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Critical Role of Regulatory T Cells in Th17-Mediated Minor Antigen-Disparate Rejection

Benoît Vokaer,* Nicolas Van Rompaey,* Philippe H. Lemaître,* Frédéric Lhomme,* Carole Kubjak,* Fleur S. Benghiet,* Yoichiro Iwakura,† Michel Petein,‡ Kenneth A. Field,§ Michel Goldman,* Alain Le Moine,* and Louis-Marie Charbonnier*†

Th17-mediated immune responses have been recently identified as novel pathogenic mechanisms in a variety of conditions; however, their importance in allograft rejection processes is still debated. In this paper, we searched for MHC or minor Ag disparate models of skin graft rejection in which Th17 immune responses might be involved. We found that T cell-derived IL-17 is critical for spontaneous rejection of minor but not major Ag-mismatched skin grafts. IL-17 neutralization was associated with a lack of neutrophil infiltration and neutrophil depletion delayed rejection, suggesting neutrophils as an effector mechanisms downstream of Th17 cells. Regulatory T cells (Tregs) appeared to be involved in Th17 reactivity. We found that in vivo Treg depletion prevented IL-17 production by recipient T cells. An adoptive cotransfer of Tregs with naive monospecific antidonor T cells in lymphopenic hosts biased the immune response toward Th17. Finally, we observed that IL-6 was central for balancing Tregs and Th17 cells as demonstrated by the prevention of Th17 differentiation, the enhanced Treg/Th17 ratio, and a net impact of rejection blockade in the absence of IL-6. In conclusion, the ability of Tregs to promote the Th17/neutrophil-mediated pathway of rejection that we have described should be considered as a potential drawback of Treg-based cell therapy.


C ontrolling alloreactive T cells remain a decisive challenge to achieve allograft tolerance. Alone or together, CD4+ and CD8+ alloreactive T cells are effectors of rejection. Because of their ability to trigger delayed-type hypersensitivity reactivity, alloreactive CD4+ Th1 cells were initially considered as master effectors of tissue damage. Afterward, Th2 cells-mediated pathways of acute and chronic allograft rejection were identified (1). Recently, IL-17–secreting CD4+ Th cells appeared as a novel and distinct Th cell lineage (Th17) (2). Although a pathogenic role for the Th17 cell subset has been well established in several experimental models of autoimmune disease or sepsis (2), their intrinsic participation in the allograft rejection process has been less established. Results from several studies have reported an accumulation of IL-17 in the context of chronic lung, liver, and kidney allograft rejection in human (3–5) as well as in a human–mouse chimeric model of vasculopathy (6). In more sophisticated models in which Th1 immune responses are impaired by the deficiency of T-bet, mouse Th17 cells mediate an accelerated form of cardiac allograft rejection accompanied with a dense neutrophil infiltration (7). Similarly, T-bet−/−CD8+ T cells mediate rejection after costimulation blockade (8, 9). However, little evidence is available about a putative IL-17–mediated pathway of allograft rejection in wild-type recipients.

Because of their ability to control both Th1 and Th2 immune responses, regulatory T cells (Tregs) are considered a potential therapy for tolerance in clinical transplantation. However, the complex interactions between Tregs and Th17 require further investigation. Indeed, Tregs have been reported to promote Th17 differentiation during in vitro polyclonal stimulation by anti-CD3 (10–13). By this way, emergent alloreactive Th17 cells could represent a limitation to Treg cellular therapy. In this study, we investigated the role of IL-17A in skin transplantation across different MHC or minor Ag disparities and tested the role played by Tregs in this pathway of rejection.

Materials and Methods

Mice

BALB/c, C57BL/6.C-H-2bm1 (bm1), C57BL/6.C-H-2bm12 (bm12), and C57BL/6 (B6) deficient for IL-4 or IL-6 (IL-4−/− or IL-6−/− B6 mice), RAG1−/− B6, and B6-Thy1.1 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Wild-type B6 mice were purchased from Harlan (Zeist, The Netherlands). IL-17A−/− B6 mice were provided by Dr. Y. Ikawura, Institute for Medical Science (Tokyo, Japan). TCR transgenic female Marilyn mice that recognize male HY peptide (NAGFNSRanceressS) (TCR Vα1.1, TCR Vβ6) presented by I-Ab (14) in the B6 RAG2−/− background, were purchased from the Transgenesis, Archiving and Animals Models Laboratory, Centre National de la Recherche Scientifique (Orléans France). B6 FoxP3GFp knockin mice that expressed FoxP3 as a FoxP3-GFP fusion protein were obtained from Prof. M. Alegre (University of Chicago, Chicago, IL) (15). Eight- and 12-wk-old animals were used, and animals were bred in our specific pathogen-free animal facility. All animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Institutes of Health (Bethesda, MD), and protocols were approved by the local committee for animal welfare.

