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JLB

Highlighted Article

Early IL-10 production is essential for syngeneic graft acceptance

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ABSTRACT

We performed a comparative study and evaluated cellular infiltrates and anti-inflammatory cytokine production at different time-points after syngeneic or allogeneic skin transplantation. We observed an early IL-10 production in syngeneic grafts compared with allografts. This observation prompted us to investigate the role of IL-10 in isograft acceptance. For this, we used IL-10 KO and WT mice to perform syngeneic transplantation, where IL-10 was absent in the graft or in the recipient. The majority of syngeneic grafts derived from IL-10 KO donors did not engraft or was only partially accepted, whereas IL-10 KO mice transplanted with skin from WT donors accepted the graft. We evaluated IL-10 producers in the transplanted skin and observed that epithelial cells were the major source. Taken together, our data show that production of IL-10 by donor cells, but not by the recipient, is determinant for graft acceptance and strongly suggest that production of this cytokine by keratinocytes immediately upon transplantation is necessary for isograft survival. ***J. Leukoc. Biol.*** **91: 000–000; 2012.**

Introduction

Inflammation is inherent in surgical trauma in organ or tissue transplantation and may significantly contribute to graft rejection. In conditions where donor-recipient MHC antigens mismatch, alloantigens play a significant role in immune-mediated tissue destruction. Amplification of the inflammatory response is, in this context, the result of activation of alloreactive T cells and recruitment of primed T cells and leukocytes into the graft [1, 2]. Studies by El-Sawy et al. [3] show that recipient's $CD8^+CD62^{low}$ alloreactive T cells migrate into cardiac allografts very early on and up-regulate the inflammatory response by an IFN-γ-mediated effect. In models of syngeneic cardiac and skin transplantation, neutrophil infiltration peaks early but subsequently decreases [3, 4]. Therefore, absence of alloreactivity in isografts favors the resolution of inflammation and consequently, graft acceptance. This phenomenon requires pro- and anti-inflammatory factors to be tightly orchestrated to guarantee modulation of the inflammatory response.

IL-10 is a regulatory cytokine produced by various immune cells, including DCs, macrophages, T and B lymphocytes, mast cells, NK cells, neutrophils and eosinophils (reviewed in ref. [5]), and other cell types, such as keratinocytes [6]. IL-10 suppresses the release of proinflammatory mediators by monocytes/macrophages and inhibits $CD4^+$ T cell activation by modulating the expression of MHC II and costimulatory molecules on APCs or acting directly by inhibiting proliferation and cytokine synthesis of these T cells (reviewed in ref. [7]). In patients undergoing liver resection, a transient increase of IL-10 was observed during surgery, suggesting a role for this cytokine in modulation of the inflammatory response during the operative period [8]. In mice, the effect of treatment with IL-10 on allograft survival is as yet unclear: some reports support a role for IL-10 in allograft acceptance [9–12], whereas other studies show no effect [13–16].

Herein, we report the observation of an early IL-10 production in syngeneic transplants compared with allogeneic grafts in a time-course study, which prompted us to investigate the role of this cytokine in isograft acceptance. Our data show that IL-10 in the donor but not in the recipient tissues is determinant and strongly suggest that production of this cytokine by keratinocytes immediately upon transplantation is necessary for isograft survival.

MATERIALS AND METHODS

Animals

Eight- to 10-week-old BALB/c ($H\text{-}2^d$), NZW ($H\text{-}2^z$), C57Bl/6 ($H\text{-}2^b$), and C57Bl/6 *Il-10*$^{-/-}$ male mice were obtained from the animal facility of the Biomedical Institute of the University of São Paulo (São Paulo, Brazil). C57Bl/6 IL-$10^{IRESeGFP}$ (IL-10 eGFP) [17] male mice were obtained from

Abbreviations: CNPq=Conselho Nacional de Desenvolvimento Científico e Tecnológico, FAPESP=Fundação de Amparo a Pesquisa do Estado de São Paulo, FCT=Fundação de Ciência e Tecnologia, GR-1=granulocyte receptor 1, IBD=inflammatory bowel disease, IL-10 eGFP=C57Bl/6 IL-$10^{IRESeGFP}$, KO=knockout/deficient, Treg=regulatory T cell

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the animal facility of Instituto Gulbenkian de Ciência (Oeiras, Portugal). All animals were maintained under specific pathogen-free conditions on standard pellet food and with water ad libitum. Experiments were performed following the guidelines for animal use, approved by the Ethics Committee on Animal Experimentation.

