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Ivan Dzhagalov, Pierre Chambon and You-Wen He

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Regulation of CD8<sup>+</sup> T Lymphocyte Effector Function and Macrophage Inflammatory Cytokine Production by Retinoic Acid Receptor γ<sup>1</sup>

Ivan Dzhagalov,* Pierre Chambon,† and You-Wen He²*</p>

Vitamin A and its derivatives regulate a broad array of immune functions. The effects of these retinoids are mediated through members of retinoic acid receptors (RARs) and retinoid X receptors. However, the role of individual retinoid receptors in the pleiotropic effects of retinoids remains unclear. To dissect the role of these receptors in the immune system, we analyzed immune cell development and function in mice conditionally lacking RARγ, the third member of the RAR family. We show that RARγ is dispensable for T and B lymphocyte development, the humoral immune response to a T-dependent Ag and in vitro Th cell differentiation. However, RARγ-deficient mice had a defective primary and memory CD8<sup>+</sup> T cell response to Listeria monocytogenes infection. Unexpectedly, RARγ-deficient macrophages exhibited impaired inflammatory cytokine production upon TLR stimulation. These results suggest that under physiological condition, RARγ is a positive regulator of inflammatory cytokine production. The Journal of Immunology, 2007, 178: 2113–2121.

Two families of nuclear receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs), act as retinoid receptors (14). The RARs (α, β, γ, and their isoforms) are activated by both ATRA and 9-cis-RA, whereas the RXRs (α, β, and γ) are exclusively activated by 9-cis-RA (4). RARγ, the third member of the RAR family, recognizes a direct repeat of AGGTCA separated by 2 or 5 nt and binds to DNA as a heterodimer with members of the RXR family (15). RARγ/RXR heterodimer binds to its responsive element constitutively, and in the absence of ATRA it represses transcription by recruiting corepressors (16). Upon binding to ATRA, RARγ/RXR dimer recruits coactivators and up-regulates transcription of target genes. In addition to functioning as a coreceptor for RARs, RXRs can also serve as heterodimeric partners for other nuclear receptors such as thyroid hormone receptors, peroxisome proliferator-activated receptors (PPARs), and nerve growth factor-induced gene B (17). The level of complexity in the formation of different types of retinoid receptors by RARs and RXRs in various cells suggests that individual RARs or RXRs may exert unique modulating function in the immune system.

To dissect the role of individual RARs in transducing retinoid signals in the immune system, we examined immune cell development and function in mice with RARγ conditionally deleted in hemopoietic cells. Our experiments were designed to examine the function of RARγ under physiological or pharmacological levels of retinoid acid stimulation. Our data show that RARγ is dispensable for the development of immune cells, but it is required for CD8<sup>+</sup> T cell IFN-γ production and effector function in response to Listeria monocytogenes infection in vivo. Unexpectedly, inflammatory cytokine production is impaired in RARγ-deficient macrophages. These data suggest that RARγ plays a nonredundant role in regulating inflammatory cytokine production in T lymphocytes and macrophages.

Materials and Methods

Mice

C57BL/6 mice were obtained from The Jackson Laboratory. The RARγ<sup>−/−</sup> VavCre mice were generated by crossing RARγ<sup>−/−</sup> mice with VavCre transgenic mice (18, 19). To determine the efficiency of Cre-mediated deletion, genomic DNA from thymus, spleen, and bone marrow (BM)
was digested with XhoI and XhoI (New England Biolabs) and probed, as described (18). RARγ, VavCre and RARγL, mices were indistinguishable from wild-type mice and used as controls in all of the experiments. All mice were fed regular diet (vitamin A sufficient), maintained under specific pathogen-free conditions at Duke University Vivarium, and used at 6-12 wk of age. All experiments were performed according to protocols approved by Duke University Animal Care and Use Committee.

RT-PCR and quantitative RT-PCR

Lymphocyte populations from the thymus and spleen of C57BL/6 mice were purified by fluorescence-activated cell sorting (>99% pure), and total RNA from 1 × 10^6 cells was extracted with RNasey Mini kit (Qiagen). First strand DNA was reverse transcribed with iScript Reverse Transcriptase kit (Bio-Rad). Quantitative and semiquantitative RT-PCR were performed with the following primers: RARγ forward, 5′-TCC TGG CTT CTA TAA GCC ATG CTT TG and reverse, 5′-TTG GAC ATG CCC ACT TCG AAA CAC; hypoxanthine phosphoribosyltransferase (HPRT) forward, 5′-GAT ACA GGC CAG ACT TTG TTG and reverse, 5′-GTT AGG AGT GCC TAT AGG CT. The quantitative RT-PCR was performed in triplicates on LightCycler (Roche). The mRNA abundance of RARγ was calculated and normalized to HPRT using Relative Expression Software Tool provided by M. Pfaffl (Technical University of Munich, Munich, Germany).

