Th17 Cells Induce a Distinct Graft Rejection Response That Does Not Require IL-17A

E. I. Agorogiannis, F. S. Regateiro, D. Howie, H. Waldmann and S. P. Cobbold*

University of Oxford, Sir William Dunn School of Pathology, South Parks Road, Oxford OX1 3RE, UK
*Corresponding author: Stephen P. Cobbold, stephen.cobbold@path.ox.ac.uk

IL-17A-producing helper T (Th17) cells have been implicated in the pathogenesis of autoimmune disease, inflammatory bowel disease and graft rejection, however the mechanisms by which they cause tissue damage remain ill-defined. We examined what damage Th17 cell lines could inflict on allogeneic skin grafts in the absence of other adaptive lymphocytes. CD4+ Th17 cell lines were generated from two TCR transgenic mouse strains, A1(M).RAG1−/− and Marilyn, each monospecific for the male antigen Dby. After prolonged in vitro culture in polarizing conditions, Th17 lines produced high levels of IL-17A with inherently variable levels of interferon gamma (IFNγ) and these cells were able to maintain IL-17A expression following adoptive transfer into lymphopenic recipients. When transferred into lymphopenic recipients of male skin grafts, Th17 lines elicited a damaging reaction within the graft associated with pathological findings of epidermal hyperplasia and neutrophil infiltration. Th17 cells could be found in the grafted skins and spleens of recipients and maintained their polarized phenotype both in vivo and after ex vivo restimulation. Antibody-mediated neutralization of IL-17A or IFNγ did not interfere with Th17-induced pathology, nor did it prevent neutrophil infiltration. In conclusion, tissue damage by Th17 cells does not require IL-17A.

Key words: graft rejection, mouse skin allograft, T-cell cytokine, T helper cells

Abbreviations: APC, antigen-presenting cell; BMDCs, bone marrow-derived dendritic cells; ChIP, chromatin immunoprecipitation; cDNA, complementary DNA; FBS, fetal bovine serum; FACS, fluorescence-activated cell sorting; CSF3, granulocyte colony stimulating factor; CSF2, granulocyte-macrophage colony stimulating factor; GVHD, graft-versus-host disease; rm, recombinant murine; H&E, hematoxylin and eosin; IHC, immunohistochemical; IFNγ, interferon gamma; IMDM, Iscove’s modified Dulbecco’s medium; MPO, myeloperoxidase; nM, nanomolar; PMA phorbol myristate acetate; PBS, phosphate-buffered saline; (RT-)PCR, reverse transcriptase polymerase chain reaction; RAG, recombination activating gene; SD, standard deviation.

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Introduction

Th17 cells can orchestrate a wide range of proinflammatory activities in both normal host defense and autoimmune disease pathogenesis (1,2). A contribution to graft rejection has been proposed (3), but despite early claims for a role of IL-17A (4–10), convincing experimental evidence for the involvement of Th17 cells in this process has only recently emerged (11–13). Further work has implicated Th17 cells in dysfunction and acute rejection of lung transplants (14–16), in chronic kidney rejection (17) and also graft-versus-host disease (GVHD) (18). Nonetheless, the redundancy of effector mechanisms able to induce transplant damage (19) has not allowed for clear evidence of a specific role of Th17 cells in this setting. Even though antagonism of IL-17A activity could interfere with forms of transplant rejection (5–7), Th17 cells are not essential to the process, as Th1 or Th2 cell lines were able to elicit graft rejection in the absence of other lymphocytes (20).

Th17 cells can mediate graft rejection of MHC-IImismatched cardiac allografts in T-bet-deficient mice (13). IL-17A produced by CD4+ T cells and neutrophils were shown to be important in this case. For rejection of fully MHC-mismatched heart transplants by T-bet-deficient mice the main source of IL-17A was CD8+ T cells (21,22). Previous findings had revealed increased neutrophil infiltration within grafts in IFNγ (23) or IL-4 deficient recipients (24), and depletion of neutrophils was associated with prolonged transplant survival. The involvement of IL-17A was not assessed. There may, however, be a necessary role for Th17 cells in rejection of male skin grafts by female C57BL/6 hosts that absolutely depended on IL-17A and neutrophil infiltration (12). Th17 cells were detected early in both skin grafts and draining lymph nodes, their abundance decreasing over time paralleled by the emergence of IFNγ-producing CD8+ T cells (12).

As previous studies did not exclude the presence of other lymphocyte populations, the exact contribution of Th17 cells to transplant rejection remained unclear. We needed
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to know whether Th17 cells could induce graft rejection in their own right or whether they merely form part of an inflammatory cascade involving other types of T cells. Here we describe the effector properties of long-term polarized, stable Th17 cells in single minor histocompatibility antigen (H-Y)-mismatched transplantation, in the absence of other T- or B-cell populations.

