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Distinct sources and targets of IL-10 during dendritic cell-driven Th1 and Th2 responses *in vivo*

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Dendritic cells (DC) can both initiate an immune response and dictate its character. Cytokines are critically involved in this process and, although interleukin (IL)-10 is known as a potent immunosuppressant, the impact of its release from DC remains unclear. Here, we transfer pathogen-conditioned murine DC *in vivo* and show that, while DC-derived IL-10 can act to limit Th1 development, it is not required for Th2 induction. In both Th2 and Th1 settings, however, IL-10 from cells other than the initiating DC dominates the regulation of the emerging effector cell populations. Surprisingly, the critical source of IL-10 in this process is neither T nor B cells. These data illustrate the distinct actions of IL-10 during differently polarised, pathogen-focussed, DC-driven immune responses *in vivo*.

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Introduction

Dendritic cells (DC) are potent antigen-presenting cells (APC) [1]. They direct both the size and the character of an immune response, using a carefully orchestrated sequence of signals including antigen dose [2], costimulation [3] and cytokine secretion [4–6] that reflects the specific activation elicited by different pathogens [7]. As such, the cytokines released by the DC and those triggered downstream are critical in determining the outcome of the immune response, and one of the key players in this process is IL-10 [8]. Here, we examine the role of IL-10 in DC-mediated induction of Th1 and Th2 development, and show that its influence is tightly and differently controlled during oppositely polarised immune responses *in vivo*.

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Abbreviations: aIL-10R: blocking antibody against the IL-10 receptor · **Pa:** heat-killed *Propionebacterium acnes* · **SEA:** soluble egg antigen from *Schistosoma mansoni*

IL-10 was originally described as a product of a Th2 T cell clone that could inhibit pro-inflammatory Th1 activity [9]. Subsequent studies have revealed a variety of sources, including DC [8], and it is now known as an important effector molecule for Tr1 [10, 11] and perhaps also $CD4^+CD25^+$ regulatory T cells [12, 13]. Mice genetically deficient for IL-10 ($IL\text{-}10^{-/-}$) develop severe inflammatory bowel disease in response to normal gut flora [14], and the ability of IL-10 to limit IFN-γ production and reduce Th1-mediated pathology has been well documented [13, 15–17]. Although direct effects have been described, its anti-inflammatory action is thought to be largely a result of its impact on APC, reducing their ability to promote Th1 development [18]. Indeed, IL-10-mediated inhibition of classical maturation is reported to render DC tolerogenic [19]. DC secretion of IL-10 can act in autocrine and paracrine routes [8] and has been associated with both suppressive [11, 20] and Th2 T cell phenotypes [21, 22].

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The influence of IL-10 appears to be critically dependent on the timing and context of its production. In schistosomiasis, a helminth infection endemic across the tropics, the absence of IL-10 allows an uncontrolled Th1 response to dominate early disease, and increased mortality ensues [23, 24]. Concurrent removal of Th1 cytokines, using IL-10/IL-12 double knockout mice, does not protect but instead results in an exacerbated Th2 response and an equally severe increase in pathology [25]. IL-10 also features in the host response to several species of bacteria and protozoa [26–29]. In these settings, IL-10 may act to benefit the host, protecting against the pathology of an unmitigated Th1 response [11], or in favour of the pathogen, minimising the immune attack that would otherwise eradicate infection [30].

To define the role of IL-10 in polarised immune responses against pathogen-derived antigens, we pulsed DC with either soluble egg antigen from the helminth *Schistosoma mansoni* (SEA), or a heat-killed bacterium, *Propionebacterium acnes* (Pa), before transferring them *in vivo*. By restricting IL-10 deficiency to either the transferred DC or the recipient mouse, we compared the impact of IL-10 made during the initiation of divergent T cell responses with that released later, during their amplification. We demonstrate that DC-derived IL-10 is not required for Th2 or Th1 induction *in vivo*. While IL-10 from transferred DC does act to restrain Th1 development, its impact is dwarfed by the dominant regulation provided by IL-10 from other cells. Selective removal of IL-10 from B and T cells does not disable this regulation, suggesting that it is IL-10 from innate or non-haematopoietic sources that controls the outcome of the DC-driven immune response.

