

# A Pivotal Role for CD40-Mediated IL-6 Production by Dendritic Cells during IL-17 Induction In Vivo<sup>1</sup>

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The costimulatory requirements for Th17 development remain to be defined. In this study, we show that CD40-deficient animals immunized with the Gram-positive bacterium *Propionibacterium acnes* were specifically impaired in their ability to mount an IL-17 response, but not that of IFN- $\gamma$ . The same cytokine imbalance resulted from in vivo priming with pathogen-pulsed, CD40-deficient dendritic cells (DC). Engagement of CD40 on *P. acnes*-conditioned DC stimulated the release of IL-12, IL-23, and IL-6, of which IL-6 alone proved essential for Th17 differentiation. Compared with wild-type DC, priming with those lacking expression of CD40 resulted in reduced disease severity during experimental autoimmune encephalomyelitis, coincident with reduced IL-17 production. Our data delineate sequential requirements for DC expression of CD40 and production of IL-6 during Th17 polarization in vitro and in vivo, and reveal distinct costimulatory requirements for Th1 vs Th17 generation. *The Journal of Immunology*, 2009, 182: 2808–2815.

The importance of the IL-17 family of cytokines in both autoimmunity and infection is becoming increasingly apparent. CD4<sup>+</sup> cells secreting IL-17A have recently been identified as a distinct subset of helper T cells, named Th17 and distinguished from both Th1 and Th2 cells by cytokine profile, transcriptional regulation, and mutually inhibitory development (1–3). Their autoaggressive action in mouse models of multiple sclerosis (4) and rheumatoid arthritis (5) offers an explanation for the fact that these diseases, initially considered to be Th1-mediated, show exaggerated pathology in mice lacking IFN- $\gamma$ , the hallmark of a Th1 response. A radical reassessment of T cell immunology has ensued, with IL-17 now a feature of allergy and infection in addition to autoimmune disease (6). An understanding of the molecular events that determine the Th17 response therefore holds significant therapeutic promise.

The emergence of Th17 cells was first associated with IL-23, originally thought to act in an analogous way to that by which IL-12 reinforces IFN- $\gamma$  production in Th1 cells (7). A number of reports have also described a critical role for TGF- $\beta$  and IL-6 in the Th17 induction process (8–10), and the current understanding is that TGF- $\beta$  and IL-6 together instruct naive T cells to become IL-17-producing effectors. This early differentiation provides IL-23 receptor on the T cell surface, allowing IL-23 to maintain and expand the developing Th17 population (9) in a process reinforced by IL-21 (11–13). The involvement of TGF- $\beta$  in both Th17 and regulatory T cell development emphasizes its duplicity of

function, able to promote both pro- and anti-inflammatory responses. The presence of IL-6 may thus be decisive (14). The source of these cytokines during IL-17 induction, and the factors that trigger their release, remain uncertain. TGF- $\beta$  can be replaced with CD25<sup>+</sup> Treg cells in vitro with equivalent efficacy (8) and, while many cell types are able to contribute TGF- $\beta$  in vivo, T cell-derived cytokine can be sufficient (15). IL-6 may (16) or may not (17) be produced by Th17 cells themselves but, at least in the initial priming of IL-17-secreting T cells, an APC source of IL-6 seems likely (8).

Of the professional APC, dendritic cells (DC)<sup>5</sup> are uniquely able to stimulate naive T cells (18). The size and the quality of the ensuing immune response is directed by DC provision of MHC: peptide (“signal 1”), costimulation (“signal 2”), and cytokine instruction (“signal 3”), each one determined by the nature of the pathogen or other activation stimulus that the DC encounters (19). Signal 3 is often regarded as the single cytokine IL-12, released by DC in response to CD40 ligation and acting directly and indirectly on T cells to promote Th1 differentiation (20). IL-12 is also secreted by DC in a CD40-independent manner, however (21, 22), and DC expression of CD40 is not always required for Th1 development in vivo (23). As models of effector cell polarization extend to include the Th17 lineage, the relationship between this key costimulatory molecule, the cytokine production it elicits, and the T cell response that results still remains to be clarified.

To examine the role of costimulatory molecules in the differentiation of IL-17 secretors, in this study we use pathogen-conditioned DC to prime both Th1 and Th17 responses in vitro and in vivo. We show that CD40 plays a pivotal role in Th17 induction, and that it acts to trigger IL-6 release from DC. In contrast to wild-type (WT) DC, and coincident with their impaired ability to elicit IL-17, CD40<sup>-/-</sup> DC do not exacerbate pathology in autoimmune encephalomyelitis. Together, these data demonstrate an

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<sup>5</sup> Abbreviations used in this paper: DC, dendritic cell; WT, wild type; Pa, a heat-killed preparation of *Propionibacterium acnes*; St, a heat-killed preparation of *Salmonella typhimurium*; MOG, myelin oligodendrocyte glycoprotein; EAE, experimental autoimmune encephalomyelitis.

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essential requirement for CD40-driven DC as the source of IL-6 in Th17 induction *in vivo*.

## Materials and Methods

### Mice

C57BL/6, CD40<sup>-/-</sup>, p40<sup>-/-</sup>, OT-II, OT-IIxCD154<sup>-/-</sup>, and 2D2 mice were bred and maintained under specific pathogen-free conditions in the animal facilities of the School of Biological Sciences at the University of Edinburgh. *p35<sup>-/-</sup>* and *IL-6<sup>-/-</sup>* bone marrows were provided by Dr. Helena Helmby and by Profs. Manfred Kopf and Richard Grencis, respectively. Animals were used at 6–12 wk of age and were age- and sex-matched within each experiment. All experiments were approved by a Project License granted by the Home Office (U.K.) and conducted in accordance with local guidelines.

