according to standard procedures. Three-micrometer-thick sections were stained with hematoxylin and eosin (H&E) and examined under a light microscope.

To measure alloreactive T cell proliferation *in vivo*, red blood cell-depleted suspensions of spleen cells from B6 mice were stained with 2 μM carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes) for 10 min at 37 °C. Recipient C.B-17-SCID (BALB/c background) beige mice (Taconic) were injected i.v. with 2×10^7 CFSE-labeled B6 cells. Three days later, recipients were sacrificed, and single cell suspensions from individual recipient spleens stained with anti-CD3e-Allophycocyanin (clone 145-2C11), anti-CD4-PE (clone RM4-5) and anti-CD8-PerCP (clone 53-6.7), all purchased from BD Pharmingen. Flow cytometry data were acquired on a FACS Calibur (BD Biosciences) using CellQuest software and analyzed with FlowJo software (Treestar, CA).

In the BMTx studies, individual reagents consistently failed to support stable hematopoietic chimerism, whereas dual combinations of anti-CD40L with either anti-LFA-1 or everolimus given around the time of BMTx promoted mostly 100% incidences of stable multi-lineage chimerism [5] and Fig. 2a–c. In direct comparison, the combination of anti-CD40L with DSG was similarly effective and resulted in 100% stable chimerism (Fig. 2d). High chimerism levels, as obtained with the dual combination anti-LFA-1 plus everolimus were also achieved when everolimus was replaced with DSG. This did not, however, increase the incidence of ~50% stable chimerism (Fig. 2e, f). Moreover, there was no further increase with the triple combination anti-LFA-1, everolimus and DSG; nor could the dual combination DSG plus everolimus support chimerism, indicating that these two drugs did not synergize in this system (Fig. 2g, h).

Upon donor BM stem cell engraftment, hematopoietic cells of both host and donor origin participate in thymic negative selection. For the BALB/c to B6 combination, this may be revealed by the depletion of host $V\beta11^+$ TCR T cells specific for a donor I–E-restricted endogenous retroviral superantigen [15,16]. An increase in the ratio of unaffected $V\beta8^+$ to donor-specific $V\beta11^+$ cells among T cells of host origin 3 months after BMTx appeared to be predictive for chimerism stability and central allograft tolerance in this model [5]. Here, we used this parameter to follow chimerism development upon BMTx under DSG-containing combination treatments. Stable multi-lineage hematopoietic chimerism correlated with a marked rise in the Vβ8/Vβ11 ratio from 3.5–4 to 8–16 among host type T cells (Fig. 3A). Stable chimerism always protected allo-BM-matched skin grafts from acute rejection, yet failed to prevent their slow deterioration and involution, as reported in earlier studies for several models, and recently for the BALB/c to B6 Tx combination by us and others (Fig. 3B) [5,17–19]. While the levels of chimerism were generally high for BM recipients with stable chimerism in the present study, our previous experiments including BM recipients with low (<1%) but stable chimerism had revealed that the stability rather than the degree of chimerism is critical to avert allospecific acute skin graft rejection in this model. Furthermore, the levels of chimerism did not correlate with the onset of chronic skin allograft rejection (Fig. 3B, Ref [5]).The symptoms of patchy dryness, dandruff-like shedding and hair loss correlated with the histopathology of selective cellular infiltrates of sebaceous glands and hair follicles. By contrast, BALB/c allografts that appeared intact by macroscopic examination were indistinguishable from isografts by histological analysis (Fig. 4).

Different efficacies of immune-modulatory treatments to promote stable chimerism should be reflected by their different capabilities to limit the quantity and/or productive quality of alloreactivity. To address the quantitative aspect, we tested the anti-proliferative effects of single and combined reagents on CFSE-labeled B6 donor spleen cells transferred to SCID/beige recipients on a BALB/c background [10]. Although the immunodeficiency of the SCID host provokes homeostatic (i.e. self-peptide MHC-dependent) proliferation of transferred syngeneic T cells, the more vigorous proliferation as well as the lack of CD8 coreceptor downregulation, a characteristic of homeostatic but not of alloreactive proliferation, revealed the alloantigen-driven nature of T cell proliferation in this transfer system [20]. SCID recipients of B6 cells were treated with individual or combined reagents for 3 days as specified in Fig. 1 (but without busulfan). Three days after cell transfer, CD4 and CD8