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Differential Requirements for Th1 and Th17 Responses to a Systemic Self-Antigen

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T cell–APC interactions are essential for the initiation of effector responses against foreign and self-antigens, but the role of these interactions in generating different populations of effector T cells in vivo remains unclear. Using a model of CD4+ T cell responses to a systemic self-antigen without adjuvants or infection, we demonstrate that activation of APCs augments Th17 responses much more than Th1 responses. Recognition of systemic Ag induces tolerance in self-reactive CD4+ T cells, but induction of CD40 signaling, even under tolerogenic conditions, results in a strong, Ag-specific IL-17 response without large numbers of IFN-γ–producing cells. Transfer of the same CD4+ T cells into lymphopenic recipients expressing the self-antigen results in uncontrolled production of IL-17, IFN-γ, and systemic inflammation. If the Ag-specific T cells lack CD40L, production of IL-17 but not IFN-γ is decreased, and the survival time of recipient mice is significantly increased. In addition, transient blockade of the initial MHC class II-dependent T cell–APC interaction results in a greater reduction of IL-17 than of IFN-γ production. These data suggest that Th17 differentiation is more sensitive to T cell interactions with APCs than is the Th1 response, and interrupting this interaction, specifically the CD40 pathway, may be key to controlling Th17-mediated autoimmunity. The Journal of Immunology, 2011, 186: 4668–4673.

Different subsets of effector CD4+ T cells, including Th1 and Th17 cells, play distinct roles in host defense against different types of microbes and in various autoimmune diseases. An important question in the field is what stimuli lead to the induction of these subsets in responses to microbial and self-antigens. Much of the work addressing this question has focused on the role of cytokines produced by APCs, leading to the conclusions that IL-12 initiates the Th1 response, whereas IL-6 and IL-23 are involved in generating Th17 responses (1–3). Additionally, the generation of Th1 and Th17 effector cells has been shown to be dependent on various other parameters including transcription factors, epigenetic modifications, and CD28 costimulation (2–5). There is also evidence for temporal differences in the appearance of Th1 and Th17 cells, with most studies showing that the Th17 response peaks early, followed by the appearance of Th1 cells (6–9). Finally, the development of these subsets is reciprocally regulated, due mainly to the fact that IFN-γ is a powerful inhibitor of Th17 differentiation (3, 10).

Signals from APCs to T cells are integral in shaping the magnitude and nature of T cell responses. The activation status of dendritic cells (DCs) is a critical determinant of the outcome of Ag recognition by T cells (11, 12). Ag presentation by immature DCs often results in tolerance, whereas mature DCs are associated with the priming of T cell populations (11, 13, 14). It is, however, unclear if there are distinct APC requirements for Th1 and Th17 differentiation.

To assess the involvement of DCs in the initiation and propagation of a T cell response in vivo, we exploit a model of systemic Ag recognition in which we can follow the T cell response to a transgene-encoded, endogenous Ag in the absence of extrinsic stimuli such as adjuvants or infections. Using this model, we have previously shown that interaction of DOI1.10 CD4+ T cells with a systemic Ag (soluble OVA; sOVA) in a lymphocyte-sufficient host (soluble OVA transgenic; sOVA-Tg) leads to profound unresponsiveness of the T cells followed by their deletion. Interaction of these Ag-specific T cells with their cognate Ag in a lymphopenic host (sOVA-Tg/Rag-/-) leads to a failure of tolerance, T cell activation, and a systemic inflammatory disease. During the propagation of this inflammatory disease there is uncontrolled activation of self-reactive DOI1.10 cells, resulting in massive proliferation and the production of IFN-γ and IL-17 (7–9). In the absence of B7 expression in the host, there is a failure to initiate an effector response from Ag-specific T cells, implying an essential contribution of B7+ APCs, presumably DCs, in the generation of cytokine-producing cells (15).