Abbreviations used in this paper: B6, C57BL/6; dLN, draining lymph node; Fem., female; GIL, graft-infiltrating lymphocyte; MFI, mean fluorescence intensity; ND, not detected; PB, Pacific blue; Treg, regulatory T cell; WT, wild-type.

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Skin grafting, Ab treatments, and T cell reconstitution

Mice were anesthetized with a mixture of 5% xylazine (Rompun) and 10% ketamine in PBS. A total of 100 μl per 20 g body weight was injected i.p. Skin grafting was performed according to an adaptation of the method of Billingham and Medawar. Briefly, skin grafting was conducted by grafting full-thickness skin (1 cm²) on the lateral flank. Grafts were monitored daily after the removal of the bandage on day 10 and considered rejected when >75% of epithelial breakdown had occurred. When recipients were co-transplanted, female and male skins were grafted on opposite flanks. When specified, recipients were neutralized or depleting Ab injections. A single dose of depleting anti-CD25 (PC61; provided by O. Leo, Institute for Medical Immunology, Université Libre de Bruxelles, Gosselies, Belgium) or isotype control (YCAT provided by S. Cobbold, Sir Dunn School of Pathology, Oxford University, Oxford, U.K.) were i.p. injected at a dose of 500 μg 6 d prior skin transplantation. The neutralizing anti–IFN-γ (clone R46A2) purchased from BioXCell (West Lebanon, NH) or the isotype control (LODNP and LO-IMEX; Université Catholique de Louvain, Brussels, Belgium) was i.p. injected at a dose of 250 μg twice per week after transplantation. Neutrophil depletion was achieved by a first i.v. injection of 200 μg of the anti-Ly6G (clone RB6-8C5; BioXCell) or control Ab (LO-IMEX; Université Catholique de Louvain) and followed by injections of 50 μg of the same amount three times per week. Depletion efficiency of 90% was measured by FACS of the remaining Ly6G+CD11b+ cells among PBMCs at day 10 posttransplantation. Three nonoverlapping fields at the same focus were used. Positive cells were counted by two independent operators, and the results were obtained by CD4+ RAG1 (purity > 95% by FACS). In the second set of reconstitution experiments, RAG1−/− mice were adoptively transferred with both 8 × 10³ CD4+ and 8 × 10³ CD8+ T cells sorted with CD4 and CD8 isolation kits from Miltenyi Biotec (Bergisch Gladbach, Germany) (purity > 95% by FACS). In the second set of reconstitution experiments, RAG1−/− mice were adoptively transferred with 4 × 10^5 Marilyn splenocytes alone or together with 4 × 10^5 Foxp3-GFP Tregs. Foxp3-GFP Tregs were obtained by CD4+ spleen cell isolation from naive Foxp3-GFP mice (CD4+ T cell isolation kit; Miltenyi Biotec), followed by a GFP+ cell sorting through a Moflo cytometer (Dakocytomation, Glostrup, Denmark). CD4+ Foxp3-GFP+ cell purity was >95% by FACS.

MLC and cytokine production

Cells isolated from spleen or draining lymph nodes (inguinal and axillary) were used as responders (2.5 × 10⁶ cells/ml) and stimulated with 2.5 × 10⁶ cells/ml male or female irradiated splenocytes (2000 rad) in 48-well flat-bottom plates (150687; Nunc, Roskilde, Denmark). Cultures were incubated at 37°C in a 5% CO₂ atmosphere in medium consisting of RPMI 1640 supplemented with 2 mM L-glutamine, 1 mM nonessential amino acids, 5% heat-inactivated FCS, and 1 mM sodium pyruvate. IFN-γ production and IL-17A production were measured in culture supernatants after 48 or 96 h using commercially available ELISA kits (Duoset; R&D Systems, Minneapolis, MN). The detection threshold was <8 pg/ml for both IFN-γ and IL-17A.

RNA extraction and real-time RT-PCR

Total RNA was extracted from skin grafts or draining lymph nodes using the MagnaPure LC RNA Isolation Kit III for tissue (Roche Diagnostics, Basel, Switzerland). Reverse transcription and real-time PCR were performed using LightCycler-RNA Master Hybridization Probes (one-step procedure) on a Lightcycler apparatus (Roche Diagnostics). β-Actin was used as RNA loading control. In Fig. 2C, gene expression is expressed as 2−ΔΔCT, in which CT represents “cycle of threshold.” ΔΔCT = ΔCTmale graft − ΔCTfemale graft, and ΔCT = CTgene of interest − CTβ-actin. Primer and probe sequences are shown in Table I.

