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Retinal Self-Antigen Induces a Predominantly Th1 Effector Response in Axl and Mertk Double-Knockout Mice

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The TAM family of receptors (Tyro3, Axl, and Mertk) plays an important role in the negative regulation of response of dendritic cells (DCs) and macrophages to pathogenic stimuli, and mice lacking this receptor family develop spontaneous lupus-like systemic autoimmunity against a variety of tissues, including retina. To study the molecular mechanism underlying the TAM regulation of APC functions and subsequent effects on the induction of an autoimmune response against the eye, we examined CD4 T cell differentiation following retinal self-antigen immunization. CD4 T cells prepared from naive or interphotoreceptor retinoid-binding protein (IRBP)1-20–immunized Axl and Mertk double-knockout (dko) mice reacted to activation using anti-CD3 and anti-CD28 Abs or to bolster by self-antigen in vitro with a predominantly Th1 effector response, as characterized by increased IFN-γ production and higher frequency of IFN-γ–positive CD4 T cells. The Th17 effector response to IRBP immunization was similar in dko mice to that in wild-type controls, as shown by ELISA measurement of IL-17A in the culture medium and flow cytometric analysis of IL-17A–secreting CD4 T cells. Interestingly, APCs or DCs isolated from IRBP-immunized dko mice exhibited a greater ability to drive the Th1 response. The production of two driving cytokines for Th1 differentiation, IL-12 and IL-18, was dramatically increased in dko DCs and macrophages, and LPS stimulation bolstered their production. The preferential development into the Th1 subset in dko mice suggests that the cytokine milieu produced by the mutant mice in vivo or by mutant APCs in vitro selectively creates a differentiation environment favoring the Th1 effector response.

On encountering cognate Ags presented by APCs, such as DCs, naive CD4 T cells differentiate into several effector subsets, including Th1, Th2, Th17, and regulatory T cells (Treg), characterized by the production of distinct cytokines and effector functions (6–10). Th1 cells produce IFN-γ and lymphotoxin, which are responsible for immunity against intracellular pathogens, and other Th1 cytokines that are responsible for autoimmune responses. Th2 cells, producing IL-4, IL-5, IL-13, and IL-25, are essential for the generation of appropriate classes of Abs and play critical roles in asthma and other allergic diseases. Th17 cells are characterized by the production of IL-17 and other cytokines primarily acting against extracellular pathogens and are associated with the pathogenesis of several organ-specific autoimmune diseases (11–13). The Treg CD4 T cell subset expresses CD25 on the cell surface and the intracellular transcription factor Foxp3 (14, 15) and acts as an inhibitory cell type by releasing inhibitory cytokines, for example, IL-10 and TGF-β, and plays a critical role in T-cell–dependent peripheral tolerance (16–19). Developmental or functional anomalies, or alteration in the number, Treg have been linked to several chronic inflammatory and autoimmune diseases, such as multiple sclerosis (20), rheumatoid arthritis (21), and systemic lupus erythematosus (22).

The cytokine milieu plays an important role in T cell polarization, and different combinations of the surrounding cytokines induce specific transcriptional factors that control T cell differentiation. For example, during Th1 cell differentiation, IFN-γ causes induction of T-bet, a master regulator of Th1 cell differentiation that promotes Th1 polarization (23, 24). For Th2 cell differentiation, activation of Stat6 is necessary and sufficient to transduce IL-4 signaling (25). The differentiation of the Th17 cell is driven and stabilized by IL-6, TGF-β, IL-21, and IL-23, and the transcription factors STAT3 and RORγt are essential for the initial differentiation of Th17 cells (26, 27).

APCs affect T cell polarization by secreting specific cytokines, a notable example of which is IL-12, which selectively enhances...
Th1 cell growth by induction of IFN-γ production through activation of Stat4 (28). IL-18, originally known as IFN-γ-inducing factor, also provides an important accelerating and amplifying signal for Th1 proliferation and IFN-γ production (29). IL-12 and IL-18 act synergistically to drive Th1 activation (30–33) and are implicated in the pathogenesis of arthritis (34). Elevated levels of IL-18 and IL-12 are often correlated with the severity of autoimmune pathologies in experimental models and in clinical situations (33). Excessive production of IL-18 is seen in the blood of patients with rheumatoid arthritis (34, 35), lupus nephritis (36), and systemic lupus erythematosus (22, 37, 38).

Experimental autoimmune uveitis (EAU), an animal model for several human uveociliary autoimmune disorders (39, 40), can be elicited by immunization with retinal Ags in CFA, adoptive transfer of retinal autoantigen-specific CD4 T cells, or adoptive transfer of DCs pulsed with specific retinal autoantigens, and can develop spontaneously in some gene knockout or transgenic mice (39, 41–45). Depending on the approach used to elicit the disease, either Th1 or Th17 effector responses can be induced and lead to the development of EAU (39, 41). Th17 cells contribute to the development of EAU induced by immunization with retinal Ags in CFA in an IL-23-dependent manner, and this requires priming by an IFN-γ–producing effector T cell (41, 46, 47). However, transfer of DCs pulsed with interphotoreceptor retinoid-binding protein (IRBP)1-20 or an uveitogenic CD4 Th1 cell line specific for IRBP1-20 induces an IFN-γ–dependent Th1 effector response, leading to development of this disease (41, 48).