Flow cytometry

Single-cell suspensions of the spleen, BM, thymus, and lymph nodes were lysed of erythrocytes, incubated with an FCr blocker (2.4G2 supernatant), and stained with fluorochrome-labeled mAbs in PBS containing 2% FCS and 0.02% sodium azide. The following Abs directly conjugated to FITC, PE, or PE/cy5 were used for flow cytometric analyses: CD3, CD4, CD8a, CD43, CD44, CD25, B220, TCRβ, TCRγδ, BP-1 (Ly51), CD24 (heat-stable Ag), IgM, IgD, and αβδγ integrin (DATK), from BD Pharmingen, eBioscience of Biolegend. Analyses were performed on a FACScan flow cytometer using CellQuest software (BD Biosciences). K^−/OVA-PE tetramers were provided by M. Bevan (University of Washington, Seattle, WA).

For cell proliferation experiments, 200 μl of single-cell suspensions from spleen at 10^6 cells/ml was stimulated in triplicates in complete RPMI 1640 medium (Invitrogen Life Technologies) with 5 μg/ml anti-CD3 (2C11) Ab or 40 μg/ml anti-IgM Ab (MP Biomedicals) for 72 h in the presence or absence of 10 nM ATRA (Sigma-Aldrich). Then the cells were pulsed with 1 μCi of [3H]thymidine (GE Healthcare). After 4 h, the cells were harvested with a Tomtec cell harvester (Tomtec) onto fiberglass filters (PerkinElmer) and incubated with Betaplate Scint scintillation liquid (PerkinElmer Wallac). The filters were read on Microbeta TriLux (PerkinElmer). In the cell stimulation experiments, total splenocytes were stimulated with 1 μg/ml anti-CD3 (2C11) Ab in the presence or absence of 10 nM ATRA (Sigma-Aldrich) for 2 days, washed, and cultured for 3 more days in the presence of 100 U/ml human IL-2 (hIL-2; Biologend).

Th1/Th2 differentiation

CD4^+ T cells were purified by depletion with a mixture of biotinylated Abs for B220, CD8, I-A^k, CD11c, and Mac-1, followed by Dynabeads M-280 streptavidin (Invitrogen Life Technologies). The purity was typically above 88%. A total of 2 × 10^6 CD4^+ T cells was incubated with the same number of irradiated (3000 rad) T cell-depleted splenocytes on anti-CD3 (5 μg/ml)-coated 24-well plates (BD Biosciences) in the presence of 100 U/ml hIL-2. For Th1 differentiation, anti-IL-4 Ab at 4 μg/ml (Biolegend) and IL-12 at 5 ng/ml (PeproTech) were added. For Th2 differentiation, anti-IFN-γ Ab at 4 μg/ml (Biolegend) and IL-4 at 40 ng/ml (PeproTech) were added. After incubation at 37°C for 5 days, the live cells were isolated by gradient centrifugation on Lymphocyte-M (Cedarlane Laboratories) and activated on anti-CD3 (5 μg/ml) plus anti-CD28 (1 μg/ml)-coated 24-well plates overnight for cytokine production, as measured by ELISA or with PMA (10 ng/ml) plus ionomycin (300 ng/ml) in the presence of GolgiStop (BD Pharmingen) for 5 h for intracellular cytokine staining.

Cytokine assays

IL-6 and TNF-α production was assayed with ELISA kits (eBioscience). IL-12, IL-4, IL-5, IL-3, and IFN-γ production was determined by ELISA using the following pairs of Abs: 2 μg/ml anti-IL-12 capture Ab with 1 μg/ml biotin anti-IL-12 Ab (Biolegend); 4 μg/ml anti-IL-4 capture Ab (11B11) with 0.5 μg/ml biotin anti-IL-4 Ab (Biolegend); 2 μg/ml anti-IL-5 capture Ab with 1 μg/ml biotin anti-IL-5 Ab (Biolegend); 1 μg/ml anti-IL-13 capture Ab with 0.2 μg/ml biotin anti-IL-13 Ab (R&D Systems); and 0.25 μg/ml anti-IFN-γ capture Ab with 1 μg/ml biotin anti-IFN-γ Ab (Biolegend). All biotinylated Abs were detected with 1/1000 dilution of streptavidin-HRP (eBioscience). The reactions were developed with TMB Peroxidase EIA Substrate kit (Bio-Rad).

