Full Development of Th2 Immunity Requires Both Innate and Adaptive Sources of CD154[1]

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The CD40-CD154 interaction is critical for Th2 response generation during helminth infection and following immunization with helminth-conditioned dendritic cells, yet the key cellular sources of these molecules have still to be defined in vivo. In this study, we demonstrate that the requirement for CD40 expression during murine Th2 response induction is restricted exclusively to the Ag-bearing dendritic cells. In contrast, development of full Th2 immunity required CD154 expression on multiple populations. In this respect, optimal production of IL-5, IL-10, and IL-13 was dependent upon CD154 expression by both $CD4^+$ T cells and non-lymphoid cells. IL-4 production had less stringent costimulatory requirements, with expression of CD154 on either non-lymphoid cells or T cells alone being sufficient to enable production of this archetypal Th2 cytokine. Disparities in CD154 requirements for T cell and B cell responses were revealed during experimental schistosomiasis where, even in the face of robust Th2 generation, B cell class-switching was entirely dependent upon expression of CD154 by the lymphoid compartment. These data help define the costimulatory interactions that occur during the generation of Th2 immunity, and challenge the widely held view that CD154 expressing T cells are the sole contributors in this process. *The Journal of Immunology,* **2008, 180: 8083–8092.**

Generation of Th2 immunity in vivo likely involves a complex series of interactions between $CD4^+$ T cells, APCs, and additional cells that provide costimulatory and/or polarizing signals. Identifying the signals and cells involved is key to understanding the process of Th2 priming. A pivotal component of leukocyte cross-talk is the TNF family member CD40 and its ligand CD154 (reviewed in Refs. 1, 2). The interaction between CD40 on dendritic cells (DC)[4] and CD154 on T cells provides signals to both cells that ultimately determine the quality of the ensuing adaptive response (1, 2). We and others have reported a critical role for CD154 in the generation of Th2 polarized responses during helminth infections (3, 4). However, the cellular distribution of CD40 and CD154 is extremely broad (reviewed in Ref. 5), and the precise sources of both molecules during Th2 polarization remain unknown.

CD40 is most highly expressed on cells with APC function, such as DC. Indeed, Th2 priming by DC exposed to helminth products is dependent upon their expression of CD40 (6, 7). This suggests that CD40 ligation permits DC to effectively prime Th2 responses, in a similar manner to that generally seen during Th1 priming by DC (8, 9). The observation that $CD154^{-/-}$ mice exhibit defective activation of their DC populations and impaired Th2 priming during schistosomiasis provides further evidence for CD40-mediated activation of DC in a Th2 setting (10). Beyond activation of DC populations, CD40-CD154 interactions may have additional functions critical for Th2 generation. For example, B cell expression of CD40 is essential not only for their ability to expand and produce Ab (11), but also to act as APC (12), while on T cells CD40 can function as a costimulatory molecule to enhance their cytokine production (13). Thus, different cell types could provide critical CD40 signals at distinct stages in Th priming, polarization, expansion, and maintenance.

CD154 was originally described on activated T cells (reviewed in Ref. 1, 5), where it is expressed rapidly following signaling through the TCR. Adoptive transfer of CD154-deficient T cells has provided direct proof for the functional importance of T cell CD154 in the generation of Th1 responses in vivo (14, 15). Similar evidence has not yet been provided in a Th2 setting in vivo. Furthermore, it remains to be shown that a T cell source of CD154 is specifically required for stimulation of DC via CD40 in vivo, particularly during generation of Th2 responses. Indeed, a wide range of non-T cells, both in the periphery and the secondary lymphoid organs, also express CD154 upon activation, including B cells, epithelial cells, endothelial cells, fibroblasts, and monocytes and their derivatives (1, 5). This broad expression profile implies that non-T cells may have the ability to provide CD154-mediated stimulation of APC in vivo. Indeed, CD40 expression by APC has been reported to be essential for their migration to the draining lymph node (16), suggesting that cells other than T cells may act as an initial source of CD154.

To identify key sources of CD40 and CD154 during Th2 generation we have used adoptive transfer techniques and bone marrow (BM) chimeric mice to restrict the expression of each molecule to specific cellular compartments. This approach has revealed the expression of CD40 to be required solely upon the Ag-bearing DC, while full Th2 differentiation depends on CD154 expression by multiple cell types, including those of innate and adaptive origin. Additionally, different sources of CD154 are required for induction of diverse components of the Th2 response, with IL-4 displaying less restricted demands than IL-5, IL-10, and IL-13. This suggests that DC require multistep,

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[4] Abbreviations used in this paper: DC, dendritic cells; BM, bone marrow; SEA, soluble egg Ag from *Schistosoma mansoni*; WT, wild type; LN, lymph node; Tg, transgenic.

multicellular interactions through CD40 to effectively prime optimal Th2 responses in vivo.

Materials and Methods

Mice, reagents, and parasites

C57BL/6, CD40$^{-/-}$, CD154$^{-/-}$, Rag$^{-/-}$, and OTII mice were bred at the animal facilities of the School of Biological Sciences at the University of Edinburgh according to Home Office guidelines. Immunodeficient mice, and their experimental controls, were maintained on Borgal supplemented water (Intervet). Animals were used when aged 6 wk or older, and were age and sex matched within experiments. *Biomphalaria glabrata* snails infected with *S. mansoni* were obtained from Dr. Fred Lewis (Biomedical Research Institute, Rockville, MD). Endotoxin-free soluble egg Ag (SEA) from *S. mansoni* was prepared in-house as previously described (17). OVA peptide was synthesized by Advanced Biotechnology Centre, Imperial College, London (a gift from Prof. D. Gray, University of Edinburgh, Edinburgh, U.K.). All Abs for flow cytometry were purchased from BD Biosciences, while αCD3 (clone 145.2C11) and αCD28 (clone 37.51) mAb were produced in-house from hybridomas.

Production and phenotypic analysis of single and mixed BM chimeric mice

Wild type (WT) C57BL/6 and CD154$^{-/-}$ mice were lethally irradiated with 1250 rads of γ radiation to deplete their hematopoietic system. The following day irradiated recipient mice were reconstituted by i.v. injection of 4×10^6 donor BM cells from C57BL/6, CD154$^{-/-}$, or Rag$^{-/-}$ mice. All BM was depleted of mature T cells before transfer using CD90 microbeads followed by negative selection using MACS (Miltenyi Biotec). To restrict CD154-deficiency to non-hematopoietic cells, single BM chimeras were created whereby CD154$^{-/-}$ recipients were given 100% C57BL/6 WT BM. To restrict CD154-deficiency to hematopoietic cells, WT mice were given 100% CD154$^{-/-}$ BM. Controls of WT or CD154$^{-/-}$ recipients receiving WT or CD154$^{-/-}$ BM, respectively, were also generated. Alternatively, to restrict CD154-deficiency to lymphoid cells alone, mixed BM chimeras were created where WT mice received donor BM comprised of 80% Rag$^{-/-}$ (unable to make B or T cells) and 20% CD154$^{-/-}$ (unable to make CD154 but sufficient to repopulate the B and T cell pool). Similarly, to restrict CD154-deficiency to B and T cells and non-hematopoietic cells, CD154$^{-/-}$ recipient mice were given donor BM comprising 80% Rag$^{-/-}$ and 20% CD154$^{-/-}$ cells. All other hematopoietic populations were derived from both donor BM populations. The assumption that 80% would be derived from CD154$^{+/+}$ and 20% from CD154$^{-/-}$ BM is supported by the observation that control mice reconstituted with 80% WT and 20% CD154$^{-/-}$ BM had ~20% fewer CD154$^+$ CD4$^+$ T cells than those reconstituted with 100% CD154$^{+/+}$ BM (data not shown). Recipient mice were left for a minimum of 8 wk to allow their hematopoietic systems to reconstitute before being used in experiments. Initial observations showed that WT mice reconstituted with 80% WT and 20% CD154$^{-/-}$ BM (to control for 20% deficiency in all CD154-dependent hematopoietic cell interactions) showed a trend for suboptimal T and B cell responses compared with those reconstituted with control mixtures of 100% WT or 80% Rag$^{-/-}$ with 20% WT BM (data not shown), and were therefore chosen as the most suitable controls for all other experiments. Splenocytes from chimeras were assessed by flow cytometry to check for full reconstitution of B cell (B220$^+$) and CD4$^+$ and CD8$^+$ T cell populations. No significant reduction in any of these populations was observed (data not shown). Preliminary results excluded the use of Rag$^{-/-}$ mice as recipients because they demonstrated severely deficient reconstitution of lymphoid organs and the CD4$^+$ compartment (data not shown). To ensure CD154-deficiency in CD4$^+$ T cells, CD154 expression on splenocytes was determined following stimulation in vitro with 0.5 μg of immobilized αCD3 mAb for 10 h (18).