Triple knockout of the Tyro3, Axl, Mertk (TAM) family of receptors causes autoimmune disorders due to two relevant events, as follows: 1) defective phagocytosis; around weaning, there is a rapid accumulation of apoptotic debris that is constantly exposed to the immune system, and 2) overreactivity of activated APCs (49–51). We previously showed that TAM triple-knockout mice have increased numbers of DCs with higher surface levels of MHC II and B7 (49, 51). The CD4 T cell population is also increased in triple-knockout mice (49). Negative regulation of activated DCs is essential to prevent overproduction of cytokines and hyperactivation of T cell responses, and the suppressor of cytokine signaling (SOCS) protein plays very important roles in the negative regulation of pathogen-induced macrophage activation (52) and the suppression of systemic autoimmune responses caused by DCs (53). DCs express both Axl and Mertk (AM), but not Tyro3 (51), and pathogen stimulation enhances expression of AM, predominantly the Axl, which in turn upregulate the expression of the inducible negative regulator SOCS during the late stage of pathogen-induced DC activation (51, 54). In the absence of SOCS, DCs are hyperactivated, show enhanced Ag presentation, and cause an increased Th1 immune response (55, 56).

Autoimmune T cells can escape central selection in the thymus, but they are normally inactive due to immunologic peripheral tolerance to self-antigens. Increased amounts of self-antigens from the accumulation of apoptotic cell (AC) debris and unrestricted Ag presentation by hyperreactive APCs can break such self-tolerance and induce pathological autoimmune responses against normal tissues that express self-antigens. Mertk has been shown to play a critical role in AC-induced inhibition of DC activation and maturation (57), most likely through inhibition of NF-κB pathway (58). Without Mertk, mice develop spontaneous autoimmune disorder (59).

We have recently shown that naïve mice lacking all three members of TAM family of receptors develop autoimmunity against the retina-specific self-antigen IRBP characterized by the presence of IRBP-specific CD4 T cells and retina infiltration of lymphocytes (60). In the current study, we further showed invasion of lymphocytes in the degenerating Axl and Mertk double-knockout (AM dko) retina and the IRBP-specific CD4 T cells also existed in the dko mice. Based on the facts that DCs predominantly express AM receptors, and both are important for constraining DC activation and effector functions (51, 57), we further set out to investigate how DCs lacking AM affect CD4 T cell polarization and how these two receptors regulate the DC response to self-antigen; we analyzed CD4 T effector responses to immunization with the peptide IRBP1-20. Our data showed that the dko mice exhibited hyperpolarization into the Th1 phenotype and prolonged effector responses following immunization with the IRBP peptide IRBP1-20. This dominant Th1 response in the dko mice is most likely attributable to increased production of proinflammatory cytokines, for example, IL-12 and IL-18, by APCs that lack AM receptors.

Materials and Methods

Animals and reagents

The AM gene knockout mice, which were created on the C57BL/6 and 129 mixed background (50), have been backcrossed to the wild-type (WT) pure C57BL/6 background for at least 11 generations in our laboratory. All animals were housed in a pathogen-free facility and were handled according to the regulations of the Institutional Animal Care and Use Committee, and all procedures adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Reagents and materials

Human IRBP1-20 peptide (aa 1–20 of IRBP: GPTHLFQPSLVDMDKVKLVLLD) was synthesized by GenScript. Recombinant mouse IL-12, IL-6, IL-4, and TGF-β were purchased from Peprotech. Mouse rIL-23, IL-12, IL-4, and IL-10 kits for IFN-γ, IL-17A, IL-4, IL-10, and TGF-β, respectively, were anti-rat mouse CD4 mAb (NK1.1– or RM4–5–, FITC–, allophycocyanin–, or PE–conjugated), anti-mouse IFN-γ Ab (clone XMG1.2, purified, PE or FITC conjugated), anti-mouse IL-4 Ab (clone 11B11, purified or PE-conjugated), anti-mouse IL-25 Ab (PC6.15–, PE–, or allophycocyanin-conjugated), anti-mouse Foxp3 Ab (clone FJK-16s, PE-conjugated), neutralizing anti-IL-12 Ab (C17.8), neutralizing rat IgG2a (eBioscience), isotype Ab control, and PE-conjugated rat IgG2a isotype Ab controls were purchased from eBiosciences (San Diego, CA). Rat anti-mouse IL-4 mAb (clone 11B11), anti-mouse IL-12, anti-mouse CD16/32 Ab (clone 2.4G2), anti-mouse IL-17 Ab (clone TC11-18H10), anti-mouse CD3 Ab (clone 17A2), and hamster anti-mouse CD28 mAb (clone 37.51) were from BD Biosciences (San Diego, CA). Rat anti-mouse IL-18–neutralizing Ab (93-1OC) was purchased from MBL-International (Wohamu, MA). Brefeldin A was obtained from Sigma-Aldrich (St. Louis, MO). Mouse rGM-CSF was purchased from R&D Systems (Minneapolis, MN).

Induction of EAU by active immunization

Two hours after i.p. injection with Bordetella pertussis toxin (0.2 μg/mouse; Sigma-Aldrich), female mice aged 6–8 wk were immunized s.c. over six spots at the tail base and the flank with 250 μg human IRBP1-20 in 0.2 ml of a 1:1 v/v emulsion in CFA containing Mycobacterium tuberculosis strain H37RA (2.5 mg/ml; Difco); then, on days 0, 14, or 21 after immunization, the retina, spleen, and draining lymph nodes (inguinal, iliac) were collected and used for RNA preparation or to prepare a single-cell suspension for flow cytometric analysis or in vitro cytokine production assays.