Materials and Methods

**Mice**

C57BL/6, CBA/Ca, Marilyn (25) bred to a C57BL/6 recombination activating gene 1 deficient (RAG1-/-) background, A1(M).RAG1-/- (20), C57BL/6.RAG1-/- and CBA.Ca.RAG1-/- mice were bred and maintained in the specific pathogen-free animal facility of the Sir William Dunn School of Pathology (Oxford, UK), and used at the age of 5-25 weeks. All experiments were performed in accordance with the Animals (Scientific Procedures) Act 1986.

**Generation of long-term CD4+ T-cell lines**

For the generation of long-term Th1 and Th17 lines, A1(M).RAG1-/- or Marilyn naive CD4+ T cells, purified using the CD4+ T cell isolation kit (Miltenyi Biotech, CD4+ cell purity >80%), were stimulated weekly in the presence of female syngeneic myelomycin C-treated T-cell-depleted splenocytes and cognate peptide (100 nM) under the following conditions: for Th1 cells 5 nM recombinant murine (rMu) IL-12, 10 nM rMuIL-2 and 10 nM anti-IL-4 (clone 11B11) (26) and for Th17 cells 2 nM recombinant human (rHu) TGF-β, 50 nM rMuIL-6, 50 nM rMuIL-21, 10 nM anti-IFNγ (clone XMG1.2) (27) and 10 nM anti-IL-4. For the preparation of T-cell-depleted splenocytes, mice were injected with 2 mg of each of YTA3.1.2 and YTS191.1.2 (anti-CD4), and YTS156.7.7 and YTS169.4.2.1 (anti-CD8) antibodies (28,29) on days −5 and −3, and spleens were harvested on day 0. Recombinant cytokines were purchased from R&D Systems and PeproTech EC. Peptide sequences recognized by T cells from A1(M).RAG1-/- and Marilyn mice were REELHOFRRSGBP (Dby08-422) in the context of H2-Kb and NAGFSNRSRSS (Dby08-422) in the context of H2-Aβ respectively (30). At the end of each activation round, live cells were separated on a histopaque gradient or total splenocytes recovered from mice injected with CD4+ T cells, were restimulated for 4–5 h with 50 nM phorbol myristate acetate (PMA) and 500 nM ionomycin, for the last 2–3 h in the presence of 10 μg/ml brefeldin A (all from Sigma-Aldrich). Restimulated cells were stained for membrane markers and subsequently fixed with formaldehyde and permeabilized with the saponin solution (Sigma-Aldrich) in PBS before staining with anticytokine antibodies. Antibodies used were anti-CD3 (clone 145–2C11), anti-CD4 (RM4–5), anti-CD8 (clone XMG1.2) (27) and 10 μg/ml anti-IFNγ (clone XMG1.2) (33) and murinized IgG104.1 (isotype control for anti-H3K4me3 antibody, UCB Celltech, Brussels, Belgium).

**Imaging of skin grafts**

Mice were anesthetized using isoflurane (IsoFlo, Abbott Animal Health, Abbott Park, IL, USA) and black and white photos of skin grafts were taken using a Maestro in vivo imaging system (CRi) weekly starting on day 7 after transplantation. The skin graft surface area was calculated using Adobe Photoshop CS2 software (version 9.0.2). Data are expressed as relative graft surface area normalized to the baseline area measured on day 7 after grafting. Color photos of skin grafts were taken with an Olympus SP-500 UZ digital camera.

**Histological analysis of skin grafts**

Skin grafts were recovered and fixed in a solution of 4% wt/vol formaldehyde in PBS. After 24 h, skin grafts were embedded in parafin and microscopic sections were stained with hematoxylin and eosin (H&E) at the histology facility of the Sir William Dunn School of Pathology. Immunohistochemical (IHC) analysis was performed with polyclonal rabbit anti-human myeloperoxidase (Dako, Glostrup, Denmark, A02398) and antihuman CD3 (clone SP7, rabbit IgG, Lab Vision). Photos of histological slides were taken with a Nikon CoolScope digital microscope (Nikon, Tokyo, Japan).