Immunization with DCs

DC were generated from WT BM cells by culture in vitro in the presence of rGM-CSF (PeproTech), as previously described (17). After 10 days cells were re-plated and cultured for a further 18 h with the Th2-conditioning helminth product SEA (25 μg/ml) or with medium alone (19). DC were then harvested and 5×10^5 injected i.p. into recipient mice. In some experiments 0.2 μM OVA peptide was added to the cells for the final 4 h of culture. Injected cells were typically >90% CD11c$^+$ MHCII$^+$ (data not shown).

In vivo OTII expansion assay

CD4$^+$ cells were purified from single cell suspensions of splenocytes and LN (lymph node) cells from naive OTII TCR transgenic (Tg) mice by positive MACS selection and 2×10^6 injected i.v. into WT or CD154$^{-/-}$ mice. Control mice were injected with PBS. Taking advantage of the Th2 adjuvant properties of SEA (19, 20), mice were immunized 1 day later with OVA pulsed SEA-treated DC. After 5 days, splenocytes were analyzed by flow cytometry to assess the frequency of CD4$^+$ cells positive for the Vα2 and Vβ5 TCR chains (expressed by OTII cells). Frequencies of naive OTII cells were determined in control mice immunized with non-OVA-pulsed DC/SEA. In agreement with previous reports (18, 21) frequency of endogenous CD4$^+$ cells did not differ between WT and CD154$^{-/-}$ recipients (data not shown).

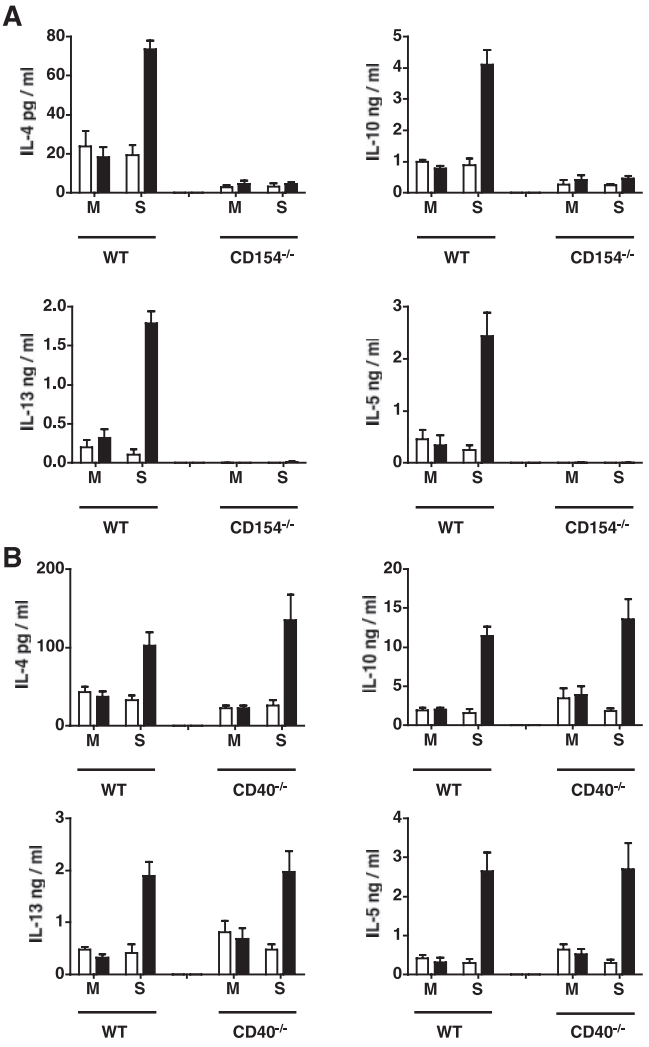

FIGURE 1. Critical requirement for recipient CD154 but not CD40 during Th2 priming by DC. Recall cytokine response by WT and CD154$^{-/-}$ mice (*A*) or WT and CD40$^{-/-}$ mice (*B*) 1 wk after i.p. immunization with 0.5×10^6 SEA-conditioned (S) or medium-conditioned (M) DC. Splenocytes were re-stimulated in vitro with medium (white bars) or SEA (black bars) and cytokine production measured by ELISA. Data are presented as mean ± SEM of 4 mice per group (*A*) or of 6–7 mice per group (*B*) and are representative of four repeat experiments each.

Recall cytokine assays

Immune responses induced in immunized or *S. mansoni* infected mice were determined by in vitro Ag-specific splenic recall assays. In DC transfer experiments, mice were culled 7 days after immunization to coincide with peak Th2 cytokine production (22). In OTII assays in vivo, mice were culled 5 days after DC transfer at which point expansion of OTII cells is reported to become dependent upon CD154 (23). In both experimental regimes, splenocytes were then re-stimulated in vitro with 15 μg/ml SEA or, in some experiments, 0.01–10 μM OVA peptide, in X-Vivo 15™ serum-free medium (BioWhittaker) supplemented with 2 mM L-glutamine and 50 μM 2-ME (Invitrogen). In some experiments, CD4$^+$ T cells were

purified by positive MACS selection and re-stimulated in vitro with 0.5 μg immobilized αCD3 mAb (clone 145.2C11) and 1 μg αCD28 mAb (clone 37.51). Culture supernatants were analyzed by ELISA at 72 h for production of IL-2, IL-4, IL-5, IL-10, IL-13, and IFN-γ by ELISA, and by ELISPOT for the number of IL-4 producing cells, as described previously (22).

S. mansoni *infection*

Mice were anesthetized before percutaneous infection with 75 cercariae. Mice were culled 7.5 wk postinfection during the period of acute Th2 response (24) but before the onset of severe pathology that occurs in CD154-deficient mice (3). Spleen and mesenteric LN cells were harvested for in vitro recall assays, and serum collected and analyzed by ELISA for IL-4 and total IgE content, using paired Abs and IgE standard (BD Biosciences). SEA-specific IgG1, IgG2a, IgG2b, and IgM content of serum was determined using 0.25 μg/well SEA capture Ag and anti-Ig detection Abs from Southern Biotechnology Associates. Plates were developed with *p*-nitrophenyl phosphate (Southern Biotechnology Associates).

Statistical analysis

The significance of the data was evaluated by Student's *t* test.