Skin transplantation

Donor tail skin was grafted on the back of recipients. Skin was excised from the donor tail, cut into pieces, 1 cm^2 in size, and kept in PBS at room temperature until use. Grafts were placed on a bed prepared by removing an area on the back dermis of the recipient, sutured, and covered with gauze. Lack of acceptance was scored when 80% of the donor skin area was destroyed.

Recovery of graft cells

Grafts were removed from the recipients, cut in four equal pieces, and placed in 0.5 mL DMEM (Gibco-BRL, Life Technologies, Grand Island, NY, USA), supplemented with 5% FBS (Hyclone, Logan, UT, USA), 10^{-5} M 2-ME (Sigma-Aldrich, St. Louis, MO, USA), 2 mM L-glutamine, 0.1 mM vitamins, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 100 μg/ml gentamicin (all from Gibco-BRL) in a 48-well plate. After incubation for 24 h, the emigrated cells were removed, washed, counted, and phenotyped using fluorescent antibodies. Histological examination at each time-point after cell emigration showed that most of inflammatory cells emigrated from the transplants. Supernatants were collected and stored at −70°C until use. For histological analysis, grafts were soaked in OCT (Tissue-Tek, Sakura Finetek USA, Torrance, CA, USA), frozen in dry ice, and stored at −80°C.

Cytokine measurement

IL-10 and TGF-β were quantified in the supernatants by two-step sandwich ELISA using commercial kits from R&D Systems (Minneapolis, MN, USA), according to the manufacturer's instructions.

Flow cytometry

Cells were incubated for 20 min on ice with Fc block (hamster anti-mouse CD16/32, clone 24G2) and subsequently labeled with fluorescent mAb against GR-1(PE), CD11b(FITC), CD3(FITC), CD4(Cy), CD8(PE), or matched isotype controls for 30 min. All antibodies were from PharMingen, Becton Dickinson (San Diego, CA, USA). Cells were washed and analyzed in a FACSCalibur (CellQuest software) cell cytometer (Becton Dickinson).

Histolological analysis of skin

Frozen skin was cut in 10 μm-thick slices using a Leica 3050S cryostat (Leica Microsystems, Germany). Sections were labeled directly with DAPI (500 μg/ml) and phalloidin-tetramethylrhodamine B isothiocyanate (5 μg/ml; both from Sigma-Aldrich) for 5 min at room temperature in a dark chamber. After three washes in PBS, slides were mounted with Mowiol mounting media (Sigma-Aldrich) and images acquired in a DMRA2 Leica microscope (Leica Microsystems) using Metamorph software.

IL-10 treatment

Commencing on the day of transplantation, C57Bl/6 mice were injected daily with 500 pg purified rIL-10 (COS cell supernatant) in PBS (100 μl/animal) at the transplant site for a total of 6 days. The control group received PBS only.

Statistical analysis

Graft survival was compared between groups by log-rank sum test; unpaired *t* tests or ANOVA with Tukey's post-test were used. Analyses were performed using Prism 4.0 software (GraphPad Software, La Jolla, CA, USA). Data were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Kinetics of inflammatory cell infiltration and anti-inflammatory cytokine production in syngeneic and allogeneic grafts