**Fluorescence-activated cell sorting (FACS) analysis**

Live cells separated on a histopaque gradient or total splenocytes recovered from mice injected with CD4+ T cells, were restimulated for 4–5 h with 50 nM phorbol myristate acetate (PMA) and 500 nM ionomycin, for the last 2–3 h in the presence of 10 μg/ml brefeldin A (all from Sigma-Aldrich). Restimulated cells were stained for membrane markers and subsequently fixed with formaldehyde and permeabilized with the saponin solution (Sigma-Aldrich) in PBS before staining with anticytokine antibodies. Antibodies used were anti-CD3 (clone 145–2C11), anti-CD4 (RM4–5), anti-VδF (RR4–7), anti-Vβ8 (F23.1), anti-IL-17A (TC11–18H10.1), anti-IFNγ (clone XMG1.2) (all from BD Biosciences, Franklin Lakes, NJ, USA) and anti-FoxP3 (FJK-16s) (eBioscience, San Diego, CA, USA). Cells were analyzed on a 4-colour FACSCalibur flow cytometer (BD Biosciences). Data were analyzed using FlowJo software version 7.6.1 (Tree Star, Ashland, OR, USA). In all FACS plots, numbers in gates represent percentages of cells.

**Analysis of gene expression**

DNase-treated total RNA was purified using the SV total RNA isolation system (Promega) from cell lysates or from skin graft samples homogenized using a FastPrep-24 system (MP Biomedicals, Irvine, CA, USA). The SuperScript III first-strand synthesis system for reverse transcriptase polymerase chain reaction (RT-PCR) with random hexamers (Invitrogen, Carlsbad, CA, USA) was used to synthesize complementary DNA (cDNA) from 1 μg of total RNA. TaqMan gene expression assays (Applied Biosystems, Foster City, CA, USA) for Hprt1 (Mm01545399_m1), Cd3g (Mm00438095_m1), Il17a (Mm00439619_m1), Ifng (Mm00801778_m1), Mpo (Mm00447886_m1), Rorc (Mm00482009_m1), and Tbx21 (Mm00438095_m1), were used for real-time RT-PCR analysis on the Applied Biosystems 7500 Fast Real-Time PCR System with SDS software v1.3.1. Using the 2−ΔΔCt method, expression of a target gene was normalized to an endogenous control gene, and data were presented relative to the same sequence in a calibrator sample (34).

**Chromatin immunoprecipitation (ChIP) analysis**

The “fast ChIP” method (33) was used for the immunoprecipitation of histone 3 trimethylated at lysine 4 (H3K4me3) or 27 (H3K27me3) with anti-H3K4me3 (17–614, Millipore, Billerica, MA, USA) and anti-H3K27me3 (17–622, Millipore) antibodies, respectively. Real-time PCR was performed at the indicated time points and concentrations. Isotype controls were antitanine antibody YCATE55 (rat IgG1) (33) and murinized IgG104.1 (isotype control for anti-H3K4me3 antibody, UCB Celltech, Brussels, Belgium).

**Measurement of cell proliferation using tritiated (3H) thymidine**

To assess T-cell proliferation, CD4+ T cells were cultured in 96-well round bottom plates at 5 × 104 per well with 1 × 106 bone marrow-derived dendritic cells (BMDCs) or 25 × 104 T-cell-depleted splenocytes in the presence of the indicated conditions. Generation of BMDCs has been described before (31). Cells were incubated for a total of 72 h, for the last 24–3 h in the presence of 0.5 μCi 3H-thymidine (Amersham, Buckinghamshire, UK) per well of culture. Incorporation of 3H-thymidine was assessed using a flat-bed assay of total RNA. TaqMan gene expression assays (Applied Biosystems, Foster City, CA, USA) was used to synthesize complementary DNA (cDNA) from 1 μg of total RNA. TaqMan gene expression assays (Applied Biosystems, Foster City, CA, USA) for Hprt1 (Mm01545399_m1), Cd3g (Mm00438095_m1), Il17a (Mm00439619_m1), Ifng (Mm00801778_m1), Mpo (Mm00447886_m1), Rorc (Mm03862796_m1), and Tbx21 (Mm00450960_m1) were used for real-time RT-PCR analysis on the Applied Biosystems 7500 Fast Real-Time PCR System with SDS software v1.3.1. Using the 2−ΔΔCt method, expression of a target gene was normalized to an endogenous control gene, and data were presented relative to the same sequence in a calibrator sample (34).

**In vivo T-cell transfers and skin transplantation**

AutoMACS-isolated naive CD4+ T cells or live-cultured CD4+ T cells separated on a histopaque gradient were resuspended at appropriate concentrations in phosphate-buffered saline (PBS) and injected in the lateral tail vein. Control mice were injected with PBS. When necessary, skin transplantation was performed 1 day after injection, as previously described (28,29). Skin grafts were considered rejected when no viable tissue was visible.

