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# A retinoic acid–dependent checkpoint in the development of CD4+ T cell–mediated immunity

Karina Pino-Lagos,[1] Yanxia Guo,[1] Chrysothemis Brown,[3] Matthew P. Alexander,[1] Raúl Elgueta,[3] Kathryn A. Bennett,[1] Victor De Vries,[1] Elizabeth Nowak,[1] Rune Blomhoff,[4] Shanthini Sockanathan,[5] Roshantha A. Chandraratna,[6] Ethan Dmitrovsky,[2] and Randolph J. Noelle[1,3]

[1]Department of Microbiology and Immunology, Dartmouth Medical School, Norris Cotton Cancer Center, Lebanon, NH 03756
[2]Department of Pharmacology and Toxicology, Dartmouth Medical School, Hanover, NH 03755
[3]Medical Research Council Centre of Transplantation, Guy's Hospital, King's College London, King's Health Partners, London SE1 9RT, England, UK
[4]Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, 0372 Oslo, Norway
[5]The Solomon H. Snyder Department of Neuroscience, Johns Hopkins University School of Medicine, Baltimore, MD 21205
[6]IO Therapeutics, Santa Ana, CA 92705

**It is known that vitamin A and its metabolite, retinoic acid (RA), are essential for host defense. However, the mechanisms for how RA controls inflammation are incompletely understood. The findings presented in this study show that RA signaling occurs concurrent with the development of inflammation. In models of vaccination and allogeneic graft rejection, whole body imaging reveals that RA signaling is temporally and spatially restricted to the site of inflammation. Conditional ablation of RA signaling in T cells significantly interferes with CD4+ T cell effector function, migration, and polarity. These findings provide a new perspective of the role of RA as a mediator directly controlling CD4+ T cell differentiation and immunity.**

CORRESPONDENCE
Randolph J. Noelle:
rjn@dartmouth.edu

Abbreviations used: $aT_{reg}$ cell, adaptive $T_{reg}$ cell; dLN, draining LN; RA, retinoic acid; RALDH, retinaldehyde dehydrogenase; RAR, RA receptor; VAD, vitamin A deficiency; WBI, whole body imaging.

The importance of vitamin A in host resistance to infectious disease is irrefutable (Semba, 1999). Up to 10 million malnourished children are at increased risk of complications and death from measles and other infectious diseases as consequences of vitamin A deficiencies (VADs). Although recent studies have revealed how retinoic acid (RA) may control the development of protective immunity, findings reported herein show that RA plays a far more fundamental role in inflammation than previously anticipated.

RA signaling to T cells imprints their homing to the mesenteric LNs and gut through the up-regulation of α4β7 and CCR9 (Iwata et al., 2004; Mora et al., 2008; Svensson et al., 2008; Wang et al., 2010) and contributes to B cell homing and isotype switching to IgA (Mora et al., 2008). Furthermore, RA at physiological concentrations has been shown to be critical for the development of Th17 (Uematsu et al., 2008; Cha et al., 2010; Wang et al., 2010). These findings provide a plausible explanation for the epidemiological findings of impaired immunity in vitamin A–deficient populations. At odds with its proinflammatory role in immunity, it has been shown that RA (at higher concentrations) can effectively interfere with the generation of inflammatory Th17 cells as well as enhance regulatory $CD4^+$ T cell ($T_{reg}$ cell) frequencies and function (Mucida et al., 2007; Schambach et al., 2007). In conjunction with TGF-β, RA enhances the expression of the transcription factor FoxP3 (Benson et al., 2007; Coombes et al., 2007; Sun et al., 2007), the master regulator for $T_{reg}$ cells, and facilitates the differentiation of $CD4^+$ effector T cells to stable, adaptive $T_{reg}$ cells ($aT_{reg}$ cells; Benson et al., 2007), likely by acting differentially in specific subsets of the $CD4^+$ T cell compartment (memory vs. naive



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populations; Hill et al., 2008). Both of these latter activities provide compelling evidence that RA may exert antiinflammatory effects within the host. Under what circumstances RA plays a proinflammatory role or an antiinflammatory role remains to be determined.

The molecular basis for RA signaling to T cells and the cellular sources of RA within the immune system have begun to resolve. Of the three RA receptors (RARs; α, β, and γ), RA has been shown to control the suppressive ($T_{reg}$) and inflammatory activities (Th17) of the $CD4^+$ T cell compartment by signaling through RAR-α (Mucida et al., 2007; Hill et al., 2008; Hall et al., 2011). Although it was originally believed that RA produced by hematopoietic cells may be limited to the gut, the production of RA by both hematopoietic and nonhematopoietic cells outside the gut has been repeatedly demonstrated (Hammerschmidt et al., 2008; Molenaar et al., 2009; Guilliams et al., 2010). The capacity of cells to produce RA is dependent on the expression of retinaldehyde dehydrogenase (RALDH) enzymes, the key family of enzymes which drive the irreversible conversion of retinal to RA (Duester, 2000). It has been shown that gut-resident $CD103^+$ DCs (Coombes et al., 2007; Sun et al., 2007), splenic DCs, and stromal cells (Hammerschmidt et al., 2008; Molenaar et al., 2009) produce RA. Within the gut, the opposing regulatory actions of RA on $T_{reg}$ cell (to mediate suppression) and Th17 cell differentiation (to suppress inflammation) have been implicated as critical actions in maintaining gut immune homeostasis (Mucida et al., 2007).

Whereas the role of RA in regulation of gut immunity has pictured RA as an important homeostatic regulator of inflammation, the findings presented in this study provide a fundamentally new perspective on the role of RA in the development of cell-mediated immunity. Using mice that report the up-regulation of luciferase as a consequence of RA signaling, this study shows that robust RA signaling occurs concurrent with the development of inflammation. In models of vaccination and allogeneic graft rejection, whole body imaging (WBI) revealed that RA signaling was temporally and spatially restricted with the site of inflammation. Conditional ablation of RA signaling in T cells arrested inflammation by altering T cell effector function, migration, and polarity. Our findings and others (Hall et al., 2011) establish that RA signaling to T cells is critical as an early mediator in the development of $CD4^+$ T cell–mediated immunity and help to explain the profound impairment of immunity in vitamin A–deficient populations.

