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Dissecting the Role of Retinoic Acid Receptor Isoforms in the CD8 Response to Infection

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Vitamin A deficiency leads to increased susceptibility to a spectrum of infectious diseases. The studies presented dissect the intrinsic role of each of the retinoic acid receptor (RAR) isoforms in the clonal expansion, differentiation, and survival of pathogen-specific CD8 T cells in vivo. The data show that RARα is required for the expression of gut-homing receptors on CD8+ T cells and survival of CD8+ T cells in vitro. Furthermore, RARα is essential for survival of CD8+ T cells in vivo following Listeria monocytogenes infection. In contrast, RARβ deletion leads to modest deficiency in Ag-specific CD8+ T cell expansion during infection. The defective survival of RARα-deficient CD8+ T cells leads to a deficiency in control of L. monocytogenes expansion in the spleen. To our knowledge, these are the first comparative studies of the role of RAR isoforms in CD8+ T cell immunity. The Journal of Immunology, 2014, 192: 3336–3344.

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The online version of this article contains supplemental material.
DEAD Fixable Near-IR Dead Cell Stain Kit (Invitrogen) was used to staining. Five-color FACS data were collected on a BD FACSCalibur flow

mAbs

The following FITC-, PE-, PerCPI-, allophycocyanin-Cy7–, or allophycocyanin-conjugated Abs were used: anti-CD8 (53-6.7), anti-CD25 (PC61), anti–TNF-α (MP6-XT22), anti–granzyme B (B111), anti-CD44 (IM7), anti–MHCII (M5), anti–IFN-γ (XM1.G1.2), anti-CD69 (H1.2F3), anti–CCR9 (242503), and anti–a4β7 (DATK32). All Abs were purchased from BioLegend, with the exception of anti-α4β7 (BD Biosciences). A LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Invitrogen) was used to exclude dead cells in FACS analysis.

T cell activation in vitro

CD8+ T cells were purified from RARα-/- mice, RARβ-/- mice, RARγ-/- mice, and control mice by Microbeads (Miltenyi Biotec) selection. For α4β7 and CCR9 measurement, CD8 T cells were activated with plate-bound anti-CD3 (2C11; 10 μg/ml) and anti-CD28 (PV1; 1 μg/ml) for 48 h in RPMI 1640 and then restimulated in fresh RPMI 1640 with 100 U/ml human IL-2 for an additional 48 h. All-trans retinoic acid (ATRA) was supplemented at the indicated concentrations. Cultured cells were harvested for analysis by 96 h in culture. For the cell-viability assay, CD8+ T cells were cultured under the same condition without exogenous ATRA. Colonies were counted 2 d later.

Cytokine secretion in vitro

CD8+ T cells were cultured in RPMI 1640 with or without 10 μg/ml anti–CD3 and with or without 1 μg/ml anti-CD28 for 24 h. Brefeldin A was applied during the final 10 h of culture. IL-2 and IFN-γ production by CD8+ T cells was assessed by intracellular staining, according to the protocol described previously (23).

Infection

Mice were infected i.v. with 5 × 105 CFU L. monocytogenes engineered to overexpress OVA (L. monocytogenes-OVA). In all infection experiments, CD4+ T cells were depleted by i.p. injection of 200 μg anti-CD4 (GK1.5; Bio X Cell) on day 0 prior to infection. Bacterial load in spleen was measured as previously described (24). In brief, a single-cell suspension was prepared on day 5 postinfection, incubated with 1% paraformaldehyde for 30 min, and plated (10 μl 1:10- and 1:100-diluted cells) onto BHI media with erythromycin. Colonies were counted 2 d later.

Analysis of MHC class I tetramer, granzyme B, IFN-γ, and TNF-α by flow cytometry

MHC class I tetramer, IFN-γ, and TNF-α staining was performed as previously described (23). Splenocytes were not restimulated ex vivo for granzyme B staining but were restimulated with 10 μg/ml SIINFEKL in the presence of 10 U/ml human IL-2 for 5 h prior to IFN-γ and TNF-α staining. Five-color FACS data were collected on a BD FACSCalibur flow cytometer and analyzed using FlowJo software.