Flow cytometry

Single-cell suspensions were prepared from the spleens and draining lymph nodes (inguinal and iliac) of naïve WT and dko mice or at day 14 post-immunization (pi) or day 21 pi after immunization with IRBP1-20 in CFA. After lysis of RBCs using ACK buffer (0.83% ammonium chloride, 0.1% potassium bicarbonate, 0.037% EDTA [pH 7.2]), Fc receptors were blocked by guest on July 29, 2017 http://www.jimmunol.org/ Downloaded from
were stained for 30 min at 4°C with anti-Foxp3 Ab or rat IgG2a isotype controls.

For intracellular cytokine staining, the cells were pretreated for 4 h at 37°C with 50 ng/ml PMMA and 500 ng/ml ionomycin, and then for 2 h at 37°C with 10 μg/ml brefeldin A (Sigma-Aldrich) prior to surface Ag staining. After surface Ag staining and washes, the cells were fixed and permeabilized for 20 min at 4°C with Cytofix/Cytoperm buffer (BD Biosciences), and then stained for intracellular cytokines with Abs against IFN-γ, IL-4, IL-17, or IL-12, and analyzed on a four-color BD FACSCalibur (BD Biosciences), the data being processed with CellQuest Pro 5.1.1 software (BD Biosciences).

Cytokine ELISAs

The pooled cells from the spleen and lymph nodes (inguinal, iliac, axillary, and submandibular) from naive or IRBP1-20-immunized WT and dko mice were initially separated by nylon-wool column filtration and then subsequently purified by EasySep mouse CD4+ positive selection kit following manufacturer’s instruction (StemCell Technologies). For Ab activation assays, the purified CD4+ T cells were stimulated with plate-bound anti-CD3 (5 μg/ml), soluble anti-CD28 (1 μg/ml) Abs; both anti-CD3 and anti-CD28 Abs were omitted from the controls. For naive CD4 T cell polarization assays, the CD4+ T cells were cocultured with gamma-irradiated splenic APCs with or without IRBP1-20 for 48 h under Th cell polarization conditions (Th1 conditions, 10 ng/ml IL-12 and 10 μg/ml anti-IL-4 Ab; Th2 conditions, 10 ng/ml IL-4, 10 μg/ml anti-IFN-γ Ab, and 10 μg/ml anti-IL-12 Ab; Th17 conditions, 20 ng/ml mouse rIL-23, 3 ng/ml TGF-β, 20 ng/ml IL-6, and 1 μg/ml anti-IL-4 Ab). Concentrations of IFN-γ, IL-4, IL-17A, IL-10, IL-12, and TGF-β were then measured in the culture medium using the relevant ELISA kits and following the manufacturer’s instruction (eBiosciences).

Preparation of bone marrow-derived DCs, bone marrow-derived macrophages, and peritoneal macrophages

For the preparation of bone marrow-derived cells, bone marrow was flushed from the femurs and tibias of WT and dko mice and red cells were removed with ACK buffer, and then 3 × 10⁶ cells in complete medium (RPMI 1640 medium containing 10% FBS, 50 mM 2-ME, 10 mM HEPES [pH 7.4], 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) were added to each well of a six-well plate. To obtain DCs, 10 ng/ml GM-CSF was added to the culture medium. On day 3, floating cells were gently removed and fresh medium added, and then, on day 7, nonadherent cells and loosely adherent proliferating DCs were collected and 3 × 10⁶ cells were placed in each well of a new six-well plate. On day 10 of culture, the cells were treated with 100 ng/ml LPS for the indicated time. To obtain bone marrow-derived macrophages, 30% L929 cell (American Type Culture Collection, Rockville, MD)-conditioned medium was added to the bone marrow cultures as a source of CSF-1, and then, 24 h later, nonadherent cells were removed and the remaining adherent cells were cultured for 6 more days in the same medium containing L929 cell-conditioned medium. On day 7, the cells were treated with 100 ng/ml LPS for the indicated time.

To obtain peritoneal macrophages, 1 ml 3% thioglycolate was injected i.p. On day 4 of induction, the cells in the peritoneum were flushed out with 9 ml cold PBS containing 0.5% FBS, and then, after a wash with cold PBS plus 0.5% FBS, 3 × 10⁶ cells were plated into each well of a six-well plate. Nonadherent cells were removed 2 h later, and the remaining adherent cells were cultured in complete medium for 3 d, and then were treated with 100 ng/ml LPS for 4 h before harvesting for flow cytometric analysis.

RNA isolation, cDNA synthesis, and real-time quantitative PCR

Total RNA was extracted from cultured cells or from the spleen, draining lymph nodes (inguinal, iliac), or retinas of naive mice or at days 14 and 21 pi after IRBP1-20 immunization using TRIzol reagent, following the manufacturer’s instruction (Invitrogen, San Diego, CA). The integrity of the RNA samples was checked by a 2:1 ratio of 28S and 18S RNA on 1% formaldehyde-denaturing RNA agarose gel and an A260/A280 ratio >1.9. Two micrograms of total RNA from each sample was treated with DNase I to remove traces of genomic DNA, and then was reverse transcribed into first-strand cDNA using a qScript cDNA SuperMix kit (Quanta Biosciences, Gaithersburg, MD) for real-time quantitative PCR (qPCR) analysis. Real-time qPCR was performed in a SYBR green-based PCR reaction mixture on a MX3005p system (Agilent Technologies, Santa Clara, CA), with a program of a 10-min initial hot-start activation of Taq polymerase at 95°C, followed by 40 cycles of amplification (95°C for 10 s, 56°C for 5 s, and 72°C for 10 s). After amplification, a melting curve was generated by