### Ags and Abs

*Propionibacterium acnes* (Pa) was obtained from American Type Culture Collection (6919) and *Salmonella typhimurium* (St) was provided by Dr. Maurice Gallagher (University of Edinburgh, Edinburgh, Scotland). Both were heat killed by incubation at 80°C for 30 min. The agonistic anti-CD40 Ab (clone FGK.45), its isotype control Ab (clone Mac-1), and agonistic Abs against CD3 (clone 145.2C11) and CD28 (clone 37.51) were produced in house.

### DC culture

DC were generated in the presence of recombinant GM-CSF (PeproTech) for 11 days, as previously described (23). Cells were >95% CD11c<sup>+</sup> MHC class II<sup>+</sup> DC, with the remainder predominantly Gr1<sup>+</sup> granulocytes. To activate the DC, cells were harvested on day 10 of culture and replated at  $2 \times 10^6$  DC/ml for 18 h in the presence of either Pa or St, both used at 10 µg/ml as measured by the Coomassie Plus Protein Assay (Perbio Science). To assess any further activation triggered by the ligation of CD40, DC were harvested after bacterial stimulation, washed, and replated at  $2 \times 10^6$  DC/ml for a further 24 h in the presence or absence of an agonistic anti-CD40 Ab or an isotype control.

### Assessment of DC activation

The expression of surface molecules on DC was assessed by flow cytometry using Abs against MHC class II (clone M5114, purified in house), CD11c, and either CD40 or CD86 (BD Pharmingen). Samples were acquired on a FACSCalibur flow cytometer and analyzed using FlowJo software (Tree Star), gating on live, CD11c<sup>+</sup> cells. Cytokine ELISAs were performed on culture supernatants using paired mAb purified in house or purchased from BD Pharmingen, and recombinant cytokine standards purchased from PeproTech or BD Pharmingen. TNF-α was measured using the DuoSet ELISA kit from R&D Systems. TGF-β was measured by bioassay.

### TGF-β bioassay

TGF-β was measured using the mink lung epithelial cell luciferase reporter bioassay, where levels of TGF-β signaling are detected by a firefly luciferase reporter gene stably transfected into mink lung epithelial cells under the control of the plasminogen activator inhibitor-1 promoter (courtesy of Daniel B. Rifkin, Department of Cell Biology, New York University Medical Center, New York, NY) (24). Cells were allowed to adhere to 96-well white fluorometer plates (BMG Biotechnologies) at  $1.6 \times 10^4$  cells/well in the presence of 200 µg/ml G418 (Invitrogen), at 37°C for 4 h. Samples were either added neat, to measure endogenous TGF-β, or first heat-activated at 80°C for 10 min and then diluted 10-fold in medium, to measure latent TGF-β levels. Samples and standards (recombinant human TGF-β; R&D Systems) were added and incubated at 37°C overnight. One hundred µl/well Bright-glo luciferase substrate (Promega) was then added and the cells were allowed to lyse for 2 min at room temperature, before reading by Luminometer (BMG Biotechnologies).

### In vivo priming

Mice were injected either *s.c.* with 25 µg of heat killed Pa or St, or *i.p.* with  $5 \times 10^5$  DC that had been cultured for 18 h in the presence of Pa, St, or medium alone. Seven days later, spleens were removed and  $2 \times 10^6$  splenocytes plated per well of a 96-well plate in X-Vivo 15 serum-free medium (Lonza Walkersville) supplemented with 2 mM L-glutamine (Life Technologies) and 50 µM 2-ME (Sigma-Aldrich). Pa and St were added to give a final concentration of 1 µg/ml. Culture supernatants were taken at 72 h to assess their cytokine content by ELISA.

To measure the *in vivo* response of CD4<sup>+</sup> T cells, splenocytes taken from DC-immunized mice on day 7 were positively selected for CD4<sup>+</sup> cells by autoMacs (Miltenyi Biotec). Resulting cells were either cultured with irradiated splenocytes at a ratio of 1 CD4<sup>+</sup> cell to 10 APC, or with plate-bound anti-CD3 (0.5 µg/well) and soluble anti-CD28 (5 µg/ml). Culture supernatants were then taken at 72 h to assess their cytokine content by ELISA.

### In vitro DC:T cell coculture

DC harvested on day 10 and replated in the presence and absence of Pa were exposed to peptide Ag during the same 18 h incubation. In OT-II experiments, the peptide was OVA 323-ISQAVHAAHAEINEAGR-339 (OVA<sub>323-339</sub>; Albachem); in 2D2 experiments, the peptide was myelin oligodendrocyte glycoprotein 35-MEVGWYRSPFSRVVHLYRNGK-55 (myelin oligodendrocyte glycoprotein (MOG)<sub>35-55</sub>; Advanced Biotechnology Centre, Imperial College London). DC were washed and plated with CD4<sup>+</sup> cells at a ratio of 1 DC to 10 CD4<sup>+</sup> cells, using CD4<sup>+</sup> cells purified from pooled spleen and lymph nodes by MACS positive selection (Miltenyi Biotec). CD4<sup>+</sup> cells were routinely >95% CD4<sup>+</sup>, culture medium was as for DC but without GM-CSF, and 2-ME was added to 50 mM (Merck). Cytokine production was measured in supernatants collected at 72 h, assessed by ELISA. To assess cytokine production by intracellular cytokine staining, PMA (50 ng/ml), ionomycin (1 µg/ml), and brefeldin A were added to cultures on day 3. After 5 h, cells were harvested, permeabilized with PBS containing 2% FCS and 0.1% saponin, then stained with Abs against IFN-γ and IL-17 (BD Pharmingen) before surface staining for CD4, fixing and acquisition (as above). Samples were analyzed using forward and side scatter characteristics to gate on live cells.