Results

CD40 is required exclusively on the Ag-presenting DC during Th2 induction in vivo

Immunization with Ag-pulsed BM-derived DC provides a robust method to study key cellular interactions during Th polarization and expansion, because deficiencies in genes of interest can be restricted either to the priming DC or the recipient mice. DC exposed to helminth products, such as schistosome SEA, drive potent Th2 responses following transfer into naive mice (19). We have previously shown that this process depends completely upon DC expression of CD40 (6, 7). To verify the role of CD154 during Th2 priming by DC, CD154$^{-/-}$ and WT control mice were immunized with SEA-conditioned WT DC (SEA-DC). WT recipients produced high levels of SEA-specific IL-4, IL-5, IL-13, and IL-10 (Fig. 1*A*); by contrast, CD154$^{-/-}$ recipients failed to make any detectable Th2 recall response (Fig. 1*A*). This demonstrated an overall deficiency in T cell priming rather than a switch in the character of the Th response, because CD154$^{-/-}$ recipients also failed to make upon challenge the low levels of SEA-specific IL-2 or IFN-γ seen in WT mice (data not shown).

Although DC expression of CD40 is critical for Th2 induction (6), it is unclear whether other CD154-CD40 interactions also contribute to the outcome of priming. To address this, we injected WT DC into CD40$^{-/-}$ mice, so that only the transferred DC were receptive to CD154-mediated signals, and CD40-CD154 interactions between recipient cells were prevented. CD40$^{-/-}$ mice receiving SEA-pulsed WT DC showed no defect in their ability to make Ag-specific Th2 cytokines (Fig. 1*B*), or IFN-γ or IL-2 (data not shown). Taken together these data strongly suggest that, during Th2 induction, CD154 is solely required for stimulating DC through CD40, and that there is no additional requirement for CD40-CD154 interactions between other cell types.

Multiple sources of CD154 contribute to optimal Th2 priming by DC

Having established that CD154-deficient recipients fail to generate Th2 responses following transfer of SEA-conditioned DC (Fig. 1*A*), we then determined whether CD154 expression on hematopoietic cells alone was sufficient for Th2 priming by transferring WT SEA-DC into CD154$^{-/-}$ mice that had been lethally irradiated and reconstituted with WT BM (Fig. 2*A*). In these single BM chimeric mice, CD154 expression on donor BM cells (Fig. 2*B*) proved sufficient for full Th2 development, with equivalent levels of IL-4, IL-5, IL-10, and IL-13 produced by splenocytes from

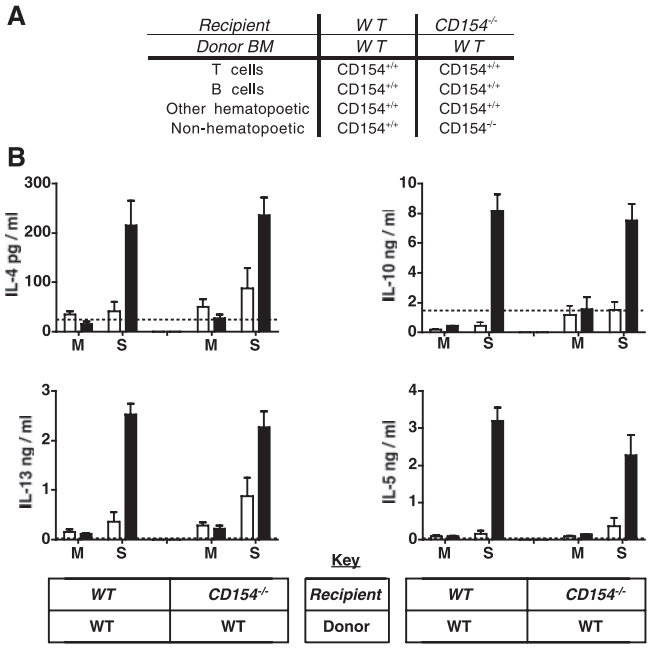

FIGURE 2. WT BM rescues defective Th2 responses in CD154$^{-/-}$ mice. WT or CD154$^{-/-}$ mice were lethally irradiated and then rescued by transfer with WT BM. *A*, Resultant genotype of each cell compartment in the chimeric mice. *B*, Following reconstitution, mice were immunized i.p. with 0.5×10^6 SEA-conditioned (S) or medium-conditioned (M) DC. Recall cytokine response by splenocytes to SEA (black bars) or medium (white bars) was measured 1 wk later. Data are presented as mean $\pm$ SEM of 3 mice per group. Dotted lines represent background cytokine levels produced upon recall of CD154$^{-/-}$ mice previously reconstituted with CD154$^{-/-}$ BM.

these mice compared with those from WT mice reconstituted with WT BM (Fig. 2*B*).

Within this hematopoietic pool, the primary candidate for the essential provision of CD154 is the CD4$^+$ T cell (14, 15). We first assessed whether expression of CD154 on T cells alone was sufficient for their expansion and polarization by transferring CD154$^{+/+}$ CD4$^+$ T cells from OTII mice into CD154$^{-/-}$ recipient mice, then immunizing with OVA peptide-pulsed SEA-DC. CD154$^{+/+}$ CD4$^+$ OTII cells (identified as being Vα2$^+$Vβ5$^+$) expanded upon immunization with OVA-pulsed SEA-DC irrespective of whether the recipient mice were CD154$^{-/-}$ or WT (Fig. 3*A*). Moreover, CD154$^{+/+}$ OTII cells primed in CD154$^{-/-}$ mice produced equivalent levels of OVA-specific IL-4 to those primed in WT mice (Fig. 3*B*), demonstrating that CD154 expression on T cells alone is sufficient for generation of IL-4 producing cells. However, OVA-specific production of IL-5, IL-13, and IL-10 was reduced by up to 50% when OTII cells were primed in a CD154-deficient environment, suggesting that sources of CD154 in addition to activated T cells are critical for optimal Th2 induction.

Although sufficient for limited Th2 development, CD4$^+$ T cell expression of CD154 proved not to be essential for optimal Th2 polarization. When CD4$^+$ cells from OTII mice on either a CD154$^{+/+}$ or CD154$^{-/-}$ background were transferred into CD154$^{+/+}$recipients, expansion of Vα2$^+$Vβ5$^+$ CD4$^+$ OTII cells occurred following immunization with OVA-pulsed SEA-DC irrespective of their ability to express CD154 (Fig. 4*A*). Moreover, the expanded CD154$^{-/-}$ OTII cells produced equivalent levels of peptide-specific Th2 cytokine as their CD154$^{+/+}$ counterparts (Fig. 4*C*). To exclude the possibly that T cell expansion and cytokine production in this transgenic system was independent of