For intracellular cytokine staining, the cells were first stained for surface CD3 or CD4 and then fixed with 2% paraformaldehyde in PBS for 20 min at 4°C. The cells were permeabilized with 0.5% saponin (Sigma-Aldrich) and stained with anti-IFN-γ FITC (BD Pharmingen) and/or anti-IL-4 biotin (Biolegend) for 30 min at 4°C, followed by streptavidin-PE (Molecular Probes, Invitrogen Life Technologies) for 20 min at 4°C. The cells were analyzed by flow cytometry.

For intracellular IFN-γ staining in Ag-specific CD4^+ and CD8^+ T cells, 4 × 10^5 splenocytes were cultured in 24-well plates in the presence of GolgiStop (BD Pharmingen) with either medium alone or 10^7 M OVA257–264 (American Peptides) for CD8^+ cells or 5 μlisteriolysin O (OVA257–264) (Invitrogen Life Technologies) for CD4^+ cells in complete RPMI 1640 medium.

Pathogen infection, immunizations, and Ab titration

The recombinant L. monocytogenes strain secreting chicken OVA (rLmOVA) was used to infect RARγ-deficient and control mice, as described (20, 21). To determine in vivo bacterial clearance after infection, mice were infected with 2.8 × 10^7 CFU of Salmonella typhimurium in Luria-Bertani medium, 2 × 10^6 CFU of group B streptococcus (GBS) grown in Todd-Hewitt medium (BD Biosciences), or 3000 CFU of rLmOVA resistant to erythromycin grown in brain-heart infusion medium (Difco) containing 5 μg/ml erythromycin (Sigma-Aldrich). The correct titer of bacteria was determined by spreading an aliquot of the inoculum. After 6 or 48 h, the spleens were harvested and homogenized in 0.1% Triton X-100 (Shelton Scientific), and different dilutions were plated on Luria-Bertani agar plates for S. typhimurium, blood agar (Difco) for GBS, and brain-heart infusion agar with 5 μg/ml erythromycin for L. monocytogenes.

Mice were immunized i.p. with 100 μg (0.2 ml per mouse) of DNP-keyhole limpet hemocyanin (KLH; Calbiochem) mixed 1:1 with alum (Fierce). Serum were collected, and isotype-specific anti-DNP Abs were determined by ELISA, as described (20, 21). The titer was expressed as relative units as compared with a master sample prepared by pooling aliquots from all samples.

31^Cr release assay

To determine the ability of splenocytes to lyse OVA257–264-loaded target cells, 2-fold serial dilutions of splenocytes were prepared in triplicates in 96-well round-bottom plates. EL-4 target cells were labeled with 250 μCi of 31^Cr with or without 10^7 M OVA257–264 peptide for 1 h at 37°C. The target cells were then washed three times and added to effector cells at 10,000 cells/well. To determine the spontaneous and maximum lysis, target cells were incubated without effector cells or lysed with 1% Triton X-100 (Shelton Scientific). After 6 h of incubation at 37°C, the plates were centrifuged at 1500 rpm for 3 min, and 50 μl of the supernatant was mixed with 100 μl of OptiPhase SuperMix (PerkinElmer) on 96-well Isolates (PerkinElmer). The samples were counted on Microbeta TriLux (PerkinElmer) to determine the amount of 31^Cr released in the supernatant. The percentage of specific lysis was calculated as 100 × (experimental cpm – spontaneous cpm)/(maximum cpm – spontaneous cpm).

Intraepithelial lymphocyte (IEL) isolation

IELs were isolated from the small intestine after Peyer’s patches removal. The intestines were flushed with medium and cut into small pieces that were stirred twice for 20 min at 37°C in 10% PBS in PBS with 20 mM HEPES and 0.1 mM EDTA. After vortexing, the pieces were allowed to settle and the supernatants were filtered and spun down. The cells were resuspended in 44% Percoll (GE Healthcare) and overlaid on 67% Percoll for 20 min spin at room temperature at 1700 rpm. The cells at the interface were collected and washed, followed by 10-min incubation with FeR block (2.4G2 supernatant) and staining with TCRβ FITC, TCRγδ PE, pro- pidium iodide, CD4 allophyocyanin, CD8β biotin, CD8α PE/Cy7, and CD45.2 allophyocyanin/cy7. The biotinylated Abs were detected by streptavidin-Texas Red (Jackson ImmunoResearch Laboratories).