Immunostaining

Five-micrometer paraffin sections were cut, deparaffinized, and rehydrated. Endogenous peroxidase activity was first quenched by H₂O₂ peroxidase loading control. In Fig. 2A, gene expression is expressed as 2−ΔΔCT, in which CT represents “cycle of threshold.” ΔΔCT = ΔCTmale graft − ΔCTfemale graft, and ΔCT = CTgene of interest − CTβ-actin. Primer and probe sequences are shown in Table I.

Flow cytometry

Pacific blue (PB)-conjugated anti-mouse CD4 (clone RM4-5), allophycocyanin-conjugated anti-mouse CD4 (clone RM4-5), PE-conjugated anti-mouse TCR Vβ6 chain (clone RB4-7), FITC-conjugated anti-Thy1.1 (clone OX-7), FITC- or PE-conjugated anti-mouse CD25 (clones 7D4 and 3C7, respectively), PerCP-conjugated and PB-conjugated anti-mouse CD3ε (clone 500A2), PerCP-conjugated anti-mouse CD8 (clone 53-6.7) and anti-mouse-CD16/CD32 (Fc block, clone 2.4G2) mAbs, and isotype controls were purchased from BD Pharmingen. Allophycocyanin-conjugated anti-mouse IL-17A (clone eBio17B7), Alexa Fluor 647-conjugated anti-mouse T-bet (clone eBio4B10), and PB-conjugated anti-mouse FoxP3 (clone FJK-16s) were purchased from eBioscience (San Diego, CA). Cytometry analysis was performed on a CyAn-LX cytometer using Summit 4.1 software (DakoCytometry). IFN-γ and IL-17A intracytoplasmic stainings were performed after cell incubation with 50 ng/ml PMA and 500 ng/ml ionomycin for 4 h with brefeldin A (10 μg/ml) in the last 2 h; then, the cells were incubated for 10 min with Fc block, stained for surface markers for 20 min, washed with 0.1% PBS/BSA/0.01% NaN₃, fixed with CytoFix/Cytoperm (BD Biosciences, San Jose, CA), permeabilized with Perm/Wash buffer (BD Biosciences), and labeled with anti-cytokine Abs. FoxP3 staining was performed after cell surface marker labeling by using eBioscience fixation/ permeabilization and permeabilization buffers, according to the manufacturer’s instructions. To isolate graft-infiltrating lymphocytes (GILs), skin grafts were minced and then incubated at 37°C for 2 h with type I collagenase at 2.5 mg/ml (Sigma-Aldrich, St. Louis, MO) and hyaluronidase at 0.25 mg/ml (Sigma-Aldrich) in a phosphate-buffered solution.

Statistical analyses

Statistical analyses of differences between groups were performed using the two-tailed Mann-Whitney nonparametric test. Graft survival curves were compared by the log-rank test. A two-tailed paired t test was used for kinetic RT-PCR experiments comparing intragraft cytokine profiles between female and male grafts (Fig. 2C). A p value < 0.05 is considered statistically significant.

Results

IL-17A neutralization does not affect MHC-mismatched skin allograft rejection

To assess the potential role of IL-17A in skin allograft rejection, B6 wild-type or IL-17A−/− recipient mice were grafted with allogeneic (clone RB4-7), FITC-conjugated anti-Thy1.1 (clone OX-7), FITC- or PE-conjugated anti-mouse CD25 (clones 7D4 and 3C7, respectively), PerCP-conjugated and PB-conjugated anti-mouse CD3ε (clone 500A2), PerCP-conjugated anti-mouse CD8 (clone 53-6.7) and anti-mouse-CD16/CD32 (Fc block, clone 2.4G2) mAbs, and isotype controls were purchased from BD Pharmingen. Allophycocyanin-conjugated anti-mouse IL-17A (clone eBio17B7), Alexa Fluor 647-conjugated anti-mouse T-bet (clone eBio4B10), and PB-conjugated anti-mouse FoxP3 (clone FJK-16s) were purchased from eBioscience (San Diego, CA). Cytometry analysis was performed on a CyAn-LX cytometer using Summit 4.1 software (DakoCytometry). IFN-γ and IL-17A intracytoplasmic stainings were performed after cell incubation with 50 ng/ml PMA and 500 ng/ml ionomycin for 4 h with brefeldin A (10 μg/ml) in the last 2 h; then, the cells were incubated for 10 min with Fc block, stained for surface markers for 20 min, washed with 0.1% PBS/BSA/0.01% NaN₃, fixed with CytoFix/Cytoperm (BD Biosciences, San Jose, CA), permeabilized with Perm/Wash buffer (BD Biosciences), and labeled with anti-cytokine Abs. FoxP3 staining was performed after cell surface marker labeling by using eBioscience fixation/ permeabilization and permeabilization buffers, according to the manufacturer’s instructions. To isolate graft-infiltrating lymphocytes (GILs), skin grafts were minced and then incubated at 37°C for 2 h with type I collagenase at 2.5 mg/ml (Sigma-Aldrich, St. Louis, MO) and hyaluronidase at 0.25 mg/ml (Sigma-Aldrich) in a phosphate-buffered solution.