Statistical analysis

Graphs were made using GraphPad Prism software, and data are expressed as mean ± SEM or mean ± SD. Differences for data with one grouping variable were analyzed by the Student t test (two groups).

Results

Phenotypic analysis of T cells in mice with conditional deletion of RARα, RARβ, or RARγ

The expression of RARα, RARβ, and RARγ mRNA in naive and anti-CD3–activated CD8 T cells was evaluated. As shown in Supplemental Fig. 1A, all three RARs are expressed in naive CD8 T cells, as well as CD8 T cells activated for 48 or 72 h. To define the different functions of RARα, RARβ, and RARγ in determining various aspects of CD8 T cell function, RARα-, RARβ-, or RARγ-transgenic (20), RARα/β-, RARα/γ-, RARβ/γ- (21), and RARγ-transgenic (22) mice were crossed with CD4+ mice to generate mice deleted of RARα, RARβ, or RARγ in T cells. These mice are known as RARα-/-, RARβ-/-, and RARγ-/-, respectively. In all experiments, littermate controls were used bearing alleles of RARα-/-, RARβ-/-, or RARγ-/-, but not CD4-/-, hereafter referred to as “control.” Specific RAR deletion in CD8 T cells was confirmed by both the detection of the Cre-dependent null allele in the genome and the absence of corresponding receptor mRNA in RARα-/-, RARβ-/-, and RARγ-/- mice. In this context, naive CD8+ T cells were purified from spleen of these mice, and PCR was performed with previously published primers to detect the null allele of RARα in RARα-/- mice (20), RARβ in RARβ-/- mice (21), and RARγ in RARγ-/- mice (22). As shown in Supplemental Fig. 1B, the null allele of RARα was detectable in CD8 T cells from RARα-/- mice but not in their counterparts from RARβ-/-, RARγ-/-, or control mice. The same is true for the specific deletion of RARβ and RARγ in CD8 T cells from RARβ-/- and RARγ-/- mice, respectively. Specific deletion by evaluation of the three isotypes expressed in naive CD8 T cells was quantified by real-time PCR. As shown in Supplemental Fig. 1C, RARα mRNA was undetectable in CD8 T cells of RARα-/- mice, but it was found at comparable levels in CD8 T cells of RARβ-/-, RARγ-/-, and control mice. Similarly, RARβ and RARγ are specifically deleted only in CD8 T cells of RARβ-/- and RARγ-/- mice, respectively. The presented data confirmed the deletion of each specific RAR in each strain and provided the opportunity to define distinct functions for RARα, RARβ, and RARγ in various aspects of CD8 T cell development and immunity.

Further studies were conducted to determine whether the deletion of specific receptors impairs T cell development under steady-state. No drastic differences among RARα-/-, RARβ-/-, RARγ-/-, and control mice were observed with regard to the proportion and total number of CD8+ T cells in the thymus, spleen, and peripheral lymph node (Supplemental Fig. 2A–C). However, a difference in the proportion of memory-like CD8+ CD44high T cells was observed across the mutant strains: RARα-/- (26.52 ± 2.23%), RARβ-/- (10.13 ± 2.70%), RARγ-/- (16.39 ± 1.8%), and control (12.93 ± 1.61%) mice (Supplemental Fig. 2D, 2E). Similarly, no dramatic differences were observed in CD4+ T cells, with the exception of a slightly lower proportion of CD4+ T cells and fewer total Tregs in the peripheral lymph node of RARα-/- mice compared with RARβ-/-, RARγ-/-, and control mice (Supplemental Fig. 2). Overall, the analysis indicated no overt T cell development deficiency due to the deletion of RARα, RARβ, or RARγ in T cells.