**FIGURE 1.** In vitro activation of CD4+ T cells by plate-bound anti-CD3 Ab and soluble anti-CD28 Ab induces more IFN-γ-secreting Th1 cells in AM dko mice than in the WT. A, Pooled spleen and lymph node CD4+ T cells were isolated from WT or AM dko mice by an initial nylon-wool column filtration and followed by EasySep mouse CD4+ positive selection kit (StemCell Technologies). A total of 8 × 10⁶ CD4+ T cells/well was plated on a 96-well plate bound with anti-CD3 (5 μg/ml) in the presence of soluble anti-CD28 (1 μg/ml) Ab. The control cells were plated on a 96-well plate in the absence of both anti-CD3 and anti-CD28 Abs. After 2 d of culture, levels of IFN-γ, IL-4, IL-17A, IL-10, IL-12, and TGF-β in the culture medium were measured by Ready-Set-Go ELISA kits following manufacturer’s instruction (eBiosciences). B, Pooled naive spleen and lymph node CD4+ T cells from WT or dko mice were cultured in 96-well plates (as in A), and then IFN-γ-secreting CD4+ T cells were analyzed by flow cytometry on a four-color BD FACSCalibur (BD Biosciences). The results are one representative of three mice in each group. C, The cell culture media prepared from A were subjected to IL-12 measurement by Ready-Set-Go ELISA kit (eBiosciences). The concentration of IL-12 is expressed as pg/ml. D, A total of 8 × 10⁶ CD4+ T cells prepared as described in A was plated on a 96-well plate and stimulated for 48 h by plate-bound anti-CD3 and soluble anti-CD28 Abs, in the presence of neutralizing Abs against IL-12 (2 μg/ml, C17.8; eBiosciences) and IL-18 (2 μg/ml, 93-10C; MBL-International); and the controls use either PBS or rat IgG1-κ isotype mAb (2 μg/ml, eBRG1; eBiosciences) in the place of neutralizing Abs. The culture medium after 48 h was subjected to IFN-γ measurement, as in A, and the concentration of IFN-γ is expressed as ×10⁵ pg/ml. E. No expression of AM in the naive WT CD4+ T cells. Pooled WT spleen and lymph node CD4+ T cells were first enriched by an initial nylon-wool column filtration and then purified on a high-speed cell sorter (MoFlo; DakoCytomation, Fort Collins, CO); 1 × 10⁶ CD4+ T cells with purity >98% were subjected to RNA isolation, and 1 μg total RNA was used for reverse transcription and real-time qPCR analysis of AM expression. The total RNA from WT control spleens was used as positive control. The results in A, C, and D are the mean ± SD for five wells per group in one experiment and are representative of those obtained in three independent experiments. *p < 0.05, ***p < 0.001 in the one-way ANOVA test using ProStat Ver 5.5.
holding the reaction at 65°C for 15 s, then heating to 95°C, with a ramp rate of 0.1°C/s. To obtain the melting temperature of each sample, the fluorescence signal was plotted against temperature. The comparative threshold cycle method normalized to β-actin was used to analyze relative changes in gene expression.

The oligonucleotides used for qPCR were 5′-CACGCAACACGC-GGGCAAGAAC3′ and 5′-CTGGACCTGTGGTGGTTGAC3′ for IFN-γ; 5′-AACATGAGTCCAGGGAAGCTCA3′ and 5′-AGTGTGTTGGACTCAGGCTG3′ for IL-17A; 5′-TGGAACCCGAGACCAGCTG3′ and 5′-CCTGGAAACGTTTCTGAAAGA3′ for IL-18; 5′-GTATGAGTGACCCTTGCTTGTGA3′ and 5′-ATTCTGAAGTGCTGTTGTAGGCGC-3′ for IL-12p35; 5′-GCAAACGACTTGAACCTCAAA3′ and 5′-GCATGTGGTGAACCTGTGAATGAF-3′ for Foxp3; 5′-GTGGGGCGAGC-TGTACATTGTACCTT3′ and 5′-TGTACCGTGTGTCAGGCTCAA3′ for TGF-β; 5′-CATGGCCCAAGAATACTCAAGGA3′ and 5′-GGAGGAATCGATGACAGCGC-3′ for IL-10; 5′-TCTACACCAGCAAGAGCGATGT-3′ and 5′-TGTTTCTACGAGGTTCCTTGAGGC3′ for Axl; 5′-GAAA-CTGCACTGTGCGGATGACA3′ and 5′-CAATGCGGCCTTGGCGGATCT-3′ for Merk; and 5′-GGGTGATATCCCTCCATCG3′ and 5′-CCAGTGTGTAACATGC-CATGT-3′ for β-actin.