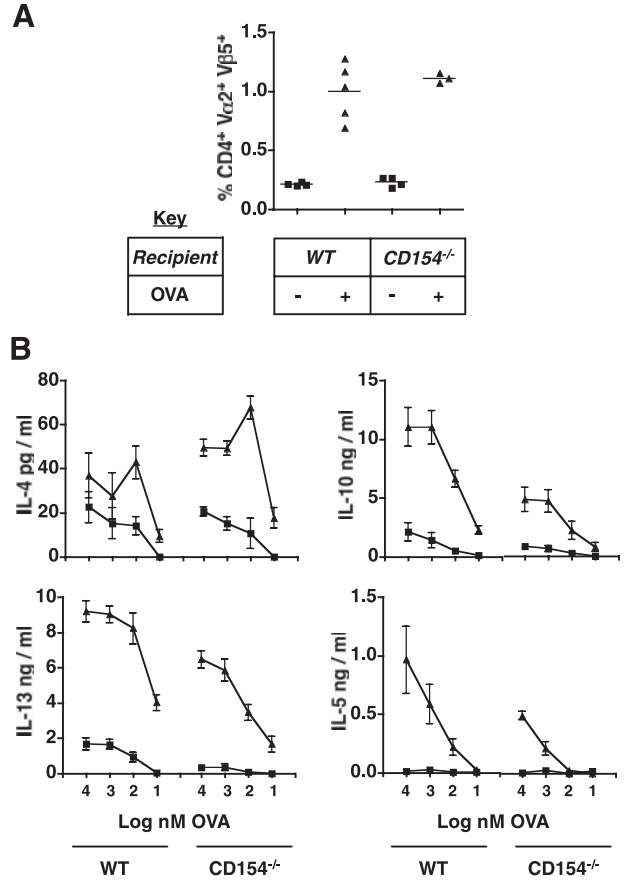

FIGURE 3. CD154 expression by recipient mice is required for optimal Th2 cytokine production by CD154$^{+/+}$ OTII CD4 T cells. WT and CD154$^{-/-}$ mice received 2 × 10^6 purified CD4$^+$ OTII T cells on a CD154$^{+/+}$ background. One day later mice were immunized with 0.5 × 10^6 SEA-conditioned DC that had been pulsed with OVA peptide (+, triangles) or medium alone (-, squares). After 5 days splenocytes were analyzed for their expression of Vα, Vβ, and CD4 by flow cytometry (*A*). Recall cytokine responses were measured after in vitro re-stimulation of splenocytes with OVA peptide (0.01–10 μM), and are presented as OVA-specific (calculated as levels produced in presence of OVA minus background levels detected after culture with medium alone) (*B*). Data are presented as values for individual mice with mean values displayed by lines (*A*), or as mean ± SEM of 3–5 mice per group (*B*). Data are from one of two repeat experiments.

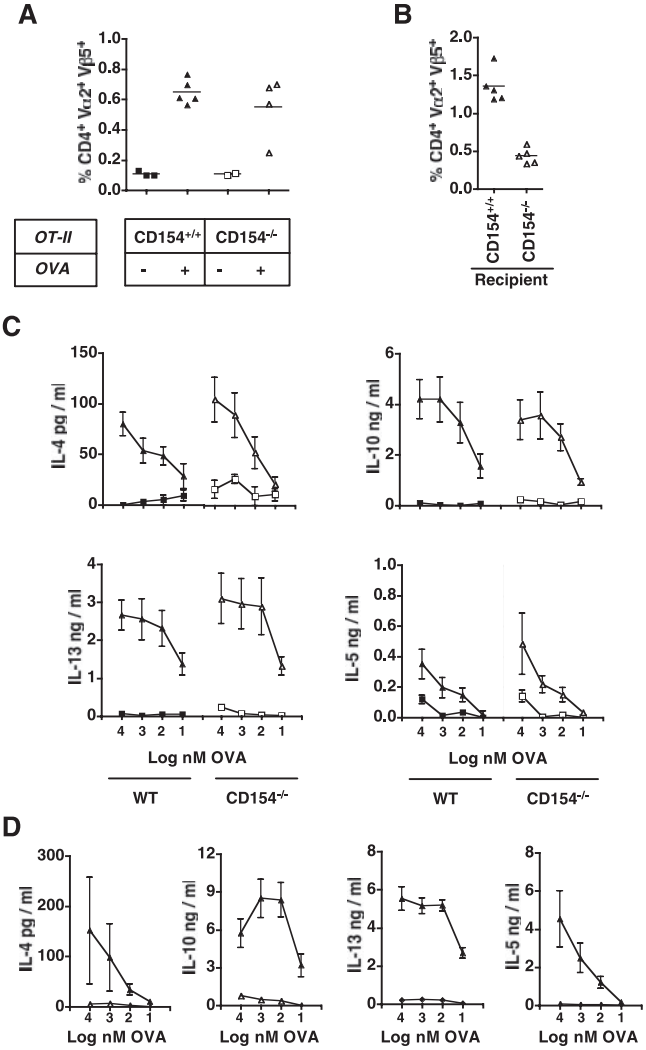

FIGURE 4. CD154 expression by OTII CD4$^+$ T cells is not essential for their expansion and cytokine production in WT mice. WT mice received 2 × 10^6 purified CD4$^+$ OTII T cells on a CD154$^{+/+}$ (filled symbols) or CD154$^{-/-}$ (open symbols) background (*A* and *C*). Alternatively, WT (filled symbols) or CD154$^{-/-}$ (open symbols) mice received 2 × 10^6 purified CD4$^+$ OTII T cells on a CD154$^{-/-}$ background (*B* and *D*). One day later mice were immunized with 0.5 × 10^6 SEA-conditioned DC that had been pulsed with OVA peptide (+, triangles) or medium alone (-, squares). After 5 days splenocytes were analyzed for their expression of Vα, Vβ, and CD4 by flow cytometry (*A* and *B*). Recall cytokine responses were measured after in vitro re-stimulation of splenocytes with OVA peptide (0.01–10 μM), and are presented as OVA-specific (calculated as levels produced in presence of OVA minus background levels detected after culture with medium alone) (*C* and *D*). Data are presented as values for individual mice with mean values displayed by lines (*A* and *B*), or as mean ± SEM of 2–5 mice per group (*C* and *D*). Data are from one of two repeat experiments.

CD154, we transferred CD154$^{-/-}$ OTII cells into CD154$^{-/-}$ mice before immunizing with OVA-pulsed SEA-DC. Under these conditions, the expansion and cytokine production of CD154$^{-/-}$ OTII cells was significantly impaired compared with that in CD154$^{+/+}$ recipients (Fig. 4, *B* and *D*).

Taken together, these data (Figs. 3 and 4) strongly suggest the critical involvement of a non-T cell source of CD154 during optimal Th2 polarization. Furthermore, they suggest that CD154 expression by individual Ag-specific T cells is not an absolute requirement for their Th2 development.

To examine these issues in a non-TCR Tg system, we generated mixed BM chimeric mice in which CD154 was selectively depleted either on both lymphoid (Rag-dependent) and non-lymphoid (Rag-independent) BM-derived cells, or only upon lymphoid cells, by reconstituting lethally irradiated mice with 100% CD154$^{-/-}$ BM, or a mix of 20% CD154$^{-/-}$ and 80% Rag$^{-/-}$ BM, respectively (Fig. 5*A*). Mice receiving Rag$^{-/-}$/CD154$^{-/-}$ BM or CD154$^{-/-}$ BM alone had greatly reduced proportions of T cells that could express CD154 compared with control mice receiving 20% CD154$^{-/-}$ and 80% WT BM (Fig. 5*B*). Strikingly, mice in which CD154-deficiency was restricted to Rag-dependent lymphoid cells (Rag$^{-/-}$/CD154$^{-/-}$ BM) were capable of mounting a Th2 response upon transfer of SEA-conditioned DC, as judged by expansion of SEA-specific IL-4 producing cells (Fig. 5*C*) and splenic IL-4, IL-10, (Fig. 5*D*), and IL-2 production (data not shown). Provision of CD154 by non-lymphoid cells also enabled induction of IL-5 and IL-13, although not to the levels detected in control mice (WT/CD154$^{-/-}$ BM) (Fig. 5*D*). In contrast, mice that were reconstituted with CD154$^{-/-}$ BM alone and lacked