Assessment of RARα, RARβ, and RARγ in T cell activation, proliferation, and survival in vitro

To elucidate the role of each RAR in T cell activation, a series of in vitro studies was executed with each of the conditionally deleted RARs mutants. The use of T cells in which there is conditional deletion only in the T cell compartment avoids any significant issues with isolating T cells from a severely compromised host in which RAR is universally deleted. RARα-knockout mice present with significant morbidities and mortality (25). First, the expression of early activation markers CD25 and CD69 was measured at different time points postactivation. As shown in Fig. 1A, at 18 h postactivation, CD8+ T cells from all RAR-deficient groups showed upregulation of CD25 and CD69 in response to anti-CD3 + anti-CD28 stimulation, which was indistinguishable from controls. We observed no significant differences in mean fluorescence intensity of CD25 and CD69 on CD8 T cells at 18 h (Fig. 1B) or other time points we measured (data not shown). This indicated that early T cell activation in the absence of RARα, RARβ, or RARγ was intact. Such intact early T cell activation excluded the possibility of
any deficiency caused by purification of T cells from different hosts per se. Second, CD8+ T cells from RARαfl/fl, RARβfl/fl, RARγfl/fl, and control mice were activated in vitro with anti-CD3, with or without anti-CD28, and the proliferation was evaluated by CFSE dilution. No differences were observed in the proliferation profile of live CD8+ T cells post–72 h activation in vitro (Fig. 1C, 1D). No difference in CD25 or CD69 upregulation or proliferation profile was observed among CD8+ T cells from RARαfl/fl, RARβfl/fl, RARγfl/fl, and control mice when soluble anti-CD3 was used with CD11c+ dendritic cells as APCs (data not shown). In addition, we observed no deficiency in CD25 and CD69 upregulation or proliferation of CD8+ T cells when exogenous RA (100 nM) was supplemented during activation (data not shown). However, we observed reduced cell survival in the RARαfl/fl group.

To gain further insight into a possible role for RARα in CD8 survival, viable cell numbers were monitored following anti-CD3 + anti-CD28 stimulation of CD8+ T cells from RARαfl/fl, RARβfl/fl, RARγfl/fl, and control mice in vitro. As shown in Fig. 2A and 2B, after 24 h of stimulation there was a 50% reduction in the percentage of viable CD8+ cells, which decreased even further at 48 h in RARα-deficient CD8+ T cells (Fig. 2B, 2C). To determine whether cytokines could rescue the apparent anti-CD3-associated defect in RARα-deficient T cells, T cells were put into 2-d culture in fresh media with high exogenous IL-2 supplementation (100 U/ml) without anti-CD3 or anti-CD28. IL-2 rescued the viability of the RARα-deficient CD8+ T cells to the same level as their counterparts from other groups, even though the total cell number did not reach the same level (Fig. 2). Our study also showed that 10 U/ml exogenous IL-2 in culture from day 0 can sustain a high viability of RARαfl/fl CD8+ T cells (data not shown).

**Impaired differentiation of RARα- and RARβ-deficient CD8+ T cells in vitro**

The prior data clearly indicated that T cell activation was not impaired in any of the RAR mutant T cells by virtue of cell activation markers or proliferation. However, RARα appeared to impact on the survival of T cells in vitro. To determine whether any of the RAR-deficient CD8+ T cells were impaired in their differentiation to effector cells, the production of effector cyto-
kines was measured in vitro. IL-2 and IFN-γ production was measured by intracellular cytokine staining of activated CD8+ T cells from control, RARαfl/fl, RARβfl/fl, and RARγfl/fl mice. There was a significantly lower proportion of IL-2 producers in RARαfl/fl and RARβfl/fl CD8+ T cells than in control and RARγfl/fl CD8+ T cells when activated by anti-CD3 + anti-CD28. The same reduction due to RARα or RARβ deficiency also was observed when activated by anti-CD3 alone, even if not statistically significant (Fig. 3A, 3C). A reduction in IFN-γ production was observed in RARαfl/fl CD8+ T cells, but not in other groups of CD8+ T cells, when activated by anti-CD3 alone (Fig. 3B, 3C). The decrease in IFN-γ production was not observed in the presence of anti-CD28 (Fig. 3B, 3C). We also measured IL-2 and IFN-γ production by CD8+ T cells stimulated with soluble anti-CD3 in the presence of CD11c+ dendritic cells. Under this condition, we also observed the decrease in IL-2 and IFN-γ production by RARα-deficient CD8+ T cells. Furthermore, exogenous RA in culture showed no impact on cytokine production (data not shown). Therefore, RARαfl/fl CD8+ T cells activated in vitro produce fewer effector molecules (IL-2 and IFN-γ). The reduced production of IL-2 likely impacts on the impaired survival of RARα-deficient CD8+ T cells in vitro. This was consistent with the fact that the short-term stimulation-induced cell death in RARα-deficient CD8+ T cells was rescued by high IL-2 (Fig. 2).