We evaluated cell infiltration and cytokine production in allogeneic and syngeneic grafts at different time-points after transplantation. BALB/c (H-2^d) recipients were grafted with the allogeneic tail skin from NZW (H-2^z) or syngeneic BALB/c (H-2^d) donors and monitored for 45 days for graft survival. Allogeneic grafts were rejected with a median survival time of 9 days in BALB/c recipients, whereas all syngeneic transplants were accepted (data not shown). For the comparative study, grafts were excised at different time-points and cultured in complete medium for an additional 24 h. Emigrating cells were collected and phenotyped for polymorphonuclear (PMN) cells ($GR1^{high}$ $CD11b^+$) and macrophages ($GR1^{low}$ $CD11b^+$) (**Fig. 1A**), and $CD4^+$ and $CD8^+$ T cells (Fig. 1B). In general, the number of cells infiltrating the grafts was similar in allogeneic and syngeneic transplants during the first 72 h (Fig. 1A and B). We observed increased infiltration of PMN cells, mainly neutrophils ($GR1^{high}$ $CD11b^+$), macrophages ($GR1^{low}$ $CD11b^+$), and $CD4^+$ and $CD8^+$ T cells in allografts compared with isografts on Day 7 post-transplantation. The number of macrophages and PMN cells decreased constantly after 72 h in isografts. These results show that the magnitude of leukocyte infiltration during the first 72 h is independent of MHC donor-recipient combinations. Previous studies have reported enhanced PMN cell infiltration in cardiac allografts compared to isografts, with a pathologic effect driven by early IFN-γ-producing $CD8^+$ T cells infiltrating the graft [3]. In our study, allogeneic and syngeneic skin grafts had high infiltration of neutrophils, 24 h after transplantation, which decreased progressively in the syngeneic combination reaching baseline values. In allogeneic skin grafts, we observed two waves of neutrophil infiltration. The second peak coincided with enhanced $CD8^+$ and $CD4^+$ T cell infiltration (Fig. 1B). In syngeneic grafts, the number of neutrophils that infiltrated was very similar to values in allogeneic transplantation. This suggests that the latter observation might be related to tissue injury rather than constituting a specific immune response. Unpublished data from our group showed that depletion of PMN cells with anti-GR-1 antibodies before allogeneic transplantation interfered with vascularization of the graft, leading to death by hypoxia. This suggests that neutrophils that infiltrate allografts in the initial phase are not deleterious to the transplant. Studies in the literature strongly support the role of neutrophils in mediating angiogenesis through expression of a wide variety of proangiogenic factors by these cells [17]. Therefore, apparently, the absence of infiltrating neutrophils in the initial phase affects neovascularization and wound healing. Moreover, continuing entry of PMN cells into the allograft, especially after 72 h, also suggests that these cells are being recruited as a result of the inflammatory context, a consequence of the alloreactive response. Decrease of neutrophil infiltrates at this time-point in syngeneic grafts is strongly suggested to be associated with modulation of inflammation. In fact, levels of IFN-γ in allografts at Day 7 post-transplantation (5200±696.8