## RESULTS AND DISCUSSION

### Immune activation elicits RA reporting on $CD4^+$ T cells

To determine whether RA production and signaling occurs during immune activation, mice were administered immune activators, and WBI was performed. The distribution and intensity of RA signaling after immune activation were determined using a RARE-Luciferase reporter mouse (DR5-Luciferase), which transcriptionally up-regulates luciferase expression upon signaling by RA (Svensson et al., 2008). Constitutive luciferase signal was routinely observed in the abdominal area of mice because of signaling emanating from the area of small and large intestines. Surprisingly, the intradermal administration of α-CD40, α-CD3, CFA, and LPS elicited a robust, enhanced luciferase signal that was localized to the site of administration as early as 24 h after immunization (Fig. 1 A). In addition, after the i.p. administration of CFA, a locally and temporally restricted luciferase signal was first induced over controls in 24 h, but the signal intensified by the following days, with a gradual decrease over the ensuing 20 d (Fig. 1 B). A more in-depth analysis of the temporal and spatial induction of RA reporting and identity of the cells reporting was undertaken after the i.p. administration of CFA and α-CD3. In this case, the administration of CFA triggered RA signaling on $CD4^+$ T cells (Fig. 1 C), but to a lesser extent than T cell activation induced by α-CD3, where a heightened activity was observed when compared with IgG control (Fig. 1 D). The in vivo administration of a RA antagonist before T cell isolation ablated the RA reporting, establishing the possibility that the enhanced reporting was specific for RA (Fig. 1, C and D). Therefore, different immune activators stimulate RA signaling in a spatially and temporally restricted manner.

In addition, RA reporting by $CD4^+$ T cells was studied in a model of allogeneic skin graft rejection. DR5-Luciferase $CD4^+$ T cells were adoptively transferred into $RAG^{-/-}$ mice before syngeneic or allogeneic skin graft transplantation. After transfer, WBI was used to assess RA reporting by the DR5-Luciferase T cells. As shown in Fig. 2 A, no signal was found at early time points (first 5 d after transplantation); however, between 7 and 21 d after transplant, enhanced RA signaling was detected in allogeneic grafts versus the syngeneic grafts. By week 3 after transplant, RA reporting declined to near baseline levels in both groups. Signaling from the transplanted allograft was associated with the presence of the allograft because RA reporting disappeared when the graft was fully rejected. In Fig. 2 B, quantified photon flux is presented for both mice transplanted with syngeneic and allogeneic grafts. Therefore, alloreactive $CD4^+$ T cells that enter an allograft are triggered by RA.

Our findings establish that RA signaling in T cells may be linked with the development of inflammation. The temporally restricted and regionally controlled signaling by RA suggests that RA regulates immunity within the inflammatory site over a prescribed period of time. In this study, polyclonal T cell activation in vivo augments RA signaling (or sensing) by $CD4^+$ T cells. Although gut-resident DCs (Coombes et al., 2007; Sun et al., 2007) have been identified as the major source of immune-derived RA, it is likely that RA production by DCs, other APCs, and even nonhematopoietic cells may be responsible for the reporting that is observed in intact mice. These observations are supported by several studies, in which not only gut-resident DCs are able to synthesize RA but also draining LN (dLN)– and skin-resident DCs (Guilliams et al., 2010), as well as nonhematopoietic cells (Hammerschmidt et al., 2008; Molenaar et al., 2009).

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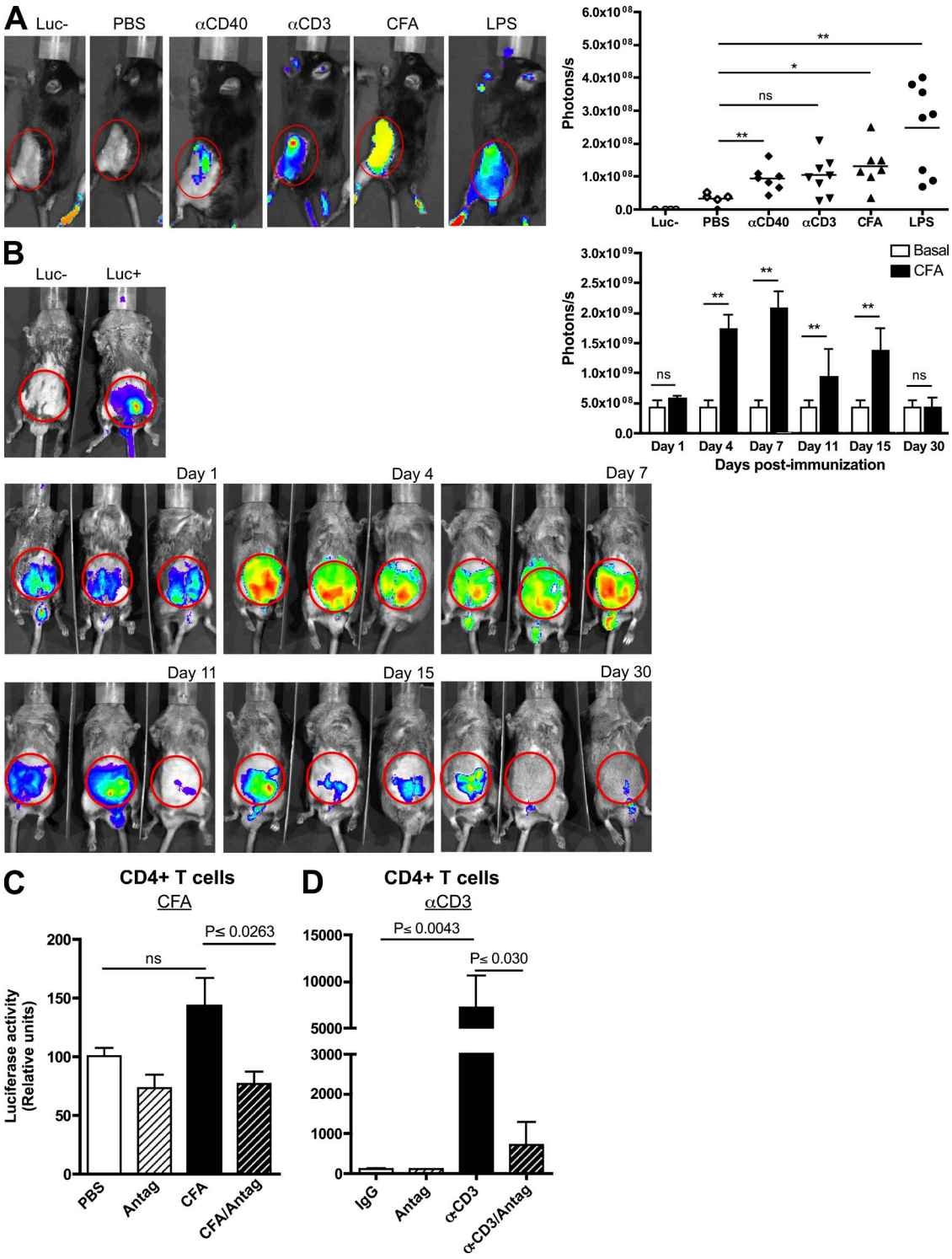


**Figure 1. Immune activation induces RA signaling.** (A) DR5-Luciferase reporter mice were immunized with the indicated activators by footpad injection. WBI was performed 24 h after immunization using IVIS technology. Quantification of photon flux is shown in the plot (right). $n = 1–3$ mice per group, and data represent three pooled independent experiments. Bars indicate the mean. (B) DR5-Luciferase reporter mice were immunized with CFA by i.p. injection, and kinetics for RA reporting was tracked by WBI. Basal reporting is shown in the top left picture (Luc+) including a negative control littermate (Luc–). Quantification of photon flux is included in the plot (right). $n = 3$ mice. (C and D) $CD4^+$ T cells were isolated from the spleens of DR5-Luciferase mice previously immunized by i.p. injection with CFA (C) or α-CD3 (D). In some mice, the RA-specific antagonist (Antag; NRX194310; 50 µg per mouse) was injected i.p. at the time of stimulus. Luciferase activity was measured in vitro as described in Materials and methods. $n = 2–3$ mice per group. Pooled data of at least two independent experiments are shown. Error bars indicate SEM. ns, nonstatistical difference; *, $P \leq 0.01$; **, $P \leq 0.05$. These findings are representative of two independent experiments.