### Induction and assessment of experimental autoimmune encephalomyelitis (EAE)

C57BL/6 mice were immunized with 100 µg MOG<sub>35-55</sub> peptide in a total volume of 100 µl CFA to induce EAE disease, injecting 50 µl subcutaneously into each hind leg. Mice also received 200 ng pertussis toxin (Health Protection Agency, Porton Down, U.K.) *i.p.* on the same day and 2 days later. Clinical signs of EAE were assessed daily using a discrete scoring system: 0, no signs; 1, flaccid tail; 2, impaired righting reflex and/or impaired gait; 3, partial hind limb paralysis; 4, total hind limb paralysis; 5, hind and fore leg paralysis; 6, moribund or dead. When given,  $5 \times 10^5$  DC were delivered *s.c.* 7 days before disease induction. To assess the cytokines associated with disease, lymph nodes draining the site of *s.c.* immunization were harvested 15 days after disease induction, the time at which clinical signs were most severe, and their cells cultured in the presence of graded doses of MOG<sub>35-55</sub>. Supernatants were collected at 72 h and their cytokine content measured by ELISA.

### Statistical analysis

Unpaired Student's *t* test was used to determine the statistical significance between two groups. Throughout, ns indicates no statistical significance; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . Differences in the total disease burdens of mice with EAE were calculated using the Mann-Whitney *U* test appropriate for data that are not normally distributed.

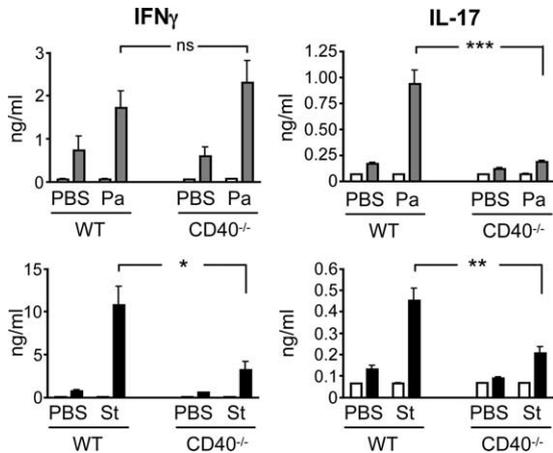
## Results

### IL-17 induction requires CD40 expression *in vivo*

The costimulatory requirements for Th17 development are currently unknown. To investigate a role for CD40-CD154 interaction, we injected naive WT or CD40-deficient mice with heat killed preparations of two different bacteria, Gram-positive Pa and Gram-negative St, and examined the resulting cytokine response. Pa-specific IL-17 was significantly reduced in CD40-deficient animals compared with WT controls (Fig. 1). In contrast, the levels of IFN-γ triggered by Pa were maintained in both the presence and absence of CD40. CD40-deficient mice immunized with St displayed impaired production of both cytokines (Fig. 1). These data promote a key role for the CD40-CD154 partnership during IL-17 induction against distinct bacteria *in vivo*.

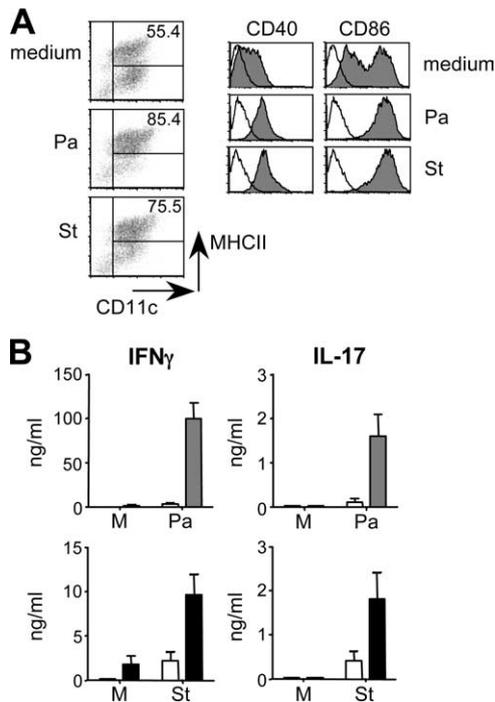
### Bacterially conditioned DC drive concurrent IFN-γ and IL-17

Pathogen-derived molecules can dictate the character of a T cell response through their conditioning of DC (19) and such an influence