Macrophage stimulation

Peritoneal macrophages were elicited by injection of 1 ml of 3% thioglycolate broth (Difco) i.p. 3 days before the experiment. The cells were recovered by peritoneal lavage with 2% FBS in PBS and let to adhere overnight in 10-cm plates (BD Biosciences). On the next day, the cells
were scrapd and live cells were purified by gradient centrifugation on Lympholyte M (Cedarlane Laboratories). The cells were resuspended at 3 × 10⁶/ml, and 0.5 ml was added to 48-well plates in triplicates. The cells were stimulated with 100 ng/ml LPS (Sigma-Aldrich), 10 μg/ml peptidoglycan (PGN) (InvivoGen), or 10 μg/ml poly(I:C) (Sigma-Aldrich) overnight. The supernatants were collected and frozen at −80°C until assayed.

Western blot
Peritoneal macrophages were seeded at 10⁶/ml in 1 ml in 24-well plates. After 3 h, the medium was replaced with prewarmed medium containing 10 μg/ml PGN (InvivoGen). At different time points, the cells were lysed with 100 μl of 1× SDS sample buffer, boiled for 5 min, and stored at −20°C until assayed. The proteins were separated on 10% SDS-PAGE and transferred on polyvinylidene difluoride membranes (PerkinElmer). For immunoblot, anti-ERK-2, anti-pERK (Santa Cruz Biotechnology), anti-IκBα, anti-pIκBα, anti-pJNK, and anti-pp38 (Cell Signaling Technology) were used. The secondary Abs were anti-mouse and anti-rabbit-HRP conjugates (Jackson ImmunoResearch Laboratories). The detection was achieved with Western Pico substrate (Pierce).

Statistical analysis
The statistical analysis was performed using unpaired two-tailed Student’s \( t \) test using the GraphPad Prizm software (GraphPad). Values of \( p < 0.05 \) were considered statistically significant.

Results
Regulated expression of RARγ in developing T lymphocytes
In a differential analysis of gene expression in developing T lymphocytes using DNA microarrays, we found that RARγ expression was differentially regulated in CD4⁺ and CD8⁺ single-positive (SP) thymocytes. CD8⁺ SP thymocytes expressed 2- to 3-fold higher levels of RARγ mRNA than CD4⁺ SP thymocytes (data not shown). We confirmed this result by quantitative real-time RT-PCR (Fig. 1A). RARγ expression was at a low level in double-positive (DP) thymocytes, but was up-regulated in SP thymocytes (Fig. 1A). The expression level of RARγ mRNA in CD8⁺ SP thymocytes and CD8⁺ mature T cells from spleen and lymph nodes was 50–150% higher than that in their CD4⁺ counterparts (Fig. 1A). These data demonstrate that RARγ expression is regulated in developing T lymphocytes and suggest that RARγ may play a role in lymphocyte development and function.

Conditional deletion of RARγ in mouse hemopoietic cells
Mice lacking RARγ exhibit growth deficiency and early lethality (22). To circumvent growth abnormalities that may indirectly affect lymphocyte development and function, we generated a mouse strain that conditionally lacked RARγ in all hemopoietic cells by crossing mice with floxed exon 8 of RARγ (RARγ⁹/⁹) to mice expressing Cre recombinase under the control of the Vav promoter. The Vav promoter drives Cre expression in all hemopoietic cells (19). Southern blot analysis demonstrated that Cre-induced deletion of the floxed RARγ alleles in the spleen and BM of RARγ⁹/⁹-VavCre mice was essentially complete (Fig. 1B). To further determine the deletion efficiency, we performed semiquantitative RT-PCR for RARγ mRNA expression in the thymus, spleen, and BM. RARγ mRNA expression in thymus of RARγ⁹/⁹-VavCre mice was reduced by >98%, whereas its expression in spleen and BM was reduced by >99% (Fig. 1C). The residual expression of RARγ mRNA in RARγ⁹/⁹-VavCre thymus, spleen, or BM may be due to the presence of nonhemopoietic cells and/or cells escaping deletion. Nevertheless, these results demonstrated that RARγ was efficiently deleted in most hemopoietic cells.

T and B lymphocyte development in RARγ⁹/⁹-VavCre mice
Given that vitamin A deficiency in mice causes lymphoid organ atrophy (6), and a recent report that RARγ has important functions in hemopoietic stem cell (23), we examined the development of T and B lymphocytes in RARγ⁹/⁹-VavCre mice. The total cellularity of thymus and spleen in RARγ-deficient mice was similar to that of control littermates (Fig. 2 and data not shown). Thymocyte development as defined by the expression of CD4 and CD8 markers proceeded normally from double-negative (DN) through DP to CD4 SP and CD8 SP cells in RARγ-deficient mice (Fig. 2A). To further characterize the early stages of T cell development in the thymus, we stained DN thymocytes for CD44 and CD25 expression. We did not find obvious defects in DN1 (CD44⁺CD25⁻),
assessed cell proliferation by [3H]thymidine incorporation. As CD3 to activate T cells or with anti-IgM to activate B cells and lymphocyte proliferation, we stimulated total splenocytes with anti-C

(Fig. 2). Taken together, these results demonstrate that RAR

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Lymphocyte proliferation and differentiation in the absence not essential for T and B lymphocyte development.