Further studies are needed to elucidate the mechanism of IL-2 regulation by RARα.

RARα is required for RA-induced gut-homing receptor α4β7 and CCR9 expression on activated CD8+ T cells

One important function that has been ascribed to RA is the imprinting of T cells to express the gut-homing receptors α4β7 and CCR9 (3, 26). Previous studies using different receptor-specific agonists/antagonists (3) and transgenic mice (27) showed that RARα is required for RA-induced α4β7 and CCR9 upregulation in CD4+ T cells upon activation. To address whether RARβ or RARγ influences RA-induced gut imprinting of CD8+ T cells, RA-induced α4β7 and CCR9 expression was evaluated in RARαfl/fl, RARβfl/fl, RARγfl/fl, and control CD8+ T cells. To this end, CD8+ T cells from different strains were stimulated in vitro with anti-CD3 + anti-CD28 for 48 h, rested with fresh media containing 100 U/ml IL-2 for another 48 h, and analyzed at 96 h postculture. ATRA was supplemented in the culture at various concentrations. This culture regimen efficiently induced α4β7 and CCR9 expression on activated CD8+ T cells in vitro (28), as well as overcome the lower viability shown in RARαfl/fl CD8+ T cells (Fig. 2). The expression of α4β7 and CCR9 was analyzed at 96 h post-activation. As shown in Fig. 4, although RA induced significant upregulation of α4β7 (Fig. 4A, 4C) and CCR9 (Fig. 4B, 4C) in
control, RARβflo/flo, and RARγflo/flo CD8+ T cells, there was very low, if any, expression of αβ7 and CCR9 in RARαflo/flo CD8+ T cells. This is true across a range of RA concentrations (1–100 nM) tested. This observation extended the prior studies showing that neither RARβ nor RARγ contributes to RA-induced imprinting and that only RARα is critical for this induction.

The essential requirement of RARα, but not RARβ or RARγ, for CD8+ T cell survival and bacterial clearance

To examine whether the impact of RAR deficiency that was manifested in vitro was recapitulated in vivo, CD8+ T cell responses were tracked and analyzed in each mutant RAR mouse strain. To this end, we analyzed the primary CD8+ T cell response to systemic infection with L. monocytogenes–OVA. CD8+ T cell responses in RARαflo/flo, RARβflo/flo, RARγflo/flo, and control mice were monitored by measurement of OVA-MHC I tetramer+ CD8+ T cell expansion and differentiation.

CD4+ T cell help was shown to be required for optimal CD8+ T cell response against L. monocytogenes–OVA infection (29), but our preliminary data showed measurable CD8+ T cell response in CD4+ T cell–depleted hosts at 5 × 104 CFU infection, even if at lower magnitude than CD8+ T cell response elicited in CD4+ T cell–sufficient hosts (data not shown). Because we wanted to unequivocally evaluate the intrinsic requirement for each RAR in the control of CD8+ T cell responses without the potential interference from possibly defective CD4+ T cell help, we depleted CD4+ T cells systemically prior to infection. RARαflo/flo, RARβflo/flo, RARγflo/flo, and control mice were infected with 5 × 108 CFU L. monocytogenes–OVA systemically. Complete systemic CD4+ T cell depletion was confirmed on day 5 postinfection (data not shown). Under this condition, peak accumulation of OVA-specific CD8+ T cell was observed on day 5 postinfection (data not shown). Thus, the following analyses were performed on day 5 postinfection. The Ag-specific CD8+ T cell response and bacterial load were analyzed. The proportion and total CD8+ T cell number in the spleen of infected RARαflo/flo mice was ∼2-fold lower compared with other groups of mice, indicating CD8+ T cell death due to infection (Fig. 5A, 5B). The lower CD8+ T cell number in RARαflo/flo hosts is consistent with in vitro results (Fig. 2A, 2B), suggesting an essential role for RARα in control of overall, polyclonal CD8+ T cell survival. The frequency and total number of OVA-specific CD8+ T cells were measured in the spleen by MHC I–OVA–tetramer staining. There was a significantly lower number of total CD44highOVA-tet+ CD8+ T cells, despite the slightly higher frequency of CD44highOVA-tet+ cells among CD8+ T cells in infected RARαflo/flo mice, as a result of massive CD8+ T cell loss in these hosts (Fig. 5A, 5C). There was a significant, but modest, reduction in both the frequency (4.481 ± 0.6829% versus 8.806 ± 0.8455%, p = 0.0008) and total number (25.74 ± 4.276 × 104 versus 46.17 ± 3.185 × 104, p = 0.0008) of CD44highOVA-tet+ CD8+ T cells in infected RARβflo/flo mice compared with control mice, indicating modestly defective Ag-