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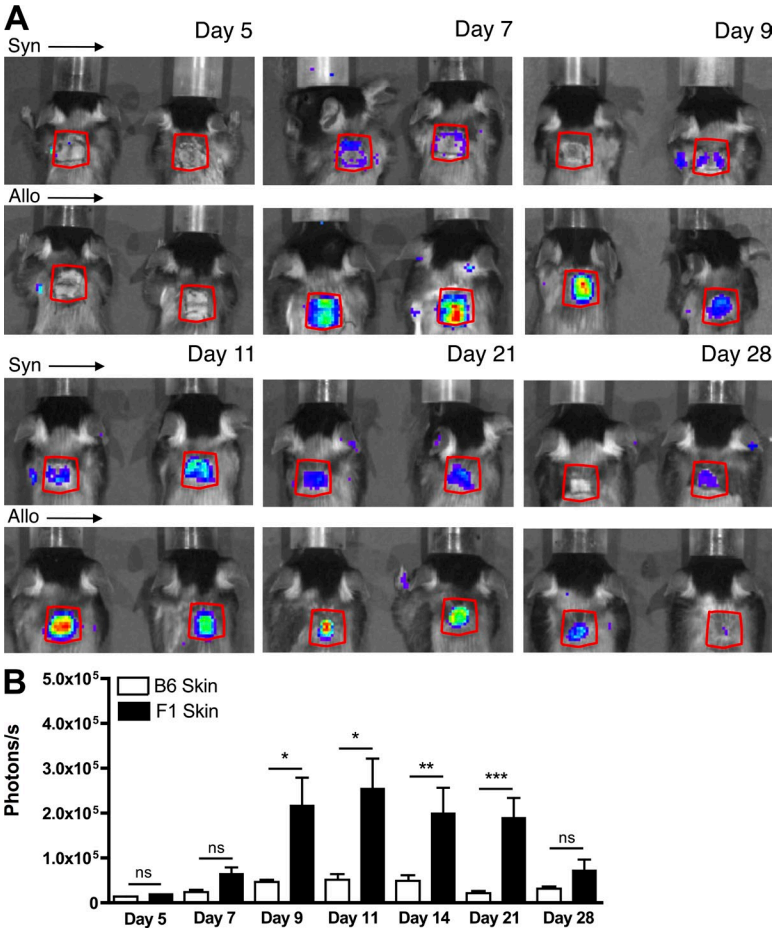


**Figure 2. Enhanced RA signaling in allogeneic skin grafts.** (A) RAG$^{-/-}$ recipient mice received DR5-Luciferase CD4$^+$ T cells and were then transplanted the next day with syngeneic (Syn; B6) or allogeneic (Allo; F1) skin grafts. Kinetics of RA reporting by CD4$^+$ T lymphocytes was tracked by WBI. $n = 4$ mice per group/experiment. (B) Graph displays kinetics of T cell–derived WBI RA reporting from skin grafts. Error bars indicate SEM. ns, nonstatistical difference; *, $P \leq 0.01$; **, $P \leq 0.05$; and ***, $P \leq 0.001$. The findings are representative of two experiments.

### Disruption of RA signaling in CD4$^+$ T cell impairs T cell tissue accumulation and effector cell function

To analyze the functional impact of intrinsic RA signaling on CD4$^+$ T cell population dynamics during graft rejection, RA signaling was selectively ablated in graft-specific T cells. A dominant-negative form of the RAR-α, RAR403 (hereafter denoted dnRAR-α; Rajaii et al., 2008), was overexpressed in T cells by interbreeding the dnRAR-α with CD4$^{Cre}$ mice.

The potential impact of *dnRAR-α* gene expression on T cell ontogeny in the dnRAR-αCD4$^{Cre}$ was addressed. Analysis of the T cell development indicated that overexpression of the *dnRAR-α* gene impacted the frequencies of thymic single-positive CD8$^+$ T cell populations, as previously described (Zhou et al., 2008), but had little effect on T cell populations in the periphery (Fig. S1). Overexpression of the *dnRAR-α* did not affect α-CD3–induced T cell proliferation in vitro or in vivo (Fig. S2) but, as anticipated, did inhibit RA-induced TGF-β–dependent FoxP3 conversion (Fig. S3; Benson et al., 2007; Coombes et al., 2007; Sun et al., 2007). To study the impact of RA signaling on graft rejection, the *dnRAR-α* gene was also overexpressed in OVA-specific OT-II TCR transgenic T cells, and mice were grafted with skin that expresses OVA as a transplantation rejection antigen (Ehst et al., 2003). C57BL/6 mice received dnRAR-α–OT-II–Ly5.2 or dnRAR-αCD4$^{Cre}$–OT-II–Ly5.2 cells by i.v. injection, and the next day, mice were transplanted with either syngeneic (B6) or allogeneic (actin-OVA) grafts. After 7 d, skin grafts and dLNs were removed and analyzed for OT-II–CD4$^+$ T cell infiltration. Fig. 3 (A and B) shows that the alteration of RA signaling in CD4$^+$ T cells impairs accumulation of T cells at the site of inflammation (skin graft). No differences in the expression of either activation markers (CD44, CD69, and CD62L) or chemokine receptors that are up-regulated upon inflammation and are associated with migration to inflamed sites (CCR4, CCR5, CCR6, CXCR3, and CCR10) were observed (Fig. S4). Therefore, early TCR-mediated events and expansion of graft-specific T cells appeared intact in the absence of RA signaling, but tissue accumulation was impaired.

