

Cutting Edge: Th2 Response Induction by Dendritic Cells: A Role for CD40¹

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We investigated the influence of dendritic cell (DC) CD40 expression on Th2 and Th1 development by in vivo transfer of Ag-pulsed bone marrow-derived DC generated from wild-type (WT) or CD40^{-/-} mice. Contrary to expectation, CD40^{-/-} DC primed with Ag that inherently induce a Th2 response (soluble egg Ag from *Schistosoma mansoni*) failed to induce a Th2 response or any compensatory Th1 response, whereas CD40^{-/-} DC primed with Ag that inherently induce a Th1 response (*Propionibacterium acnes*) generated a competent Th1 response. Thus, DC expression of CD40 is a prerequisite for initiation of Th2, but not Th1, responses by these Ag. Consistent with this, CD154^{-/-} mice, unlike WT mice, failed to mount a Th2 response when directly injected with schistosome eggs but mounted a normal Th1 response after challenge with *P. acnes*. CD40-CD154 interaction can therefore play a major role in Th2 response induction. *The Journal of Immunology*, 2002, 168: 537–540.

Dendritic cells (DC)⁴ possess the ability to potently activate T cells, but the mechanisms underlying this process remain an issue of some contention (1–3). The current understanding is that a series of defined signals must be delivered to naive T cells to initiate the adaptive immune response. Of key importance in the process of Ag presentation via MHC class II to CD4⁺ Th cells (signal 1) is the supply of appropriate costimulation (signal 2). Other facets of this interaction that may subsequently influence T cell effector class (signal 3) are less well defined (4).

In comparison with the increasing awareness of the mechanisms by which inflammatory pathogens such as bacteria, protozoa, or viruses activate DC to drive Th1 responses (5), little is known

about how DC interpret pathogen-inherent information to induce Th2 responses. Available data suggest that DC respond to Th2-polarizing pathogens with few of the activation-associated changes that accompany exposure to Th1-driving pathogens (6, 7). Despite this, DC primed with *Schistosoma mansoni* egg Ag (SEA), an inherently Th2 response-inducing Ag, capably induce Th2 responses both in vivo and in vitro (7) via a mechanism that depends on MHC class II expression by the Ag-pulsed DC.

A pivotal role in the activation and function of DC is thought to be played by the CD40-CD154 partnership (8, 9). DC that have been exposed to proinflammatory Th1 Ag display elevated levels of expression of CD40 (10), and increased ability to produce IL-12 in response to CD40 engagement (7, 11). Further, DC IL-12 production has been established as being contributory to Th1 response development (12, 13), and the ability of DC to produce IL-12 in response to certain pathogens, and then again during communication with T cells via CD40-CD154 interaction, provides an attractive mechanism for the augmentation of the developing Th1 response (14–16). Consistent with this, several experimental systems have shown the importance of CD40-CD154 for Th1 response development during infection (17), but the role of these molecules in Th2 response development is much less clear.

To analyze the role of CD40-CD154 in the induction of Th responses by DC, we transferred murine bone marrow-derived wild-type (WT) and CD40^{-/-} DC that had been primed with Ag that induce strong Th2 or Th1 responses (SEA and *Propionibacterium acnes*, respectively) into WT recipient mice. Our data show that, unexpectedly, CD40 plays a fundamental role in DC-driven Th2, but not Th1, response development in vivo.

Materials and Methods

Animals and reagents

WT C57BL/6 mice were purchased from Taconic Farms (Germantown, NY), and CD40-deficient and CD154-deficient B6 mice were from The Jackson Laboratory (Bar Harbor, ME). For each experiment, mice were age and sex matched. For a Th2 Ag, we used *S. mansoni* eggs and SEA, which are highly effective Th2 inducers (18–20). Endotoxin-free SEA was prepared as previously described (7, 21, 22). For a Th1 stimulus, we used heat-killed *P. acnes* (a Gram-positive bacterium previously known as *Corynebacterium parvum*) (23), a stock of which was kindly provided by the Trudeau Institute (Saranac Lake, NY).