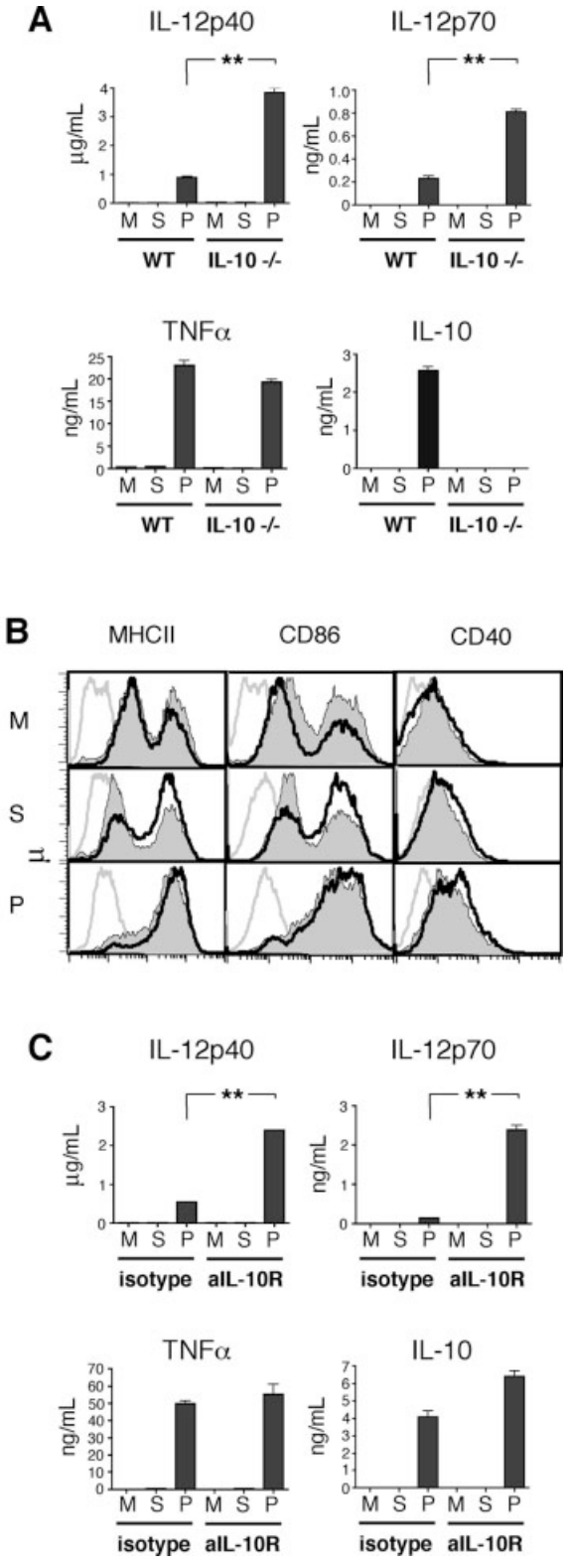

Figure 1. Autocrine effects of DC-derived IL-10. WT or IL-$10^{-/-}$ DC were cultured overnight in medium alone (M), with SEA (S) or *P. acnes* (P). (A) Cytokine production. (B) Surface expression of activation markers. Gray lines indicate isotype controls; filled histograms, WT DC; thick black lines, IL-$10^{-/-}$ DC. (C) Cytokine production by WT DC when either aIL-10R or control antibody was included in the overnight culture. All graphs are representative of four experiments. Error bars indicate SEM of triplicate wells; ** $p < 0.01$.

Results

Autocrine effects of DC-derived IL-10

Reis e Sousa and colleagues described an IL-10/IL-12 axis of DC maturation: Pathogen products that elicit high levels of IL-12 from DC generate strong Th1 responses, and those that trigger IL-10 do not [21]. We exposed DC to either bacterial (Pa) or helminth (SEA) preparations, shown previously to drive polarised Th1 and Th2 responses respectively [31], and instead found that IL-10 was induced in DC pulsed with Pa, the same pattern as shown by the pro-inflammatory cytokines IL-12 and TNF-α (Fig. 1A). Pa potently stimulated both WT and IL-$10^{-/-}$ DC, driving high expression of MHC

class II, CD86 and CD40 and significant release of IL-12 and TNF-α (Fig. 1A, B). Against this strong activation, the enhanced response of IL-10$^{-/-}$ DC was clear only in their increased IL-12 production. Neither TNF-α nor IL-6 release was elevated above the WT comparison (Fig. 1A and data not shown) and the surface phenotype of both IL-10$^{-/-}$ and WT populations was similar (Fig. 1B). Neither WT nor IL-10$^{-/-}$ DC pulsed with SEA secreted any more IL-12 or TNF-α than DC left in medium alone (Fig. 1A).

Previous work has suggested that IL-10 acts in an autocrine manner to dampen DC activity [32, 33], and indeed the influence of IL-10 seen here was exerted during activation, rather than development, since the phenotype of IL-10$^{-/-}$ DC was reproduced by blocking the IL-10 receptor during stimulation of WT cells (Fig. 1C) [34].

DC-derived IL-10 is dispensable for Th2 induction *in vivo*

When transferred *in vivo* into naive, WT recipient mice, WT DC generated either an SEA-specific Th2 response, with clear production of IL-4, IL-5 and IL-13 but no IFN-γ, or a Pa-specific Th1 response dominated by IFN-γ (Fig. 2 and data not shown) [31]. The Th2 response was not significantly altered when the priming DC were IL-10$^{-/-}$, and neither WT nor IL-10$^{-/-}$ DC induced any measurable SEA-specific IFN-γ. In contrast, the Pa-specific IFN-γ response was markedly exaggerated when transferred DC were IL-10$^{-/-}$ (Fig. 2).