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Skin from a panel of different mouse strains. First, skin from BALB/c, bm1, or bm12 donors was transplanted as full MHC mismatch (class I + II), single class I, or class II MHC mismatches, respectively (Fig. 1). For all of the allogeneic combinations tested, no significant improvement of allograft survival was observed in IL-17A−/− mice compared with wild-type littermates. In both groups, survival was comparable whether skin graft tissue was from wild-type or IL-17A−/− donors (data not shown).

We next assessed whether T cells from skin grafted female mice could be primed for cytokine production. For this purpose, cells from graft draining lymph nodes were stimulated 12 d after transplantation in MLC with either female or male syngeneic-irradiated spleen cells (Fig. 2B). IL-17A and IFN-γ were undetectable in culture supernatants of naive mice (ungrafted animals), whereas significant amounts of IL-17A and to a lesser extent IFN-γ were detected in grafted animals.

Because IFN-γ and IL-17A were produced by draining lymph node cells, intragraft mRNA expression of Th1 (IFN-γ) and Th17 cytokines (IL-17A, IL-17F, and IL-22) was measured at days 7, 12, and 19 posttransplantation (Fig. 2C, Table I). For each recipient, a female skin graft served as control (background of gene expression). A clear upregulation of transcripts encoding the Th17 cytokines (IL-17A, IL-17F, and IL-22) was noticed at the early-phase posttransplantation (days 7 and 12 postgraft). Thereafter, the production of these cytokines dropped below background levels, measured on day 19. In an opposite manner, IFN-γ mRNA expression increased from background levels 7 d after transplantation up to 100 times more at days 12 and 19.

Flow cytometry analysis of both GILs and skin-draining lymph node T cells was performed. An absolute count of GILs per 100 mg grafted tissue revealed that both CD4+ and CD8+ T cells increased to 100 times more at days 12 and 19. Thus, skin grafting seemed to affect the functionality of both T-cell subsets, because upon IL-17A deficiency delays rejection of minor mismatched skin grafts

We then tested the role of IL-17A in a weaker antigenic combination by using the H-Y minor Ag disparity model. Male B6 skins were transplanted into syngeneic female recipients (Fig. 2A). Interestingly, IL-17A deficiency significantly delayed graft rejection because up to 50% of IL-17A−/− recipients retained their graft for >45 d, whereas all grafts were rejected at this point in wild-type female recipients. The ability of the donor skin tissue to produce IL-17A was not required for rejection, because male skin graft survival was comparable whether skin graft tissue was from wild-type or IL-17A−/− donors (data not shown).

A two-tailed paired t test was used to compare ∆CTmale versus ∆CTfemale for each time point and cytokine. ND, not detected.

### Materials and Methods

#### Table I. Oligonucleotide sequences used for PCR

<table>
<thead>
<tr>
<th>Oligonucleotide, 5′-3′</th>
<th>Sense</th>
<th>Antisense</th>
<th>Probe</th>
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<td>Mouse β-actin</td>
<td>CCGAAGCGGACTACTATGCTA</td>
<td>TTTTCATAGATGGCGTTGTTG</td>
<td>6FAM-ATCGGTGGCTCCATCCTGGC-TAMRA</td>
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<tr>
<td>Mouse IL-17A</td>
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<td>CTGGCCTCAGCTGATG</td>
<td>6FAM-ACCTCAAGCTTCCACCGCCCTCC-TAMRA</td>
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<tr>
<td>Mouse IL-17F</td>
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<td>GCTGGTACCTTTCCTGACCACTCGAGACCC-TAMRA</td>
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<td>Mouse IFN-γ</td>
<td>GGAACCATTAAGATATTGCC</td>
<td>GTCTTGAGGTCAACAACCCACAGG-TAMRA</td>
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<tr>
<td>Mouse RORγt</td>
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<td>TCCACAGTGGTGCCGAC</td>
<td>6FAM-TCATCACTAAGCAAGGCGGA-TAMRA</td>
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<td>Mouse T-bet</td>
<td>CAAGTTCAACCAGCAAGCAAGA</td>
<td>CACATCCACAGAAACATTCCCG</td>
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</table>