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gan of the tested mice. The results are representative of at least three inde-

pendent experiments.

FIGURE 2. Lymphocyte development in RARγ-deficient mice. A, FACS profile of total thymocytes (upper panels) and DN thymocytes (lower panels) from RARγL/LVavCre and littermate control mice. Numbers indicate the percentage of cells in each subset. B, FACS profile of early stages (upper panels) and later stages (lower panels) of B cell development in the BM of RARγL/LVavCre and littermate control mice. C, FACS profiles of total splenocytes (upper panels) and splenic T cells (lower panels) from RARγL/LVavCre and littermate control mice. Splenic T cells were gated on CD3+ cells. The numbers in parentheses indicate the absolute number of cells in the respective organ of the tested mice. The results are representative of at least three independent experiments.

DN2 (CD44+CD25+), DN3 (CD44-CD25+), and DN4 (CD44-CD25-) cells (Fig. 2A).

We next analyzed B cell development in the BM of RARγ-deficient mice. The earliest stages of B cell development that are characterized by CD43 and B220 expression can be subdivided into fractions A, B, C, and C’ based on heat-stable Ag and BP-1 expression (24). The more mature stages do not express CD43, but still express B220. They can be divided into fractions D, E, and F by IgD and IgM expression. B cell development at these different stages in the BM of RARγ-deficient mice was comparable to that in littermate controls (Fig. 2B). Furthermore, peripheral T and B cell compartments in RARγ-mutant mice were apparently normal (Fig. 2C). Taken together, these results demonstrate that RARγ is not essential for T and B lymphocyte development.

Lymphocyte proliferation and differentiation in the absence of RARγ

Although high levels of retinoic acid modulate lymphocyte proliferation and differentiation, it is not known which receptors are used in these cells. Some previous studies, using selective agonists or antagonists, have suggested that retinoic acid exerts its effects on Th1/Th2 differentiation and expression of homing receptors through RARα or RARβ (25, 26). However, it is not clear what role RARγ may play in these processes, especially when retinoic acid is at physiologic levels. To determine the role of RARγ in lymphocyte proliferation, we stimulated total splenocytes with anti-CD3 to activate T cells or with anti-IgM to activate B cells and assessed cell proliferation by [3H]thymidine incorporation. As shown in Fig. 3A, both T and B cells from RARγ-deficient mice divided at the same rate as control cells. Similar results were observed after 2 days of stimulation and after stimulation of T cells with PMA plus ionomycin or of B cells with LPS (data not shown). In agreement with previous studies (7, 8), the addition of ATRA to the culture inhibited the proliferation of both T and B cells in response to anti-CD3 and anti-IgM (Fig. 3A). Importantly, the effects of ATRA on lymphocyte proliferation were still observed in the absence of RARγ. These results demonstrate that RARγ is not required for the proliferation of T and B lymphocytes. Furthermore, RARγ is not essential for the anti-proliferative action of high concentration ATRA on lymphocytes.

Given that ATRA can modulate Th cell differentiation (25, 27), we determined Th cell differentiation in the absence of RARγ. CD4+ T cells produced only IFN-γ, but not IL-4, IL-5, or IL-13 when cultured under Th1 conditions, and produced IL-4, IL-5, and IL-13, but not IFN-γ when cultured under Th2 conditions (Fig. 3B). The polarization of RARγ-deficient CD4+ T cells to Th1 or Th2 lineage was not obviously impaired (Fig. 3, B and C). These results demonstrate that RARγ deficiency did not result in impairment of in vitro CD4+ T cell differentiation.

Humoral immune response in RARγ-deficient mice

We further examined the in vivo function of B cells and CD4+ Th cells in RARγ-deficient mice by testing the humoral immune response in these mice. RARγL/LVavCre and control mice were immunized with the T-dependent Ag DNP-KLH and boosted 28 days
after the primary immunization. Anti-DNP-specific Abs were measured by ELISA. The Ag-specific Abs were similarly detected in RAR<sup>L/LVavCre</sup> and control mice after primary and secondary immunization (Fig. 4), indicating that RAR<sup>L/LVavCre</sup> is not essential for Ig secretion and class-switching by B cells.