It appeared that clonal T cell proliferation was intact in the absence of RA signaling, and so additional experiments were designed to evaluate the impact of RA signaling on T cell differentiation. Because of the difficulty of recovering sufficient numbers of transferred CD4$^+$ T cells when the host is on the WT C57BL/6 background, skin-grafted RAG$^{-/-}$ mice were used as recipients to ensure a high recovery and purity of OT-II–CD4$^+$ T cells. dnRAR-α–OT-II–Ly5.2 or dnRAR-αCD4$^{Cre}$–OT-II–Ly5.2 cells were transferred to RAG$^{-/-}$ recipient mice and grafted the next day. dLN-resident CD4$^+$ T cells were isolated after 7 d and co-cultured with unpulsed or ISQ-pulsed DCs. After 72 h, cytokine and chemokines were quantified. As shown in Fig. 4 A, the production of Th1-type cytokine IFN-γ and IL-17 was significantly reduced (~60% for both, with $P \leq 0.022$ [IFN-γ] and $P \leq 0.028$ [IL-17]) when dLN-resident dnRAR-αCD4$^{Cre}$–OT-II–Ly5.2 T cells were recalled with their cognate antigen, as compared with their counterpart dnRAR-α–OT-II–Ly5.2 T cells. Chemokine production by recalled dnRAR-αCD4$^{Cre}$–OT-II–Ly5.2 and dnRAR-α–OT-II–Ly5.2 T cells was also evaluated. As depicted in Fig. 4 B, the interruption of RAR-α signaling in CD4$^+$ T cells blocks the production of Th1-linked chemokines such as MIP-1α ($P \leq 0.014$) and MIP-1β ($P \leq 0.008$), including a down-regulation in the production of RANTES (Fig. S5; Cook, 1996). Moreover, we observed a tendency to a Th2-skewed/suppressive phenotype characterized by the up-regulation in the production of IL-4 ($P \leq 0.037$), IL-9

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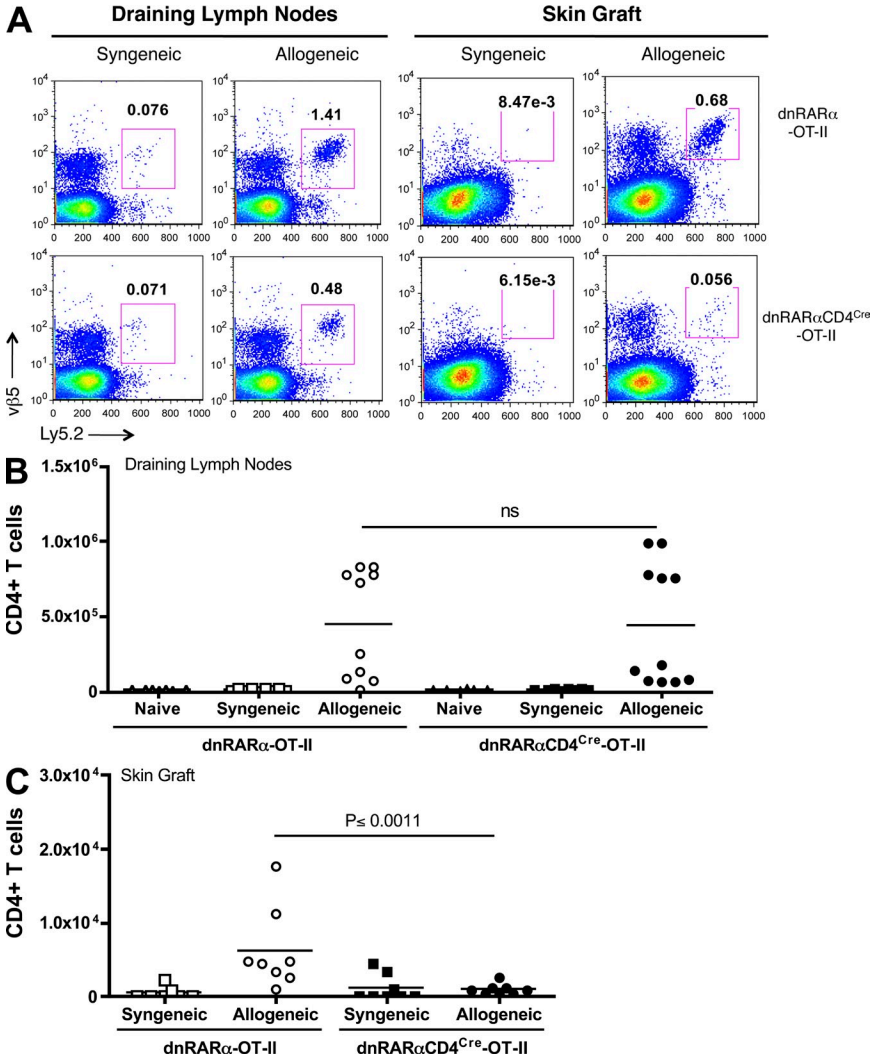


**Figure 3. RAR-α signaling blockade in CD4⁺ T cells compromises migration to the allograft.** (A) dnRAR-α–OT-II–Ly5.2 or dnRAR-αCD4$^{Cre}$–OT-II–Ly5.2 cells were transferred into C57BL/6 recipients, and T cell infiltration in dLNs and skin graft was analyzed by flow cytometry. $n$ = 2–4 mice per group. (B and C) Graphs depict the number of transferred cells infiltrating the dLNs (B) and skin grafts (C). Pooled data from three independent experiments are shown. Bars indicate the mean. ns, nonstatistical difference.

(P ≤ 0.026; Fig. 4 C), and IL-10 (P ≤ 0.128), although for IL-10 this difference was not statistically significant (Fig. S5). The impairment of Th1-linked chemokines by graft-specific T cells may contribute to the transcriptional program required for Th1/Th17 cell differentiation and the development of CD4⁺ T cell–mediated inflammation.

To evaluate the functional significance of RA signaling to graft rejection, T cell–mediated graft rejection was studied in mice in which RA signaling was ablated chemically and genetically. Mice receiving syngeneic (B6) or allogeneic (actin-OVA) skins were treated with a RAR pan-antagonist (NRX 194310). As shown in Fig. 5 A, the blockade of RAR signaling resulted in complete acceptance of the skin allograft (open red circles). Experiments then evaluated the involvement of RA signaling in CD4⁺ T cells during graft rejection. WT, dnRAR-α, and dnRAR-αCD4$^{Cre}$ were grafted with syngeneic or allogeneic skin, and the kinetics of graft rejection was evaluated over time. As depicted in Fig. 5 B, dnRAR-α mice reject the allograft following the same kinetics previously described for C57BL/6 (Ehst et al., 2003). However, rejection in the dnRAR-αCD4$^{Cre}$ mice was completely abolished. Therefore, experiments with an RAR pan-antagonist and overexpression of the *dnRAR-α* gene in T cells demonstrate that RA signaling is required for T cell–mediated graft rejection.