FIGURE 3. Impaired IL-2 and IFN-γ production by RARαflo/flo CD8+ T cells upon activation. Representative FACS plots of IL-2 (A) and IFN-γ (B) in CD8+ T cells by intracellular staining at 24 h postactivation with no stimulation (left panels), anti-CD3 stimulation (middle panels), or anti-CD3 + anti-CD28 stimulation (right panels). Data shown are gated on live CD8+ T cells. Numbers in boxes indicate percentage of IL2+ (A) or IFNγ+ (B) CD8+ T cells. (C) Quantification of percentage of IL2+ (left panel) or IFNγ+ (right panel) among CD8+ T cells. Data shown are mean ± SEM pooled from two experiments with n = 5–6 mice/group.
specific CD8+ T cell expansion in response to L. monocytogenes–OVA infection. On the contrary, RARγfl/fl mice did not show any significant differences from the control group with regard to either the frequency or total number of OVA-specific CD8+ T cells in these studies, with the exception of a slight increase in the percentage of CD8+ T cells (Fig. 5A–C). Our measurement of CD8+ T cell response in the liver revealed the same decrease in OVA-specific CD8+ T cells in infected RARβfl/fl mice, suggesting a systemic deficiency in Ag-specific CD8+ T cell accumulation (data not shown).

The ability of CD8+ T cells to produce effector molecules was measured by intracellular staining of granzyme B (without ex vivo stimulation), IFN-γ, and TNF-α (post–ex vivo stimulation with SIINFEKL). Given the lower number of polyclonal CD8+ T cells in RARαfl/fl mice, there were fewer total IFN-γ-, granzyme B–, and TNF-α–producing CD8+ T cells in RARαfl/fl mice compared with the control group (Fig. 5D, Supplemental Fig 3). In agreement with previous results, the overall expansion of Ag-specific CD8+ T cells and the accumulation of IFN-γ–producing CD8+ T cells do not always mirror each other during infection (30). A modest decrease in the number of granzyme B–producing, but not IFN-γ– or TNF-α–producing, CD8+ T cells was observed in infected RARβfl/fl mice (Fig. 5D, Supplemental Fig 3), despite the reduced accumulation of CD44highOVA-tet+CD8+ T cells (Fig. 5C). On the contrary, infected RARγfl/fl mice showed no deficiency in the production of any effector molecule, as evaluated above, suggesting that CD8+ T cell–intrinsic RARγ is dispensable for Ag-specific CD8+ T cell expansion and differentiation in our studies.