Activating or maturing DCs by administering agonistic anti-CD40 Abs has been shown to break tolerance (16), and the presence of CD40 on mature APCs has been noted during the initiation of T cell-dependent autoimmune responses (17, 18). CD40 activation induces IL-12 production on DCs, resulting in the induction of Th1 responses (19–21). The role of CD40 in Th17 responses is less well studied, but data suggest that CD40 is involved in the induction of IL-6 and subsequent IL-17 production (22). Our experimental system allows us to address the requirements for the generation of Ag-specific Th1 and Th17 effector cells within the same host. Using this experimental model, we have asked what happens to the effector response if we manipulate CD40 expression and the early T cell–APC interaction.

In these studies, we demonstrate that DCs have an activated phenotype in mice undergoing an inflammatory reaction. Upon administration of an agonistic anti-CD40 Ab to tolerant sOVA-Tg hosts, tolerance is broken, resulting in a specific increase in IL-17...
production. Conversely, the absence of CD40L on donor DO11.10 T cells leads to a reduction in the amount of IL-17 produced by effector cells and significantly increases the survival time of lymphopenic sOVA-Tg/Rag<sup>−/−</sup> recipients. Notably, transient blockade of MHC class II in recipient mice results in a specific decrease in IL-17 production, indicating that the signals received during the initial T cell–APC interactions are especially important for the Th17 response. These results demonstrate that the activation state of APCs has a much greater impact on Th17 than on Th1 responses and suggest strategies for selectively suppressing the development of pathogenic Th17 cells.

Materials and Methods

Mice

All experimental mice were used at 5–8 wk of age. Wild-type (WT) BALB/c mice were obtained from Charles River Laboratories. Transgenic mice expressing the DO11.10 TCR were obtained from Dr. K. Murphy (Washington University, St. Louis, MO) and were crossed onto a Rag2<sup>−/−</sup> background for use as sources of donor cells for adoptive transfer. sOVA-Tg mice expressing a soluble form of OVA have been described previously (15). sOVA-Tg mice were also used on a Rag2<sup>−/−</sup> background as described (23). CD40L<sup>−/−</sup> mice were obtained from Dr. R. Flavell (Yale University, New Haven, CT) and crossed onto the DO11.10 Rag2<sup>−/−</sup> background. IL-12p40<sup>−/−</sup> mice were obtained from The Jackson Laboratory and crossed onto the sOVA-Tg background. All mice were bred and maintained in a specific pathogen-free facility in accordance with the guidelines of the Laboratory Animal Resource Center of the University of California San Francisco.

Ab treatments and flow cytometry

Fixed and permeabilized cells were prepared for flow cytometric analysis after intracellular cytokine staining as previously described (9). The Abs anti–IL-6 (MP5-20F3) and anti-CD40 (FJK-4) were obtained from the University of California San Francisco Monoclonal Antibody Core. MHC class II IA<sup>β</sup> blocking Ab (MKD6) was purchased from American Type Culture Collection (Manassas, VA). Anti–IL-6, 0.5 mg, was administered i.p. at the time of T cell transfer and every other day posttransfer. A single dose, 250 μg, of anti-CD40 or 1 mg MHC class II blocking Ab was administered i.p. at the time of T cell transfer.

Cell preparations, purifications, culture, and adoptive transfer

CD4<sup>+</sup> T cells for adoptive transfer were purified from spleens and lymph nodes of DO11.10 Rag2<sup>−/−</sup> or CD40L<sup>−/−</sup> DO11.10 Rag2<sup>−/−</sup> mice using the EasySep Mouse CD4<sup>+</sup> T cell enrichment kit (StemCell Technologies). T cells (0.5 × 10<sup>6</sup>) were transferred i.v. into recipient mice. For in vitro proliferation assays, CD4<sup>+</sup> T cells were stimulated on BALB/c mitomycin-treated splenocytes with 1 μg/ml OVA peptide (323–339). T cells (1 × 10<sup>5</sup>) and APCs (9 × 10<sup>5</sup>) were cultured for 48 h with titrated concentrations of MHC class II blocking Ab (MKD6) starting at 1 mg/ml and 0.125 μg/ml well. [3H]Thymidine was added during the final 12 h of culture.