**In vivo antibody treatment**

Neutralizing antibodies against IFNγ (clones XMG1.2 and R46-42, both rat IgG1) (27,32) and IL-17A (ab13, UCB Celltech) were injected intraperitoneally...
using SYBR green master mix (Applied Biosystems) on the Applied Biosystems 7500 fast real-time PCR system with SDS software v1.3.1. The density of an immunoprecipitated factor (target) at a locus was expressed relative to 10% input DNA according to the equation “relative density = 2^(Ct(input)−Ct(target))”, where Ct(input) and Ct(target) indicate the threshold PCR cycles for amplification of input DNA and immunoprecipitated factor-associated DNA respectively. The following primers were used for real-time PCR analysis; RORγt promoter: 5′-CATTTCTAGGCCCCTGGAA-3′, 5′-GGGACACACCTGCACAAAG-3′ (38); Tbet promoter: 5′-TGGGCCATACAGGAGGCGACA-3′, 5′-TCGCTTTTGTGAGGACTGAA-3′ (37).

**Statistical analysis**
Where indicated, statistical analysis was performed using GraphPad Prism (version 5.01) for Windows (GraphPad Software, San Diego, CA, USA) with unpaired two-tailed Student’s t-test. p-Values were classified as not significant (ns; p > 0.05), significant (*p < 0.05), very significant (**p < 0.01), extremely significant (***p < 0.001).

**Results**

**Prolonged culture under polarizing conditions confers greater stability of IL-17A expression**
Previous studies have highlighted the propensity of Th17 cells, generated in short-term cultures, to lose IL-17A expression and up-regulate IFNγ following transfer in vivo (38-43). Although such phenotypic flexibility might be a physiological property, it does not allow a clear interpretation of adoptive transfer experiments. We hypothesized that repetitive stimulation in the presence of polarizing conditions would enable the generation of Th17 cells with a more stable phenotype. As shown in Figure 1A and B, long-term culture of either A1(M).RAG1^−/−^ or Marilyn CD4^+^ T cells in the presence of TGFβ, IL-6, IL-21 plus neutralizing anti-IFNγ and anti-IL-4 antibodies, led to a persistently high production of IL-17A with variable levels of IFNγ co-expression. Importantly, even after 160 days of culture, cells still maintained specific antigen-dependent proliferation. When transferred into female Rag1^−/−^ hosts, lacking any endogenous B or T cells, these long-term Th17 lines were able to maintain expression of IL-17A in the majority of cells and did not generate any detectable IL-17A^-IFNγ^- cells (Figure 1C), suggesting they could be used for in vivo transfer studies.

**Th17 cells induce a slow skin graft rejection**
In order to test the direct influence of Th17 cells on graft survival, female C57BL/6.RAG1^−/−^ hosts were infused with Marilyn “long-term” Th17 cells, and subsequently grafted with male C57BL/6.RAG1^−/−^ skin. As positive controls we monitored graft rejection caused by naive Marilyn CD4^+^ T cells or “long-term” Th1 cells. Flow cytometry analysis validated the Th1 and Th17 cells as properly polarized (Figure 2A). As shown in Figure 2B, both Th1 and Th17 cells were able to enter skin grafts, as transplants recovered on day 7 from Th1- or Th17-injected hosts had increased levels of CD3γ mRNA, compared to PBS-injected mice. Nevertheless, grafts on Th1-injected hosts had higher levels of CD3γ transcripts, suggesting a more efficient migration and/or increased proliferation of Th1 cells (Figure 2B). The total numbers of Vβ6^+^ cells detected in spleens of graft recipients on day 22 after transplantation were also higher when Th1 cells were injected (Figure S1), suggesting that Th17...
cells might also have a reduced ability to homeostatically expand in lymphopenic recipients compared to Th1 cells.

As expected, intragraft levels for IL-17A and IFNγ mRNA were higher after transfer of Th17 and Th1 cells respectively (Figure 2B). Myeloperoxidase (MPO) expression at this time point, a measure of neutrophil infiltration, was not increased by the transfer of either Th1 or Th17 cells above the background levels induced by nonspecific inflammatory changes associated with skin grafting (Figure 2B). Similar findings were observed following the transfer of A1(M).RAG1+/− Th17 cells into female CBA/Ca.RAG1+/− hosts and transplantation with male CBA/Ca.RAG1+/− skin (Figure S2A).

Both Th1 and naive CD4+ T cells induced acute rejection of male skin grafts (Figure 2C and D), manifesting as massive epithelial necrosis and scab formation within 2 weeks after grafting, followed by contraction and replacement by scar tissue. Male grafts on PBS-injected hosts remained healthy, similar to female grafts on Th17-injected mice (Figure 2C and D). Nevertheless, Marilyn Th17 cells did not induce a classic acute rejection, but caused a number of
rapid pathological changes in male skin grafts (Figure 2C), characterized by hair loss and focal erythema that soon resolved, and succeeded by a gradual loss of graft volume that could be followed over time (Figure 2D). A similar pattern of rejection was induced by A1(M).RAG1−/− Th17 cells (Figures S2B and C). Forty-eight days after transplantation, residual tissue could still be observed in skin grafts on hosts that received Th17 cells (not shown), so we could not define a specific time of rejection, but the absence of any normal skin texture (e.g. hair or dermal ridges) was proof for the potential of transferred Th17 cells to induce antigen-specific tissue damage in the absence of other lymphocyte populations.