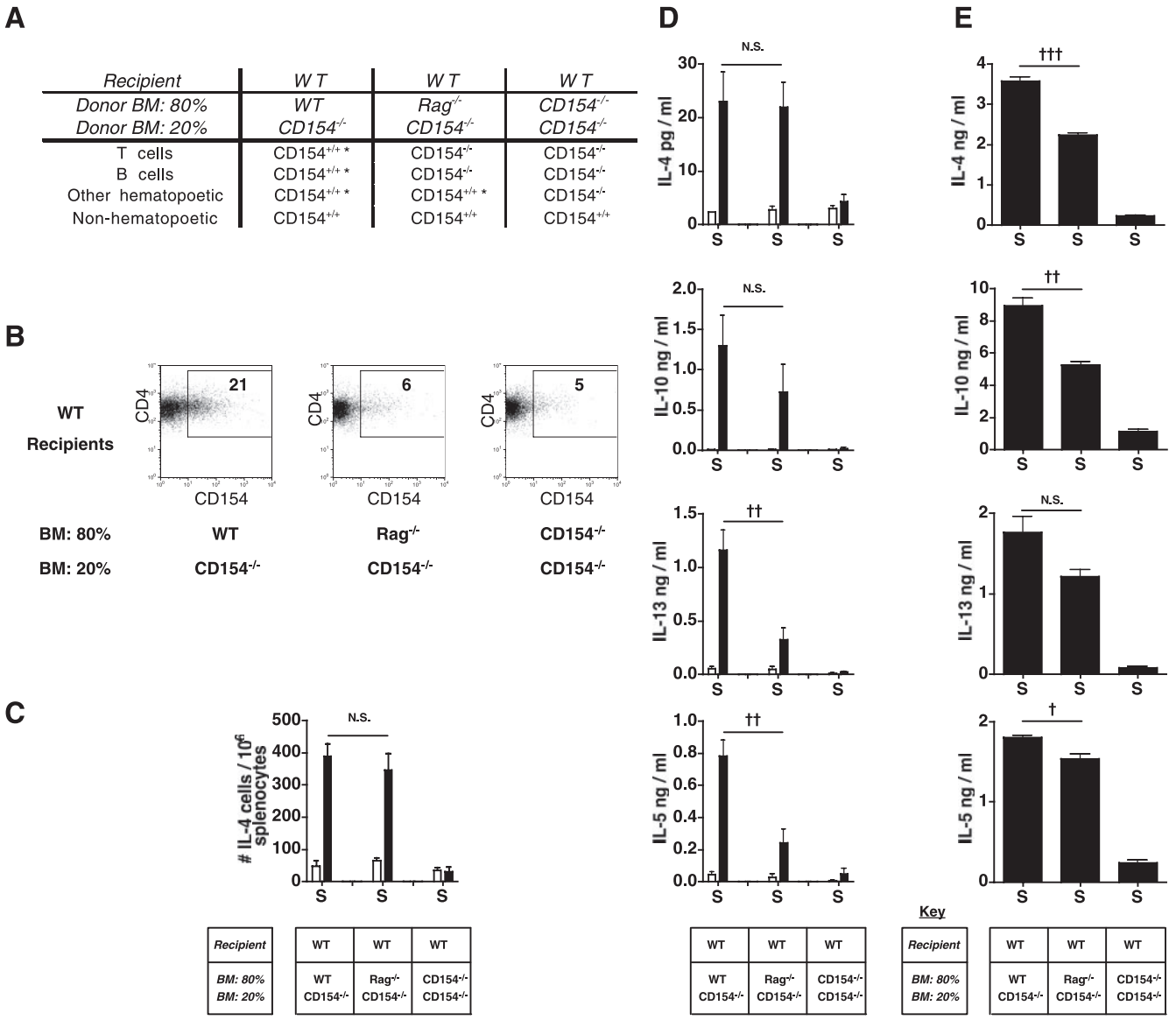

FIGURE 5. Induction of Th2 responses by DC in mixed BM chimeric mice. BM chimeras were generated by lethally irradiating WT mice followed by reconstitution with 4×10^6 BM cells comprised of 80:20% mixtures of WT and $CD154^{-/-}$ or $Rag^{-/-}$ and $CD154^{-/-}$ cells, or of 100% $CD154^{-/-}$ cells. *A*, Resultant genotype of cell compartments of the chimeric mice. * = majority (80 vs 20%) of cells. *B–E*, Following reconstitution mice were immunized i.p. with 0.5×10^6 SEA-conditioned (S) DC and sacrificed 1 wk later. *B*, Representative plots of splenocytes that were cultured with αCD3 mAb and then analyzed by flow cytometry to determine expression of CD154 on $CD4^+$ T cells. The percentage of $CD4^+$ T cell expressing CD154 is shown in the gated area, and is from one representative mouse per BM chimera group. *C*, The number of IL-4 producing splenocytes were measured by ELISPOT following in vitro re-stimulation with SEA (black bars) or medium (white bars). *D*, Cytokine responses measured by ELISA following re-stimulation of splenocytes with SEA (black bars) or medium (white bars). *E*, Cytokine responses by purified splenic $CD4^+$ T cells were measured following stimulation in vitro with αCD3 and αCD28 mAbs. Data are presented as mean ± SEM of 4–6 mice per group (*D*), or mean ± SEM of triplicate (*C*) or quadruplicate (*E*) wells of pooled cells, and are representative of two experiments. †, $p < 0.05$; ††, $p < 0.01$; †††, $p < 0.001$; N.S., $p > 0.05$.

lymphoid and non-lymphoid hematopoietic expression of CD154 were unable to develop Th2 responses upon DC transfer (Fig. 5, *C* and *D*). This demonstrates that a non-lymphoid (Rag-independent) BM-derived source of CD154 contributes directly to Th2 priming. Purified $CD4^+$ T cells from mice reconstituted with $CD154^{-/-}$ BM also exhibited a marked inability to respond to re-stimulation ex vivo with αCD3 and αCD28 mAb, compared with cells from mice in which all hematopoietic cells could express CD154 (WT/$CD154^{-/-}$) or whose hematopoietic expression of CD154 was restricted to non-lymphoid cells ($Rag^{-/-}$/$CD154^{-/-}$) (Fig. 5*E*). This emphasizes the generalized defect in priming of T cell responses in vivo in these mice, rather than simply a requirement for CD154-CD40 interactions during ex vivo re-stimulation. These data reiterate the significant contribution of CD154 on non-lymphoid donor BM-derived cells during Th2 priming in vivo (Figs. 3 and 4), whereas T cell and B cell derived CD154 is largely dispensable. An optimal Th2 response, however, does require expression of CD154 on lymphoid (Rag-dependent) cells, because some elements of the Th2 cytokine response were significantly reduced in mice whose hematopoietic expression of CD154 was restricted to non-lymphoid cells ($Rag^{-/-}$/$CD154^{-/-}$) in comparison to WT/$CD154^{-/-}$ controls (Fig. 5, *D* and *E*).

Given that WT donor BM could restore full Th2 development in vivo in $CD154^{-/-}$ recipients (Fig. 2), we tested whether the provision of CD154 by non-lymphoid BM-derived cells alone was sufficient to restore Th2 priming in $CD154^{-/-}$ mice (Fig. 6*A*). As