Results

Naive CD4 T cells from AM dko mice are preferentially polarized into Th1 effector T cells

Although the naive mice lacking all three members of the TAM family of receptors produce retinal Ag-specific CD4 T cells and develop spontaneously autoimmunity against the eye (60), mice without AM receptors exhibited similar lymphocyte infiltration into the degenerating retina; those included CD3-positive T cells and F4/80-positive macrophages (Supplemental Fig. 1); and these AM dko mice also generated CD4+ T cells specifically recognizing retinal-specific autoantigen, IRBP (Supplemental Fig. 2). We then asked which T effector responses were involved and dominated in those dko mice. On stimulation with plate-bound anti-CD3 Ab and soluble anti-CD28 Ab, naive CD4 T cells proliferate, differentiate into one of three major effector T cell subsets, and secrete signature cytokines. We therefore prepared CD4+ T cells from a pooled spleen and lymph node cell suspension from naive WT or AM dko mice, activated them using plate-bound anti-CD3 Ab and soluble anti-CD28 Ab without polarizing cytokines, and then measured cytokines released into the culture medium after 48 h by ELISA. As shown in Fig. 1A, in the absence of polarizing conditions, CD4+ T cells from dko mice produced dramatically higher amounts of IFN-γ (4- to 5-fold increase), slightly less IL-17A, and more TGF-β than those of WT mice. Interestingly, only a small amount of IL-4 was produced by the dko cells, compared with the 80 pg ml−1 secreted by the WT cells. In the absence of activation, levels of all of these cytokines were very low or undetectable for both WT and dko cells. To confirm the preferential Th1 differentiation in the mutant mice, we measured levels of IFN-γ-producing CD4+ T cells in the in vitro cultured T cells by flow cytometry and found that, after 2 d of activation by anti-CD3 and anti-CD28 Abs, 17.7% of CD4+ T cells were IFN-γ producing in dko mice compared with 8.5% in the WT, although both groups showed an increase compared with the unstimulated groups (WT 1.8% and dko 2.6%) (Fig. 1B).

In vitro differentiation of naive CD4 T cell into Th1 population is frequently conditioned by the presence of IL-12. Given the higher level of IFN-γ released from the T cells activated by plate-bound anti-CD3 and soluble anti-CD28 Abs, we then asked whether IL-12 was present in the culture medium and found that there was indeed a small amount of IL-12 existing in both genotypes; but the mutant contained higher level of this cytokine compared with WT

![Figure 2](http://www.jimmunol.org/)  
**FIGURE 2.** IRBP1-20 immunization elicits a dominant Th1 effector response. WT and AM dko mice were immunized with 250 µg per animal of IRBP1-20 peptide in CFA, and then Th1 and Th17 effector T cell subsets in the CD4+ T cells were prepared at days 0, 14, or 21 pi from the spleen (top), and lymph nodes (LN, bottom) were analyzed by FACS for IL-17A (A, B, and D) or IFN-γ (A, B, and C) versus CD4 expression with the gates on the CD4+ T cell population. The percentages represent the percentage of cytokine-expressing CD4+ T cells in the total CD4+ T cells of the WT and AM mice (n = 6).
control (Fig. 1C). Neutralization with anti–IL-12 as well as anti–IL-18 Abs decreased the IFN-γ release into the culture medium by ∼25% compared with the unneutralizing conditions, but the Ab-neutralized mutant cells still produced significantly more IFN-γ over controls (Fig. 1D).

Abnormal polarization of CD4 T cells in the dko may result from the receptor deficiency in the CD4+ T cells; however, the real-time qPCR measurement of AM mRNA in the sorted CD4+ T cells (>98%) yielded undetectable signals (Fig. 1E), which was in agreement with previous publications (49, 61, 62). This suggests that abnormal CD4 T cell responses in the mutants might be caused by other cell types that normally express Axl and Mertk.

**Immunization with the retinal autoantigen IRBP1-20 drives a dominant Th1 effector response**

The existence of CD4 T cells with Th1 potential in the dko mice prompted us to investigate differences between the WT and dko mice in response to self-antigen immunization and to examine the Th subsets that are preferentially induced in vivo. To assess T cell polarization in response to IRBP1-20 immunization, we analyzed effector T cell subsets by flow cytometry at days 0, 14, and 21 pi. As in a previous report that IRBP1-20 immunization invokes Th1 and Th17 responses in WT mice (41), we observed increases in both cell types in the dko mice, with a peak at day 14 pi; however, the increase in Th1 cells was greater (Fig. 2A, 2B). It is generally considered that the T cell response decreases gradually after reaching a peak at 2 wk after immunization, and this was true for our WT mice, in which the spleen and lymph nodes contained, respectively, 14.5 and 6.8% IFN-γ–secreting cells at day 14 pi, and 3.1 and 2.1% at day 21 pi (Fig. 2B, 2C). However, at day 21 pi, the AM dko mice still had large numbers of Th1 T cells (Fig. 2C). These results suggest that the absence of AM receptors on APCs affects the duration and degree of the Th1 effector response.

**T cells from IRBP1-20–immunized dko mice produce more IFN-γ in vitro than WT mice**

Preference differentiation into the Th1 lineage in the mutant mice in response to IRBP1-20 immunization was further demonstrated by the cytokine release profile of the in vitro cultured CD4 T cells. CD4+ T cells were prepared at different times postimmunization from WT and dko mice immunized with IRBP1-20 and cocultured with irradiated APCs from the same mice in the presence of IRBP1-20, and then, 48 h later, cytokines released into the culture medium were measured by ELISA. As shown in Fig. 3A, IFN-γ released from cells prepared at day 14 pi from immunized WT and dko mice (Fig. 3A, middle panel) was markedly increased compared with that seen with nonimmunized mice (left panel). Consistent with the flow cytometry result, the dko mice produced more IFN-γ–secreting Th1 T cells in response to IRBP1-20 immunization (Fig. 2B), and the CD4+ T cells prepared from dko mice at day 14 pi secreted more than twice as much IFN-γ into the culture medium as WT cells (Fig. 3A, middle panel). Although IFN-γ secretion was markedly decreased in both genotypes at day 21 pi, dko T cells still released ∼5 times more IFN-γ than WT T cells (Fig. 3A, right panel). Overproduction of IFN-γ mRNA in the spleens and lymph nodes of dko mice immunized with IRBP1-20 at days 14 and 21 pi was confirmed by real-time qPCR (Fig. 3B).