**CD8<sup>+</sup> T cell responses in RARγ-deficient mice**

Because RARγ is expressed at higher levels in CD8<sup>+</sup> T cells than CD4<sup>+</sup> T cells, we tested the ability of RARγ-deficient CD8<sup>+</sup> T cells to mount an immune response using a model pathogen, rLmOVA (28). RARγ<sup>L/LVavCre</sup> and control mice were infected with 10,000 CFU of rLmOVA, and 7 days later the CD8<sup>+</sup> T cells from RARγ<sup>L/LVavCre</sup> and littermate control mice. Shown are percentages of CD4<sup>+</sup> cells expressing either IFN-γ or IL-4. The results in Fig. 3, B and C, are representative from four independent experiments.

**FIGURE 3.** Lymphocyte proliferation and Th1/Th2 differentiation in the absence of RARγ. A, T and B cell proliferation after anti-CD3 (5 μg/ml) or anti-IgM stimulation (40 μg/ml) in the presence or absence of 10 nM ATRA. Total splenocytes were stimulated for 3 days and then pulsed with [3H]thymidine for 4 h. The cells were harvested, and the amount of the incorporated [3H]thymidine was determined as a measure for cell proliferation. The graph shows the mean and SD of triplicates of an individual mouse in a group of three. B, In vitro Th1/Th2 differentiation. Purified CD4<sup>+</sup> T cells from RARγ<sup>L/LVavCre</sup> and littermate control mice were polarized for 5 days, washed, and restimulated. The amounts of IL-4, IL-5, IL-13, and IFN-γ in the supernatants were determined by ELISA. C, Intracellular cytokine staining for IFN-γ and IL-4 in the above treated CD4<sup>+</sup> T cells from RARγ<sup>L/LVavCre</sup> and littermate control mice. Shown are percentages of CD4<sup>+</sup> cells expressing either IFN-γ or IL-4. The results in Fig. 3, B and C, are representative from four independent experiments.

**FIGURE 4.** Humoral immune response in RARγ-deficient mice. RARγ<sup>L/LVavCre</sup> and littermate control mice were immunized with DNP-KLH in alum by i.p. injection. Serum anti-DNP-specific Abs were determined by ELISA. The relative units were determined by comparing the titer from each mouse with a master sample prepared by pooling aliquots from all the samples. Shown are mean ± SD from five mice per group.
IEL development in RARγ-deficient mice.

A recent report showed that ATRA imprints gut-homing specificity on memory T cells (26). ATRA produced from gut dendritic cells (DCs) stimulates T cells to express the gut-homing $\alpha_4\beta_7$ integrin. These T cells activated in the gut go into circulation and, upon re-encounter of their cognate Ag, home preferentially back to the gut. We investigated whether ATRA-induced $\alpha_4\beta_7$ integrin expression depends on RARγ. Anti-CD3 stimulation of CD4+ and CD8+ T cells up-regulated $\alpha_4\beta_7$ integrin expression, and this effect was further enhanced by ATRA (Fig. 6A and data not shown). Up-regulation of $\alpha_4\beta_7$ integrin expression in RARγ-deficient CD4+ or CD8+ T cells by anti-CD3 or anti-CD3 plus ATRA was similar to that on control T cells (Fig. 6A and data not shown), indicating that RARγ is not essential for the expression of gut-homing receptors on T cells.

We further examined the gut lymphocyte compartment in RARγ-deficient mice. The IELs consist of both conventional CD4+ TCRαβ+ and CD8αβ+ TCRαβ+ T cells and several non-conventional T cell subsets. The most abundant of the latter are the γδ T cells, which comprise approximately half of IELs. In addition, there are also CD4+CD8−TCRαβ+ T cells, as well as CD8αα−TCRαβ− T cells. As shown in Fig. 6B, the percentages of TCRαβ+ and TCRγδ+ IELs were similar between control and mutant mice. Furthermore, IEL subsets corresponding to CD4+CD8+, CD4−CD8α+, and CD4−CD8− T cells were comparable in RARγ-deficient and littermate control mice (Fig. 6B). The absence of any effect of the RARγ deficiency on the composition of the IEL compartment further indicates that RARγ is dispensable for the ATRA effect on T cell gut migration.
We examined the role of RAR for Western blot analysis. Total ERK-1/2 serves as a loading control. The result is representative of two independent experiments.