Earlier studies (31) showed that CD8+ T cells are required for full clearance of L. monocytogenes–OVA from the infected spleen. Therefore, the bacterial burden in different groups of mice postinfection was measured. Although L. monocytogenes–OVA was completely cleared from the spleen of RARβfl/fl, RARγfl/fl, and control mice on day 5 postinfection, RARαfl/fl mice failed to clear the bacteria completely (Fig. 5E). However, the decrease in only granzyme B production in RARβfl/fl mice kept the host intact in bacterial clearance. Consistent with the dominant role of innate immunity in L. monocytogenes clearance of liver (32), L. monocytogenes–OVA were cleared from the liver in all strains of mice at the same level on day 5 postinfection, despite the slightly dampened CD8+ T cell response in the liver of RARαfl/fl mice (data not shown).
Discussion
To our knowledge, this is the first report to elucidate the distinct functions of each RAR isoform in the control of CD8+ T cell immunity through conditional deletion of specific RAR isoforms in T cells. The key findings are that RA signaling is not required for CD8+ T cell activation and proliferation; RA signaling mediated by RARα, but not RARβ or RARγ, is required for upregulated expression of the gut-homing receptors α4β7 and CCR9 on activated CD8+ T cells; RARα is required for CD8+ T cell survival and expression of effector molecules; RARβ may be involved in Ag-specific CD8+ T cell expansion in response to L. monocytogenes infection; and RARαfl/fl mice are incompetent in bacterial burden control.

Previous studies (3, 26) revealed the role of RA in imprinting the gut-homing receptors α4β7 and CCR9 on activated CD8+ T cells. In this study, the role of each RAR isoform in the intrinsic control of α4β7 and CCR9 upregulation was addressed. The exclusive requirement of RARα in the control of gut-homing receptor expression supports the following conclusions. First, the functional distinction between RARs is not determined solely by the expression pattern, as indicated in other studies (reviewed in Ref. 33), because all three RARs are expressed in CD8+ T cells.

FIGURE 5. Defective CD8+ T cell response against L. monocytogenes–OVA infection and bacterial burden control in RARαfl/fl mice. (A) Representative FACS plots of MHC-I OVA-tetramer staining in naive and infected control, RARαfl/fl, RARβfl/fl, and RARγfl/fl mice splenocytes. Representative profile of CD8+MHCII− T cells gated on live cells (upper panels). Representative profile of CD44 and OVA-tet on gated CD8+MHCII− T cells (lower panels). Gating strategy was determined with naive mice as negative control. (B) Quantification of proportion (left panel) and total number (right panel) of CD8+ T cells in spleens of infected mice on day 5 postinfection. (C) Proportion of CD44highOVA-tet+ CD8+ T cells (left panel) and total number of CD44highOVA-tet+ CD8+ T cells (right panel) in the spleen of infected mice on day 5. (D) Total number of CD8+ granzyme B+ (upper left panel), CD8+IFNγ+ (upper right panel), and CD8+TNFα+ (lower left panel) T cells in the spleen of infected mice on day 5. (E) Bacterial load in spleen of day-5 infected mice. Data shown in (B), (C), (D), and (E) are pooled from four independent experiments (n = 11 mice/group). Horizontal lines in (B)–(D) stand for mean value in corresponding strain. Dashed lines in (E) indicate lowest number of bacteria that can be detected in our system.
Second, the exclusive requirement of RARα in control of αβ7 and CCR9 argues against the well-established notion of functional redundancy among RARs. Numerous studies (34–36) suggested redundancy among these receptors because of the need to produce double- or triple-receptor knockout to recapitulate (or add to) VAD phenotype. Our data also corroborated the recent finding that BATF is required for normal αβ7 and CCR9 expression in CD8+ T cells by regulation of RARα binding to the regulatory regions of Itgα4 and ccr9 genes (37).

Previous studies (12) showed that RA signaling is required for proliferating CD8+ T cell survival at the effector phase in both tumor and anti-CD40/TLR agonist immunization-induced CD8+ T cell responses without any impact on early cell division per se. Using both in vitro and in vivo CD8+ T cell–activation studies, we found that RARα-deficient, but not RARβ- or RARγ-deficient, CD8+ T cells showed survival deficiency upon activation. The significantly lower IL-2 production may partially lead to the defective viability of RARα-deficient CD8+ T cells in vitro. This essential role for RARα-mediated IL-2 production in CD8+ T cell survival also was supported by the fact that the short-term anti-CD3 + anti-CD28 stimulation-induced cell death in RARα-deficient CD8+ T cells was rescued by high IL-2 in the following culture. Further studies are needed to elucidate the molecular mechanism of IL-2 regulation by RARα.