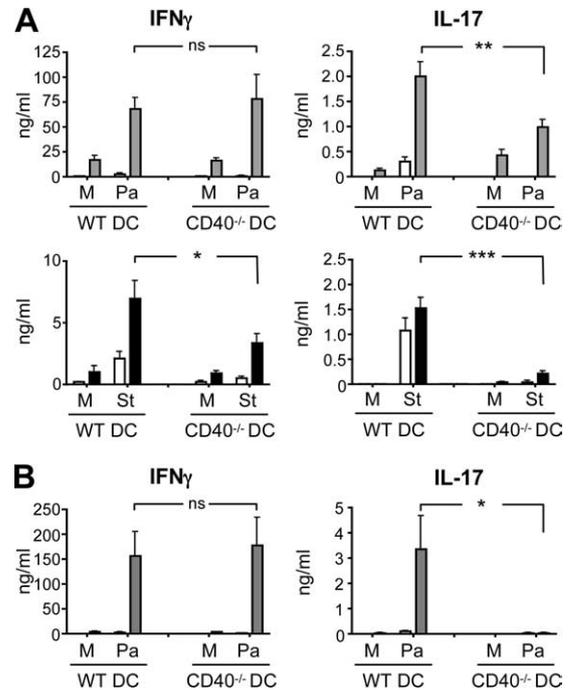


**FIGURE 1.** IL-17 induction requires CD40 expression *in vivo*. Heat killed preparations of *P. acnes* (Pa) or *S. typhimurium* (St) were injected s.c. into WT or CD40-deficient mice. Control mice received PBS alone (PBS). Seven days later, splenocytes were cultured in medium (□), with Pa (▤), or with St (■), and their levels of cytokine production measured by ELISA. Data are representative of two experiments and error bars indicate SEM of five mice per group.

on IL-17 induction has been suggested (25). To assess directly the balance of the cytokine response generated following pathogen activation of DC, we compared the conditioning of DC by either Pa or St, and examined their ability to polarize T cells *in vivo*. Both bacteria triggered phenotypic maturation of DC, up-regulating surface expression of MHC class II and of the costimulatory molecules CD40 and CD86 (Fig. 2A). When transferred into WT



**FIGURE 2.** Concurrent induction of IFN- $\gamma$  and IL-17. WT DC were cultured overnight in medium alone (M), with *P. acnes* (Pa) or with *S. typhimurium* (St). A, Surface expression of activation markers. Open histograms indicate isotype controls. B, DC were injected into naive, WT mice. Seven days later, splenocytes were cultured in medium (□), with Pa (▤), or with St (■), and their levels of cytokine production measured by ELISA. Data are representative of at least four experiments. Error bars indicate SEM of four mice per group.

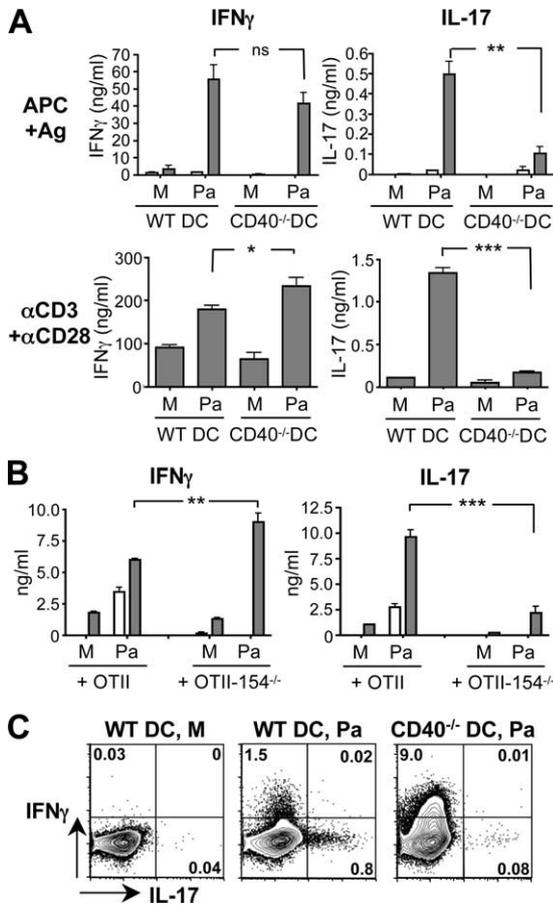


**FIGURE 3.** CD40 is essential for IL-17 induction *in vivo*. WT or CD40<sup>-/-</sup> DC were cultured overnight in medium alone (M), with *P. acnes* (Pa), or with *S. typhimurium* (St) and injected into naive WT (A) or CD40<sup>-/-</sup> (B) mice. Seven days later, splenocytes were cultured in medium (□), with Pa (▤), or with St (■), and their levels of cytokine production measured by ELISA. Data are representative of five (A) and two (B) experiments. Error bars indicate SEM of four to six mice per group.

naive recipient mice, DC activated by either Pa or St generated both IFN- $\gamma$  and IL-17 responses against their respective bacterial Ags. Interestingly, even though evidence exists to suggest the mutual inhibition of IFN- $\gamma$  and IL-17 *in vitro* (1, 2), this was not apparent *in vivo*. The IFN- $\gamma$  response to Pa-pulsed DC was consistently stronger than that induced by DC exposed to St, whereas similar levels of IL-17 were elicited by DC activated with either bacteria (Fig. 2B).