Recipient cells, not the initiating DC, dominate the IL-10 response

Two recent reports have described the ability of IL-10-producing, CD25^{+} and CD25^{-} T cells to suppress Th1 development and consequent pathology in schistosomiasis [12, 35]. To assess the role of IL-10 derived from cells other than the initiating DC, we injected WT DC pulsed with either Pa or SEA into IL-10-deficient recipients. The result was striking: levels of Pa-specific IFN-γ in IL-10$^{-/-}$ recipients were dramatically exaggerated (Fig. 3). This pattern was consistent with the elevated Th1 response seen in the absence of DC-derived IL-10, but the scale was greatly amplified (compare Fig. 2 and Fig. 3). No further increase in IFN-γ was seen

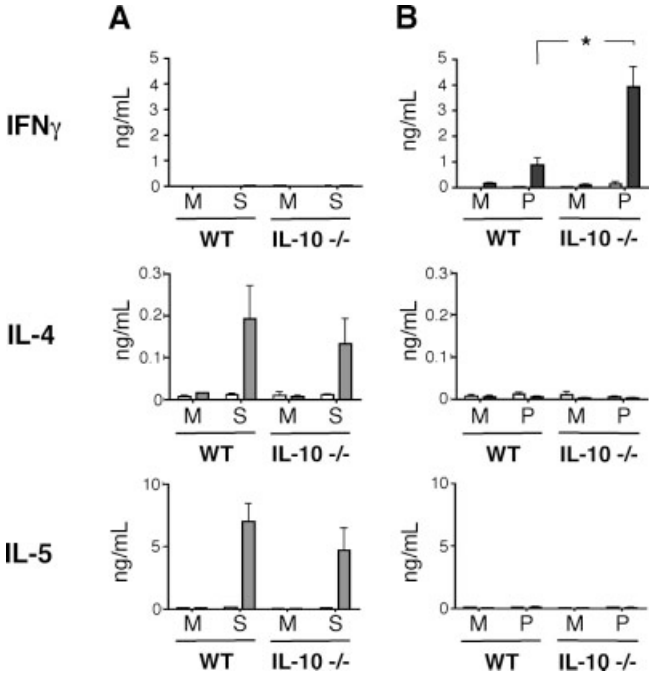

Figure 2. DC-derived IL-10 is dispensable for Th2 induction *in vivo*. WT or IL-10$^{-/-}$ DC were incubated in medium (M), with SEA (S) or *P. acnes* (P) and injected into naive, WT mice. After 7 days, splenocytes were cultured in medium (white bar), with SEA (gray) or with Pa (black), and their levels of cytokine production were measured by ELISA. (A) SEA-specific responses; (B) Pa-specific responses. Data shown is representative of three experiments. Error bars indicate SEM of four mice per group; * $p < 0.05$.

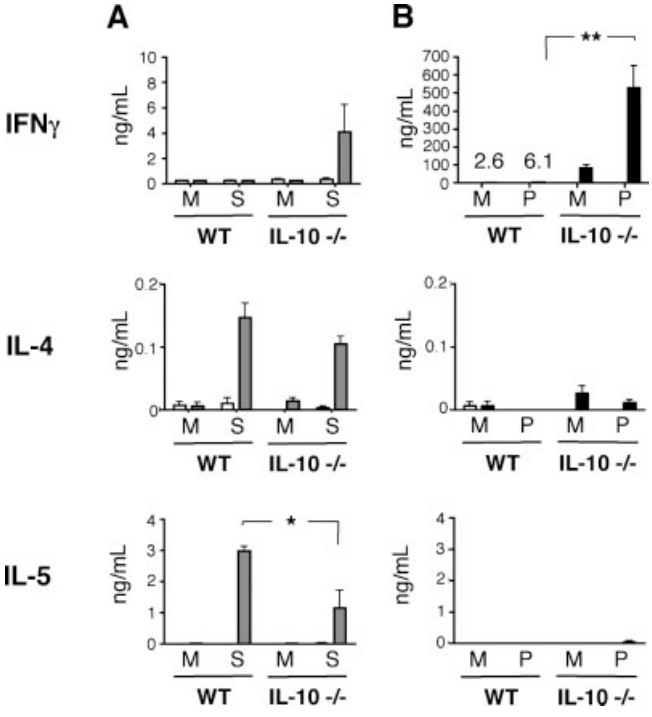

Figure 3. Cells other than the initiating DC dominate the IL-10 response. WT DC were incubated in medium (M), with SEA (M) or *P. acnes* (P) and injected into WT or IL-10$^{-/-}$ recipients. After 7 days, splenocytes were cultured in medium (white bar), with SEA (gray) or with Pa (black). Cytokine production was measured by ELISA. (A) SEA-specific responses; (B) Pa-specific responses. Data shown is representative of four experiments and error bars indicate SEM of at least three mice per group. * $p < 0.05$, ** $p < 0.01$. Numbers give the mean concentration of the black bars above which they are situated.

in IL-10$^{-/-}$ recipients when the transferred DC were also IL-10 deficient (data not shown).

In contrast to the apparent lack of influence of IL-10 from injected DC in the SEA-specific response, the impact of IL-10 from recipient cells was clear: after transfer of WT DC into IL-10$^{-/-}$ mice, levels of IL-4, IL-5 and IL-13 were reduced below those of WT recipient controls (Fig. 3 and data not shown). This reduction corresponded with the ability of SEA-pulsed WT DC to elicit a small but detectable IFN-γ response only in the absence of recipient IL-10 (Fig. 3), in keeping with data from active schistosome infection of IL-10$^{-/-}$ mice [24, 25]. The impairment in Th2 cytokines was not absolute; IL-10-deficient recipients remained capable of mounting a robust Th2 response to SEA-pulsed DC. Indeed, neither the reduction in IL-4 nor the emergence of SEA-specific IFN-γ reached statistical significance (Fig. 3).