**Th17 cells in AM dko mice exhibit normal responses to IRBP1-20 induction**

Because the Th17 cell has been demonstrated to be associated with several autoimmune disorders (11–13), we tested the Th17 response to IRBP1-20 immunization in both WT and AM dko mice. Consistent with the notion that immunization with IRBP1-20 in CFA mainly provokes a Th17 cell response (41, 46, 47), Th17 cells were induced in both WT and dko mice (Fig. 2B, 2D), suggesting that the Th17 response is normal in dko mice. To further test Th17 cell polarization during IRBP1-20 immunization, we isolated and cultured CD4 T cells from WT and dko mice immunized with IRBP1-20 at day 0 and days 14 and 21 pi in the presence of IL-23, anti–IFN-γ, and anti–IL-4 Abs and gamma-irradiated APCs from the same immunized mice for 48 h, and then measured IL-17A released into the culture medium by ELISA and found that IL-17A levels were increased by IRBP1-20 immunization, with a peak at day 14 pi and a gradual decrease thereafter by day 21 pi (Fig. 3C). These data indicate that AM dko mice have a normal Th17 response to autoantigen immunization.

**High levels of Treg are induced in dko mice immunized with IRBP1-20 at day 21 pi**

Given the facts that TAM triple-knockout mice develop spontaneous autoimmune disorders (49, 51) and that Treg inhibit autoimmunity (20–22), we then examined whether the Treg frequency in IRBP1-20–immunized dko mice was decreased compared with that in WT mice. However, surprisingly, after immunization by days 14 and 21 pi, the dko mice produced more CD4+CD25+Foxp3+ Treg than the WT controls (Fig. 4A–C). Consistent with this, dko mice, as well as the CD4+ T cells prepared from dko mice, produced increased amounts of IL-10 protein (Fig. 4D) and IL-10 mRNA (Fig. 4E), implying there was an increased frequency of Treg in the dko mice at day 21 pi. To serve as a control, CD4+ T cells isolated from naive WT and dko mice at 4–5 wk of age were activated by anti-CD3 and anti-CD28 Abs, IL-10 levels in the culture medium were measured by ELISA, and the cells...
from the two types of mice were found to produce similar amounts of IL-10 (Fig. 4F). An increased frequency of Treg in the dko mouse spleen and lymph node might possibly inhibit the CD4+ Th response. However, we noted previously (60) that, at day 21 after immunization with a low dose (100 μg) of IRBP1-20, a large number of CD3-positive T cells was still present in the mutant retina, so we therefore measured Foxp3 mRNA levels by qPCR in the WT and dko retina. As shown in Fig. 4G, there was less Foxp3 transcript in the dko retina than in the WT retina, although increased Foxp3 mRNA levels were detected in dko spleens and lymph nodes compared with the WT (Fig. 4H, right). In contrast, increased IFN-γ mRNA levels were detected in both retina and lymphoid tissues in the dko mice compared with the WT (Fig. 4G, 4H).

**AM dko APCs have a greater ability to drive T cells to develop into the Th1 lineage**

DCs, as a major APC cell type, play a critical role in driving adaptive immune responses. In the immune system, the TAM family of receptor tyrosine kinases is expressed mainly on myeloid cells and monocytes and their derivatives and not on T and B lymphocytes (49, 61, 62). We therefore examined whether the aberrant T cell activation in dko mice was caused by dysfunctional APCs. We tested CD4 T cell polarization in an in vitro culture system in which purified CD4+ T cells from day 14 pi mice were cocultured with CD11c+ DCs derived from bone marrow progenitors and found that LPS immunization with a low dose (100 μg) of IRBP1-20, a large number of CD3-positive T cells was still present in the mutant retina, so we therefore measured Foxp3 mRNA levels by qPCR in the WT and dko retina. As shown in Fig. 4G, there was less Foxp3 transcript in the dko retina than in the WT retina, although increased Foxp3 mRNA levels were detected in dko spleens and lymph nodes compared with the WT (Fig. 4H, right). In contrast, increased IFN-γ mRNA levels were detected in both retina and lymphoid tissues in the dko mice compared with the WT (Fig. 4G, 4H).