Our data demonstrate that RA signaling to CD4⁺ T cells is essential for the inflammatory responses that mediate the rejection of allogeneic skin grafts. The absence of RA signaling in CD4⁺ T cells alters T cell polarity, resulting in a shift from a Th1/Th17 to a Th2 phenotype. The selective impact of RA on T cell differentiation and not clonal expansion is consistent with the well-described role of RA as a differentiation factor in developmental biology (Maden, 2007). With the present data considered, it is clear that RA exerts both proinflammatory and anti-inflammatory effects on cell-mediated immunity. Numerous studies have implicated RA in facilitating immune suppression via the enhanced differentiation of $aT_{reg}$ cells (Benson et al., 2007; Coombes et al., 2007; Sun et al., 2007) and the suppression of Th17 cell differentiation (Mucida et al., 2007; Elias et al., 2008). In fact, it has been shown that RA agonists can suppress several murine autoimmune diseases, including systemic lupus erythematosus and experimental autoimmune encephalomyelitis (Yamauchi et al., 2005). In the latter, reduced differentiation of Th17 was attributed to the resolution in disease. Furthermore, a major emphasis for the role of RA in immunity has been placed on its role in gut homeostasis. Studies have shown that CD103⁺ DCs from the gut can potentiate the differentiation of $aT_{reg}$ cells through their capacity to express RALDH enzymes required for RA synthesis (Coombes et al., 2007; Sun et al., 2007). Although its role in the gut is no doubt critical, the findings reported herein provide a completely new perspective of the role of RA in immunity. In this regard, our preliminary results show that stimuli like T cell activation by α-CD3 antibody treatment increases the frequencies of DCs expressing RALDH. In the skin transplantation model, although we observed heightened RA signaling in CD4⁺ T cells during

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the rejection process, we did not see a significant difference in the numbers of RALDH$^+$ DCs between the syngeneic and allogeneic groups (either dLNs or skin grafts; unpublished data). Thus, signaling of RA to CD4$^+$ T cell may not be correlated with differential production from other cell types (APCs and stromal cells). It is known that RA signaling can be regulated by the expression of RA transport proteins expressed by the target cell. Alternatively, RA concentrations in vivo can be regulated by its catabolism by the catabolic enzyme, Cyp26. In this regard, future experiments involving Cyp26, the protein which degrades RA in the cytosol, will be pursued to understand its role in immunity. In contrast, the molecular mechanisms underlying the ability of RA to control the lineage commitment of T cells in vivo are unresolved. Our data show that ablation of RA signaling in vivo reduces the expression of Th1- and Th17-associated cytokines and chemokines. This effect is not seen in vitro when dnRAR-αCD4$^{Cre}$ T cells are stimulated with α-CD3 (unpublished data). The role of RA in eliciting Th1 and Th17 inflammatory T cells may be through its capacity to influence the expression of master transcription factors that regulate the fate of Th cells, such as T-bet or ROR-γt. Alternatively, RA may control T cell plasticity in vivo, and dnRAR-αCD4$^{Cre}$ may have impairments in lineage commitment. Studies are underway to evaluate these hypotheses.

In addition, our data show that the disruption of RA signaling in the CD4$^+$ T cell compartment impairs T cell infiltration of the skin graft. Although the expression of the major skin/inflammation-related chemokine receptors was not compromised, the added effect of reduced Th1 cytokines and chemokines may cooperate for the poor recruitment of CD4$^+$ T effector cells to the allograft. We observed that dnRAR-αCD4$^{Cre}$ CD4$^+$ T cells produce less Th1-linked chemokines, which can explain in part the lack of CD4$^+$ T cell accumulation within the graft if chemokine migration by infiltrating T cells amplifies the recruitment of additional T cells to the inflammatory site.

A recent publication (Hall et al., 2011) has highlighted the role of RA signaling of T cells in the mucosal immune response to *Toxoplasma gondii*. These investigators have shown that VAD impairs IFN-γ recall responses in immune mice. Furthermore, they show that T cells derived from an RAR-α$^{-/-}$ mouse display abnormalities in cell growth and $Ca^{2+}$ mobilization and produce less IFN-γ in response to vaccination with OVA protein and *Escherichia coli* toxin. In contrast to these findings, we show that T cells that are unresponsive to RA via overexpression of a *dnRAR-α* proliferate in response to antigen in vivo and in vitro. An apparent explanation to the differences is not clear at this time. It is possible that T cells

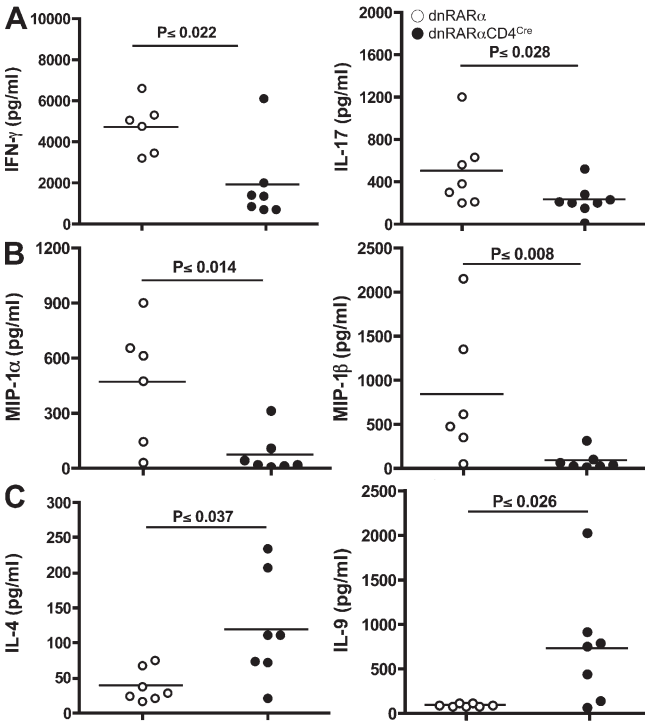


**Figure 4. RAR-α signaling is required for effector function on CD4$^+$ T cells.** RAG$^{-/-}$ recipient mice received dnRAR-α-OT-II-Ly5.2 or dnRAR-αCD4$^{Cre}$-OT-II-Ly5.2 cells 1 d before B6 or actin-OVA skin graft transplantation. After 7 d, CD4$^+$ T cells were isolated from dLNs and co-cultured with ISQ-pulsed splenic CD11c$^+$ cells. (A and C) Supernatants were collected to measure Th1/Th17 (A)- and Th2-type (C) cytokines by ELISA and Luminex. (B) Chemokine secretion was also quantified by Luminex. $n$ = 2–3 mice per group. Graphs depict pooled data from three independent experiments. Bars indicate the mean.