IL-10 targets different effector populations during Th1 and Th2 responses

To identify the cells responsible for the elevated IFN-γ seen in IL-10$^{-/-}$ recipients, spleens were removed from mice immunised with WT DC and their cytokine profile examined by intracellular staining. Splenocytes were incubated with either SEA or Pa to induce cytokine release; no mitogenic stimulus was used, so the small percentages of cytokine-positive cells measured represent a polyclonal, antigen-specific response. In splenocytes from mice receiving Pa-pulsed DC, IFN-γ was detected in CD4$^+$, CD8$^+$ and, interestingly, B220$^+$ cells (Fig. 4A). In IL-10$^{-/-}$ recipients, the exaggerated Th1 response measured by ELISA (Fig. 3) was reflected in an increase in the proportion of IFN-γ-producing cells in all three lymphocyte populations (Fig. 4B, top row). In contrast, the SEA-specific IFN-γ response seen in IL-10$^{-/-}$ hosts could only be detected in CD4$^+$ cells (Fig. 4B, bottom row). Although there was some suggestion that CD8$^+$ cells may contribute, this did not reach statistical significance. NK1.1$^+$ cells also featured as a source of IFN-γ only in spleens from mice given Pa-pulsed DC. Unlike the lymphocyte populations, these IFN-γ$^+$ NK1.1$^+$ cells were not further expanded in the absence of recipient IL-10 (Fig. 4C).

In the Th2 setting, the reduction in SEA-specific IL-4 and IL-5 in IL-10$^{-/-}$ recipients measured as secreted cytokine (Fig. 3) was shown to correlate with decreased IL-4 and IL-5 production by CD4$^+$ cells (Fig. 5). Neither cytokine was detected in CD8$^+$ or B220$^+$ cells (data not shown).

IL-10 acts on host APC

IL-10 has been reported to regulate IFN-γ production by CD4$^+$ cells both directly and indirectly through its action