Early non-Ag–specific T cell depletion in lymphoid organs was reported during the acute phase of L. monocytogenes infection (38, 39). Clearly, deletion of RARα, but not RARβ or RARγ, exacerbated the activation-induced cell death seen upon L. monocytogenes infection. The unaltered proportion of Ag-specific CD8+ T cells in these hosts indicated that the reduced number of total CD8+ T cells may be due to deficiency in overall survival instead of Ag-specific expansion during the priming phase. The reduced CD8+ T cell survival following L. monocytogenes infection in RARα−/− mice is consistent with in vitro results, confirming the essential role of RARα in control of CD8+ T cell survival. Further studies are needed to elucidate how RARα regulates CD8+ T cell survival during L. monocytogenes infection.

Our data showed a significant, but modest, decrease in Ag-specific CD8+ T cell expansion in the absence of RARβ. No other dramatic biological impact of RARβ deficiency has been noted either in vitro or in vivo. Furthermore, the reduced expansion of L. monocytogenes–specific CD8+ T cells had no impact on L. monocytogenes clearance or burden control, despite decreased granzyme B production. Although primary clearance of L. monocytogenes is not solely dependent on Ag-specific CD8+ T cell expansion, clearance in response to bacterial challenge is heavily influenced by Ag-specific CD8+ T cells (40). Whether RARβ deficiency impacts on memory responses to L. monocytogenes is currently being examined. Nonetheless, the data clearly demonstrate the contrasting roles of RARα and RARβ in CD8+ T cell function.

Cytokines produced by polyclonally activated CD8+ T cells facilitate innate immunity–mediated clearance of L. monocytogenes during primary infections (41, 42). The number and frequency of L. monocytogenes–induced effector CD8+ T cells were reduced in RARα−/− mice. Thus, the lower number of activated CD8+ T cells and, consequently, fewer effector molecule–producing CD8+ T cells may directly render RARα−/− hosts unable to facilitate bacterial clearance during primary infection. Various effector molecules are redundant in bacterial clearance. Previous studies demonstrated that CD8+ T cells lacking either IFN-γ (43) or TNF-α (44) were equally potent in protection against L. monocytogenes as their wild-type counterparts. Therefore, unsurprisingly, a decrease in granzyme B production alone by CD8+ T cells still allowed for intact bacterial clearance in RARβ−/− hosts.

This study extends our understanding of RA signaling in the control of Ag-specific CD8+ T cell survival during expansion (12) and differentiation in tumor (12) and vaccinia virus infection (13) by showing the exclusive control of polyclonal CD8+ T cell survival by RARα both in vitro and during intracellular pathogen infection. In addition, our study clearly demonstrated the requirement of CD8+ T cell–intrinsic RARα for primary L. monocytogenes infection clearance, as opposed to a previous study (45) illustrating the requirement of RARγ for proinflammatory cytokine production by macrophages and T cells, without any impact on disease control. Together, these studies enable us to better understand how RA and RAR(s) may regulate adaptive immunity directly or indirectly in different disease models.

Our studies focused on the different functions of RARα, RARβ, and RARγ in the control of CD8+ T cell immunity via genetic deletion of each specific isoform. An RA-independent impact of RAR deletion was noted previously in a number of in vivo models, in particular in neutrophil development (46–48), and, furthermore, with other nuclear receptors, such as thyroid receptor (49). A recent study (50) suggested that RAR also may control the CpG methylation status of specific promoter regions in a ligand-independent manner. The above findings suggest that caution is needed because RAR knockout mice may reveal both RA-dependent and -independent function of RARs. Studies are underway to dissect such differences.

In summary, our study revealed for the first time, to our knowledge, the essential requirement of RARα, but not RARβ or RARγ, for CD8+ T cell gut imprinting, survival, and bacterial clearance. Given the increased susceptibility to infectious diseases among children with VAD, this is important for specific targeting of each receptor for more efficacious vaccines against infection.

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Disclosures

The authors have no financial conflicts of interest.