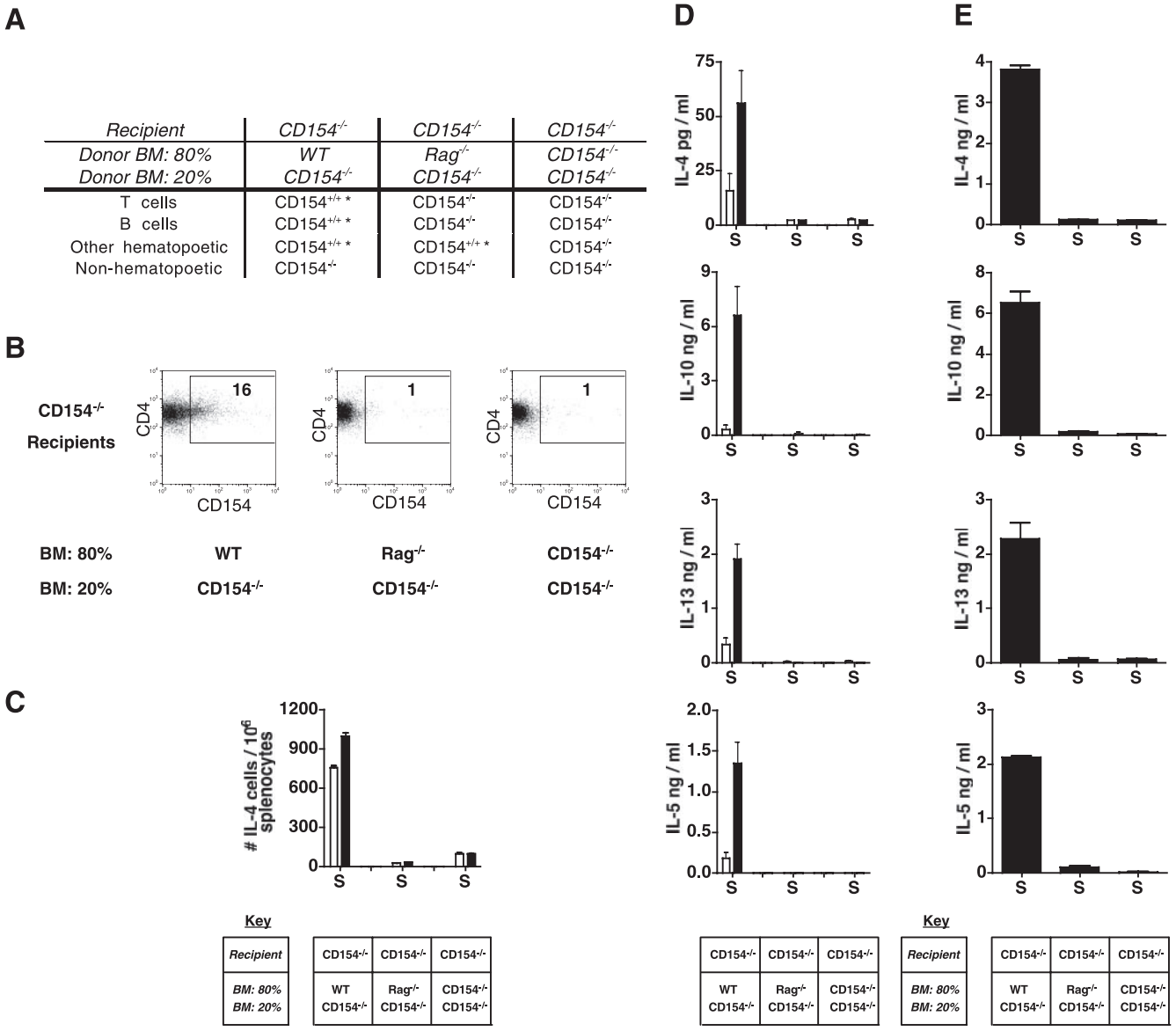

FIGURE 6. Induction of Th2 responses by DC in mixed BM chimeric mice. BM chimeras were generated by lethally irradiating $CD154^{-/-}$ mice followed by reconstitution with 4×10^6 BM cells comprised of 80:20% mixtures of WT and $CD154^{-/-}$ or $Rag^{-/-}$ and $CD154^{-/-}$ cells, or of 100% $CD154^{-/-}$ cells. *A*, Resultant genotype of cell compartments of the chimeric mice. * = majority (80 vs 20%) of cells. *B–E*, Following reconstitution for no <8 wks mice were immunized i.p. with 0.5×10^6 SEA-conditioned (S) DC and sacrificed 1 wk later. *B*, Representative plots of splenocytes that were cultured with αCD3 mAb and analyzed by flow cytometry to determine expression of CD154 on $CD4^+$ T cells. The percentage of $CD4^+$ T cell expressing CD154 is shown in the gated area, and is from one representative mouse per BM chimera group. *C*, The number of IL-4 producing splenocytes was measured by ELISPOT following in vitro re-stimulation with SEA (black bars) or medium (white bars). *D*, Recall cytokine responses measured by ELISA following re-stimulation of splenocytes with SEA (black bars) or medium (white bars). *E*, Cytokine responses by purified splenic $CD4^+$ T cells from mice immunized with SEA-treated DC were measured following stimulation in vitro with αCD3 and αCD28 mAbs. Data are presented as mean ± SEM of 4–6 mice per group (*D*), or mean ± SEM of triplicate (*C*) or quadruplicate (*E*) wells of pooled cells, and are representative of two experiments.

expected, $CD4^+$ T cell expression of CD154 was absent in $CD154^{-/-}$ mice reconstituted with $Rag^{-/-}/CD154^{-/-}$ BM (Fig. 6*B*). Surprisingly, these mice demonstrated the same phenotype as $CD154^{-/-}$ mice reconstituted with $CD154^{-/-}$ BM alone, failing to mount a detectable Th2 response (Fig. 6, *C* and *D*). Again, this was a defect in T cell priming in vivo, as purified $CD4^+$ cells from these mice also exhibited a marked inability to respond to re-stimulation ex vivo with αCD3 and αCD28 mAb (Fig. 6*E*). The disparity in ability of WT (Fig. 5) but not $CD154^{-/-}$ (Fig. 6) recipients reconstituted with $Rag^{-/-}/CD154^{-/-}$ mice to mount Th2 responses suggests that CD154 on donor non-lymphoid cells alone is unable to support T cell priming, and that additional host-derived $CD154^+$ populations are required for this process.

Lymphoid cell expression of CD154 is critical for Ab production but dispensable for Th2 induction during experimental schistosomiasis

Having revealed that multiple sources of CD154 are required for induction of Th2 responses in a DC-driven model of T cell polarization, we wanted to address the functions and provision of CD40 and CD154 in a Th2-biased infection setting. $CD40^{-/-}$ mice mounted Th2 responses that were impaired to the same degree as

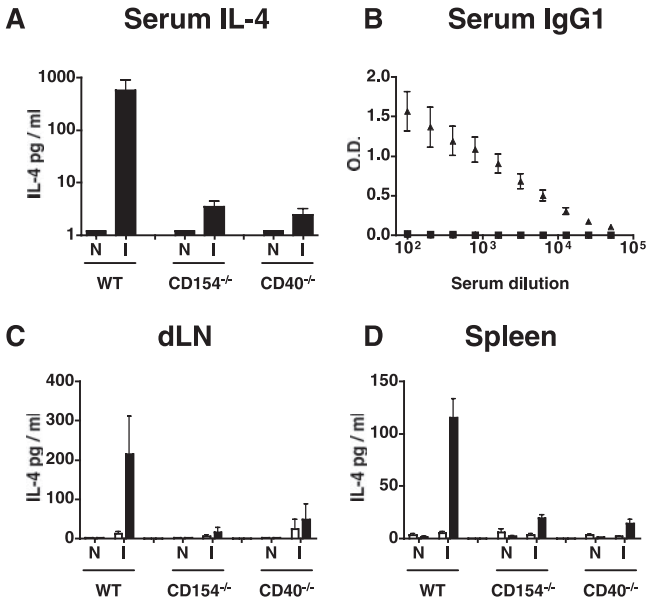

FIGURE 7. A critical requirement for host CD40 during experimental schistsomiasis. WT, CD154$^{-/-}$, and CD40$^{-/-}$ mice were infected with 75 *S. mansoni* cercariae. After 7.5 wk mice were killed and serum analyzed by ELISA for IL-4 (*A*) and SEA-specific IgG1 (*B*). In addition, recall cytokine responses were measured after in vitro re-stimulation of mesenteric LN cells (*C*) or splenocytes (*D*) with SEA (black bars) or medium alone (white bars). Data presented as mean ± SEM of 4–7 mice per group, and are representative of two repeat experiments. I = mice infected with *S. mansoni*, N = naive controls. For Ab data, WT = triangles, CD154$^{-/-}$ = squares, and CD40$^{-/-}$ = diamonds, and either infected (filled symbols) or naive (open symbols).