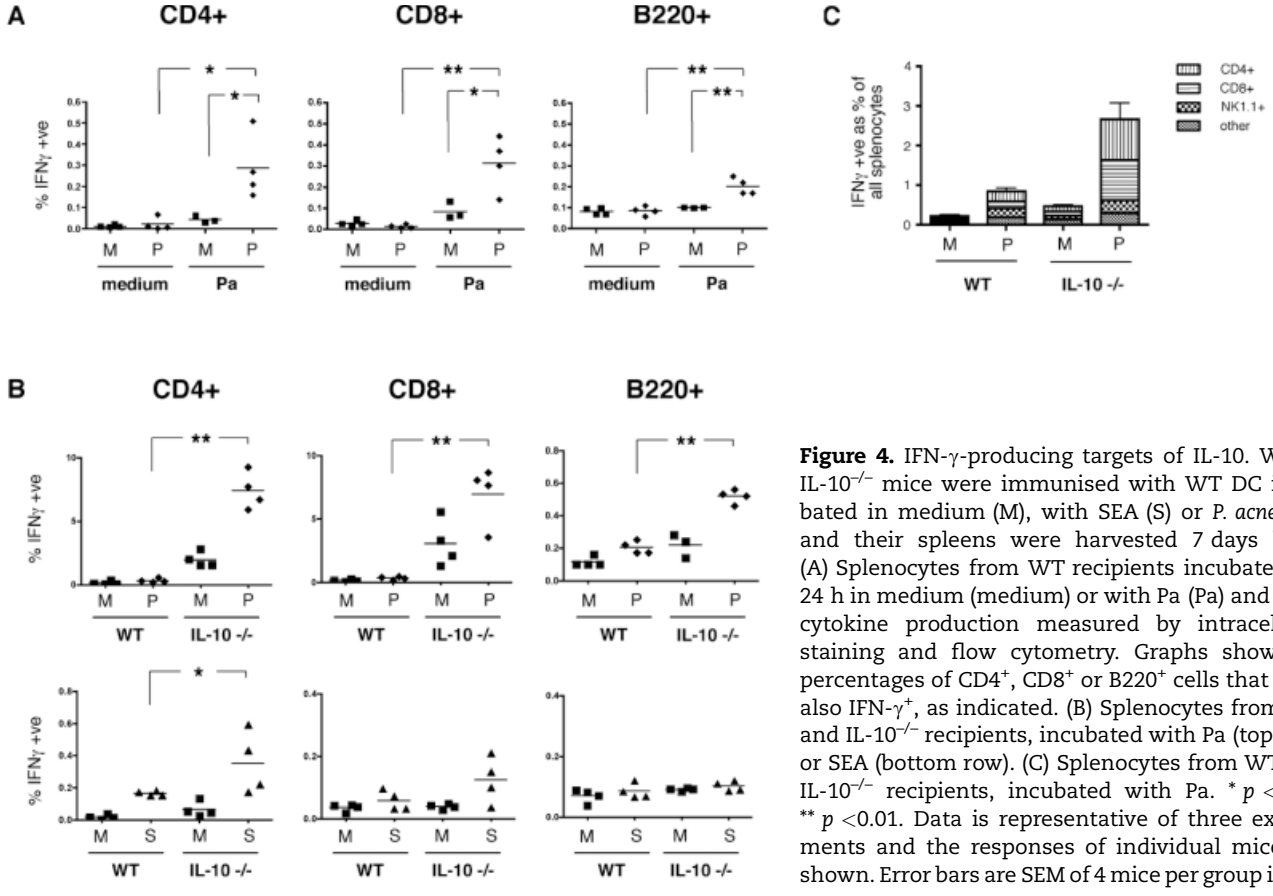

Figure 4. IFN-γ-producing targets of IL-10. WT or IL-10$^{-/-}$ mice were immunised with WT DC incubated in medium (M), with SEA (S) or *P. acnes* (P), and their spleens were harvested 7 days later. (A) Splenocytes from WT recipients incubated for 24 h in medium (medium) or with Pa (Pa) and their cytokine production measured by intracellular staining and flow cytometry. Graphs show the percentages of CD4$^+$, CD8$^+$ or B220$^+$ cells that were also IFN-γ$^+$, as indicated. (B) Splenocytes from WT and IL-10$^{-/-}$ recipients, incubated with Pa (top row) or SEA (bottom row). (C) Splenocytes from WT and IL-10$^{-/-}$ recipients, incubated with Pa. * $p < 0.05$, ** $p < 0.01$. Data is representative of three experiments and the responses of individual mice are shown. Error bars are SEM of 4 mice per group in (C).

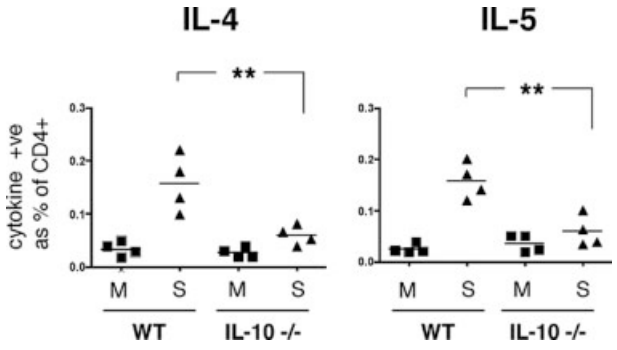

Figure 5. Targets of IL-10 during a Th2 response. WT and IL-$10^{-/-}$ mice were immunised with DC incubated in medium (M) or with SEA (S), and their spleens were harvested 7 days later. Cytokine production was measured after overnight culture in SEA, using intracellular staining and flow cytometry. Graphs give the percentage of $CD4^+$ cells that were also IL-4^+ or IL-5^+, as indicated. ** $p < 0.01$. Data is representative of three experiments and the responses of individual mice are shown.

on APC populations [8]. We assessed APC activation during the Th1 response by measuring IL-12 release after immunisation with WT DC, in the presence or absence of IL-10. IL-12 was not detected in either WT or IL-$10^{-/-}$ recipients of SEA-pulsed DC (data not shown). However, the exaggerated IFN-γ release seen in IL-$10^{-/-}$ recipients of Pa-pulsed DC (Fig. 3) was accompanied by elevated levels of both IL-12 p40 and p70 (Fig. 6).

Neither T nor B cells are the decisive source of IL-10 during Th1 or Th2 induction by DC

The above experiments established a critical role for IL-10 from cells other than the initiating DC in shaping T cell responses. To identify the dominant source, we generated mixed bone marrow chimeric mice in which IL-10 deficiency was restricted either to B cells alone or to B and T cells together (Fig. 7A). Release of Pa-specific IFN-γ after immunisation with WT DC was unaffected by the absence of B cell IL-10 and, although exaggerated when both B and T cells were IL-10 deficient, still did not reach the levels seen in animals in which all cells were IL-$10^{-/-}$ (Fig. 7B; compare with Fig. 3). The Th2 cytokines IL-4 and IL-5 were unchanged between both types of chimeras and their WT controls (Fig. 7B and data not shown), and SEA-specific IFN-γ was not evident in any group (data not shown). Thus, it was IL-10 from non-B, non-T cell sources that played the dominant role in regulating DC-driven Th1 and Th2 responses *in vivo*.

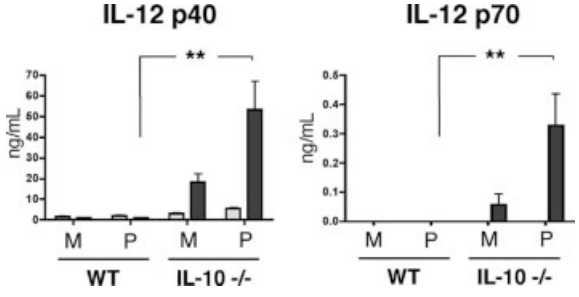

Figure 6. IL-10-deficient recipients release exaggerated levels of IL-12 during a Th1 response. WT DC were incubated in medium (M) or with *P. acnes* (P) and injected into WT or IL-$10^{-/-}$ recipients. After 7 days, splenocytes from these mice were cultured in medium (white bars) or with Pa (black). Cytokine production was measured by ELISA. Data shown is representative of three experiments. Error bars indicate SEM of four mice per group; ** $p < 0.01$.