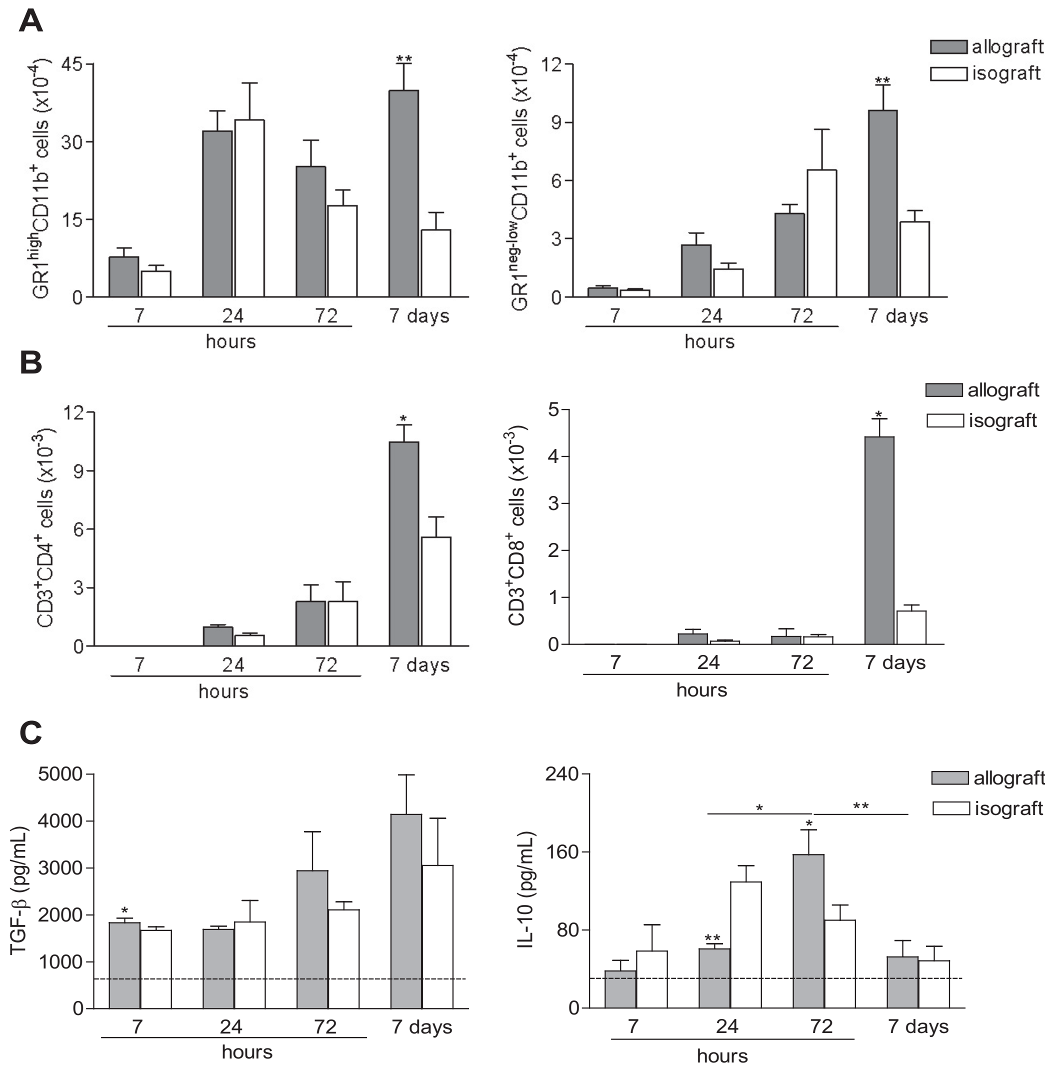

Figure 1. Kinetics of inflammatory cell migration and cytokine production after allogeneic or syngeneic skin transplantation. BALB/c mice were grafted with NZW or syngeneic tail skin. Grafts were excised from the hosts at different time-points and cultured in complete medium for 24 h. Total cells were collected, counted, and phenotyped by flow cytometry. Cytokine content of supernatants was measured by ELISA. (A) Number of GR-1^{high} and GR-1$^{neg-low}$ CD11b^{+} cells. (B) Number of CD3^{+}CD4^{+} and CD3^{+}CD8^{+} cells. (C) TGF-β and IL-10 quantification. Results are expressed as mean ± SEM. *$P < 0.05$; **$P < 0.01$; compared with isograft within the same time-point or where indicated. Results are representative of three independent experiments.

pg/ml) revealed a 19-fold increase compared with the previous time-point (72 h: 267.4±165.5 pg/ml). In syngeneic grafts, cytokine levels were similar to allografts at 72 h (137.0±52.6 pg/ml), decreased to undetectable levels on Day 7 (33.3±8.8 pg/ml), and were significantly lower compared with allografts (P=0.0018).

Regarding anti-inflammatory cytokines (Fig. 1C), TGF-β was already detected in supernatants within 7 h after transplantation; except for this time-point, no differences were observed comparing allografts and isografts. Interestingly, IL-10 peaked earlier in syngeneic grafts (at 24 h) and was at a higher level in comparison with allogeneic grafts. This prompted us to investigate the influence of IL-10 on the outcome of syngeneic skin transplantation.

IL-10 production by epidermal cells

We investigated the source of IL-10 production in the skin. For this, we used the IL-10 eGFP mouse, where the eGFP coding sequence was gene-targeted to the *Il-10* locus just downstream of the translation stop [18]. C57Bl/6 mice were grafted with IL-10 eGFP or WT tail skin for 24 h. The graft was then excised from the recipient and cultured in complete medium for an additional 24 h to remove most of the inflammatory cell infiltrates. Frozen sections were stained with DAPI and phalloidin-rhodamine for nuclei and actin detection, respectively. We observed GFP-positive cells in the epidermal layer and surrounding the hair follicles of IL-10 eGFP skin but not in WT grafts (**Fig. 2A**); fluorescent cells colocalized with cytoskeleton (Fig. 2B) but not cell nuclei (Fig. 2C), suggesting that IL-10 has been produced by these cells and could be detected in the cytoplasm. As keratinocytes are the dominant cell type of the epidermis [19], our data strongly suggest that these cells constitute the major population that secretes IL-10 in the skin. Keratinocytes activated by UV radiation have already been shown to be involved in immune suppression via production of IL-10 [6, 20–22]. It is possible that in our model, IL-10 is being produced within a few hours after transplantation. This would be crucial to control PMN influx at earlier time-points (72 h; Fig. 1A), macrophages, and CD4^{+} and CD8^{+} T cells in a later phase (7 days; Fig. 1A and B). Moreover, mac-