**Th17 cells promote unique pathological changes and neutrophil infiltration**

The differential rejection phenotypes induced by Th1 and Th17 cells were further investigated by histological analysis. As shown in Figure 3A, PBS-injected hosts retained healthy grafts, whereas both Th1 and Th17 cells were able to induce distinct pathological patterns explaining the macroscopic changes. As early as day 7 after transplantation, Th1 cells had elicited dermal hemorrhage, signaling imminent scab formation, and epithelial damage had led to epidermal thinning (Figure 3A). On the contrary, skin grafts on Th17-injected hosts showed epidermal hyperplasia, loss of skin appendages (hair follicles and sebaceous glands) and extensive dermal inflammation (Figure 3A), without any obvious signs of inflammatory involvement of the vasculature. Such histological findings were reproducible in the A1(M).RAG1−/− transplantation system (Figure S2D). In order to further characterize the Th17-mediated inflammatory reaction, IHC staining for CD3 and MPO was performed in grafts from mice injected with Th17 cells. Figure 3B shows that on day 48 after transplantation dense MPO+ cell infiltrates were present in these grafts, suggesting neutrophils as mediators of Th17 effector function, whereas the presence of CD3+ cells verified efficient homing of Th17 cells into grafted tissue. As no difference was observed in MPO mRNA levels between grafts from PBS- and Th17-injected mice on day 7 (Figures 2B and S2A), it is likely that neutrophil infiltration remains active at later stages during Th17-mediated rejection.

**Transferred Th17 cells maintain memory of IL-17A expression in vivo**

We subsequently examined the phenotype of transferred cells in spleens of grafted mice. Th1 cells maintained their phenotype, and naive CD4+ T cells differentiated into Th1 cells expressing high levels of IFNγ, but no IL-17A (Figure 4A). IL-22 or IL-4 (data not shown). Most Marilyn-derived Th17 cells maintained IL-17A expression. There was, however, an increase in the percentage of IL-17A−IFNγ+ cells that represented only a minor fraction prior to transfer (Figure 4A). This contrasted with A1(M).RAG1−/− Th17 cells that maintained IL-17A expression without up-regulation of IFNγ (Figure S3A). The phenotype of transferred Marilyn-derived T cells was also examined after in vitro restimulation in the presence of Dby608–622 peptide. After 4 days of culture, the vast majority of CD4+ and Th1 cells expressed IFNγ (Figure 4B). The expression of both IL-17A and IFNγ by Th17 cells increased, and IL-17A−IFNγ+ cells now represented the most abundant population (Figure 4B). A small percentage of Th17 cells were seen to up-regulate FoxP3 in vivo, although they lacked any FoxP3 expression before transfer (Figure S4). It is unclear whether these FoxP3+ cells can interfere with Th17-mediated rejection.

As Th1 lines maintained memory of IL-17A expression in vivo, unlike short-term cultured cells in previous
studies (38,40), we tested transferred cells for histone 3 modifications in the promoters of RORγt (Rorc+5.0) and T-bet (Tbx21), two gene loci regulating fidelity to the Th17 phenotype. RORγt drives the differentiation program of Th17 cells and induces IL-17A expression (44), whereas T-bet has been implicated in the phenotypic instability of Th17 cells (38,40,45). ChIP analysis was performed on Marilyn long-term Th17 lines stimulated for 7 days in vitro in the presence or absence of polarizing cytokines. As shown in Figure 4C, withdrawal of polarizing conditions was associated with an increase in the H3K4me3/H3K27me3 ratio for Tbx21, but despite this the permissive modifications in the RORγt promoter were maintained. In contrast, Th1 cells cultured for the same duration showed predominance of repressive marks in the RORγt promoter and a predominance of H3K4me3 in Tbx21 as previously described (46). Similar findings were observed for A1(M).RAG1−/− Th17 cells activated under various conditions (Figure S3B), and importantly histone 3 modifications in the promoters of RORγt and Tbx21 mirrored gene expression (Figure S3C). These results indicate that activation of polarized Th17 cells in the absence of Th17-inducing cytokines, as may have occurred within skin grafts, should facilitate stable expression of IL-17A while allowing up-regulation of IFNγ.