Discussion

We have shown that the role of IL-10 in shaping polarised T cell responses generated by DC *in vivo* is

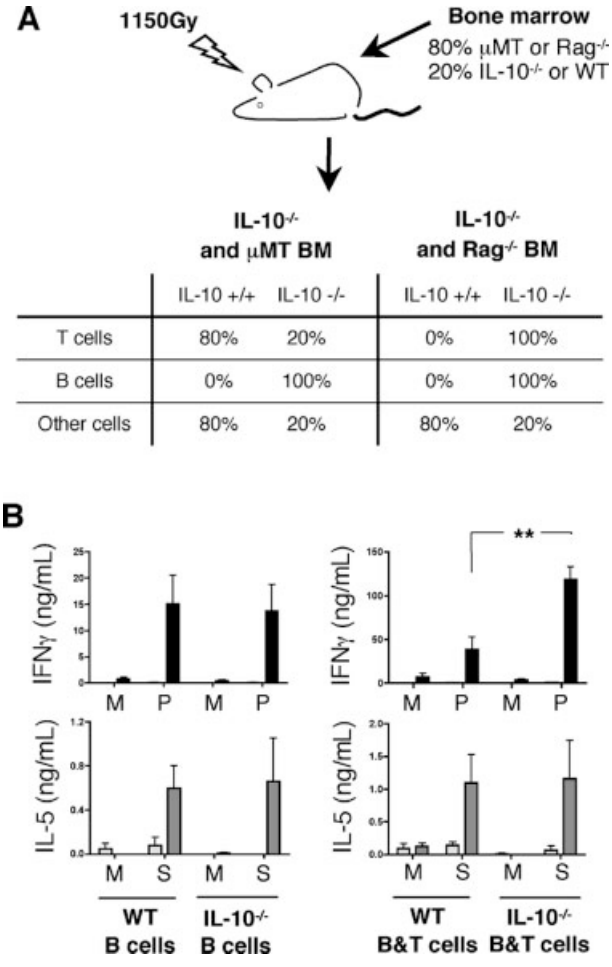

Figure 7. Non-lymphocyte sources of IL-10 control Th1 and Th2 responses. (A) Construction of chimeric mice in which IL-10 deficiency was restricted to either B cells alone or to both B and T cells. (B) These mice were immunised with WT DC incubated in medium (M), with SEA (S) or *P. acnes* (P). After 7 days, splenocytes were cultured in medium (white bars), with SEA (gray) or with Pa (black). Cytokine production was measured by ELISA. Data shown is representative of three experiments. Error bars represent SEM of four mice per group; ** $p < 0.01$.

critically influenced by the nature of the pathogen that drives the immune response. Pa-pulsed DC release both IL-10 and IL-12 and elicit IFN-γ; SEA-conditioned DC make no detectable IL-10 and yet generate strong Th2 responses. The outcome of the developing immune response is decisively influenced by IL-10 not from the initiating DC, however, but from other innate or non-haematopoietic sources. These data suggest that IL-10 from the priming DC is dispensable for the induction of both Th1 and Th2 immunity *in vivo*, and that the IL-10 that critically regulates the emerging response is not exclusively provided by lymphocytes.

Several subpopulations of DC exist and recent work has revealed that, although they may differ in the limits of their possible function, all are capable of assessing the nature and context of a given pathogen and tailoring the immune response appropriately [6, 7]. The cytokines that DC release are of key importance in this process and early experiments suggested a clear distinction in the production of IL-10 and IL-12: antigenic stimuli appeared to elicit either IL-10 or IL-12 and only the latter generated Th1-type immunity [7]. In contrast, our data and those of others working with infection models indicate that pathogens that elicit IL-12 secretion from DC and drive strong Th1 responses in their host can also trigger simultaneous IL-10 release (Fig. 1) [13, 27]. This concomitant production implies that the balance of IL-12 and IL-10 offers finer control over the developing immune response than regulation by IL-12 alone. Indeed, DC-derived IL-10 has been shown to limit Th1 expansion not only through its autocrine inhibition of IL-12 production [32] but also by its management of chemokine expression by APC [36] and its ability to stimulate IL-10 release from T cells later in the immune response [11].

The effects of DC-derived IL-10 have been associated with both Th2 promotion [22, 33, 37] and T cell tolerance [38]. We show here that IL-10 from DC is not required for Th2 induction to SEA *in vivo*. No detectable IL-10 was released by DC exposed to SEA (Fig. 1) [31, 34], and SEA-pulsed, IL-10-deficient DC were equally able to generate Th2-polarised immunity as their WT equivalents (Fig. 2). Interestingly, even the contribution of IL-10 from cells other than the initiating DC appears not to be an absolute requirement for the development of the Th2 response *in vivo*: in the absence of recipient IL-10, IL-4 and IL-5 were muted but not absent (Fig. 3).