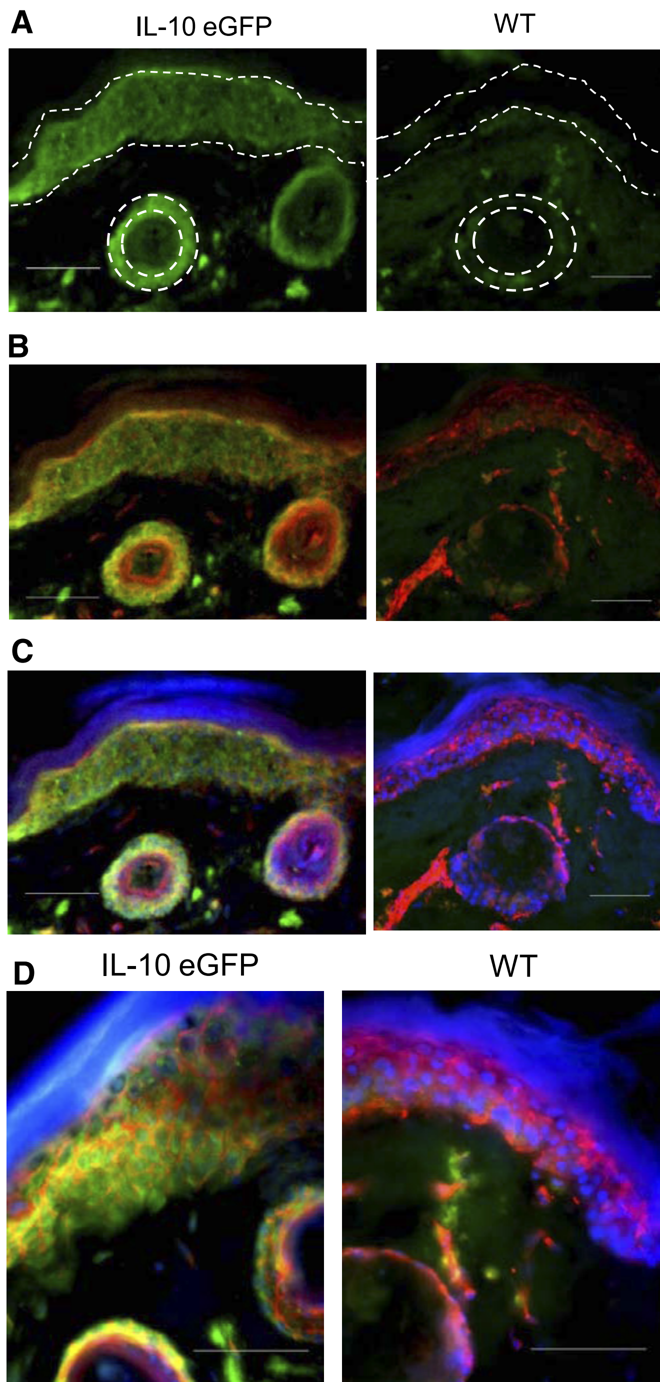

Figure 2. Cells from the epidermis are the main IL-10 producers in the skin. Tail skin from IL-10 eGFP or WT mice was transplanted onto WT recipients for 24 h. Grafts were then removed from recipients, cultured for an additional 24 h to remove inflammatory cells, frozen, and sectioned. Sections were labeled with DAPI (blue) and phalloidin rhodamin (red) to stain cell nuclei and actin, respectively. (A) GFP^+ cells can be seen in the epidermis, which also surrounds a hair follicle (dotted lines), and colocalize with actin (B) and cell nuclei (C). (D) Merged images are magnified. Scale bars: 60 μm. Results are representative of a group of five transplanted mice.

rophages and T cells infiltrating syngeneic grafts may also be IL-10 producers contributing to graft acceptance.