**FIGURE 4.** IRBP1-20 immunization induces more Treg production in dko mice than in the WT. A–C, Flow cytometric analysis of the CD4+CD25+/Foxp3+ Treg subset in the pooled splenic and lymph node CD4+ T cells prepared from WT and dko mice at day 0 pi (A), day 14 pi (B), and day 21 pi (C) with IRBP1-20. The result is one representative of four to six mice in each group. C, ELISA measurement of IL-10 in the culture medium after 2-d coculture of CD4+ T cells and syngeneic APCs in the presence of IRBP1-20. E, qPCR quantification of IL-10 mRNA in the spleen and lymph nodes of WT and dko mice at day 21 pi. F, Pooled spleen and lymph node CD4+ T cells from WT or AM dko mice were plated on a 96-well plate bound with anti-CD3 Ab in the presence of soluble anti-CD28 Ab (+Ab). The control cells were plated on a 96-well plate in the absence of both anti-CD3 and anti-CD28 Abs (−Ab). After 2 d of culture, levels of IL-10 in the culture medium were measured by ELISA (eBiosciences). G and H, qPCR measurement of Foxp3 mRNA (G) and IFN-γ (H) levels in the spleen (Sp), lymph node (LN), and retina of WT and dko mice at day 21 pi. The results in D and E are the mean ± SD for five wells per group in one experiment and are representative of those obtained in three independent experiments. The results in F–H are the mean ± SD of three mice in a single experiment and are representative of those in three independent experiments. *p < 0.01, **p < 0.001 in the one-way ANOVA test using ProStat Ver 5.5.
stimulation caused a rapid increase in both IL-12p35 and IL-18 with a peak at 6 h of treatment, followed by a drop to basal level within 24 h in both macrophages (Fig. 6C) and DCs (Fig. 6D). As expected, LPS stimulation induced a greater IL-12p35 and IL-18 response from the bone marrow–derived dko macrophages (Fig. 6C) and DCs (Fig. 6D) compared with WT. These data suggest that overproduction of IL-12 and IL-18 might be, at least in part, responsible for favoring Th1 polarization in AM dko mice.

Discussion

Previous studies have shown that mice lacking TAM receptors develop spontaneous autoimmune disorders (49, 59) due to unrestricted activation of cytokine production by DCs or macrophages (51, 54). Moreover, TAM triple-knockout mice generate autoantigen-specific CD4 T cells and have a much higher susceptibility to development of autoimmune disease on immunization with a retina-specific Ag (60). We, in the current study, further showed that AM dko mice also exhibited spontaneous lymphocyte infiltration into the degenerating retina and produce retina Ag-specific CD4 T cells. This is consistent with previous published observation that AM dko as well as Merk single-knockout mice show autoimmune disorders, although they are not as severe as the TAM triple-knockout mice (49, 59). Because T cells do not express TAM receptors (49, 61, 62), abnormal T cell differentiation is considered to be caused mainly by DCs, a potent APC, that normally only express AM, but not Tyro3 (51). To investigate the T cell effector response to retinal autoantigen in these mutant mice, we therefore focused on the AM dko mice and studied CD4 T cell polarization following IRBP1-20 immunization. Our results showed that mice lacking AM receptors responded to IRBP1-20 immunization with activation of both the Th1 and Th17 pathways, but the Th1 response was more dominant and hyperreactive. The CD4 T cell response to immunization is generally considered to reach a peak in 2 wk. At this time (day 14 pi), the number of Th1 T cells was twice as high in the dko mice as in the WT mice, and this dominant Th1 population was still present at day 21 pi, at which time the Th1 phenotype was near to basal levels in the WT controls. Such excessive Th1 response was not unique to IRBP immunization; CD4 T cells from naive dko mice generated increased numbers of Th1 cells and released more IFN-γ into culture medium upon activation by plate-bound anti-CD3 and soluble anti-CD28 Abs even at the absence of the conditioning cytokines. Preferential polarization into the Th1 lineage may be due to the presence of IL-12-secreting cells, such as DC, in the cell preparation. However, neutralizing Abs against IL-12 and IL-18 failed to completely restore the IFN-γ-secreting Th1 cells to the WT level, suggesting that those naive CD4 T cells from mutants might contain higher frequency of Th1-committed progenitors or memory T cells. The memory T cells have been demonstrated to be more susceptible to anti-CD3 and anti-CD28 activation in production of cytokines and T cell polarization (65, 66). We have previously shown that TAM knockout mice contained more memory T cells (60), which might give to Th1 cells upon Ab activation.

Th17 cells specific for self-antigens are responsible for a severe autoimmune response in several animal models (11–13), including EAU (41, 46, 47). In agreement with these results, we observed a normal Th17 response in the mutant mice, although the mutant mice showed a slightly increased response at day 14 pi. It has been well established that both Th1 and Th17 effector responses can be induced by immunization with IRBP and that the dominance of one response over the other is dependent on the method of immunization (39, 41). In WT mice, immunization with IRBP1-20 in CFA elicits a dominant Th17 response, whereas transfer of IRBP1-20–specific CD4 T cells or IRBP1-20–pulsed DCs leads to a dominant Th1 response (41, 48). However, in the current study, we demonstrated that immunization with the retinal autoantigen, IRBP1-20, in CFA elicited a dominant Th17 response, whereas transfer of IRBP1-20–specific CD4 T cells or IRBP1-20–pulsed DCs leads to a dominant Th1 response. This is probably due to overproduction of IFN-γ in the mutants (49), which is able to inhibit commitment to the Th17 phenotype (12).