#### IL-17 induction is dependent upon CD40 expression by DC

When we injected bacterially pulsed, CD40<sup>-/-</sup> DC into WT, naive recipients, the resulting IL-17 recall response from cultured splenocytes was significantly reduced in comparison to CD40-sufficient DC controls (Fig. 3A). This result was true for both Pa- and St- stimulated DC. In contrast, the ability of DC to elicit IFN- $\gamma$  following *in vivo* transfer was less reliant on CD40 expression. Pa-activated, CD40<sup>-/-</sup> DC displayed no significant alteration in their ability to provoke IFN- $\gamma$  production (Fig. 3A) (26). St-treated, CD40<sup>-/-</sup> DC consistently showed a small but significant impairment in their ability to trigger IFN- $\gamma$ , as might be predicted by reports previously describing a critical involvement for CD40 in driving IL-12 release and subsequent IFN- $\gamma$  production in response to LPS signaling (27, 28). Thus, we reveal that the induction of IL-17 is critically dependent on DC expression of CD40. When CD40<sup>-/-</sup> DC were transferred into recipient mice that also lacked CD40 (Fig. 3B), the requirement for its expression on the priming DC was absolute. This further emphasizes the importance of CD40 in the generation of an IL-17 response, suggesting that the initiating DC is supported by CD40 expression on a network of other cells, sufficient to

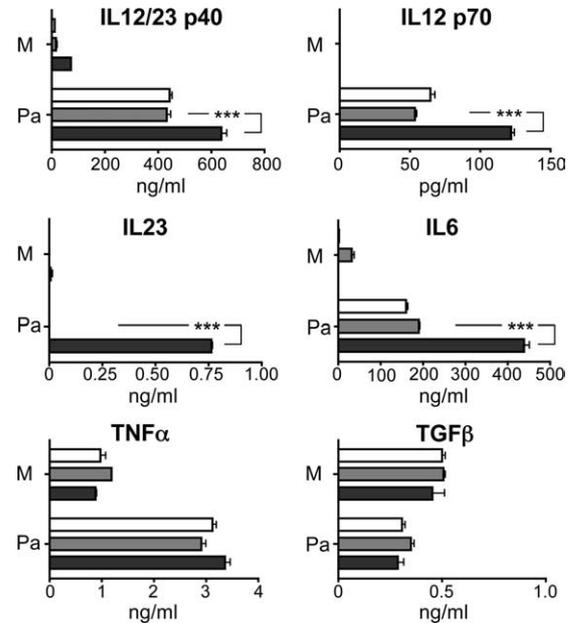


**FIGURE 4.** CD40 determines IL-17 expression by CD4<sup>+</sup> cells. **A**, WT or CD40<sup>-/-</sup> DC were cultured overnight in medium alone (M) or with *P. acnes* (Pa) and injected into naive, WT mice. Seven days later, splenocytes were collected and CD4<sup>+</sup> cells purified and either plated with irradiated APC and cultured in medium (□) or with Pa (■), or stimulated with Abs against CD3 and CD28. Their levels of cytokine production were measured by ELISA. **B**, WT DC were cultured overnight in medium alone (M) or with *P. acnes* (Pa) in the presence (■) or absence (□) of OVA peptide. DC were washed and plated with CD4<sup>+</sup> T cells from either WT OT-II or CD154<sup>-/-</sup> OT-II mice. Resulting cytokine production was measured by ELISA at 72 h. **C**, Intracellular cytokine staining was performed on WT CD4<sup>+</sup> OTII cells cultured with either WT or CD40<sup>-/-</sup> DC, pulsed with medium (M) or *P. acnes* (Pa), and OVA peptide as in (B). Numbers indicate percentage within indicated gate. Data in all parts are representative of at least two experiments and error bars indicate SEM of four mice per group (A), SEM of triplicate culture wells (B), and representative plots from triplicate culture wells (C).

sustain limited expansion of IL-17-secreting cells in the absence of CD40 on the DC themselves.

*CD40:CD154 interaction determines IL-17 production by CD4<sup>+</sup> cells*

Because IL-17 can be produced by several distinct cell populations, we then assessed the capacity of purified CD4<sup>+</sup> T cells to mount a response of a Th17 nature, in the context of CD40-dependence. CD4<sup>+</sup> cells were isolated from the spleens of mice immunized with DC and their cytokine production measured after restimulation with Pa and APC, or activation with anti-CD3 and anti-CD28. In both cases, polyclonal CD4<sup>+</sup> cells from mice injected with CD40<sup>-/-</sup> DC proved fully competent in IFN- $\gamma$  production but significantly impaired in their IL-17 secretion (Fig. 4A). We confirmed that the CD4<sup>+</sup> T cell compartment is

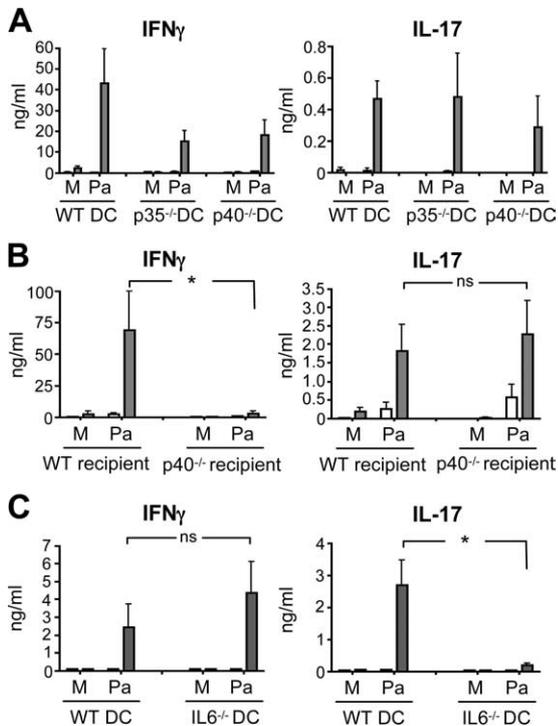


**FIGURE 5.** CD40 ligation of DC releases IL12, IL23, and IL6. WT DC were cultured overnight in medium alone (M) or with *P. acnes* (Pa), then washed and recultured for a further 16 h in medium (□) or with anti-CD40 (■) or a control Ab (□). Cytokine production was measured either by ELISA or, for TGF- $\beta$ , by bioassay. Data are representative of three experiments and error bars indicate SEM of triplicate culture wells.