Evaluation of the importance of IL-10 in syngeneic graft acceptance

To evaluate if early IL-10 production could be involved in modulation of the inflammatory response in syngeneic grafts, we transplanted C57Bl/6 WT or C57Bl/6 *Il-10*$^{-/-}$ (IL-10 KO) tail skin onto IL-10 KO recipients; in a reciprocal set-up, skin from KO mice was grafted into WT recipients (**Fig. 3A** and **B**). Grafts from IL-10 KO mice showed a lack of acceptance by ~60% of IL-10-competent hosts (KO→WT; $n=15$), whereas in controls, all grafts were accepted (WT→WT; $n=11$). When skin from WT mice was transplanted onto IL-10 KO recipients, 80% of individuals accepted the graft (WT→KO; $n=13$). The incidence of graft acceptance in the total absence of IL-10 (KO→KO; $n=17$) was very similar to that observed when only recipients lacked IL-10 (KO→KO vs. KO→WT, $P=0.41$; KO→KO vs. WT→WT, $P=0.0004$; KO→KO vs. WT→KO, $P=0.004$; WT→WT vs. WT→KO, $P=0.18$; WT→WT vs. KO→WT, $P=0.002$; WT→KO vs. KO→WT, $P=0.02$). To evaluate if rejection was associated with an indirect effect as a result of absence of IL-10 production by donor cells, we treated donor IL-10 KO skin immediately after transplantation and for 6 consecutive days with mouse rIL-10. Results showed that treatment partially restored graft survival in WT recipients, as measured by percentage of accepted skin area compared with PBS-treated mice (Fig. 3C). Although IL-10 KO grafts were not accepted completely when transplanted into WT recipients, there was an increase of skin area accepted compared with controls. IL-10 appears to be important in this phenomenon at the very initial stages of the inflammatory process. Our observations are in accordance with various in the literature, which suggests a tolerogenic role for IL-10 before and during the first priming events in organ transplantation (reviewed in ref. [23]).

Previous studies have shown accelerated wound closure in IL-$10^{-/-}$ mice and an enhanced number of macrophages in inflammatory infiltrates compared with IL-10-competent controls [24]. These data suggest that in the absence of IL-10, these infiltrating macrophages release TGF-β1, which mediates myofibroblast differentiation and rapid wound contraction. In fetal wounds, IL-10 KO grafts showed enhanced inflammation and increased collagen deposition compared with WT wounds, suggesting a role for this cytokine in regulating matrix deposition, as well as the expression of proinflammatory cytokines [25].

Analysis of cellular infiltration in grafts from IL-10 KO mice transplanted onto IL-10-competent recipients shows enhanced numbers of PMN cells and a slight decrease in infiltration of macrophages, 7 days after syngeneic transplantation compared with WT grafts transplanted onto WT recipients (Fig. 3D). We observed no difference in numbers of $CD4^+$ and $CD8^+$ T cell infiltrates or IFN-γ levels among these groups (data not shown). These results suggest that lack of acceptance of IL-10 KO grafts by WT recipients is mostly probably dependent on innate immune mechanisms rather than the adaptive compartment. Nevertheless, we do not exclude the possibility that $CD4^+CD25^+$ Tregs could be impaired in IL-10 KO skin, although we have not determined the Treg/$CD4^+$ effector T cell ratio in the grafts. As in