DC generation

DC were generated from bone marrow cultured in the presence of GM-CSF (PeproTech, Rocky Hill, NJ) for 11 days as previously described (7, 24). Cultured cells comprised 95% DC (class II⁺CD11c⁺CD8⁻), with the remainder of the cells being predominantly granulocytes. No contaminating B cells, macrophages, CD4 or CD8 T cells, or CD8⁺ DC were generated under these conditions, as determined by FACS using mAbs specific for B220, F4/80, CD4, and CD8 α (not shown). For activation of DC, cells

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⁴ Abbreviations used in this paper: DC, dendritic cells; WT, wild type; SEA, soluble egg Ag from *Schistosoma mansoni*; LN, lymph nodes; o/n, overnight.

were pulsed with the appropriate Ag (50 $\mu\text{g}/\text{ml}$ SEA, or 5 $\mu\text{g}/\text{ml}$ *P. acnes*) for the final 18 h of incubation (7).

Determination of DC activation state and priming ability

Expression of surface molecules on DC was quantified by flow cytometry using FITC- or PE-conjugated Ab (I-A^b, B220, CD4, CD8 α , CD11c, CD80, CD86), all purchased from BD PharMingen (San Diego, CA). Samples were analyzed using a FACSCaliber flow cytometer and CellQuest software (BD Biosciences, Franklin Lakes, NJ). Cytokine ELISA were performed on culture supernatants using paired mAb purchased from BD PharMingen or purified from hybridoma supernatants in our laboratory. For *in vivo* transfer experiments, mice were injected i.p. with 5×10^5 DC or DC that had been pulsed overnight (o/n) with SEA or *P. acnes* (as above). After 7 day, spleens were removed aseptically, and splenocytes (2×10^6) incubated in DMEM, 2 mM L-glutamine (Life Technologies, Gaithersburg, MD), 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin (Life Technologies), 50 μM 2-ME (Sigma, St. Louis, MO), and 3% normal mouse serum (Cedarlane Laboratories, Hornby, Ontario, Canada), alone or with SEA (50 $\mu\text{g}/\text{ml}$), *P. acnes* (5 $\mu\text{g}/\text{ml}$), or mAb anti-CD3 (0.5 $\mu\text{g}/\text{well}$, plate bound; BD PharMingen). For direct *in vivo* administration of Ag, WT or CD154^{-/-} mice were injected in each hind footpad with 2500 *S. mansoni* eggs or 50 μg *P. acnes*, in 50 μl sterile PBS. Popliteal lymph nodes (LN) were removed 1 wk later from immunized animals, single-cell suspensions were prepared, and cells (5×10^5) were stimulated with SEA, *P. acnes* or plate-bound anti-CD3 as above. Cytokine levels in 18-h DC supernatants or 24-h (IL-2) or 72-h splenocyte or LN supernatants were measured by ELISA.

Statistical analysis

Student's *t* test was used to determine the statistical significance between groups. *p* < 0.05 was considered to be a significant difference.

Results

Activation of murine DC from WT and CD40^{-/-} mice with Th2 or Th1 Ag

To investigate the role of DC CD40 expression in induction of Th2 and Th1 responses, murine bone marrow-derived DC from WT or CD40^{-/-} mice were cultured overnight in medium alone or in medium with SEA or *P. acnes*, and their resultant activation states were assessed using flow cytometry (Fig. 1, A and B) and ELISA (Fig. 1C). Consistent with previously published observations, continuous culture of murine bone marrow cells for 11 days with medium and GM-CSF resulted in the generation of a mixed population of immature DC (MHC II^{low}CD11c⁺) together with mature DC (MHC II^{high}CD11c⁺). DC generated from either WT or CD40^{-/-} mice displayed low level expression of MHC class II, CD80, CD86, and CD40, none of which was significantly up-regulated after exposure to SEA (Fig. 1, A and B). Additionally, SEA did not induce DC IL-12 production (Fig. 1C). In contrast, exposure of DC generated from either genotype to *P. acnes* resulted in DC activation. This was evident by the increased proportion of MHC II^{high} CD11c⁺ cells present after *P. acnes* treatment (Fig. 1A); increased expression of CD80, CD86, and (for WT DC) CD40 (Fig. 1B) on DC; and induction of IL-12 production (Fig. 1C). These data underscore the ability of DC to respond differently to Th2 or Th1 Ag and indicate that DC generated from either WT or CD40^{-/-} mice exhibit similar activation characteristics both before and after exposure to Ag. They also demonstrate that endogenous expression of CD40 is not required for appropriate development of murine DC from bone marrow precursors *in vitro* and that cells thus generated are as receptive as WT DC to stimulation with the Th1-inducing Ag *P. acnes*.