**Figure 4:** Maintenance of permissive histone modifications in the Rorc locus allows transferred Th17 cells to retain IL-17A expression, while up-regulating IFNγ, in recipients of male skin grafts. (A) Female mice injected with Marilyn CD4+ (n = 4), Th1 (n = 3), or Th17 (n = 7) cells, and grafted with male skin as described in Figure 2, were killed on day 48 after transplantation, and FACS analysis was performed on splenocytes restimulated with PMA and ionomycin. Expression of IL-17A and IFNγ by Vβ6+ cells is shown. (B) Total splenocytes from (A) were stimulated at a density of 2.5×10⁵ cells per well in 96-well plates with 100 nM Dby606–622 in IMDM. After 4 days, cells were restimulated with PMA and inomycin and analyzed by flow cytometry for expression of IL-17A and IFNγ. All FACS plots are gated on live Vβ6+ cells. Numbers next to gated populations indicate percentages presented as means ± SD. (C) ChIP analysis of long-term cultured Marilyn Th17 cells stimulated for 7 days in the presence (Th17) or absence (Th17 medium) of polarizing conditions. As control, long-term cultured Marilyn Th1 cells were analyzed. Real-time PCR was performed using DNA from samples immunoprecipitated with antibodies against H3K4me3 and H3K27me3. Data are normalized to 10% input DNA and presented as ratios of H3K4me3 to H3K27me3 density at the promoters of RORγt (Rorc+5.0) and T-bet (Tbx21) genes. Means and SD of n = 3 technical replicates from one experiment (representative of two) are shown.

**Th17-induced rejection proceeds independently of IL-17A and IFNγ**

We sought to determine whether neutralization of IL-17A or IFNγ could have an effect on Th17-induced rejection. IL-17A is a potent inducer of neutrophil mobilization (1, 2) and Figure 3B clearly indicated participation of neutrophils in the response to the graft. Additionally, because long-term Th17 lines produced IFNγ as an inherent feature (Figures 1A and B), the significance of this cytokine for Th17 effector function needed clarification. Exposure to IFNγ can up-regulate MHC molecules by nonimmune cells (47,48), and could thus enhance antigen presentation and the propagation of Th17-associated tissue damage. A combination of two antibodies, XMG1.2 (27) and R46-A2 (49), known to neutralize in vivo IFNγ activity, and confirmed by ourselves to block an IFNγ-specific ELISA in vitro (data not shown), was used.
Figure 5: Th17-mediated pathology proceeds independently of IL-17A or IFNγ neutralization. A total of $10 \times 10^6$ long-term cultured Marilyn Th17 cells were transferred into female C57BL/6.RAG1$^{-/-}$ hosts on day $-1$. Mice were grafted with male C57BL/6.RAG1$^{-/-}$ tail skin on day 0 and treated either with IgG ($n = 8$) or anti-IL-17A antibody ($n = 10$) (3 doses × 0.75 mg, weekly, starting on the day of grafting), or with IgG1 ($n = 4$) or anti-IFNγ antibodies ($n = 8$) (8 doses × 1 mg of each R46-A2 and XMG1.2, every other day, starting on the day of grafting). (A) Relative surface area on day 21 is presented as mean and SD for male grafts on antibody-treated recipients as described above or female grafts ($n = 4$). Statistical analysis was performed using unpaired two-tailed Student's t-test. (B) H&E and IHC stainings for MPO (in brown) were performed on grafts from IgG- or anti-IL-17A-treated mice killed on day 22. Note that the abundance of MPO$^+$ cells remains unchanged despite IL-17A neutralization. The arrows indicate the border between host and grafted skin. (C) Grafted mice were killed on day 22 after transplantation, and FACS analysis was performed on splenocytes restimulated with PMA and ionomycin. Expression of IL-17A and IFNγ by Vβ6$^+$ cells is shown. Statistical analysis was performed using unpaired two-tailed Student's t-test in order to compare relevant groups for the same gated population ($**0.001 < p < 0.01$, highlighted in red). (D) Expression of IL-22 by Vβ6$^+$ cells is shown like in (C). Statistical analysis was performed using unpaired two-tailed Student's t-test.

Neither IL-17A nor IFNγ neutralization interfered with Th17-mediated graft contraction on day 21, which was a consistent finding in both the Marilyn (Figure 5A) and A1(M).RAG1$^{-/-}$ (Figures S2B and C) systems. Despite an apparent increase in graft size in the control IgG-compared to anti-IL-17A-treated Marilyn groups (Figure 5A), histological analysis confirmed the absence of any biological difference between them (Figure 5B and Figure S2D). Neutrophil infiltration, as judged by IHC staining for MPO, was not influenced by IL-17A neutralization (Figure 5B), suggesting compensatory activity by other Th17-derived molecules such as IL-17F (50).