CD154$^{-/-}$ mice during infection with *S. mansoni*, as judged by levels of serum IL-4, SEA-specific IgG1, and total IgE, and SEA-specific IL-4, IL-5, and IL-13 production by splenocytes and mesenteric LN cells (Fig. 7, *A–D*, and data not shown). The indistinguishable responses of these strains strongly suggest that the requirement for CD40 and CD154 during Th2 induction to schistosomes is primarily for their cointeraction, rather than with any possible alternative ligands (25–27).

Next, we generated mixed BM chimeric mice to determine the importance of lymphoid and non-lymphoid sources of CD154 for Th2 induction and development during active schistosome infection. Further, because CD154 expression by T cells is recognized as the major mechanism by which they drive T-dependent humoral responses (11), we also asked whether lymphoid cell expression of CD154 was critical for B cell class-switching and Ab production during Th2 disease. As expected, control (WT) mice that had been reconstituted with a mixture of WT/CD154$^{-/-}$ BM cells showed the characteristic Th2 polarized cytokine response induced following the onset of schistosome egg production, as judged by elevated serum IL-4, and strong SEA-specific splenocyte production of Th2 cytokines (Fig. 8, *B* and *C*). These mice also produced high levels of SEA-specific serum IgG1, IgG2a, IgG2b, and IgM, and total IgE (Fig. 8, *D* and *E*, and data not shown). In contrast, and in keeping with the data generated following DC transfer or using a TCR Tg system (Figs. 3–6), chimeras that lacked T and B cell expression of CD154 (Rag$^{-/-}$/CD154$^{-/-}$ BM recipients) retained the ability to mount overt Th2 responses following infection (Fig. 8, *B* and *C*). Despite this, no Ag-specific IgG1, IgG2b (Fig. 8*E*), or IgG2a (data not shown) were detectable in the serum of these mice. SEA-specific IgM was also greatly reduced, and no significant elevation in total serum IgE was seen (Fig. 8, *D* and *E*). As expected, CD154$^{-/-}$ mice reconstituted with CD154$^{-/-}$ BM were unable to generate Th2 responses during schistosome infection and did not produce any Ag-specific IgG1, IgG2a, IgG2b, or IgM, or elevated levels of serum IgE (Fig. 8, *B–E*, and data not shown). These infection data suggest that T cell expression of CD154 is not required for Th2 development in a physiologically relevant infection system in vivo. Furthermore, the data illustrate that lymphocyte expression of CD154 is critical for B cell responses even in the face of robust Th responses, drawing a distinct line between costimulatory requirements for T cell vs B cell responses in a Th2 environment.

Discussion

CD154 plays a critical role in Th2 immunity, as shown by the marked inability of CD154 deficient mice to mount polyclonal Th2 responses during schistosomiasis (3), trichinosis (4), and following immunization with SEA-conditioned DC (Fig. 1*A*). Under such Th2 conditions it acts as a costimulatory, not polarizing, signal, demonstrated by impairment of both Th1- and Th2-associated cytokines and Ab isotypes (data not shown). We present novel evidence that CD154 provision by diverse populations is critical for full development of adaptive immune responses during Th2 dominated immunity.

Many studies have demonstrated that CD4$^+$ T cell expression of CD154 is important in their expansion (14, 15) and induction of disease (28) in Th1 dominated settings. We show CD154 expression by CD4$^+$ T cells also functions to permit priming of Th2 polarized responses. Indeed, expression of CD154 exclusively by Ag-specific CD4$^+$ T cells was sufficient for their expansion and production of IL-4 in vivo (Fig. 3). More surprisingly, we provide definitive evidence that CD154 expression by BM-derived non-lymphoid (innate) cells contributes directly toward Th2 induction. This is demonstrated by the fact that irradiated WT mice reconstituted with CD154$^{-/-}$ BM could not mount a Th2 response upon SEA-DC transfer, whereas appropriate levels of IL-4 production and expansion of IL-4 producing cells were observed in mice reconstituted with a mixture of Rag$^{-/-}$ and CD154$^{-/-}$ BM (Fig. 5). A role for non-T cell provision of CD154 in induction and/or re-stimulation of Th2 cells is also strongly suggested by the observation that CD154$^{+/+}$ Ag-specific OTII T cells primed in a CD154$^{-/-}$ environment display defective IL-5, IL-10, and IL-13 production (Fig. 3). Importantly, mixed BM chimeric mice generated by reconstituting CD154$^{-/-}$ recipients with a mixture of Rag$^{-/-}$ and CD154$^{-/-}$ BM showed a complete absence of Th2 development (Fig. 6), demonstrating that provision of CD154 by non-lymphoid BM-derived cells alone is not sufficient for the generation of Th2 responses. Non-lymphoid hematopoietic cells must therefore act in concert with another CD154$^+$ population. Given that WT BM transferred into CD154$^{-/-}$ recipients provides an environment that is sufficient to support Th2 induction by DC (Fig. 2), these CD154$^+$ cells could be either lymphoid (Fig. 2) or of recipient origin (Figs. 5 and 6). CD40 stimulation of DC by non-CD4$^+$ T cells is not unprecedented. CD8 T cells can provide CD154 to DC in the absence of CD4$^+$ T cells (29). However, in our system potential non-lymphoid candidates include mast cells and NK cells, because both can express CD154 (5) and are known to contribute to the induction of Th1 responses by DC (30, 31). Furthermore, macrophages and NK cells communicate via CD154 following exposure to schistosome glycans (32).

Our data also show that CD154 expression by individual Ag-specific CD4$^+$ T cells, despite being sufficient for their expansion and production of IL-4, is dispensable for this process. We found that CD154$^{-/-}$ OTII Tg CD4$^+$ cells underwent equivalent expansion and produced equal levels of cytokine compared with their