To determine the basis for the decreased inflammatory cytokine production in RARγ-deficient macrophages, we infected RARγ/L-/VavCre mice with different bacterial pathogens, measured the amount of proinflammatory cytokines in the serum 2 h after the infection, and determined the bacterial burden in the spleen 48 h. We used G⁺ bacteria S. typhimurium, G⁺ extracellular bacteria GBS, and G⁻ intracellular bacteria L. monocytogenes. Because the preliminary experiments showed that GBS are rapidly cleared after infection, we did not observe statistically significant differences between control and RARγ/L-/VavCre mice in the number of viable bacteria recovered from their spleens (Fig. 8A) and the amount of IL-12 or IL-6 in the serum (Fig. 8B and data not shown). We also examined the expression of TLRs and found no difference in the expression of TLR2 and 4 in macrophages from RARγ-deficient and control mice (Fig. 7B). We then examined the signaling pathways in pathogen-associated molecular pattern-stimulated macrophages. As shown in Fig. 7C, the phosphorylation of IκBα, JNK, p38, and ERK in RARγ-deficient macrophages was largely comparable to that in control cells. These results demonstrate that the membrane-proximal signaling events may exert its effect on inflammatory cytokine production in the nucleus.

Inflammatory cytokine production in RARγ-deficient macrophages

ATRA and its derivatives are widely used in clinical treatment of acne (12, 13), partly due to its inhibition of inflammatory cytokine production. Moreover, RARγ is expressed in macrophages (29, 30), suggesting that ATRA may mediate its effect through RARγ. We examined the role of RARγ in inflammatory cytokine production in macrophages. Elicited peritoneal macrophages were stimulated with LPS, PGN, or poly(I:C) in the presence or absence of ATRA. The supernatants were assessed for the production of IL-6, IL-12, and TNF-α by ELISA. As expected, the addition of ATRA decreased the production of most cytokines by 30–50%, with the exception of IL-6 induced by PGN (Fig. 7A). Surprisingly, RARγ-deficient macrophages exhibited defective production of IL-6, IL-12, and TNF-α upon LPS, PGN, and poly(I:C) stimulation (Fig. 7A). Moreover, the addition of ATRA further decreased the production of these inflammatory cytokines (Fig. 7A). These findings suggest that RARγ is required for TLR ligand-induced inflammatory cytokine production, and that the anti-inflammatory action by ATRA does not depend on RARγ. To determine the basis for the decreased inflammatory cytokine production in RARγ-deficient macrophages, we first examined whether RARγ-deficient macrophages differ phenotypically from their wild-type counterparts. No difference was found in their cell size, granularity, and expression of the macrophage marker Mac-1 between RARγ-deficient and control macrophages (Fig. 7B and data not shown). We also examined the expression of TLRs and did not find any difference in the expression of TLR2 and 4 in macrophages from RARγ-deficient and control mice (Fig. 7B). We then examined the signaling pathways in pathogen-associated molecular pattern-stimulated macrophages. As shown in Fig. 7C, the phosphorylation of IκBα, JNK, p38, and ERK in RARγ-deficient macrophages was largely comparable to that in control cells. These results demonstrate that the membrane-proximal signaling events were not obviously changed in RARγ-deficient macrophages and suggest that RARγ may exert its effect on inflammatory cytokine production in the nucleus.

Innate immune response in RARγ-deficient mice

To examine whether RARγ-deficient mice have defective innate immune responses, we infected RARγ/L-/VavCre mice with different bacterial pathogens, measured the amount of proinflammatory cytokines in the serum 2 h after the infection, and determined the bacterial burden in the spleen after 48 h. We used G⁺ bacteria S. typhimurium, G⁺ extracellular bacteria GBS, and G⁻ intracellular bacteria L. monocytogenes. Because the preliminary experiments showed that GBS are rapidly cleared after infection, we determined the bacterial burden of this microbe after 6 h. We did not observe statistically significant differences (p > 0.05) between control and RARγ/L-/VavCre mice in the number of viable bacteria recovered from their spleens (Fig. 8A) and the amount of IL-12 or IL-6 in the serum (Fig. 8B and data not shown). TNF-α was under
the detection limits in all cases. These data suggest that whereas RARγ plays important roles in regulating the production of proinflammatory cytokines by macrophages, its absence in vivo can be compensated by other cellular components.

Discussion

Although it is well established that vitamin A and its derivatives modulate immune responses and regulate lymphocyte function, its receptor use in various immune cells is not clear. Furthermore, because higher than physiological levels of retinoid acids were used in many of the in vitro studies, the outcome of interaction between retinoid acids and their receptors in vivo remains unknown. To address these issues, we induced RARγ deletion in vivo and examined the development and function of lymphocytes as well as the inflammatory cytokine production in macrophages from RARγ-deficient mice. Our results support two major conclusions, as follows: first, RARγ is dispensable for the normal development of lymphocytes, but is required for CD8+ T effector differentiation and IFN-γ production; second, RARγ functions as a positive regulator of proinflammatory cytokine production in macrophages.