Blockade of IL-17A activity did not alter the expression of IL-17A or IFNγ by Th17 cells (Figure 5C and Figure S3A), whereas IFNγ neutralization reduced the frequency of IL-17A$^+$IFNγ$^+$ cells amongst transferred Marilyn Th17 cells (Figure 5C). Antigen exposure was responsible for driving IFNγ up-regulation by Th17 cells, as Vβ6$^+$ splenocytes recovered from female graft recipients did not show increased expression of IFNγ (Figure 5C). Finally, IL-22 expression by transferred cells remained unchanged despite antibody treatments (Figure 5D).

Discussion

Despite previous reports implicating Th17 cells in transplant rejection (51,52), the exact dimensions of such involvement remain unclear. To our knowledge, our study is
the first to investigate the individual contribution of Th17 cells to graft rejection in the absence of other adaptive lymphocytes.

It is still uncertain whether Th17 cells can arise endogenously in response to grafted tissues in wild-type recipients. The implication of IL-17A and Th17 cells in transplant rejection by T-bet-deficient mice (13,21,22), unable to mount efficient Th1 responses, may suggest a redundant role for Th17 cells in wild-type hosts. Moreover, transferred naive Marilyn CD4 T cells did not seem to differentiate into Th17 cells (Figure 4A), unless regulatory T cells were also present (12). Even if Th17 cells have little or no involvement in graft rejection in unmanipulated animals, it is possible they might participate in patients receiving immunosuppression that inhibits other effector populations, or where graft antigens are recognized by preexisting memory Th17 cells acquired through heterologous immunity (53).

Th17 lines with sustainable IL-17A expression were generated after long-term culture under polarizing conditions. Cells polarized either with IL-23 or without IL-21 displayed comparable phenotypes, and variable levels of IFNγ expression remained an inherent property of these Th17 lines even after neutralization of both IL-12 and IL-23 (with anti-IL-12p40 antibody) (data not shown). Marilyn Th17 cells were found to generally produce higher levels of IFNγ compared to A1(M).RAG1−/− both in vitro and after in vivo transfer. The dose of antigen has been shown to influence the level of IFNγ expression of Th17 cells (43) and the higher surface expression of the TCR by Marilyn compared to A1(M).RAG1−/− T cells (54) may have a similar consequence.

Importantly, epigenetic stability in the Rorc locus following withdrawal of polarizing conditions indicated that long-term lines can maintain their core Th17 differentiation status and expression of IL-17A, despite enrichment for permissive modifications in the T-bet promoter and subsequent IFNγ expression. Overall, while these data might be explained by plasticity in the Th17 lineage (38–43), an alternative view would be that production of IFNγ is an inherent effector property of Th17 cells elicited following recognition of antigen. In any case, as tissue damage was not ameliorated following IFNγ neutralization and as the graft pathology induced by Marilyn and A1(M).RAG1−/− Th17 cells was indistinguishable, it is unlikely that IFNγ expression by Th17 cells plays an essential part in this model of graft rejection.

Injected Th17 cells were able to enter skin grafts and induce rapid pathological changes evident as early as day 7 after grafting. Epidermal hyperplasia (acanthosis), loss of skin appendages and dermal inflammation with neutrophil infiltration were consistent, suggesting parallels with psoriasis models (55,56), although Munro’s microabscesses were absent. A murine model of Th17-induced GVHD was also associated with epidermal hyperplasia (57), and long-term Th17 lines grown with additional IL-23 +/− IL-1β, induced identical pathology (unpublished). These changes were distinct from Th1-induced acute rejection that was characterized by epidermal thinning and dermal hemorrhage.

Th17-induced pathology was not prevented following IL-17A or IFNγ neutralization, suggesting redundancy of effector mechanisms. For example, IL-17F is also known to promote neutrophil recruitment (50), and IL-17A-deficient mice show increased neutrophil counts associated with increased IL-17F and granulocyte colony stimulating factor (CSF3) plasma concentrations (68). It is also possible that neither IL-17A nor IL-17F are essential to the rejection process mediated by Th17 cells (59), with granulocyte-macrophage colony stimulating factor (CSF2) (60,61) and IL-22 (62) as other candidates to be excluded. Indeed, IL-22 has been recognized as an important player in tissue-specific inflammation (63, 64), and IL-22 (65), but not IL-17A (66), is involved in IL-23-induced acanthosis, and in a mouse model of psoriasis IL-22 neutralization prevented disease manifestations (63).

In summary, our data indicate that Th17 cells can induce graft damage in their own right, as is the case for Th1 and Th2 cells (20), but with a distinct pathology due to different underlying effector mechanisms (67–69). Our study confirms IL-17A-independent pathways to Th17-induced inflammation (59–61) and suggests new experimental opportunities to resolve mechanisms by which these cells mediate tissue damage.