#### FIGURE 2. IL-17A deficiency delays rejection of minor Ag disparate skin grafts. A, Syngeneic male skin grafts were performed on either wild-type (WT) or IL-17A−/− B6 female recipients. Graft survivals was compared by using the log-rank test (p = 0.02). B, IL-17A and IFN-γ production in MLC. Draining lymph node cells from naive (n = 3) and grafted (n = 12) WT mice were individually stimulated for 4 d with either male or female (Fem.) irradiated B6 spleen cells. Results are expressed as mean ± SEM; *p < 0.05, **p < 0.01. Results are representative of two independent experiments. C, Kinetics of intragraft cytokine mRNA level. Female and male skins were cotransplanted on opposite flanks of the same WT female recipient and analyzed by quantitative RT-PCR. Mice were sacrificed at days 7 (n = 6), 12 (n = 7), and 19 (n = 6) after transplantation. Cytokine mRNA levels were normalized using β-actin mRNA as reference. Results are expressed as 2-ΔΔCT based on gene expression in female skin grafts (see Materials and Methods). A two-tailed paired t test was used to compare ∆CTmale versus ∆CTfemale for each time point and cytokine. ND, not detected.
IFN-γ-producing CD4+ and CD8+ T cells appeared later (Fig. 3B). In parallel, the percentage of IL-17A–producing CD4+ T cells in graft-draining lymph nodes increased 7 d after transplantation and then decreased gradually. Also, IFN-γ–producing CD4+ T cells appeared later in lymph nodes from days 7 to 19 (Fig. 3C). Nevertheless, virtually no IL-17A was observed among the CD8+ T cells in draining lymph nodes (0.09 ± 0.03% as mean ± SD), whereas significant and increasing amounts of IFN-γ–producing cells were detected with time (data not shown).

We next determined whether rejection of male skin grafts by female mice was restricted to Th17-dependent mechanisms by neutralizing Th2 or Th1 cytokines. Neither IL-4 (Fig. 4A) nor IFN-γ (Fig. 4B) neutralization affected the kinetic of male skin graft rejection, indicating that the pathway of male skin graft rejection is dominated by Th17-dependent mechanisms. Because IL-17 can be produced by many different cell types (16), we looked specifically at T cell-derived IL-17A. For this purpose, female RAG-deficient mice were reconstituted with either wild-type or IL-17A−/−CD4+ and CD8+ female T cells 30 d before male skin grafting. Although T cell reconstitution was similar in both groups as measured by flow cytometry (data not shown), only the wild-type T cell adoptive transfer elicited graft rejection (Fig. 4C). This highlights the critical role played by T cell-derived IL-17A in the rejection process.

Tissue neutrophil recruitment is considered one of the IL-17–dependent mechanisms of immune responses (7, 17, 18). We therefore assessed the impact of recipient IL-17A deficiency on neutrophil infiltration into the graft. Immunostaining with the neutrophil-specific Ly-6G Ab revealed that IL-17A deficiency nearly completely prevented neutrophil infiltration (Fig. 4D). Next, we wondered whether neutrophils might be the effector of rejection downstream of T cell-derived IL-17A. Neutrophils were depleted through multiple injections of the anti-Gr1 mAb (clone RB6-8C5). This led to a significant delay of rejection in neutrophil-depleted animals, as compared with control littermates (Fig. 4E). Altogether, these results reveal a dominant Th17-dependent neutrophil-mediated pathway of rejection in minor histocompatibility.

**Tregs promote the Th17-mediated pathway of allograft rejection**

Tregs are known to suppress Th1- and Th2-mediated allograft rejection (19, 20). Nevertheless, the relationship between Tregs and Th17 cells is still debated. We and others (10–13, 20, 21) have observed that Tregs do not prevent Th17 cell-mediated responses but rather favor them in vitro or in vivo. Therefore, we questioned a possible role for Tregs in the Th17 polarization of male skin graft rejection by female T cells. In a first set of experiments, wild-type female B6 recipients were depleted of Tregs through a single injection of the anti-CD25 mAb 6 d prior to a male skin graft. The depletion of CD4+CD25+Foxp3+ cells was assessed by flow cytometry analysis of PBMCs at the time of grafting (data not shown). The depletion of Tregs significantly reduced the level of IL-17A mRNA both within grafts and draining lymph nodes and marginally increased the level of IFN-γ mRNA. The intragraft expression of specific Th1 (T-bet) and Th17 (RORγt) transcription factors followed a comparable profile (Fig. 5, Table I). The decrease of RORγt mRNA expression in lymph nodes did not reach statistical significance, whereas T-bet mRNA remained unmodified in lymph node cells (Fig. 5). The apparent discrepancy between graft and lymph nodes might reflect different kinetics of recruitment into these two locations.