The expression of RARγ mRNA is tightly regulated during T cell development. However, its ablation did not have any noticeable effects on thymocyte development. This is somewhat unexpected, because the expression of RARγ under the control of the Lck promoter has been shown to increase the percentage of CD8 SP thymocytes (31). Moreover, there is evidence that retinoids can influence the process of negative selection (9–11). The most likely explanation for the lack of thymocyte abnormalities is that another closely related molecule substitutes for RARγ in its absence. The best candidate is RARα, which is closely related to RARγ and is ubiquitously expressed (32). RARβ has not been detected in lymphocytes (33). In addition, our results also show that RARγ is not essential for lymphocyte proliferation and Th cell differentiation in vitro as well as CD4+ effector differentiation and Ab production in vivo. ATRA-induced integrin expression in T cells does not depend on RARγ. These results suggest that RARα and RXRs are sufficient to compensate for the loss of RARγ in vivo. Alternatively, RARγ may not be used temporally and spatially in these processes. Future studies using double deletion of RARα and RARγ in mice will address whether there is a redundancy of these receptors in lymphocyte development and function.

Our result demonstrated that the CD8+, but not CD4+ T cell response was defective in RARγ−/− VavCre mice. This result is consistent with the higher expression level of RARγ in CD8+ than CD4+ T cells. The lower number of CD8+ effector and memory T cells in RARγ−/− VavCre mice after Listeria infection may be due to a role of RARγ in activating IFN-γ production. A bioinformatics search did not reveal RARγ binding sites in the IFN-γ promoter, suggesting that RARγ regulates the expression of IFN-γ indirectly. Alternatively, the impaired CD8+ T effector and memory cell differentiation may be due to the lowered inflammatory cytokine production by innate immune cells. This is unlikely because infections with S. typhimurium, GBS, and L. monocytogenes, as well as in vivo administration of PGN did not result in a lowered IL-6 and IL-12 production in RARγ−/− VavCre mice (Fig. 8 and our unpublished observations), suggesting that the production of the inflammatory cytokines in cell types other than macrophages does not depend on RARγ. In addition, the normal CD4+ response and Ab production in RARγ−/− VavCre mice further suggest that the decreased production of IFN-γ by CD8+ T cells is not due to abnormalities in their innate immune system, but a separate defect. Thus, we favor a role of RARγ in activating IFN-γ production in CD8+ T lymphocytes.

An unexpected finding from our study is the impaired inflammatory cytokine production by RARγ-deficient macrophages. This result suggests that vitamin A, at physiological levels in serum and culture medium, engages RARγ to activate IL-6, IL-12, and TNF-α production. The mechanisms by which RARγ regulates these inflammatory cytokines are not clear. Our data have ruled out an effect of RARγ on the proximal signaling upon TLR stimulation. Like many other nuclear receptors, such as GR, PPARs, and liver X receptor (LXR) (34–37), RARγ may regulate the transcription of these cytokines by either direct binding to the regulatory elements of these genes or indirect activation/inactivation of other nuclear proteins. Examination of the promoter regions of IL-6, IL-12, and TNF-α did not reveal any obvious RARγ binding sites, suggesting an indirect role of RARγ.

Our results also show that ATRA-mediated inhibition of inflammatory cytokine production by RARγ-deficient macrophages is not impaired, suggesting that RARγ is not required for the inhibitory function of ATRA. High levels of ATRA may activate other retinoid acid receptor such as RXR and mediate its inhibitory function. The abilities of RXRs to suppress inflammation are well documented, and several mechanisms have been proposed. RXRs, for example, can interact directly with NF-κB and inhibit its activity (38). In addition, RARs has been demonstrated to be a negative regulator of AP-1-responsive genes (39). Both AP-1 and NF-κB
are critical for the expression of IL-6, IL-12, TNF-α, and IFN-γ. We speculate that RARγ binding to RXRs can titrate out the inhibitory complexes in which RXRs participate (RARα/RXR, LXR/RXR, PPAR/RXR) and alleviate the suppression of cytokine transcription.

Despite the impaired cytokine production by RARγ-deficient macrophages, RARγ−/−VavCre mice had normal responses to bacterial infections. They cleared the pathogens at the same rates as control mice, and surprisingly, produced similar amounts of proinflammatory cytokines. One possible explanation for the in vivo results is that cell types other than macrophages do not depend on RARγ for their cytokine production. For example, IL-6 can be produced by a wide variety of cells (40), and it is possible that the defect in macrophages can be masked by the normal production of other cell types. Similarly, macrophages are not the only source of IL-12 (41). Neutrophils, astrocytes, and DCs can also produce IL-12. Moreover, optimal production of IL-12 by macrophages requires costimulation with IFN-γ or IL-4, whereas DCs do not need additional stimuli. Thus, it is possible that normal production of IL-12 by other cells in vivo effectively compensates for the defect in macrophages.

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Disclosures
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References