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Disclosure

The authors of this publication have no conflicts of interest to disclose as described by the American Journal of Transplantation.

References

Supporting Information

Additional supporting information may be found in the online version of this article.

This section contains four supplementary figures (S1–S4).

Figure S1: Reduced expansion of Th17 cells in female lymphopenic recipients of male skin grafts compared to Th1 and naive CD4+ T cells. Female mice injected with Marilyn CD4+ (n = 4), Th1 (n = 3) or Th17 (n = 7) cells, and grafted with male skin as described in Figure 2, were killed on day 48 after transplantation, and FACS analysis was performed on splenocytes. Total numbers of Vβ8+ cells within recovered spleens are shown.

Figure S2: A1(M).RAG1−/− Th17 cells enter skin grafts and induce transplant pathology independently of IL-17A, similar to Marilyn Th17 cells. A total of 10 × 106 long-term cultured A1(M).RAG1−/− Th17 cells were transferred into female CBA/Ca.RAG1−/− hosts on day −1. Other mice were injected only with PBS. Mice were grafted with male CBA/Ca.RAG1−/− tail skin on day 0. Th17-injected mice were left untreated (n = 3), or injected with IgG (n = 5) or anti-IL17A antibody (n = 5) (3 doses x 0.75 mg, weekly, starting on the day of grafting). (A) Real-time RT-PCR analysis for the expression of CD3γ, IL17A, IFNγ and MPO in male skin grafts recovered on day 7. Data are normalized to expression of HPRT1 and presented as means and SD of n = 4 (PBS) or 3 (Th17) mice/group. Grafts on PBS-injected mice were used as calibrator.
Statistical analysis was performed using unpaired two-tailed Student’s t-test. (B) Representative photographs (not to scale) of male skin grafts on day 21. White-dashed rectangles are indicative of the position of the graft only, and do not represent the measurements described in (C). (C) Relative surface area is presented at the indicated time points as mean ± SD for male grafts on PBS- or Th17-injected hosts. Statistical analysis was performed using unpaired two-tailed Student’s t-test in order to compare male grafts on IgG- and anti-IL-17A-injected mice. (D) H&E staining was performed on grafts from mice killed on day 22. Normal appearance of epidermis and dermis in grafts on PBS-injected hosts contrasts with epidermal hyperplasia, dermal inflammatory cell infiltration and loss of skin appendages observed in grafts from recipients of Th17 cells, regardless of IL-17A neutralization. The arrow indicates the border between host and grafted skin, whereas the arrowheads show fibrin aggregates interspersed with neutrophils.

**Figure S3: A1(M).RAG1−/− Th17 cells maintain IL-17A expression without upregulation of IFNγ, in recipients of male skin grafts.** (A) Female CBA/Ca.RAG1−/− mice injected with A1(M).RAG1−/− Th17 cells were grafted with male skin and either left untreated (n = 3) or treated with IgG (n = 5) or anti-IL-17A antibody (n = 5), as described in Figure S2. Mice were killed on day 22 after transplantation, and FACS analysis was performed on splenocytes restimulated with PMA and ionomycin. Expression of IL-17A and IFNγ by Vγ8+ cells is shown. (B) ChIP analysis of long-term cultured A1(M).RAG1−/− Th17 cells stimulated for 7 days in the presence of different conditions. Real-time PCR was performed using DNA from samples immunoprecipitated with antibodies against H3K4me3 and H3K27me3. Data are normalized to 10% input DNA and presented as ratios of H3K4me3 to H3K27me3 density at the promoters of the RORγt (Rorc+5.0) and T-bet (Tbx21) genes. Means and SD of n = 3 technical replicates from one experiment (representative of two) are shown. (C) Cells from (B) were analyzed for expression of Rorc and Tbx21 mRNA. Data are normalized to expression of HPRT1 and presented as means and SD of n = 3 biological replicates for each different condition, except for cells activated in the presence of Th17-polarizing conditions (n = 4). CBA/Ca splenocytes were used as calibrator.

**Figure S4: Th17 cells upregulate FoxP3 expression in female lymphopenic recipients of male skin grafts.** (A) Expression of FoxP3 by Marilyn and A1(M).RAG1−/− Th17 cells before in vivo transfer. (B) Female mice injected with Marilyn CD4+ (n = 4), Th1 (n = 3) or Th17 (n = 7) cells, and grafted with male skin as described in Figure 2, were killed on day 48 after transplantation, and FACS analysis was performed on splenocytes. Expression of FoxP3 by CD4+ cells is shown.

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