In a second set of experiments, we further investigated the role of Tregs in promoting the de novo differentiation of naive allogeneic CD4+ T cells toward a Th17 phenotype. For this purpose, T cell-deficient RAG−/− mice were reconstituted with female splenocytes from RAG−/− HY-specific TCR β6pos transgenic Marilyn mice. Because female Marilyn mice only contain naïve T cells (14), Th differentiation can be easily investigated without interference of cross-reactive memory T cells. RAG−/− mice were adoptively transferred with 3 × 105 Marilyn splenocytes alone or cotransferred with 6 × 105 CD4+ Tregs sorted from Foxp3-GFP transgenic mice. The reconstituted mice were then grafted with male skin. After 10 d, draining lymph nodes cells were isolated and stimulated with PMA/ ionomycin. We performed intracytoplasmic cytokine staining together with cell surface Ags that allowed us to distinguish Marilyn cells from cotransferred Tregs (by using the TCR β6 staining). We found that Th17 differentiation of Marilyn cells (CD4+Vβ6pos GFPpos) was significantly increased in Treg-cotransferred recipients (Fig. 6A). Interestingly, we found that IL-17A was also produced by cotransferred Tregs themselves (CD4+Vβ6neg GFPneg) after losing or not losing their Foxp3-GFP expression (Fig. 6B). Thereby, the respective origin of IL-17–producing CD4+ cells was as follows: 21% for Marilyn cells, 28% for Foxp3/GFP+ Tregs, and 51% for Foxp3/ GFP− cells that were originally Tregs (Supplemental Fig. 1A). Consistently, intragraft mRNA quantification revealed increased IL-17A and RORγt transcripts in Treg-cotransferred recipients.

**FIGURE 3.** Early and sustained IL-17A–producing T cell recruitment in male skin grafts and draining lymph nodes (dLNs). A–C, Male skin grafts and dLNs were harvested on days 7 (n = 6), 12 (n = 6), and 19 (n = 8) posttransplantation. GILs and dLNs cells were isolated and stimulated for 4 h with PMA and ionomycin and stained for flow cytometry. A, Absolute counts of CD3+CD4+ (•) or CD3+CD8+ (○) cells in the graft (number of cells per 100 mg graft tissue). Three untransplanted skin samples served as negative control (day 0). Results are expressed as mean ± SEM. Statistical analysis revealed a significant increase between timings for both CD4+ and CD8+ T cells. B, Representative plots of IL-17A and IFN-γ by CD3+CD4+ or CD3+CD8+ GILs are shown. Pools of individual mice are expressed as mean ± SEM (n = 6–8 mice/group). C, Plots represent IL-17A and IFN-γ expression by CD3+CD4+ cells from graft dLNs harvested from the same mice shown above (n = 6–8 mice/group) or naïve mice (n = 3) (day 0). Results are expressed as mean ± SEM.
compared with Marilyn cells alone. In addition, we detected fewer copies of IFN-γ and T-bet mRNA (Fig. 6C, Table I). The T-bet expression in CD4+Vb6posGFPneg was assessed by flow cytometry analysis. As shown in Fig. 6D, the mean fluorescence intensity of T-bet was enhanced in adoptively transferred Marilyn cells after male skin graft compared with naive cells. In addition, this T-bet upregulation was significantly reduced with Treg cotransfer (Fig. 6D). To examine the allospecificity of the IL-17 production in response to male Ags, recipient T cells were also stimulated in MLC by irradiated female or male splenocytes. As shown in Fig. 6E, male but not female stimulators induce IL-17A production only in Treg-cotransferred mice but not in case of Marilyn T cell transfer alone. IFN-γ production was reduced by 50% in Treg-cotransferred conditions, but this did not reach statistical significance. Altogether, these results show that Treg-mediated Th17 bias is associated with a Th1 downregulation of the antimale response.

**IL-6 neutralization prevents Th17 differentiation and promotes Treg suppression of allograft rejection**

Because IL-6 is important for Th17 cell differentiation, as well as for the conversion of Tregs into Th17 cells (13, 21, 22), we tested the outcome of male skin grafts in the absence of IL-6. We compared male skin grafts in female recipients, both donor and recipient combination being either wild-type or IL-6−/− (Fig. 7A). IL-6 deficiency prevented skin graft rejection 40 d after transplantation. This was accompanied by an impairment of Th17 cell differentiation as attested by GILs or draining lymph nodes (Fig. 7B). In parallel, the ratio of foxp3+ T cells was considerably increased in the IL-6−/− donor-recipient combination (Fig. 7B). Interestingly, IL-6 seemed not to influence the IL-17 production by foxp3+ T cells. Further experiments revealed that a donor IL-6 deficiency alone was not sufficient to delay skin graft rejection, highlighting the critical role of recipient-derived IL-6 in this experimental setting (Supplemental Fig. 2).