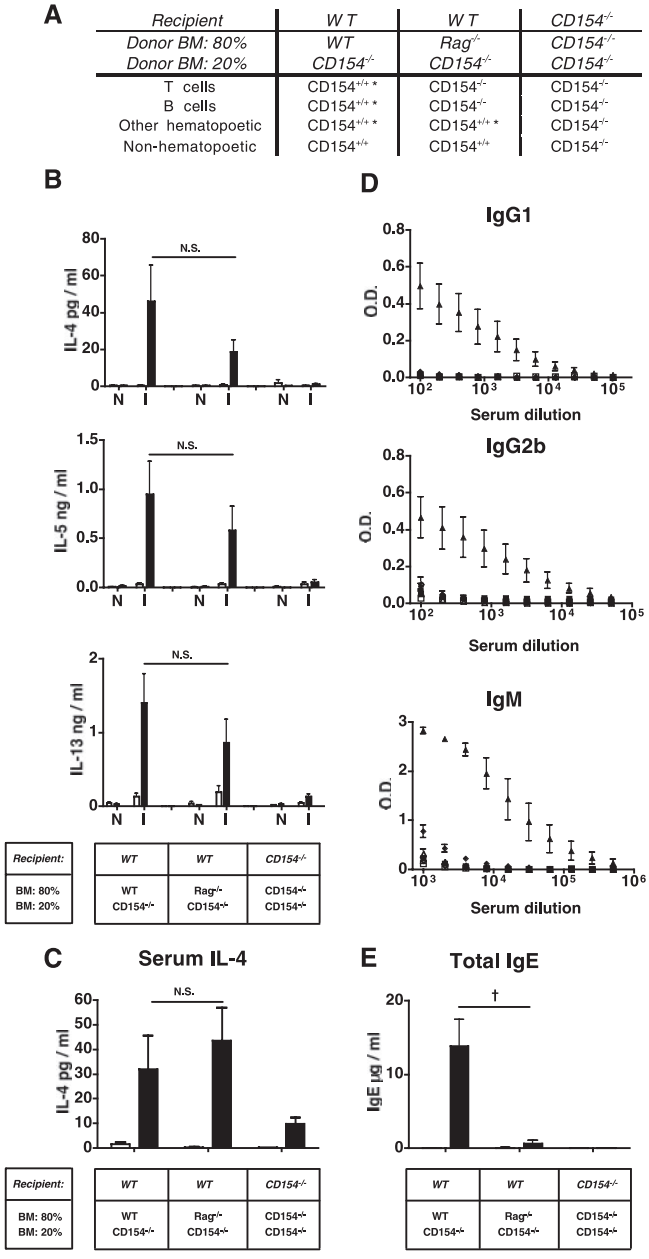

FIGURE 8. Ab production and generation of Th2 responses in mixed BM chimeric mice infected with *S. mansoni*. BM chimeras were generated by lethally irradiating WT or CD154$^{-/-}$ mice followed by reconstitution with 4×10^6 BM cells comprised of 80:20% mixtures of WT and CD154$^{-/-}$, or Rag$^{-/-}$ and CD154$^{-/-}$ cells, or of 100% CD154$^{-/-}$ cells. *A*, Resultant genotype of cell compartments of the chimeric mice. * = majority (80 vs 20%) of cells. *B–E*, Following reconstitution mice were infected with 75 *S. mansoni* cercariae. After 7.5 wk, mice were sacrificed and recall cytokine responses were measured after in vitro re-stimulation of splenocytes with SEA (black bars) or medium alone (white bars) (*B*). Serum was analyzed by ELISA for IL-4 (*C*), SEA-specific IgG1, IgG2b, and IgM (*D*), and total IgE (*E*). Data are presented as mean ± SEM of 4–6 mice per group, and are representative of two repeat experiments. I = mice infected with *S. mansoni*, N = naive controls. For Ab data, WT recipients of WT/CD154$^{-/-}$ = triangles, WT recipients of Rag$^{-/-}$/CD154$^{-/-}$ = diamonds, and CD154$^{-/-}$ recipients of CD154$^{-/-}$ = squares, and either infected (filled symbols) or naive (open symbols). †, $p < 0.05$; N.S., $p > 0.05$.

CD154$^{+/+}$ counterparts, when primed within a CD154 sufficient environment (Fig. 4), although it is possible that endogenous SEA-specific T cells may have provided a source of CD154 in these experiments. Certainly, a >70% reduction in the number of polyclonal T cells able to express CD154 did not affect IL-4 production or expansion of IL-4 producing cells following immunization with SEA-conditioned DC or infection with schistosomes (Figs. 5 and 8). This contrasts documented responses in NOD mice, in which a 50% reduction in the number of T cells expressing CD154 leads to the complete absence of the onset of diabetes (28). Given that signaling through CD154 has been reported to lead to T cell IL-2 production (33) and result in the induction of regulatory T cell activity (34), the absence of T cell CD154 in our system could theoretically release the pressure from regulatory T cells, effectively masking any defect in Th generation. This is unlikely, however, because we see no effect of depleting CD25$^+$ regulatory T cells on the generation of Th responses primed by SEA-conditioned DC (22).

CD40$^{-/-}$ and CD154$^{-/-}$ mice displayed equivalent defects in their ability to mount Th2 responses during patent schistosome infection (Fig. 7). The generation of SEA-specific Th2 responses upon immunization with SEA-conditioned DC depends upon DC expression of CD40 (6) and CD154 expression by recipient mice (Fig. 1*A*). These data strongly suggest that it is the interaction between CD40 and CD154, rather than with any alternative ligands (26, 27, 35), that is the predominant requirement for effective Th2 priming. The unimpaired Th2 responses observed following transfer of WT DC into CD40$^{-/-}$ mice shows that CD40 expression is required on the Ag-loaded DC alone (Fig. 1*B*), contrasting the significant contribution to microbial-specific Th17 (G. Perona-Wright, S. J. Jenkins, R. A. O'Connor, H. J. McSorley, R. M. Maizels, S. M. Anderton, and A. S. MacDonald, submitted for publication) and Th1 (13) that CD40 provision by other cells can make. Our data also imply that retrograde CD154 signaling to T cells is not vital for their production of Th2 cytokines in vivo, contrasting previously published in vitro work (36). We conclude that the primary function of CD154 in the generation of Th2 cells is to stimulate APC through CD40 for effective Th2 priming and not for costimulatory cross-talk between other cell types. We have recently shown that up-regulation of OX40L is a major downstream consequence of CD40 ligation on SEA-treated DC, and that anti-OX40 mAb treatment rescues defective priming by CD40$^{-/-}$ SEA-DC (22). Together with our present findings, this suggests that CD154 controls Th2 induction to schistosomes ultimately through provision of OX40 signals.

Intriguingly, we have found that costimulatory requirements distinguish the different Th2 cytokines. IL-4 appears to have the least stringent requirements for CD154, because expression restricted to Ag-reactive T cells alone (Fig. 3) was sufficient for expansion of IL-4 producing cells but not for optimal secretion of IL-5, IL-13, or IL-10. This cytokine profile was mirrored when expression of CD154 was restricted to predominantly non-lymphoid BM-derived cells (Fig. 5*C*). However, in this experiment IL-4 may partly be provided by non-T cells, because production of all Th2 cytokines (including IL-4), was suboptimal following polyclonal activation of purified CD4$^+$ T cells (Fig. 5*D*). Given that CD40 is required only on Ag-bearing DC for Th2 induction (Fig. 1), we envisage that DC require a minimum of one single costimulation event (that of DC CD40 by cognate T cell CD154) to become competent inducers of IL-4, whereas they require multistep "licensing" by successive sources of CD154 to prime full type 2 immune responses. However, we cannot yet rule out that the alternative ligands of CD154 (26, 27) act downstream of CD40-activation of DC in the initiation of IL-5, IL-13, and IL-10 production. Either way, this suggests that T cells require quantitatively or qualitatively greater input to differentiate to IL-5,

IL-13, and IL-10 producers. This hierarchy is less evident during experimental schistosomiasis (Fig. 8), which may reflect repeated exposure to Ag and Ag-bearing DC in the chronic disease setting.

In addition to exhibiting defects in the generation of Th responses, a major phenotype of CD154 and CD40 deficiency in both humans and mice is severely impaired T-dependent B cell responses (3, 18, 21, 37) (Figs. 7 and 8). We demonstrate that CD154 expression by lymphoid cells is critical for class switching during schistosomiasis and cannot be substituted by expression on innate cells (Fig. 8). This requirement for lymphoid CD154 is subsequent to Th2 expansion (Fig. 8) and, therefore, most likely supports follicular homing of T cells (38) or provision of CD40 signals to B cells (11). T cells are the probable lymphoid source of CD154 in our experiments, because CD154 expression by B cells is reported to be dispensable, yet that of $CD4^+$ T cells is sufficient, for class-switching following immunization with Ag in alum (11). Although the requirement for B cells per se during Th2 induction by schistosomes is at present controversial (39, 40), we can exclude a major role for B cell Ab production in this process, expanding on previous findings that FcRγ-chain signaling does not contribute to Th2 polarization during schistosomiasis (40).

Our data define the multistep cellular interactions that occur in the generation of Th2 immunity. Just as effective Th1 priming by DC conditioned with Th1-associated pathogens requires the input of additional cell types such as NK cells (31), we envisage a similar multicellular network in the initiation of Th2 responses. We have already identified that an additional cell type to T cells, B cells, and DC acts as the main source of IL-10 involved in Th2 polarization (41). We now highlight CD154 provision as central to this network, being a factor that can delineate processes that lead to Th2 subset development, and propose that $CD4^+$ T cell provision of CD154 can be dispensable for this process. The demarcation of cells and factors involved in the generation of specific Th2 cytokine subsets has important implications for disease therapy whereby beneficial or harmful effects of Th2 cytokines could ultimately be selectively promoted or inhibited.

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Disclosures

The authors have no financial conflict of interest.

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