CD40 expression is vital for Th2, but not Th1, induction by DC

Consistent with previous work (7), transfer of SEA-pulsed WT DC i.p. into WT mice induced a strong Ag-specific Th2 response, whereas transfer of *P. acnes*-pulsed DC induced a strong Ag-specific Th1 response (Fig. 2). Splenocytes from mice injected with SEA-pulsed DC made IL-4, IL-5, and IL-13 but no IFN- γ in re-

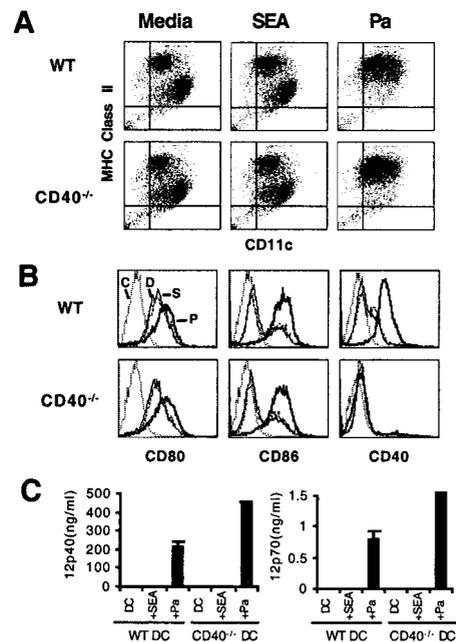


FIGURE 1. Phenotype of WT and CD40^{-/-} DC. A, Cell surface expression of MHC class II and CD11c; B, CD80, CD86, and CD40; C, levels of IL-12 produced by WT or CD40^{-/-} DC either unstimulated (media) or stimulated o/n with 50 $\mu\text{g}/\text{ml}$ SEA or 5 $\mu\text{g}/\text{ml}$ *P. acnes* (Pa). For dot plots, control Ab staining is indicated by the quadrant boundaries. For histograms, data are presented as fluorescent intensity of staining vs cell number. Curve C, Negative control staining; curve D, DC alone; curve S, DC plus SEA; curve P, DC plus *P. acnes*. IL-12 production was measured by ELISA in culture supernatants from DC that had been stimulated o/n with medium, SEA, or *P. acnes* before supernatant harvest.

sponse to stimulation with SEA, whereas cells from mice that had been injected with *P. acnes*-pulsed DC made IFN- γ but no IL-4, IL-5 or IL-13 in response to stimulation with *P. acnes* (Fig. 2). Immature DC generated for this study expressed low but detectable levels of CD40, and elevation of these levels occurred after DC exposure to *P. acnes* but not SEA (Fig. 1B). Further, DC primed with *P. acnes*, but not SEA, become receptive to a second round of stimulation via CD40 to produce substantial amounts of IL-12 (7). On the basis of these data, we expected that CD40^{-/-} DC that had been pulsed with *P. acnes* would exhibit a reduced ability to initiate a Th1 response *in vivo*. Contrary to expectation, this proved not to be the case, with splenocytes from mice injected with *P. acnes*-pulsed CD40^{-/-} DC producing levels of IFN- γ on restimulation *in vitro* that were similar to those made by splenocytes from mice injected with *P. acnes*-pulsed WT DC (Fig. 2). Most surprisingly, however, given that SEA-pulsed DC failed to exhibit any major changes in CD40 expression over immature DC, mice that had received SEA-pulsed CD40^{-/-} DC failed to mount a measurable Th2 response (Fig. 2), as well as failing to produce significant amounts of IL-2 in comparison to mice that had received SEA-pulsed WT DC (84 ± 18 pg/ml as compared with 720 ± 295 pg/ml).