Real-time PCR

CD4<sup>+</sup>KJ-126<sup>+</sup> cells were purified by high-speed cell sorting from recipient mice. Total RNA was extracted (Qiagen) and converted to cDNA using SuperScript III reverse transcriptase (Invitrogen). PCR amplification was performed with SYBR Green master mix (Applied Biosystems). Ct values were normalized to hypoxanthine-guanine phosphoribosyltransferase levels and fold induction shown relative to controls isolated from WT BALB/c mice.

Statistical analysis

All statistical analysis was done using Prism software (GraphPad). The p values were calculated using a two-tailed unpaired t test.

Results

Maturation state of DCs in tolerant and autoimmune mice

To examine the role of DCs in the propagation of an autoimmune response, we first compared the activation status of DCs under conditions of tolerance and autoimmunity. DO11.10 cells were transferred into Ag-expressing mice under tolerogenic (sOVA-Tg recipients) or activating (sOVA-Tg/Rag<sup>−/−</sup> recipients) conditions. CD11c<sup>high</sup> conventional DCs (cDCs) were evaluated by flow cytometry 3 d posttransfer. In sOVA-Tg mice, under tolerogenic conditions, cDCs had similar levels of CD80, CD86, CD40, and MHC class II on their surfaces as did cDCs from WT BALB/c control mice (Fig. 1). In contrast, cDCs from sOVA-Tg/Rag<sup>−/−</sup> recipients had increased levels of all the activation markers assayed (Fig. 1). These data indicate that DCs present during an ongoing autoimmune response have a mature phenotype compared with that of those found during the induction of tolerance and suggest that the DCs may be an initiating factor in the autoimmune reaction.

CD40 activation specifically induces an IL-17 response

To determine if the maturation state, and more specifically the increased expression of CD40 on DCs, contributes to the effector T cell response in vivo, we treated sOVA-Tg recipients with an agonistic anti-CD40 Ab at the time of DO11.10 cell transfer and assessed cytokine production 5 d later. In untreated sOVA-Tg mice, DO11.10 cells failed to produce high levels of cytokines, as previously reported (7, 8, 24) (Fig. 2A, 2B). Upon treatment of sOVA-Tg mice with the anti-CD40 Ab, DO11.10 cells produced small amounts of IFN-γ, but had a marked increase in the percentage of IL-17-producing cells, an even higher amount than that found in CD11c<sup>high</sup> tolerant cDCs from DO11.10 Rag2<sup>−/−</sup> recipients (Fig. 2A, 2B). These data were confirmed at the transcript level, with real-time PCR showing a specific increase in IL-17A and retinoic acid-related orphan receptor γt from DO11.10 cells sorted from sOVA-Tg or sOVA-Tg/IL-12p40<sup>−/−</sup> mice treated with anti-CD40 compared with that of untreated controls (Fig. 2C). Therefore, CD40 activation promoted a significant increase in IL-17 production even under conditions when T cells are normally rendered tolerant. These results suggest a specific role
for CD40 ligation in the initiation of IL-17 production from self-reactive T cells.

**Eliminating CD40L leads to a decrease in IL-17 production**

To determine the importance of the CD40 pathway in promoting cytokine production under conditions of systemic autoimmunity, we transferred WT or CD40L−/− DO11.10 cells into recipient sOVA-Tg/Rag−/− mice, assayed for cytokine production, and monitored disease progression. As shown in previous studies (7–9, 24), DO11.10 cells transferred to sOVA-Tg/Rag−/− mice and restimulated ex vivo produce both IFN-γ and IL-17 (Fig. 3A, 3B). In the absence of CD40–CD40L interactions, IFN-γ production was not significantly altered, whereas IL-17 production was greatly decreased (Fig. 3A, 3B). This decrease in IL-17 production was seen for both single positive (IL-17+) and double positive (IL-17+IFN-γ+) cell populations (Supplemental Fig. 1A). In addition, the absence of CD40L engagement prolonged the survival time of the sOVA-Tg/Rag−/− mice that received DO11.10 cells (Fig. 3C), suggesting that...
the maturation state and expression of the costimulatory molecule CD40 on DCs is integral for the induction of an IL-17–dependent pathologic immune response.