The reduction in Th2 cytokine released by IL-10$^{-/-}$ mice immunised with SEA-pulsed DC seen here (Fig. 3, 5) reinforces, with pathogen-derived antigens, the data provided by Moser and colleagues in their work with model antigens [39]. It could reflect the ability of IL-10 to promote the Th2 response either through direct action on CD4^{+} Th2 cells or, as an indirect effect, *via* its impact on other cells. We found that the diminished Th2 reaction correlated with the emergence of SEA-specific IFN-γ, suggesting that IL-10 may enhance Th2 development by negating the counter-regulatory influence of Th1 cytokines. The induction of IL-10 in recipients of SEA-pulsed DC (Fig. 3) then indicates not a passive emergence of Th2 character, but an active and carefully regulated response. This contrasts with the proposal that Th2 development occurs only in the absence of cytokines that drive a Th1 response, an idea known as the default hypothesis [40]. In support, several other reports have recently described active Th2 induction by pathogen preparations [34, 41, 42].

To distinguish the role of IL-10 during the initiation of an immune reaction from that supplied during its amplification and differentiation, we compared the responses generated by transfer of IL-10-deficient DC with those observed in IL-10-deficient recipients. Our data indicate that, while DC-derived IL-10 does restrain Pa-specific Th1 development, in both Th1 and Th2 settings the effect of IL-10 released from other cells is much more dramatic. We used mixed bone marrow chimeric mice to restrict IL-10 deficiency to either B cells alone or to both B and T cells and revealed that IL-10 production by both B and T cells is dispensable in the regulation of Th2 effector cytokines after DC transfer *in vivo* (Fig. 7B). Many cells other than lymphocytes have the capacity to release IL-10, including macrophages, DC, mast cells, keratinocytes and epithelial cells [8]. Epithelial sources may be particularly relevant in an immune response initiated by SEA, an antigenic mix derived from a parasite stage whose aim is to escape from the periportal vasculature into the gut lumen, a journey necessitating the breach of epithelial barriers. Indeed, Hesse *et al.* recently reported a non-lymphocyte source of IL-10 during active murine schistosomiasis [35]. The requirement for IL-10 in survival of schistosomiasis, shown to be due to its ability to suppress Th1 dominance and to allow a protective Th2 response to establish [25, 35], is consistent with the data presented here.

The mixed bone marrow chimeras also demonstrated the involvement of T cells and, more influentially, of non-T, non-B cells in the regulation of IFN-γ during the Pa-specific Th1 response (Fig. 7). Several reports have documented a role for IL-10-producing T cells in curtailing T cell responses, both in model systems [39] and infectious disease [12, 13, 35, 43], and B cell-derived IL-10 has been ascribed regulatory function in two examples of autoimmunity [44, 45]. The discrepancy might indicate that distinct sources of IL-10 dominate at different stages of an immune response: our data reflect the early induction and stabilisation of T cell responses, while B cell production of IL-10 was reported later in established disease [44–46]. It also reinforces the suggestion that a network of cellular interactions both supports and controls polarised T cell development. IL-10 instruction may occur between lymphocytes and

other APC, for example, as the immune response develops. Continued APC input is required not just for the initiation of T cell proliferation and differentiation but also for its maintenance [47]. We have shown that the absence of IL-10 in recipients of WT DC pulsed with Pa results in increased IL-12 production (Fig. 6), suggesting that IL-10 may suppress the Th1 response by curtailing APC support. Whether this represents the sole or only a partial mechanism is currently under investigation.

To identify the effector cells targeted by IL-10, we used antigen-specific, intracellular cytokine detection and revealed exaggerated IFN-γ production by $CD4^+$, $CD8^+$ and $B220^+$ cells (Fig. 4). B220 is displayed on the surface of plasmacytoid DC [48] and some NK cells [49], but strong expression is commonly used to identify B cells [50]. B cell lines that release IFN-γ have been reported [51] and IFN-γ^+ $CD19^+$ cells were observed in a mouse model of Lyme disease [52], but the importance of B cell cytokines remains poorly understood. Harris *et al.* described polarisation of B cells to either an IFN-γ-secreting 'Be1′ or an IL-4 producing 'Be2′ phenotype *in vitro* [53]; our data suggest that B cells contribute IFN-γ during Th1 but not Th2 development in DC-driven immune responses *in vivo*. We could not detect IL-4 or IL-5 production by B cells even in the strongly Th2-polarised response to SEA-pulsed DC.

The Pa-specific Th1 response was also characterised by IFN-γ production from $NK1.1^+$ cells (Fig. 4C). In C57BL/6 mice, NK1.1 is a marker of NK and some NKT cells [54, 55], both of which are key sources of IFN-γ *in vivo* [56] and have been shown to interact closely with DC [57]. Their potential involvement in the IFN-γ that characterises the recall responses of mice primed with Pa-pulsed DC was reinforced by a recent report of NK participation in adaptive immunity [58]. Interestingly, unlike the lymphocyte sources, the proportion of $NK1.1^+$ cells staining positive for IFN-γ appeared not to expand in the absence of IL-10; our analysis therefore suggests that the principal targets of the IL-10 elicited by Pa-pulsed DC are $CD4^+$ and $CD8^+$ T cells (Fig. 4C).