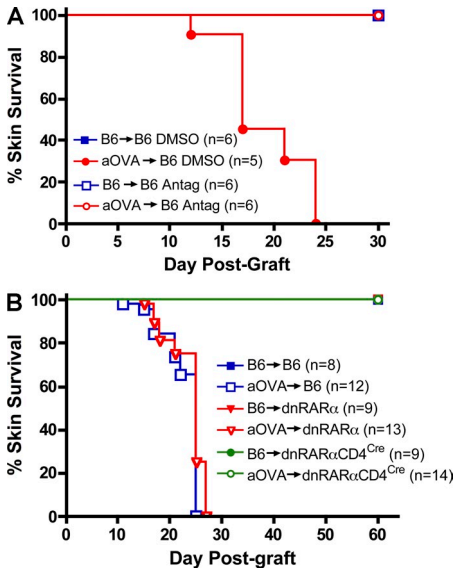


**Figure 5. RA signaling to CD4$^+$ T cells is essential for allograft rejection.** (A) C57BL/6 mice were transplanted with syngeneic (B6) or allogeneic (actin-OVA) skin grafts. RAR pan-antagonist (Antag; NRX 194310) was given three times per week at 50 µg/mouse, and skin graft survival was monitored. (B) C57BL/6, dnRAR-αCD4$^{Cre}$, and dnRAR-α were grafted with either B6 or actin-OVA skin grafts. Skin graft survival was monitored over time. The number of mice per group is indicated in each graph. Pooled data from two representative independent experiments are shown.

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derived from RAR-$\alpha^{-/-}$ are developmentally impaired. Alternatively, it is possible that the signaling function of the RAR-α molecule may still be intact in the *dnRAR-α*–expressing T cells. Future studies with conditional deletion of each of the RAR will help resolve these issues and shed insights into the role of other RAR in immunity. Nonetheless, collectively, the study by Hall et al. (2011) and our study on the role of RA in immunity provide new insights into the function of this mediator in inflammation.

With regard to cell differentiation and stability, data from experiments examining the role of RA in $aT_{reg}$ cell generation have implicated RA as an important differentiation factor for T cells, which may impact their plasticity. Data show that RA-treated natural $T_{reg}$ cells are lineage committed and virtually irreversible in their phenotype, even in the face of extremely proinflammatory conditions (Zhou et al., 2010). As such, RA controls the plasticity of $aT_{reg}$ cells (Benson et al., 2007; Nolting et al., 2009) and perhaps Th1 and Th17 cells. The enhanced $aT_{reg}$ cell differentiation by RA has been recently resolved to show that RA enhances the association of the RAR/RXR receptor to the FoxP3 enhancer, leading to enhanced histone acetylation and enhanced binding of phosphorylated Smad3 (Xu et al., 2010). For nearly a century, it has been clear that VAD compromises immunity, and these new findings on the role of RA in controlling T cell–mediated differentiation provide a rational, scientific basis for the increased susceptibility to infectious disease in vitamin A–deficient individuals.

## MATERIALS AND METHODS

**Animals.** C57BL/6, RAG$^{-/-}$, CD4$^{Cre}$, and actin-OVA (Ehst et al., 2003) mice were purchased from the Jackson Laboratory. The DR5-Luciferase reporter (Svensson et al., 2008) and dnRAR-α (Rajaii et al., 2008) have been described previously. Animal experiments were approved by the Institutional Animal Care Use Committee of Dartmouth Medical School. All animals were maintained in a pathogen-free facility at Dartmouth Medical School.

**Immunizations.** C57BL/6 or DR5-Luciferase reporter mice were immunized with PBS1X, CFA (mixed 1:1 with PBS1X; DIFCO), 2.5 µg control IgG, 2.5 µg α-CD3 (clone 2c11), 50 µg LPS (Sigma-Aldrich), and 50 µg α-CD40 (BioXCell) by i.p. (200 µl) or footpad (20 µl) injections (as indicated).

**Imaging.** Mice received 3 mg D-Luciferin (Gold Biotechnology) in 200 µl DPBS (Cellgro) by i.p. injection and were imaged in the IVIS 2000 (Xenogen Corp.) according to the manufacturer's instructions. Analysis and images were obtained using the Living Image Software (version 2.6.1; Caliper Life Sciences).

**T cell phenotyping.** Thymus, peripheral LNs, spleen, and mesenteric LNs were removed from WT, dnRAR-α, or dnRAR-αCD4$^{Cre}$ mice. Cell suspensions were prepared, and staining for CD4 and CD8 T cells was performed and analyzed by flow cytometry.

**Proliferation assay in vitro.** CD4$^+$ T cells were isolated from the spleens of dnRAR-α or dnRAR-αCD4$^{Cre}$ mice and labeled with 5 µM CFSE (Invitrogen). In parallel, T cell–depleted splenocytes (APC) were prepared, and $0.15 \times 10^5$ APCs were co-cultured with $0.5 \times 10^5$ CD4$^+$ T cells in the presence of soluble α-CD3 (clone 2C11). CFSE dilution was evaluated by flow cytometry at day 3.

**Conversion assay in vitro.** CD4$^+$ T cells were isolated from the spleens of dnRAR-α or dnRAR-αCD4$^{Cre}$ mice and stimulated with 10 µg/ml plate-bound α-CD3 (clone 2c11) and 1 µg/ml α-CD28 (clone PV-1) plus the addition of 100 U/ml IL-2, 10 ng/ml TGF-β, and different concentrations of RA (Sigma-Aldrich; as indicated). After 4 d in culture, the expression of FoxP3 was evaluated by intracellular staining (eBioscience) and analyzed by flow cytometry.

**Quantification of luciferase activity in vitro.** CD11c$^+$ and CD4$^+$ T cells were isolated from the spleens of immunized DR5-Luciferase reporter mice. $0.5 \times 10^6$ cells were resuspended in 100 µl DPBS, and the procedure was performed according to the manufacturer's instructions (SteadyLite kit; Roche).

**Skin grafting.** Skin grafting was performed as described previously (Quezada et al., 2005). For tracking RA signaling on CD4$^+$ T cells, splenic CD4$^+$ T cells were isolated from the DR5-Luciferase reporter mouse, and $3 \times 10^6$ cells were transferred into RAG$^{-/-}$ recipients by i.v. injection. The next day, syngeneic or allogeneic skin grafts were transplanted. For the antagonist treatment regimen, when indicated, recipient mice were treated three times/week with control vehicle (DMSO) or 50 µg/mouse of the RAR pan-antagonist (NRX 194310; NuRx Pharmaceuticals).

**Skin-infiltrating cell isolation.** Skin transplants were removed from animals at the indicated day in the figure legends. The transplants were incubated in HBSS media with 4 mg/ml DNase, 4 mg/ml Liberase, and 10 mg/ml Collagenase D for 3 h at 37°C (all from Roche). After this, the tissue was disintegrated using a mortar and pestle and filtered through a 100-µm nylon strainer. Cell suspension was stained for flow cytometric analysis.

**Recall experiments.** At day 0, RAG$^{-/-}$ mice received $10^6$ OT-II–dnRAR-α–OT-II–Ly5.2 or dnRAR-αCD4$^{Cre}$–OT-II–Ly5.2 cells by i.v. injection. The next day, recipient mice were grafted with syngeneic (C57BL/6) or allogeneic (actin-OVA) skins. After 7 d, CD4$^+$ T lymphocytes were isolated from skin graft dLNs (L3T4 kit; Miltenyi Biotec) and co-cultured at a 3:1 ratio with naive or ISQ-pulsed splenic CD11c$^+$ cells (10 µg/ml New England Peptide and CD11c microbeads; Miltenyi Biotec).