**IL-17 production is IL-6 dependent**

IL-6, TGF-β, and IL-23 are key cytokines in the generation of IL-17–producing effector cells (1). We have shown that IL-23 is not essential for the Th17 response induced by systemic Ag with CD40 activation (Fig. 2). DCs can also produce IL-6 upon activation (25). To determine if IL-17 production in this model of systemic autoimmunity is IL-6 dependent, we transferred DO11.10 cells into sOVA-Tg and sOVA-Tg/Rag−/− mice and adoptively transferred i.v. into sOVA-Tg and sOVA-Tg/Rag−/− recipients. CD4+ T cells were isolated on day 5, restimulated, stained, and analyzed as in Fig. 2. A, Representative flow-cytometric plots of IL-17A and IFN-γ are shown. FACS data are gated on donor CD4+ KJ-126+ DO11.10 cells. B, Cumulative data from three experiments. C, Survival curve of mice after transfer of DO11.10 T cells: CD40L−/−DO11.10 Rag−/− into sOVA-Tg (n = 4), CD40L−/−DO11.10 Rag−/− into sOVA-Tg/Rag−/− (n = 6), and DO11.10 Rag−/− into sOVA-Tg/Rag−/− (n = 5).

**Blocking T cell–APC interactions attenuates IL-17 production by self-reactive T cells**

Because IL-17 production was specifically altered when we targeted CD40, a costimulatory molecule on APCs, we next asked what effect blockade of the interaction of T cells with APCs would have on the production of different effector cytokines. To do this,
we transiently blocked the initial T cell–APC interaction using an anti-IAd MHC class II-specific Ab. To verify that treatment with the anti-MHC class II Ab would inhibit T cell–APC interactions, we cultured DO11.10 cells with APCs and OVA peptide and measured proliferation. There was a dose-dependent decrease in the proliferation of OVA-specific T cells upon addition of the MHC class II blocking Ab (Fig. 5A). We then blocked T cell–APC interactions in vivo by treating sOVA-Tg/Rag2/2 mice with 1 mg of the anti-MHC class II Ab i.p. at the time of DO11.10 cell transfer, and assessed cytokine production 5 d later. A single treatment with the MHC class II blocking Ab resulted in a marked decrease in IL-17 production, while IFN-γ was not consistently altered (Fig. 5B,5C, Supplemental Fig. 1B). These experiments were also performed using an increased dose of MHC class II blocking Ab (5 mg), and even under these conditions IFN-γ production was not markedly reduced (data not shown). These data suggest a specific role for APCs in the generation of IL-17-producing effector cells, one that is not required to generate Th1 effectors.

Discussion

Our data reveal a pivotal role for APC activation in the generation of an IL-17 response during the initiation and propagation of autoimmune responses. T cell–APC interactions occurring during the establishment of uncontrolled CD4+ T cell effector responses were shown to be essential in promoting IL-17 production. Additionally, cells normally rendered tolerant after self-antigen recog-
addition of an agonistic anti-CD40 Ab to sOVA-Tg mice led to a significant increase in IL-17 production from Ag-specific DO11.10 cells while only low levels of IFN-γ were generated. In addition, transferring CD40L-deficient DO11.10 cells to sOVA-Tg/Rag−/− mice led to a specific decrease in IL-17 production. These results suggest a specific role for APC activation in the initiation of IL-17 production from self-reactive T cells.