The differentiation of naive CD4 T cells into different lineages of effectors is largely dependent upon the production of cytokines...
by activated APCs. The cytokines secreted by APCs create an environment that is critical for directing the differentiation of activated Ag-specific lymphocytes into a distinct effector T cell subset. Because T cells do not express TAM receptors, the abnormalities in the T cell responses in the AM double-mutant mice might be due to DCs or macrophages lacking this family of receptors. AM play negative regulatory roles in DCs and macrophage upon pathogen stimulation or AC encounter, by upregulation of the inhibitory SOCS expression (51), or by inhibition of NF-κB activation (58) and constraining DC activation and maturation (57), respectively. Without AM, DCs produce increased levels of proinflammatory cytokines (51, 58). We therefore hypothesized that AM receptors negatively regulate the expression of distinct cytokines that drive Th1 polarization, and that APCs lacking AM receptors produce altered levels or a different profile of cytokines that drive a specific reaction for induction of effector T cell polarization. We focused our study on two cytokines, IL-12 and IL-18, as these cytokines are secreted by APCs and, in turn, activate Th1 development by upregulating the synthesis of inflammatory IFN-γ (30, 31, 63), whereas mice deficient in IL-12 and IL-18 are resistant to experimental autoimmune encephalomyelitis and collagen-induced arthritis (67, 68) and show reduced Th1 cell development. IL-12 is considered to play a major pathogenic role in autoimmune diseases, such as EAU and experimental autoimmune encephalomyelitis, by promoting the generation of IFN-γ-producing Th1 effector cells (63, 64). Increased inflammatory IFN-γ production is seen in naive dko mice (49). The data presented in this study showed that both DCs and macrophages lacking AM receptors were hyperreactive to LPS stimulation in terms of production of both IL-12 and IL-18. This result was in contradiction of what was observed on the NOD mice lacking Merk expression, in which the NOD-Merk−/− DCs produced equal amount of IL-12 as the control cells after 72 h of LPS treatment, unless the DCs had been previously treated with AC (57). Such difference may come from the duration of LPS treatment and the genetic background from which the DCs were derived. Interestingly, AC-induced inhibition of IL-12 production during pathogen-stimulated DC activation is indeed mediated by Merk signaling (57). It is conceivable that AM dko mice, which have accumulated massive AC debris caused by a defective phagocytic activity in the phagocytes, produce elevated level of IL-12 and IL-18, perhaps other cytokines too, due to loss of Merk inhibition of DC activation and maturation. These increased levels of IL-12 and IL-18 are probably responsible for the increased Th1 response in the AM dko mice. These results suggest that AM receptors regulate APC cytokine production that drives the Th1 effector response.

In addition, we observed less IL-4 secretion by dko CD4+ T compared with WT cells after in vitro activation by anti-CD3 and anti-CD28 Abs, which may result from a higher percentage of IFN-γ-secreting Th1 cells in the total CD4 T cell population.

The fourth subset of T cells studied in this investigation was Treg. The frequency of CD4+CD25+Foxp3+ Treg was increased in AM dko mice at days 14 and 21 pi compared with WT mice. This much higher Treg population after self-antigen immunization may simply reflect overreaction to immunization in general or may be due to the increased secretion of TGF-β in the mutants. It is well known that the regulatory functions mediated by Treg, as well as Treg active proliferation, are dependent on stimulation with Ags, including tissue-specific self-antigens (69–72). Deposition of retinal self-antigens during photoreceptor degeneration or overreaction to IRBP1-20 immunization in the dko mice may contribute to the increased frequency of Treg. Interestingly, although we observed an increased Treg population in mutant peripheral lymph tissues, we indeed found a decreased level of Treg-specific Foxp3 in the mutant retina. Many clinical observations show that patients with autoimmune diseases have lower level of circulating Treg (73). Whether or not the mutant mice are resistant to secondary immunization by the same Ag or the Treg in the mutants are not functionally active remains for further investigation.

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Disclosures

The authors have no financial conflicts of interest.

References


Retinal self-antigen induces a predominantly Th1 effector response in Axl and Mertk double knockout mice

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Supplemental Figures.

**FIGURE S1.** Lymphocytes infiltrate into degenerating AM dko retina. A-C, Paraffin sections through the retinas of 6-week-old WT (A); and 4-week-old (B) and 6-week-old (C) of AM dko mice. Arrows indicate infiltrated lymphocytes. D and E, the paraffin-embedded retina sections from WT (D) and AM dko (E) mice at ages of 5 weeks old were immunostained red with anti-CD3 antibody. Arrow shows CD3-positive T cells. F and G, the paraffin-embedded retinal sections from 5 week-old of
WT (F) and AM dko (G) were immunostained with antibody against F4/80 (Green) to show macrophages. ONL, outer nuclear layer; OS, outer segment. Bars, 80 μm for all pictures.

**FIGURE S2.** IRBP-specific T cells are present in naïve AM dko mice. The T cells (4x10⁴) used for each ³H-thymidine incorporation assay were isolated from pooled spleens and lymph nodes (inguinal, iliac, axillary and submandibular) of three naïve WT or dko female mice at ages of 6-8 weeks by nylon wool column filtration method and expanded for 72 hours on γ-irradiated syngeneic splenic APCs (1x10⁵) in the medium containing no antigen ((-)IRBP1-20, left) or IRBP1-20 (((+)IRBP1-20, 10μg, right) at concentrations of 10 μg/ml. The WT T cells were co-cultured with WT APC (WT-T::WT-APC) and the dko T cells were plated on dko APC (dko-T::dko-APC). In the last 8 hr of co-culture, 0.5 μCi of [methyl-³H]-thymidine were added into each well. ³H-thymidine incorporation into the responder T cells was measured as count per minute (cpm). The data shown is representative of those obtained in three
independent experiments. The bars are the ±SD for n = 8 wells per group in each experiment. ***p<0.001 by one way ANOVA tests created with ProStat ver 5.5.