**Cytokine and chemokine secretion.** Supernatants were collected 72 h after culture and analyzed using ELISA (mouse IL-17A kit; BioLegend) or Luminex (Bio-Rad Laboratories), as indicated. The Luminex assay was performed by the Immune Monitoring Core (Dartmouth College).

**Flow cytometry.** The following FITC-, PE-, PerCP-, or APC-conjugated antibodies were used: α-Ly5.2 (A20), α-CD4 (L3T4), α-CD8 (53-6.7), α-CD11c (N418), α-CD62L (Mel-14), α-CD11b (M1/70), α-CCR4 (2G12), α-CCR5 (7A4), α-CCR6 (29-2L17), α-CCR10 (248918), α-CXCR3 (CXCR3-173), and αvβ5 (MR9-4). All antibodies except αvβ5 (BD) and α-CCR10 (R&D Systems) were purchased from BioLegend. Data acquisition was performed on a FACScan (BD), and analysis was performed using FlowJo software (Tree Star).

**Statistical analysis.** Differences between the means of experimental groups were analyzed using the Mann-Whitney test. Data analysis was performed using Prism software (version 4.0; GraphPad Software). A p-value ≤0.05 was considered significant.

**Online supplemental material.** Fig. S1 shows the T cell frequencies and phenotype from different organs in WT, dnRAR-α, and dnRAR-αCD4$^{Cre}$ mice. Fig. S2 shows that CD4$^+$ T cells from the dnRAR-αCD4$^{Cre}$ mice do not convert to FoxP3 cells in vitro. Fig. S3 shows that the ablation of RA signaling on CD4$^+$ T cells does not affect the expression of chemokine receptors or activation markers. Fig. S4 shows that the lack of RAR-α signaling promotes the generation of Th2/suppressive CD4$^+$ T cells. Fig. S5 shows that

disruption of RAR-α signaling on CD4+ T cells skews T cell polarity toward the Th2 phenotype. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20102358/DC1.

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Author contributions: K. Pino-Lagos designed and performed the experiments, interpreted and analyzed data, and wrote the manuscript. Y. Guo contributed on imaging-reporting experiments and discussion on the manuscript. C. Brown contributed with surgeries, imaging and recall experiments, and discussion on the manuscript. M.P. Alexander contributed with imaging experiments and mice screening. R. Elgueta contributed with BM-DC experiments (response to reviewers). K.A. Bennett performed surgeries and maintained mouse colonies. V. De Vries performed surgeries. E. Nowak standardized RAR pan-antagonist administration. S. Sockanathan provided the dnRAR-α mice and advised on screening and use. R. Blomhoff provided the RARE-Luc mice and advised on screening and use of mice. R.A Chandraratna provided us with the RAR pan-antagonist and guidance on in vivo treatments. E. Dmitrovsky advised on analysis of RAR function and assisted with data interpretation and analysis. R.J. Noelle designed the study, interpreted data, and wrote the manuscript.

The authors declare no competing financial interests.

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## SUPPLEMENTAL MATERIAL

**Pino-Lagos et al., http://www.jem.org/cgi/content/full/jem.20102358/DC1**

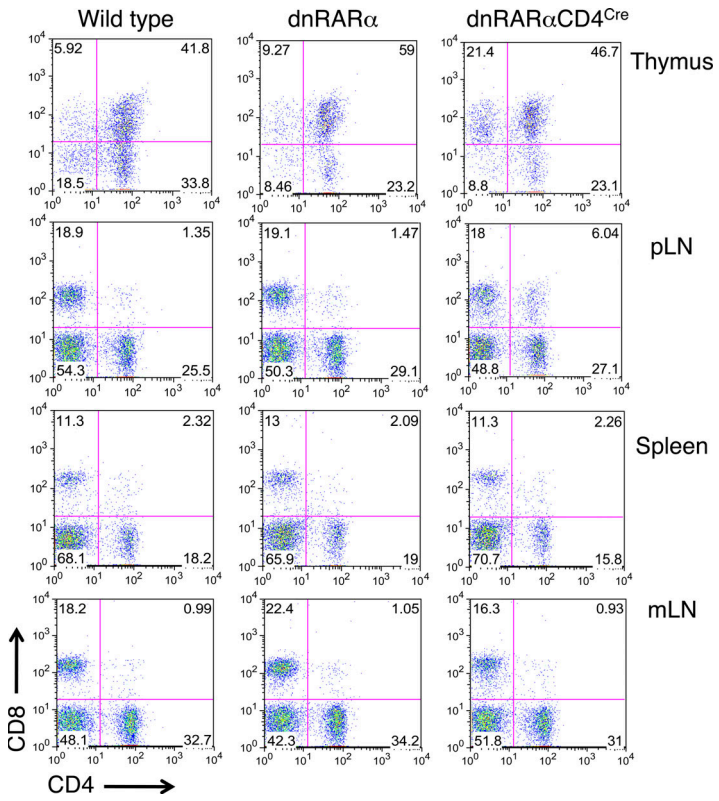


**Figure S1. Overexpression of the dnRAR-α gene in the T cell compartment.** Thymus, pLNs, spleen, and mesenteric LNs (mLN) were removed from WT C57BL/6, dnRAR-α, and dnRAR-αCD4$^{Cre}$ mice and stained for CD4 and CD8 to analyze by flow cytometry T cell distribution in the different organs. Representative staining of two independent experiments is shown.