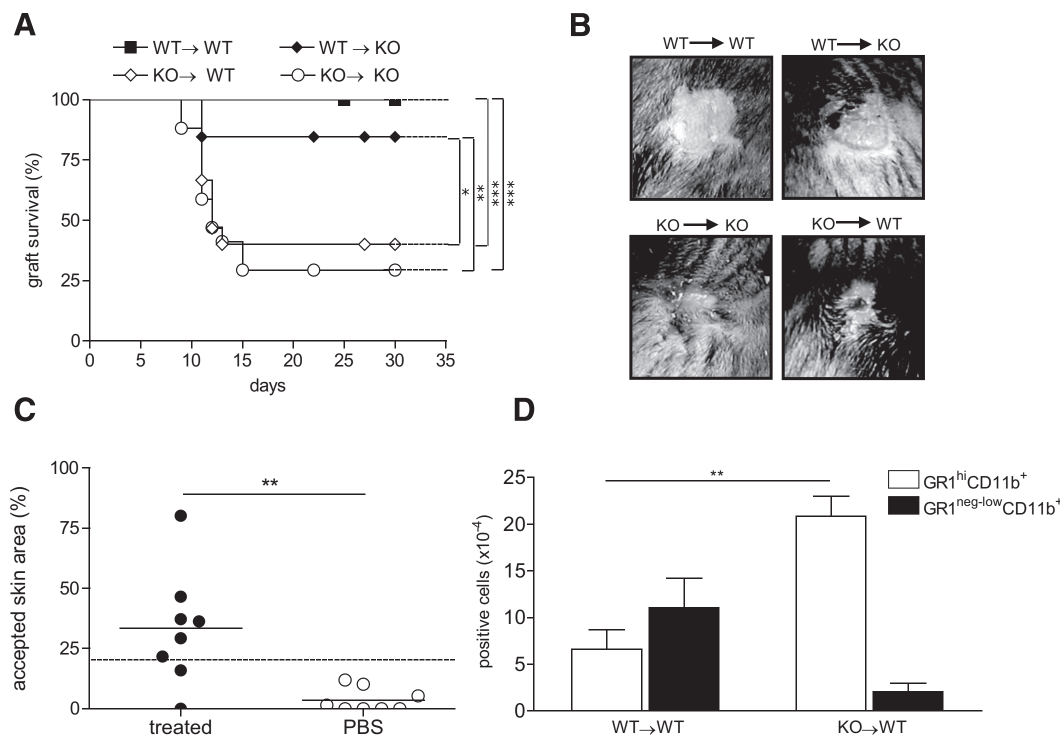

Figure 3. IL-10 from the donor determines isograft survival. (A) Survival of WT or IL-10 KO tail skin transplanted into WT or KO recipients [WT→WT (n=11); WT→KO (n=13); KO→WT (n=15); KO→KO (n=17)]. (B) Morphological appearance of accepted grafts (WT→WT; WT→KO) and scars from nonaccepted grafts (KO→KO; KO→WT). (C) Percentage of accepted skin area was measured after daily treatment (for 6 days) at the site of the transplant with rIL-10 (500 pg/mice), commencing on the day of transplantation. Control group received daily injection of PBS. Individuals below the dotted line had total graft loss. (D) PMN and macrophage infiltration on Day 7 post-transplantation in IL-10 KO or WT grafts transplanted to WT recipients. Results are expressed as mean ± SEM and representative of three independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

autoimmunity, the lack of isograft acceptance could rely on impaired modulation of responder T cells to self-antigens. The implication of $CD4^+$ and recently, $CD8^+$ Treg subsets in suppressing effector T cell responses has been very well documented in several experimental models of autoimmunity (reviewed in ref. [26]). Although the degree of Treg involvement in skin isograft tolerance is not clear, Tregs in the human skin have been shown to exhibit proliferative and suppressive properties and could regulate the response to autoantigens exposed during injury [27]. A decreased percentage of these cells in the LNs was associated with impaired skin isograft acceptance in mice selected for minimal inflammatory response [4].

Our results strongly suggest an important role for IL-10 in syngeneic graft acceptance and that the relevant source of IL-10 is donor epidermal cells. Although cells from the recipient migrate into donor tissue early on, IL-10 production by these leukocytes does not seem to be sufficient to control tissue destruction when donor skin lacks this cytokine. Differently from our results and in another setting, responder allogeneic T cells have been shown to be the major source of IL-10 production when cultured with autologous epidermal cells [28]. In this case, autologous keratinocytes were the potent inducers of suppression to alloreactivity by generating IL-10-secreting T cells; the contribution of keratinocytes themselves was marginal. Allogeneic skin sheets containing tiny pieces of autologous skin epidermis have been used successfully to treat patients with severe burns [29, 30]. This indirect keratinocyte-derived effect does not explain our results on WT isograft acceptance in IL-10 KO recipients. In this case, the involvement of host-derived, IL-10-secreting responder T cells is lacking as a result of IL-10 KO reinforcing the direct contribution of keratinocyte-induced IL-10 production to isograft tolerance.

Our data do not support the use of systemic of IL-10 in patients undergoing autologous skin transplantation as a result of the small contribution of systemic IL-10 to skin isograft tolerance. Nevertheless, it would be interesting to evaluate IL-10 production in the donor skin during the initial phase of transplant, and local IL-10 administration could be considered. In line with this idea, some animal studies and even a clinical trial involving local administration with IL-10 have been conducted, such as in IBD, where systemically administered IL-10 has limited distribution to the colonic site of inflammation [31]. Oral administration of genetically modified *Lactococcus lactis*, which secretes IL-10 directly in the gut, has been shown to improve IBD in a mouse model and was being tested recently in a phase I clinical trial [32, 33].

The mechanisms by which IL-10 exerts its healing effects in our model will require further investigation.

AUTHORSHIP

T.T. conceived of and designed research/study, performed experiments, analyzed data, and wrote the paper. C.E.T. performed experiments, analyzed data, and wrote the paper. L.V.R. wrote the paper. L.V.d.M. conceived of and designed research/study, performed experiments, analyzed data, and wrote the paper.

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