**Discussion**

We have described an IL-17A-dependent and neutrophil-mediated pathway of allograft rejection favored by Tregs. Although many different and redundant cytokine-driven mechanisms of rejection...
FIGURE 6. Tregs promote Th17 differentiation of anti-male naive T cells. T cell-deficient B6 RAG−/− mice were reconstituted with 3 × 10⁵ splenocytes from female RAG−/− TCR Vβ6pos-transgenic Marilyn mice alone or together with 6 × 10⁵ CD4+ Tregs sorted from foxp3-GFP transgenic mice. Two days later, reconstituted mice were grafted with a male skin and sacrificed 10 d after transplantation. Ex vivo flow cytometry analysis of draining lymph nodes cells was performed. Results of five mice per group were pooled. A, Plots represent intracellular expression of IL-17A in CD4+Vβ6posGFPneg cells from each group. Values represent percentages and are expressed as mean ± SEM; p = 0.03. B, The plot represents IL-17A and FoxP3-GFP expression by CD4+ TCR Vβ6neg cells corresponding to initially cotransferred Tregs. Results of five individual mice in the Treg cotransferred group are shown. Values represent percentages and are expressed as mean ± SEM. C, Intragraft IL-17, IFN-γ, RORγt, and T-bet mRNA expression. Total RNA was extracted from male skin allografts and analyzed by real-time RT-PCR. Levels were normalized using β-actin mRNA. Results represent the mean ± SEM and are representative of two independent experiments (*p < 0.05; **p < 0.01). D, On the left, scatter plot represents T-bet expression in CD4+Vβ6posGFPneg cells from each group. T cells from untouched Marilyn mice were used to assess basal T-bet expression of naive T cells (*p < 0.05). Values are expressed as mean fluorescence intensity (MFI). On the right, histogram shows T-bet expression in Marilyn cells of one representative mouse from each group. E, IL-17A and IFN-γ production in MLC. Spleen cells from reconstituted mice were stimulated by female (N) and male (n) B6 splenocytes. Supernatants were harvested 48 h later, and cytokines were measured by ELISA. Results are expressed as mean ± SEM and are representative of two independent experiments (*p < 0.05). ND, not detected.
mice were harvested on day 13 posttransplantation. GILs and dLNs were stimulated with PMA/ionomycin stimulation. Plots and histograms represent IL-17A.

also tested the role of IL-17 in costimulation blockade-resistant allograft rejection by comparing BALB/c skin graft survival in wild-type recipients MHC molecules on APCs as well as graft cells. The role potentially reduces the frequency of alloreactive T cell precursors, but it could also promote Treg interactions with matched donor and recipient mice. Survival was analyzed using the log-rank test. One representative experiment of two is shown.

Collectively, these studies suggest that minor Ag disparities lead to a Th17-mediated response. We postulate that the Treg/Teffector ratio is critical for driving the immune response toward a specific Th phenotype. Indeed, we previously showed in vitro that the immune response is driven to Th1 and Th2 cytokine production when a Treg/Teffector ratio is in favor of effectors. In contrast, the enrichment of Tregs dampened Th1 and Th2 while promoting Th17 cells (10). In the current study, we used well established models of skin allograft rejection, starting from multiple MHC-mismatched combinations to minor Ag disparity. Although the last combination could be considered more permissive, it remains a well established model of allograft rejection involving antidonor CD4+ and CD8+ T cells (34). Significantly, minor Ag disparities are frequent in clinical situations, such as bone marrow transplantation (35, 36), and could also be perceived as mimicking a situation in which the bulk of the alloreactive repertoire has been inactivated or deleted by thera- peutic interventions. Of note, the important role of IL-17 in mediating allograft rejection across minor Ag disparities was confirmed in a different mouse model (37, 38), whereby naive mice were grafted with a syngeneic skin from a GFP-transgenic mouse (Supplemental Fig. 3A/B).

Collectively, these studies suggest that minor Ag disparities lead to a Th17-mediated response. We postulate that the Treg/Teffector ratio is critical for driving the immune response toward a specific Th phenotype. Indeed, we previously showed in vitro that the immune response is driven to Th1 and Th2 cytokine production when a Treg/Teffector ratio is in favor of effectors. In contrast, the enrichment of Tregs dampened Th1 and Th2 while promoting Th17 cells (10). In case of only minor Ag disparity, we hypothesized that a lower proportion of allloreactive T cell precursors are in the presence of a relatively high proportion of Tregs (this is attested by T cells analysis shown in Fig. 7). Not only does the lack of MHC mismatch potentially reduces the frequency of allloreactive T cell precursors, but it could also promote Treg interactions with matched donor and recipient mice. The role of Tregs in the minor mismatched-related Th17 differentiation is
supported by Treg depletion in wild-type recipients and Treg adoptive cotransfer experiments in RAG\(^{-/-}\) recipients. The role of a low precursor frequency in determining human Th17 alloreactive response recently highlighted by Litjens et al. (39) is in agreement with this hypothesis.