markedly deficient in IL-17 production in the absence of CD40 signaling by testing the in vitro response of TCR transgenic CD4<sup>+</sup> cells from OT-II mice that recognize the 323–339 peptide of OVA (OVA<sub>323–339</sub>). WT OT-II cells responded to DC pulsed with both Pa and OVA<sub>323–339</sub> by releasing both IFN- $\gamma$  and IL-17; when the responding OT-II cells lacked CD154 and were therefore unable to give or receive CD40-mediated signals, IFN- $\gamma$  production was maintained but IL-17 was strikingly reduced (Fig. 4B). Intracellular cytokine staining confirmed this defective IL-17 response in the absence of DC CD40, and showed that production of IL-17 and IFN- $\gamma$  was from distinct populations of single-positive Th cells (Fig. 4C). Together these data demonstrate a fundamental requirement for CD40:CD154 interaction for CD4<sup>+</sup> Th17 development in vitro and in vivo.

*CD40 stimulation of bacterially primed DC elicits IL-12, IL-23, and IL-6*

The costimulatory dialogue between CD40 and its ligand, CD154, is thought to enhance DC activation particularly by increasing IL-12 secretion, a proinflammatory cytokine associated with Th1 polarization (29). To investigate the mechanism behind the dependence of the Th17 response on CD40, we analyzed the cytokines released by DC after stimulation with an agonistic Ab specific for CD40. DC production of both the p40 subunit of IL-12 and the bioactive p70 heterodimer was significantly increased by CD40 ligation, and its effect on the related cytokine, IL-23, was yet more dramatic (Fig. 5). CD40 signaling also triggered IL-6 release. This response was not shared by all cytokines assessed: CD40 ligation elicited no increase in TNF- $\alpha$  production and, importantly, there was no change in the levels of active TGF- $\beta$  measured after DC stimulation with either Pa or anti-CD40 (Fig. 5). Greater quantities of TGF- $\beta$  were detected after heat activation of latent protein, but again CD40 ligation elicited no more TGF- $\beta$  than was produced in response to the control Ab (data not shown). The cytokines IL-12, IL-23,



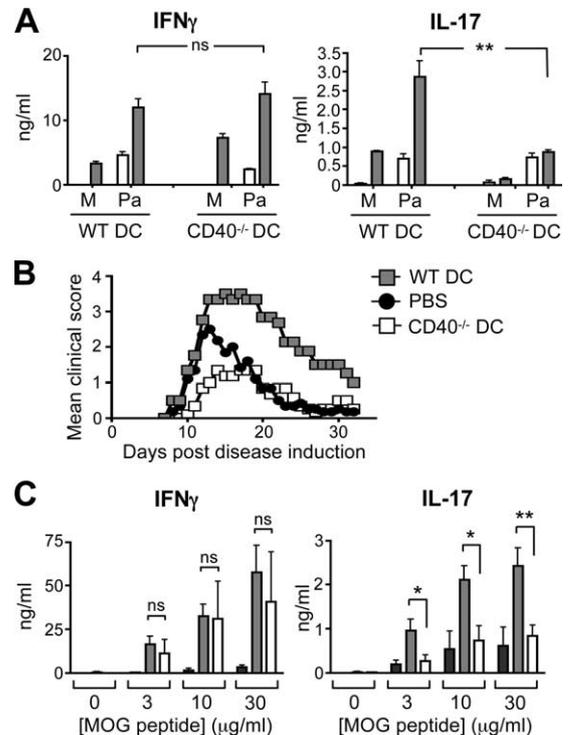
**FIGURE 6.** DC-derived IL-6 drives IL-17 induction in vivo. *A*, WT, IL-12p35<sup>-/-</sup>, or IL-12/23p40<sup>-/-</sup> DC were cultured overnight in medium alone (M) or with *P. acnes* (Pa) and injected into naive WT mice. *B*, WT DC were similarly cultured in medium or with *P. acnes* before being injected into naive WT or IL-12/23p40<sup>-/-</sup> recipients. *C*, WT or IL-6<sup>-/-</sup> DC were cultured overnight in medium alone (M) or with *P. acnes* (Pa) and injected into naive, WT mice. In all cases, splenocytes were collected 7 days later, cultured in medium (□) or with *P. acnes* (■) and their levels of cytokine production measured by ELISA. Each data set is representative of three experiments. Error bars indicate SEM of five mice per group.

and IL-6 were therefore identified as candidates that might mediate the CD40 dependence of Th17 induction.

#### DC-derived IL-6 is required for IL-17 induction in vivo

Because IL-12, IL-23, and IL-6 were elicited in DC by CD40 stimulation, these three cytokines were tested for their ability to drive IL-17 production in our in vivo system. Both p35<sup>-/-</sup> DC (which lack only IL-12) and p40<sup>-/-</sup> DC (which lack both IL-12 and IL-23) were impaired in their ability to drive Pa-specific IFN- $\gamma$  in vivo (Fig. 6A), although the response was notably not absent. In contrast, both sets of gene-deficient DC were fully competent in their ability to elicit IL-17 (Fig. 6A), suggesting that DC-derived IL-23 is dispensable in the priming of this cytokine. To ascertain the role of IL-23 in the later expansion and establishment of IL-17 production, we instead transferred WT DC into p40<sup>-/-</sup> recipient mice in which neither IL-12 nor IL-23 could be made by any cell. In this setting Pa-specific IFN- $\gamma$  was abolished, consistent with previous observations (23). Pa-specific IL-17 was unaffected (Fig. 6B). This was not due to residual IL-23 production by transferred DC, because the same result was seen in the complete absence of this cytokine, when p40<sup>-/-</sup> DC were injected into p40<sup>-/-</sup> recipients (data not shown). This suggests that neither IL-23 nor IL-12 is essential for either the initiation or amplification of Pa-specific IL-17 secretion in vivo.