Taken together, these data emphasise the influence of context on the function of IL-10 and reiterate its importance in achieving equilibrium between protective immunity and immunopathology. This balance has been exploited by bacteria and parasites to promote their survival and persistence [13, 30, 59], illustrating co-evolution of host and pathogen [60]. Here, we have shown that IL-10 is critical in shaping polarised immune responses and is precisely controlled. DC-derived IL-10 is dispensable in the induction of DC-driven Th1 and Th2 responses *in vivo*, and IL-10 from innate or non-haematopoietic sources dominates the regulation of these responses as they develop.

Materials and methods

Mice

C57BL/6, IL-10-deficient [14], μMT [61] and Rag-1-deficient [62] 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. Animals were used at 6–12 wk of age and were age- and sex-matched within each experiment.

Antigens and antibodies

Endotoxin-free SEA was either prepared in-house [31] or provided by Prof. Mike Doenhoff, University of Bangor. *P. acnes*, a Gram-positive bacterium, was obtained from American Type Culture Collection (#6919; ATCC, Manassas, VA). The blocking anti-IL-10 receptor antibody (aIL-10R), clone 1B1.3, was purchased from BD PharMingen (Oxford, UK). Isotype control antibody was produced in-house, and no significant difference was found between cells cultured with control antibody and those in medium alone.

Dendritic cell culture

DC were generated in the presence of recombinant GM-CSF (Peprotech, London, UK) for 11 days, as previously described [31]. Cells were >95% $CD11c^+$ $MHCII^+$ DC, with the remainder predominantly Gr-1^+ granulocytes. To activate the DC, cells were harvested on day 10 of culture and replated at 2×10^6 DC/mL in the presence of either SEA (50 μg/mL) or *P. acnes* (10 μg/mL) (both as measured by the Coomassie Plus Protein Assay; Perbio Science UK Ltd., Cramlington, UK) for their final 18 h of incubation. When aIL-10R was included, it was added only during this activation step and was used at 1 μg/mL, a dose optimised by titration against recombinant IL-10.

Assessment of DC activation

The expression of surface molecules on DC was assessed by flow cytometry using antibodies against MHC class II (clone M5114, purified in-house), CD11c and either CD80, CD86 or CD40 (PharMingen). Samples were acquired on a FACSCalibur flow cytometer and analysed using FlowJo software (TreeStar, Ashland, OR), gating on live, $CD11c^+$ cells. Cytokine ELISA were performed on culture supernatants using paired mAb purified in-house or purchased from PharMingen and recombinant cytokine standards purchased from Peprotech or PharMingen. TNF-α was measured using the DuoSet ELISA kit from R&D Systems (Abingdon, UK).

In vivo priming by DC

Mice were injected i.p. with 5×10^5 DC that had been cultured for 18 h in the presence of SEA, Pa or medium alone. After 7 days, spleens were removed and 1×10^6 splenocytes plated per well of a 96-well plate in X-Vivo 15 serum-free medium (Cambrex, Wokingham, UK) supplemented with 2 mM L-glutamine (Gibco, Paisley, UK) and 50 μM 2-ME (Sigma, Poole, UK). SEA was added to give a final concentration of 25 μg/mL

and Pa at 5 μg/mL. Culture supernatants were taken at 72 h to assess their cytokine content by ELISA.

Ex vivo cytokine staining

Splenocytes recovered from mice immunised with DC 7 days earlier were plated at 5×10^6 cells/well in a 48-well plate, with or without SEA or Pa for 18 h. GolgiStop (PharMingen) was added at 1 : 1500 for the last 4 h of culture. Cells were then stained for CD8, CD4 and B220 (PharMingen) before being fixed and permeabilised (BD Cytofix/Cytoperm kit; PharMingen) and stained with antibodies against IFN-γ, IL-4 or IL-5 (PharMingen). Samples were analysed using forward and side scatter characteristics to gate on live cells.

Mixed bone marrow chimeras

WT C57BL/6 mice were exposed to a lethal dose of 1150 Gy γ-irradiation to deplete their haematopoietic system, and rescued 24 h later with a transfusion of 5×10^6 bone marrow cells from mixed donors, administered i.v. in 200 μL PBS. Mature T cells were removed from all bone marrow preparations using anti-Thy1.2 MACS beads (Miltenyi Biotec, Germany). To restrict IL-10 deficiency to B cells only, donor bone marrow comprised 80% μMT cells (unable to provide B cells) and 20% IL-$10^{-/-}$ cells (unable to provide IL-10, and sufficient to populate the whole B cell pool) [44]. Similarly, a combination of 80% Rag-$1^{-/-}$ bone marrow with 20% IL-$10^{-/-}$ generated mice in which both B and T cells were IL-10 deficient, but not other cell types. Control groups received 20% WT bone marrow in place of the IL-$10^{-/-}$ component. Chimeras were left for at least 8 wk before use in experiments, to allow full reconstitution of their immune systems.

Statistical analysis

Student's *t*-test was used to determine the statistical significance difference between two groups.

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