Tregs could promote Th17 differentiation by multiple mechanisms. Possible mechanisms include the TGF-\(\beta\) production by Tregs or by other bystander cells, which allows naive T cells to differentiate into Th17 in the presence of IL-6 (12, 13). This mechanism is consistent with our results in IL-6\(^{-/-}\) mice. Another nonmutually exclusive possibility relies in the Treg-mediated suppression of Th1 cells, which have been described as inhibiting Th17 differentiation (40–42). This is supported by two results in our study. First, by the increased IFN-\(\gamma\)/IL-17 and T-bet/ROR\(\gamma\)t ratios, we observed after CD25 depletion in wild-type recipients and, second, by the adoptive transfer experiments of Marilyn cells alone or Marilyn plus Tregs. The latter results appeared more clearly probably because of the Ag restriction of TCR transgenic T cells and their naive state before adoptive transfer. Note, however, that blocking Th1 during in vitro stimulation did not induce IL-17 production by Marilyn T cells (Supplemental Fig. 4). This suggests that Th1 inhibition may not be sufficient for promoting Th17 development. A third possible scenario consists in a direct conversion of Tregs into Th17 cells. Indeed, we clearly observed V\(\beta\)6-negative T cells still expressing or not GFP (surrogate marker of Foxp3) and positive for IL-17 staining (Supplemental Fig. 1A). This observation is in line with studies showing the phenotypic conversion of natural Tregs into Th17 cells after Foxp3 downregulation by IL-6 (13, 21, 43, 44). Of note, in cotransfer experiments, a sizeable percentage of CD4\(^+\)V\(\beta\)6\(^{-}\) cells, which were initially pure Tregs, was able to produce IL-17 after losing Foxp3 expression. Moreover, some cotransferred Tregs expressed simultaneously Foxp3 and IL-17A. These double-positive cells were still found in the absence of IL-6, suggesting a redundant role for IL-1\(\beta\) in this conversion. Indeed, a recent study has shown that IL-1\(\beta\) signaling is critical for inducing IL-17A production independently of Foxp3 downregulation (45). Nevertheless, we clearly observed a key role mediated by IL-6 in the rejection process. Indeed, the results in IL-6\(^{-/-}\) mice demonstrated the regulatory role of this cytokine in the Treg/Th17 balance during antidonor immune response. High amounts of IL-6 are released in response to the surgical procedure, after TLR activation in the context of bacterial contamination and/or ischemia-reperfusion injury (29, 46–49). IL-6 can also be produced by dendritic cells in an Ag-specific manner through a dendritic/T cell cross-talk via the CD40-CD154 interactions (50, 51). Consistent with this, we observed higher amounts of IL-6 mRNA in male skin graft compared with female to female grafts (data not shown). Of note, IL-17 induces the production of IL-6 by fibroblasts (2), triggering an amplificatory loop of cytokine production. Finally, another possible mechanism favoring Th17 differentiation in the presence of high ratio of Tregs relies in the ligation of B7 molecules by CTLA4 largely expressed on Tregs, as it has been reported by Bouguermouh et al. (11). The concept of “class”-specific effects of Tregs in controlling immune responses has recently emerged (52, 53). Indeed, a Treg-selective ablation of STAT3, a critical transcription factor for Th17 cells, induces a fatal Th17-mediated colitis in mice (53). This was partly related to a defect of Treg homing and Th17 suppression as a result of an impaired expression of CCR6, a chemokine receptor expressed by both Tregs and Th17 cells. This feature might appear to be in discrepancy with our own results because they clearly show a control of a Th17-mediated disease by Tregs expressing STAT3. Although we did not investigate the expression of STAT3 in our Tregs, we did observe a large number of foxp3-expressing CD4\(^+\) cells in skin grafts undergoing Th17-biased rejection ruling out a homing defect (Supplemental Fig. 1B). Furthermore, in our experiments, heightened amounts of CCR6 (as well as CCL20) mRNA were detected in skin grafts from Treg-cotransferred mice compared with Marilyn cells alone (data not shown), although this still does not preclude a Treg-specific defect of STAT3/CCR6 expression. Another important difference between the two experimental models resides in the peculiar capacities of STAT3-deficient Tregs to produce high amounts of IL-6, TGF-\(\beta\), and vasoactive intestinal peptide that promote Th17 responses (53).

In summary, we showed that Tregs can promote a Th17-mediated neutrophil-dependent pathway of graft rejection. Our results highlight a potential risk of developing Th17-mediated inflammation during Treg-based therapy, such as in bone marrow transplantation, solid organ transplantation, or even autoimmunity and suggest that targeting IL-17 or the upstream IL-6 could provide a useful therapeutic target.

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Disclosures

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References