Of the three candidates identified as cytokines released by DC after stimulation through CD40 (Fig. 5), only IL-6 thus remained a possible requirement for IL-17 induction. Immunization of naive



**FIGURE 7.** CD40-deficient DC polarize autoreactive CD4<sup>+</sup> cells toward Th1 and fail to potentiate autoimmune disease. *A*, WT or CD40<sup>-/-</sup> DC were cultured overnight in medium alone (M) or with *P. acnes* (Pa) in the presence (■) or absence (□) of MOG peptide. DC were washed, plated with CD4<sup>+</sup> cells from 2D2 mice, and the resulting cytokine production was measured by ELISA after 72 h. Data are representative of two experiments and error bars indicate SEM of triplicate culture wells. *B*, WT or CD40<sup>-/-</sup> DC were cultured overnight in the presence of both *P. acnes* and MOG peptide before being injected into naive, WT mice 7 days before immunization to induce EAE. Control mice received PBS instead of the DC injection but were still immunized to elicit disease. Data is shown as the mean disease score for the six mice in each group and the graphs are representative of three experiments. The total disease burdens of mice that received WT or CD40<sup>-/-</sup> DC differed with a Gaussian-approximated *p* value of <0.0001. *C*, Mice were taken at day 15 of EAE disease and their LN cells cultured for 72 h with graded doses of MOG peptide (*x*-axis). Cytokine production was measured by ELISA. Error bars indicate SEM of four mice per group.

recipients with Pa-pulsed, IL-6<sup>-/-</sup> DC resulted in complete abrogation of the WT IL-17 response (Fig. 6C). In contrast, the concomitant, Pa-specific IFN- $\gamma$  response was unaffected by IL-6 deficiency, suggesting that DC-derived IL-6 selectively directs IL-17 production.

#### DC-driven IL-17 determines severity of autoimmune encephalomyelitis

To assess the importance of a CD40-mediated control of IL-17 in a pathophysiological setting, we used EAE, in response to MOG<sub>35-55</sub>, in which IL-17 has been described as a critical determinant (10, 25, 30). We first made use of TCR transgenic CD4<sup>+</sup> cells from 2D2 mice, which recognize MOG<sub>35-55</sub> (31). Consistent with our findings with OT-II cells (Fig. 3), deficiency in CD40-mediated costimulation caused a marked reduction in IL-17 production by 2D2 cells in response to MOG<sub>35-55</sub>, whereas the peptide-specific IFN- $\gamma$  response remained intact (Fig. 7A).

In vivo, the priming of naive recipients with WT DC copulsed with both Pa and MOG<sub>35-55</sub> before the active induction of EAE resulted in a clear exacerbation of both the severity and duration of

autoimmune disease (Fig. 7B). In contrast, priming with similarly pulsed, CD40<sup>-/-</sup> DC caused no such exaggeration of morbidity. The total disease burden of control mice (PBS), which received no DC, was not significantly different from that of those into whom CD40<sup>-/-</sup> DC were injected (Fig. 7B). This equivalence in clinical outcome occurred despite clear enhancement of the MOG<sub>35-55</sub>-specific IFN- $\gamma$  response in mice primed with the CD40-deficient DC (Fig. 7C), an exaggeration of the Th1 response as strong as that caused by the WT DC. The key difference between the two groups that received DC was that only the WT DC increased the MOG<sub>35-55</sub>-specific IL-17 response (Fig. 7C). Thus IL-17, but not IFN- $\gamma$ , triggered by CD40 expression on the priming DC was essential for potentiation of autoimmune disease *in vivo*.

## Discussion

The importance of Th17 cells in both autoimmune disease and infection means that an understanding of their generation and an ability to manipulate their development are now key priorities for immunotherapy. In this study, we have detailed an essential requirement for CD40 expression on DC for optimal Th17 induction *in vitro* and *in vivo*. CD40-ligation triggers IL-6 release by the DC, which is fundamental to their ability to elicit an IL-17 response *in vivo*. In comparison to WT DC, CD40<sup>-/-</sup> cells exhibited an impaired ability to prime MOG<sub>35-55</sub>-specific IL-17, despite fully competent IFN- $\gamma$  induction. This correlated with the abolition of their capacity to exacerbate symptoms of EAE. These data highlight the distinct molecular requirements for the induction and development of Th17 vs Th1 responses *in vivo*, and the potential impact they may have on immune-mediated disease.

Gram-positive and Gram-negative bacteria differ in their surface structure, their activation of DC, and their consequent influence on a T cell response (32, 33). Much is known about the interaction between DC and Gram-negative species, or their associated LPS molecules (27, 28, 34); the effect of Gram-positive bacteria, which include human pathogens such as methicillin-resistant *Staphylococcus aureus*, is less well understood. We show that stimulation of DC with heat-killed preparations of either the Gram-positive Pa or Gram-negative St grants them the ability to drive concurrent Th1 and Th17 responses *in vivo*. The concomitant development of both IFN- $\gamma$  and IL-17 producers, despite the apparent uniformity of our DC populations, suggests that the mutual exclusion of Th1 and Th17 development may not be as absolute at a whole organism level as at a cellular one, *in vitro* (1, 2). Indeed, *in vivo* Th17 cells have been proposed to be promoters rather than inhibitors of the Th1 response, acting to provide the chemokines necessary to attract IFN- $\gamma$ -secreting effectors into the inflammatory site (35).