References


Evidence that specific RAR deletion is Cre-dependent and produced a null allele. A. RARs expression in naïve and activated CD8\(^+\) T cells. CD8\(^+\) T cells were isolated from spleen of naïve C57BL/6 mice, and activated with 10\(\mu\)g/ml αCD3 for 0hr, 48hr and 72hr before analysis. RAR\(\alpha\), RAR\(\beta\) and RAR\(\gamma\) mRNA was measured in naïve and αCD3-activated CD8\(^+\) T cells by RT-PCR. +RT: with reverse transcriptase; -RT: without reverse transcriptase. B-C. CD8\(^+\) T cells were isolated from RAR\(\alpha^{\text{fl/fl}}\), RAR\(\beta^{\text{fl/fl}}\), RAR\(\gamma^{\text{fl/fl}}\) and control mice for genomic DNA or RNA purification. B. Previously published primers were used for PCR to amplify the null allele of RAR\(\alpha\) (316bp), RAR\(\beta\) (383bp) and RAR\(\gamma\) (495bp), and Cre in genomic DNA of CD8\(^+\) T cells from each strain. Lane 1: control; Lane 2: RAR\(\alpha^{\text{fl/fl}}\); Lane 3: RAR\(\beta^{\text{fl/fl}}\); Lane 4: RAR\(\gamma^{\text{fl/fl}}\). C. qRT-PCR was performed on CD8\(^+\) T cells from each strain for the expression of RAR\(\alpha\), RAR\(\beta\) and RAR\(\gamma\). In each isoform, the expression level was normalized to GAPDH. Data shown is representative of 2 experiments with similar results (pooled 2 mice/group in each experiment).
Supplemental Figure 2

A Thymus

B Spleen

C PLN

D

E

Control

RARα(III)

RARβ(III)

RARγ(III)

Control

CD3

CD44

RARα(III)

RARβ(III)

RARγ(III)

Control
Phenotype analysis of RARα<sup>fl/fl</sup>, RARβ<sup>fl/fl</sup>, RARγ<sup>fl/fl</sup> and control mice in thymus, spleen and peripheral LN. A. Top: CD4⁺%, CD8⁺%, and CD4⁺CD8⁺% in thymus of all strains. Bottom: Total number of CD4⁺, CD8⁺, and CD4⁺CD8⁺ T cells in thymus. B. Top: CD4⁺%, CD8⁺% of total and FoxP3⁺% of CD4⁺ T cells in PLN. Bottom: Total number of CD4⁺, CD8⁺, and CD4⁺FoxP3⁺ T cells in spleen of the same mice. C. The same analysis as in B, but in spleen. D. Top: Representative staining of CD44 on peripheral CD8⁺ T cells in all strains. Shown is pregated on CD8⁺ T cells. E. Quantification of CD44<sup>hi</sup>% among CD8⁺ (top) and CD4⁺ (bottom) T cells. Data shown is pooled from 2 experiments with n≥6 mice/group.
Supplemental Figure 3

A. Live cells were gated on CD8+ MHCII− T cells (top), and analyzed for Granzyme B expression (bottom). Numbers indicate Granzyme B+ % of CD8+ MHCII− T cells.

B. Live cells were gated on CD8+ T cells (top), and analyzed for IFNγ (middle) or TNFα (bottom) expression. Numbers indicate the IFNγ+ % or TNFα+ % of gated CD8+ T cells, respectively. Granzyme B was analyzed without ex vivo re-stimulation, whereas IFNγ and TNFα were analyzed post ex vivo restimulation with SIINFEKL.

Representative FACS plot of Granzyme B (A), IFNγ and TNFα (B) on CD8+ T cells in the spleen from infected mice. A. Live cells were gated on CD8+ MHCII− T cells (top), and analyzed for Granzyme B expression (bottom). Numbers indicate Granzyme B+ % of CD8+ MHCII− T cells. B. Live cells were gated on CD8+ T cells (top), and analyzed for IFNγ (middle) or TNFα (bottom) expression. Numbers indicate the IFNγ+ % or TNFα+ % of gated CD8+ T cells, respectively. Granzyme B was analyzed without ex vivo re-stimulation, whereas IFNγ and TNFα were analyzed post ex vivo restimulation with SIINFEKL.