CD40-CD154 interaction is required for Th2, but not Th1, response development *in vivo*

Unexpectedly, our data supported an important role for DC CD40 in Th2, but not Th1, response development. To test this hypothesis formally, we compared Th2 and Th1 response development in WT vs CD154^{-/-} mice injected in the footpad with *S. mansoni* eggs or *P. acnes*. WT PLN cells isolated 1 wk after injection of *S. mansoni* eggs made substantial amounts of SEA-specific IL-4, IL-5, and

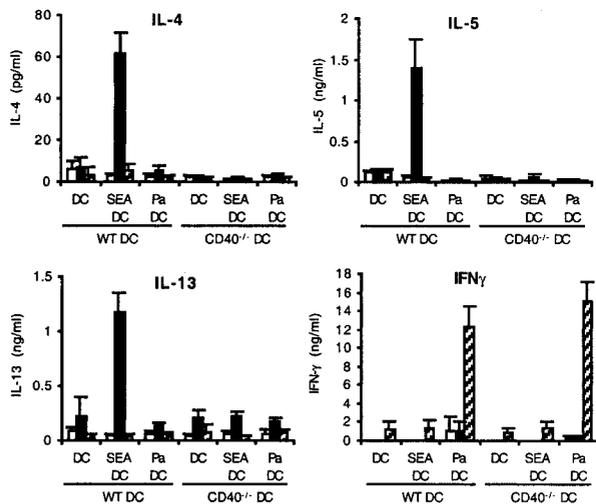


FIGURE 2. T cell polarizing ability of WT and CD40^{-/-} DC in vivo. Cytokine production as measured by ELISA in culture supernatants from spleen cells taken from C57BL/6 mice that had been injected i.p. 7 days previously with DC generated from WT or CD40^{-/-} mice that were unstimulated, SEA pulsed, or *P. acnes* (Pa) pulsed. Splenocytes were then stimulated in vitro with medium (□), SEA (■) or *P. acnes* (▨) for 72 h before supernatant harvest. Data are mean ± SD of triplicate wells from three to five mice per group that were individually assayed.

IL-13 on stimulation with SEA in vitro, whereas CD154^{-/-} mice failed to make measurable amounts of any of these mediators (Fig. 3). In contrast to this, PLN cells isolated from both WT and CD154^{-/-} animals that had been injected with *P. acnes* made significant amounts of IFN-γ on stimulation with *P. acnes* in vitro. The same pattern of cytokine production was evident after stimulation of PLN cells with anti-CD3 (not shown). Thus, consistent with our observations with transfer of differentially activated DC, animals that lacked CD154, and therefore lacked CD40-CD154 interaction, failed to mount a Th2 response but were able to mount a capable Th1 response in vivo.

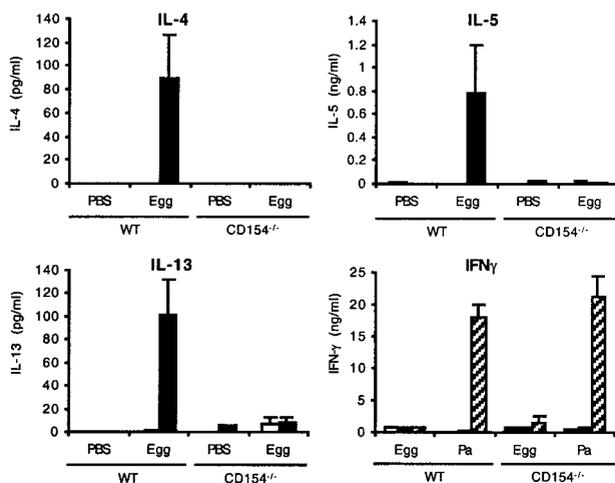


FIGURE 3. CD40-CD154 interaction is required for Th2 but not Th1 development in vivo. Cytokine production as measured by ELISA in culture supernatants from popliteal LN cells taken from WT or CD154^{-/-} mice that had been injected in the footpad 7 days previously with PBS, *S. mansoni* eggs, or *P. acnes* (Pa). Cells were then stimulated in vitro with medium (□), SEA (■), or *P. acnes* (▨) for 72 h before supernatant harvest. Data are mean ± SD of triplicate wells from three to five mice per group that were individually assayed.

Discussion

Here we describe the novel finding that the CD40-CD154 receptor-ligand pair is required for initiation of the immune response to Ag that inherently induce very strong Th2 responses. This is illustrated by the lack of Ag-specific immunological responsiveness in WT mice injected with SEA-pulsed CD40^{-/-} DC, and in schistosome egg-injected CD154^{-/-} mice. In marked contrast, use of *P. acnes* as Ag in these types of experiment led to the development of strong Th1 responses in the absence of CD40-CD154 interaction.