Heterogeneity in the involvement of CD40:CD154 interactions in establishing appropriate immunity against diverse pathogens is a hallmark of this costimulatory pair (36), and Pa and St differ in their requirement for CD40 expression on DC to elicit IFN- $\gamma$  production. However, both bacteria revealed a fundamental role for DC-derived CD40 in Th17 induction. These data promote the hypothesis that there are downstream consequences of CD40 signaling in DC in addition, or in alternative, to the triggering of IL-12 that are implicit in the ability of DC to stimulate IFN- $\gamma$  release. Indeed, we and others have previously reported IL-12-independent IFN- $\gamma$  production (22, 23, 35, 37, 38) and two discrete pathways of IFN- $\gamma$  induction have been recently described: one IL-12 dependent and the other instead requiring CD70 expression on the initiating DC (22). Even in an IL-12-dependent setting, however, this study noted that IL-12 supplied by sources extrinsic to the Ag-presenting DC was critical. This implies that IL-12 can function

outside of the DC:T cell synapse and, indeed, IL-12p40 has been reported to influence immunity by directing DC migration rather than T cell polarization (39).

Consistent with this, we find that both Pa and St elicit more IL-12 from DC than is released in response to subsequent stimulation through CD40 (26), suggesting that the dominant role of IL-12 may be at the site of bacterial encounter rather than in the draining lymph node, where the DC meets its specific T cell. A similar idea has been proposed for IL-23, whose ability to promote Th17 expansion might, at least in part, be mediated via APC rather than by direct action on T cells (35). In the context of current evidence supporting a role for IL-23 in the maintenance or expansion of Th17 cells, we were surprised to find that IL-23, whether from the initiating DC or from other cells, is entirely dispensable for a fully competent Pa-specific IL-17 response. It is tempting to speculate that stimulation of DC with whole bacteria elicits a sufficiently potent cytokine mixture to render IL-23 redundant. Cytokines other than IL-23 that may support the late stages of Th17 development remain to be identified: IL-1(40) and IL-21 (11, 12) are possible candidates, although the latter may reinforce rather than replace IL-23 (11–13).

Underscoring the physiological relevance of our observations, we show that the inability of CD40<sup>-/-</sup> DC to prime an IL-17 response negates the capacity of WT cells to potentiate EAE disease, despite the increase in MOG<sub>35-55</sub>-specific IFN- $\gamma$  that both WT and CD40<sup>-/-</sup> DC populations elicit. Disrupting the CD40-CD154 interaction *in vivo* has been shown previously to be therapeutic in EAE models, correlating with reduced CNS inflammation (41, 42), although these studies were performed and interpreted before Th17 cells were known to contribute to disease. In our system, the absence of CD40 on the transferred, Pa-pulsed DC did not allow them to fully prevent pathology, which may reflect a requirement for systemic delivery of CD40<sup>-/-</sup> DC to establish effective T cell hyporesponsiveness in autoimmunity (28, 43). In infection, CD40 ligation has been shown to be dispensable for the control of *Mycobacterium tuberculosis* (44), a disease in which protection is still associated with a Th1 response (35). Intact CD40-CD154 signaling is essential to restrict pathogen replication and permit host survival in several other infections, however, including *Salmonella* (45), *Cryptosporidium* (46), and *Candida spp.* (47). An investigation of the involvement of IL-17 in immunity against these pathogens will be illuminating.

During DC:T cell interaction, CD40 functions upstream of other costimulatory receptors and ligands such as OX40L in addition to its action in provoking cytokine production. Understanding the hierarchy of these signals, both surface molecules and cytokines, will provide specific targets with which to manipulate the character of the developing T cell response. The reported involvement of B7:CD28 ligation during Th17 induction (1) might indicate the strength of TCR engagement needed to trigger CD40 signaling (48) and downstream cytokine release. Potential exists for positive feedback reinforcement of this interaction, because IL-17 and CD40L have been shown to act synergistically in their promotion of IL-6 production by primary human kidney cells (49).

Our data suggest that a key feature of DC activation through CD40 following bacterial exposure is not IL-12 secretion but rather heightened IL-6 release. We emphasize the fundamental role of APC production of IL-6 in Th17 polarization of naive T cells *in vivo*, supporting recent work that has suggested this possibility *in vitro* (8, 10). The data presented in this study suggest that the TGF- $\beta$  also involved in this process (8–10) is not elicited by the same signals. Thus, it seems likely that T

cells can provide an autologous source of TGF- $\beta$  during Th17 induction (8, 15, 50), whereas IL-6 is an essential requirement of DC. It is also possible that DC-derived IL-10 plays an important role in regulating IL-17 production, and we are currently investigating if this is the case.

The outcome of an immune response reflects the nature of the target Ag, the context in which it is presented and the cell types recruited to effect its removal. DC are fundamental in the orchestration of this process and the signals that DC give and receive determine the quantity and character of the T cell repertoire. This study reveals Th17 development to be critically dependent upon CD40 expression and IL-6 production by the initiating DC and, by their determination of the severity of clinical signs in a model of CNS autoimmune disease, these data demonstrate the clear therapeutic promise of manipulating this pathway.

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## Disclosures

The authors have no financial conflict of interest.

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