These data confirm the role of the maturation state of the DC and more specifically CD40 expression as contributing factors to the generation of IL-17–producing cells in an in vivo model of systemic autoimmune disease. The inflammatory reactions studied in this report develop in a lymphopenic environment lacking all endogenous T and B cells. This reductionist approach has allowed us to clearly dissect the requirements for development of different self-reactive effector cell populations in vivo. Importantly, the finding that the agonistic anti-CD40 Ab preferentially promotes IL-17 production even in lymphocyte-replete mice (sOVA-Tg; Fig. 2) indicates that the role of CD40 in the Th17 response is not restricted to the lymphopenic situation.

Upon administration of a MHC class II blocking Ab, again, there is a specific decrease in IL-17 production (Fig. 5). These results suggest that the interaction between self-reactive CD4+ T cells and APCs presenting cognate Ag is especially important for IL-17 autoimmune reaction, resulting in less effector cytokine production due to the importance of the APC in the initiation and propagation of the Th17 response. These results demonstrate the key role of CD40–CD40L interactions, and more broadly the T cell–APC interactions, as these responses are greatly reduced if the amplification of Th17 responses is prohibited.

The differential role of the CD40 pathway is consistent with previous studies demonstrating that T cells, upon initial activation by APCs, produce IFN-γ and IL-12, which in turn educate the T cell to differentiate into either a Th1 or Th2 phenotype. The Th17 phenotype is not restricted to the lymphopenic situation.

Importantly, the finding that the Th17 response is not restricted to the lymphopenic situation. Upon administration of a MHC class II blocking Ab, again, there is a specific decrease in IL-17 production (Fig. 5). These results suggest that the interaction between self-reactive CD4+ T cells and APCs presenting cognate Ag is especially important for IL-17 production during systemic autoimmune disease. It is surprising that binding class II MHC has a modest and inconsistent effect on IFN-γ production, as all CD4+ T cell responses require the recognition of peptides displayed by class II molecules. A plausible explanation is that the class-II Ab as administered is an incomplete blocker of the initial T cell–APC interaction, which is clearly enough to largely abrogate the Th17 response but not the Th1 response. It may be that cytokines produced by APCs, such as IL-12, are able to compensate for the weak initial T cell–APC interactions and drive Th1 differentiation even in the presence of class II MHC blockade.

Our data draw the unexpected conclusion that the Th17 response is much more dependent on T cell–APC interactions and CD40-mediated APC activation than is the Th1 response. This direct comparison was possible because in the experimental model we have established, both Th1 and Th17 responses occur in the same T cell population after Ag recognition without the need for extrinsic stimuli such as adjuvants, resulting in an unbiased effector response. The differential role of the CD40 pathway is consistent with the greater dependence of Th17 responses on T cell–APC interactions, as these responses are greatly reduced if the amplification function of CD40 is eliminated. Our results suggest that CD40–CD40L interactions, and more broadly the T cell–APC interaction, may be much more important for Th17- than for Th1-dependent T cell-mediated inflammatory disorders.

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Disclosures
The authors have no financial conflicts of interest.

References
Supplemental Figure 1: Decreased IL-17 production from double positive IL-17^IFN\_\gamma^ cells.

A) 0.5x10^6 CD4^+ T cells were purified from WT or CD40L^−/− DO11.10 Rag^−/− mice and adoptively transferred i.v. into sOVA-Tg/Rag^−/− recipients. B) 0.5x10^6 CD4^+ T cells were purified from WT DO11.10 Rag^−/− mice and adoptively transferred i.v. into sOVA-Tg/Rag^−/− recipients. Mice were treated with or without 1 mg of MHC Class II blocking antibody i.p. CD4^+ T cells were isolated on day 5, re-stimulated, stained, and analyzed as in Fig. 2. Representative flow-cytometric plots of IL-17A and IFN\_\gamma are shown. FACS data are gated on donor CD4^+ KJ-126^+ DO11.10 cells.