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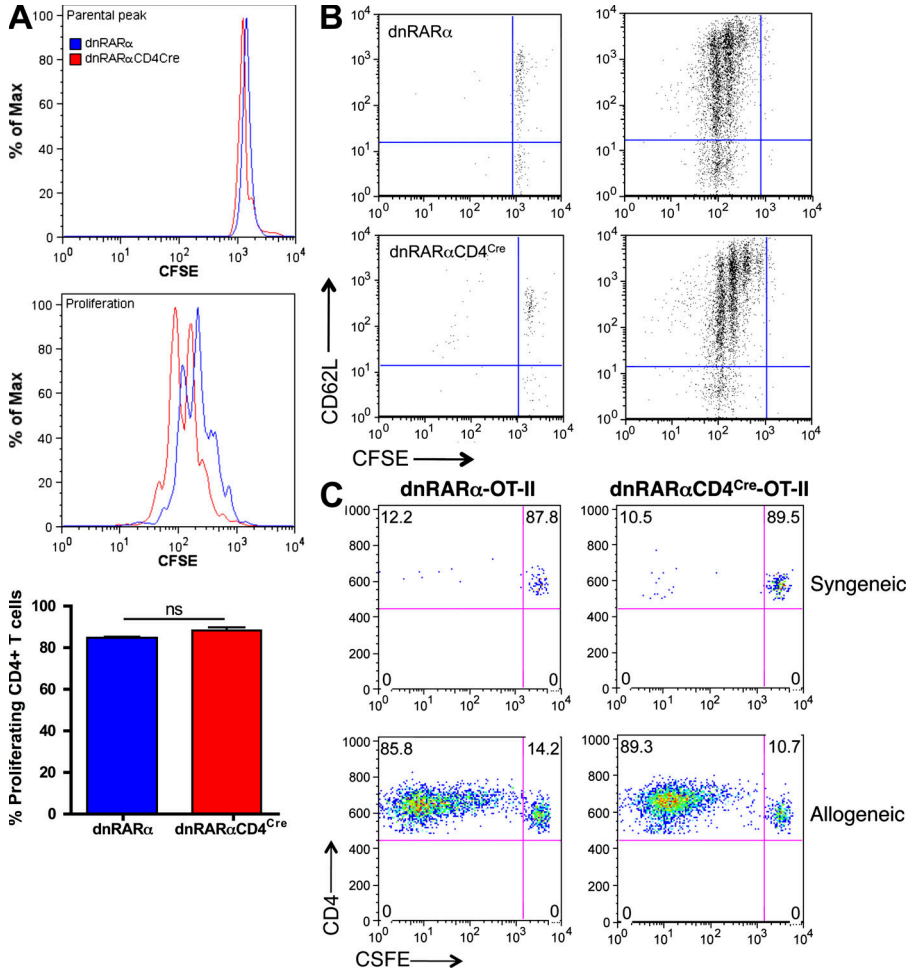


**Figure S2. Overexpression of the dnRAR-α in the T cell compartment does not alter CD4⁺ T cell proliferation in vitro.** Splenic CD4⁺ T cells were isolated from dnRAR-α and dnRAR-αCD4$^{Cre}$ and labeled with CFSE. CFSE-labeled CD4⁺ T cells were plated alone or together with T cell–depleted splenocytes (APC) plus α-CD3 for 3 d. Proliferation was analyzed by flow cytometry. (A) CFSE dilution and percentage of cells proliferating are depicted. Error bars indicate SEM. ns, nonstatistical difference. (B) CFSE dilution and CD62L expression on the CD4⁺ T cell population are shown. (C) For in vivo analysis, C57BL/6 recipient mice received either CFSE-labeled dnRAR-α–OT-II–Ly5.2 or dnRAR-αCD4$^{Cre}$–OT-II–Ly5.2 cells, and the next day, transplantation of B6 or actin-OVA skin grafts was performed. After 5 d, skin dLNs were removed, and CFSE dilution was analyzed by flow cytometry. *n* = 3 mice per group. Representative staining of at least two independent experiments is shown.

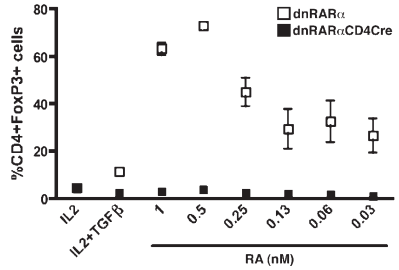


**Figure S3. Disruption of RAR-α signaling on CD4⁺ T cells blocks RA-mediated FoxP3 conversion.** Splenic CD4⁺ T cells isolated from dnRAR-α and dnRAR-αCD4$^{Cre}$ were stimulated polyclonally (α-CD3 plus α-CD28) under the conditions indicated. After 4 d in culture, intracellular staining for FoxP3 was performed, and its expression was analyzed by flow cytometry. Data are representative of two independent experiments. Error bars indicate SEM.

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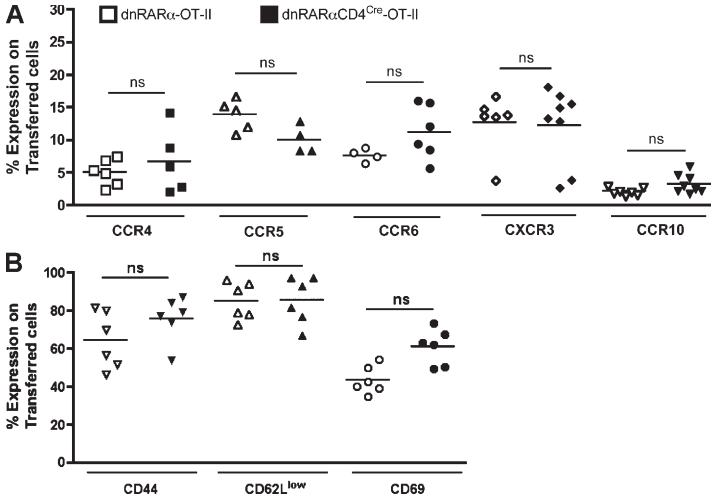


**Figure S4. Disruption of RAR-α signaling on CD4⁺ T cells does not alter the expression of activation markers and chemokine receptors.** (A and B) dnRAR-α–OT-II–Ly5.2 or dnRAR-αCD4$^{Cre}$–OT-II–Ly5.2 cells were transferred into RAG$^{-/-}$ mice, which received syngeneic (B6) or allogeneic (actin-OVA) skin grafts 1 d after T cell transfer. After 7 d, dLNs were removed, and cell suspension was prepared. Cell surface staining for chemokine receptors (A) and activation markers (B) was analyzed by flow cytometry. *n* = 2–4 mice per group. Graphs depict pooled data from two independent experiments. ns, nonstatistical difference.

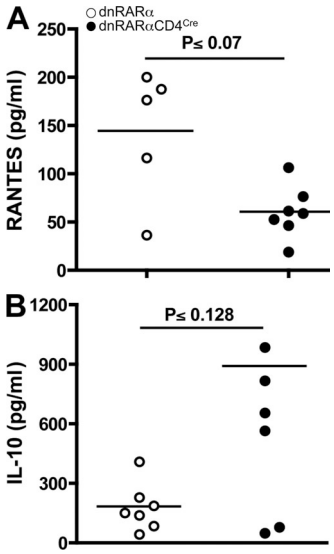


**Figure S5. Disruption of RAR-α signaling on CD4⁺ T cells skews T cell polarity toward the Th2 phenotype.** (A and B) dnRAR-α–OT-II–Ly5.2 (open circles) or dnRAR-αCD4$^{Cre}$–OT-II–Ly5.2 (closed circles) cells were transferred into RAG$^{-/-}$ mice, which received an allogeneic (actin-OVA) skin graft 1 d after T cell transfer. After 7 d, dLN CD4⁺ T cells were isolated and co-cultured with ISQ-pulsed DCs for 3 d. Supernatant was harvested and analyzed for cytokine/chemokine production using Luminex. *n* = 2–3 mice per group. Graphs depict pooled data from three independent experiments.