Although a wide range of cell types can express CD154 (8, 9), the assumption is that DC CD40 primarily encounters CD154 on T cells and that it is this interaction that stimulates further activation of DC via CD40. Because we have previously shown in transfer experiments of the type described here that DC-driven Th2 and Th1 responses also depend on MHC class II expression by the injected DC (7), it seems likely that CD4 T cells are the important CD154-bearing candidate cell. Importantly, this class II dependence also suggests that Ag is being presented by the transferred DC and not by resident cells that acquire Ag from these DC, although we have not formally ruled out the possibility that class II/peptide complexes may be transferred from injected to recipient DC (25, 26). Another possibility is that the CD40-CD154 interaction may be a two-way conversation, with direct T cell activation occurring via CD154. Indeed, T cell stimulation via CD154 'ligation' has previously been implicated in Th2 development (27). It has previously been shown that coinjection of anti-CD40 Ab with *S. mansoni* eggs results in development of a drastically reduced Th2 response, with the proposed mechanism being inhibition of Th2 development via CD40-driven production of proinflammatory cytokines (28). Re-evaluation of this study in light of our results using egg injection of CD154^{-/-} mice suggests that the loss of Th2 cytokines observed after anti-CD40 Ab treatment may have been primarily due to inhibition of CD154 ligation, rather than stimulation of APC via CD40.

Our data indicate that, irrespective of any role played by the growing range of costimulatory molecules such as CD80, CD86, OX-40L, DC-SIGN, or LIGHT (29–32), the absence of CD40 alone negates the ability of DC to induce a response to SEA. The implication of this is that expression or function of other costimulatory molecules, which may well be important for induction of the immune response by DC, occurs downstream to events that are implicitly reliant on DC CD40. These data, therefore, help establish a hierarchy of importance for costimulation in Th2 response induction by DC, with CD40 expression being key.

The current view of CD40 function is that its ligation on DC via CD154 elicits expression of IL-12, production of which is essential for Th1 response induction (15, 33). Our data partially contradict this prevailing view and suggest that expression of CD40 by DC is not necessary for Th1 induction by DC that have undergone classical activation by proinflammatory Th1 Ag. This finding is particularly surprising given that DC that have been exposed to such Ag display elevated levels of CD40 and become receptive to further stimulation via CD40 to produce IL-12 (7, 11). Our data indicate that even in the absence of DC CD40, and therefore the absence of the ability of DC to produce additional IL-12 on encountering CD154, a Th1 response can still be efficiently initiated. Supportive of these data, CD154-independent IL-12 production can be seen after systemic challenge with Ag from the protozoan parasite *Toxoplasma gondii* (34). Because DC have been reported to produce a range of proinflammatory cytokines including IL-18, IFN-γ, and TNF-α on activation with Th1 Ag, it is possible that a combination of production of such cytokines, together with elevated expression of costimulatory and adhesion molecules, compensates in the absence of further activation via CD40 to effect Th1 response induction. Preliminary work in our laboratory suggests

that DC activation is related to *P. acnes* concentration. Therefore, we expect that at low Pa concentrations where DC are not overtly activated, CD40 might play a more important role.

It is unlikely that Th2 induction in all cases is solely dependent on DC CD40 expression. In transfer of DC, alternative mechanisms of response induction usually initiated by pathogens during active infection are likely bypassed. However, the fact that CD154^{-/-} animals failed to generate a Th2 response after in vivo injection of schistosome eggs suggests that this interaction is physiologically relevant. In comparison with the case for Th2 development, CD40-CD154 interaction does not seem to be a requirement for Th1 induction by DC. It is important to note, however, that a marked heterogeneity is seen in the requirement for CD40-CD154 for appropriate immune response development, depending on the nature of the pathogen that is examined (17). It is conceivable that the requirement for this interaction depends on the outcome of DC activation by a particular pathogen. For example, DC infected in vitro (35) or in vivo (36) with *Leishmania* spp. exhibit a low activation phenotype and produce IL-12 in a CD154-dependent manner (36). Coincidentally, mice deficient in either CD40 or CD154 fail to control active leishmanial infection (37–39). In contrast to these complex proinflammatory eukaryotic pathogens, DC exposed to bacterial Ag become classically activated. In cases of this type, e.g., infection with *Mycobacterium tuberculosis* (40) or *Listeria monocytogenes* (17), deficiency in CD154 exhibits no major effect. Thus, reliance on DC CD40 for appropriate immune response development could depend on the activation status of DC after pathogen exposure and may not be restricted to Th2 development.

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