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Original Article

Blocking the CD154–CD40 interaction with anti-CD154 antibody differentially regulates interleukin-4 synthesis in T cells and IgE production in B cells

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Abstract

Using severe combined immunodeficiency mice engrafted with peripheral blood mononuclear cells from atopic patients, we analyzed the regulatory effects of anti-CD154 antibody on the *in vivo* induction of human interleukin (IL)-4 and IgE expression. Although anti-CD154 treatment of engrafted mice abrogated mature Cε transcription and IgE production, IL-4 mRNA levels were enhanced by the treatment. In addition, anti-CD154-induced enhancement of intracellular IL-4 synthesis was detectable in both $CD4^+$ and $CD8^+$ T cell subsets. Furthermore, upregulation of germline Cε transcription could be seen in anti-CD154-treated mice. These results demonstrate that blocking the CD154–CD40 interaction with anti-CD154 differentially regulates IL-4 synthesis in T cells and IgE production in B cells. Our data also indicate that antibody ligation of CD154 on T cells causes enhanced synthesis of IL-4, thereby contributing to upregulation of germline Cε transcription in B cells.

Key words: CD154, CD40, germline Cε, IgE, interleukin-4.

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Introduction

T cell help required for the induction of human IgE synthesis in B cells is mediated through interleukin (IL)-4 or IL-13 and CD154 provided by activated $CD4^+$ T cells. Interleukin-4 and IL-13 direct IgE synthesis in B cells by initiating germline Cε transcription. Such overlapping effects of IL-4 and IL-13 are brought about by using the IL-4 receptor α-chain that functions as a common component of the receptors of both cytokines.[1,2] Ligation of the counter-receptor for CD154 (i.e. CD40) in conjunction with the presence of either IL-4 or IL-13 induces IgE isotype switching in B cells, allowing mature Cε transcription and IgE synthesis. Because T cell-dependent B cell responses, including proliferation, isotype switching and differentiation, can be blocked by soluble forms of CD40 or by anti-CD154 antibodies,[3,4] the CD154–CD40 interaction is central to the delivery of contact-dependent T cell help to B cells.

Although signals initiated through the T cell receptor (TCR)/CD3 complex and CD28 are required for T cell activation and cytokine production, the role of CD154 expressed on activated T cells in regulating cytokine production is poorly understood. Only a few reports have shown that *in vitro* costimulation of CD154 with CD40 on B cells or with an anti-CD154 antibody enhances T cell proliferation and Th2 cytokine production.[5,6] In the present study we analyzed the regulatory effects of anti-CD154 monoclonal antibody (mAb) on the *in vivo* expression of both human IL-4 and IgE in severe combined immunodeficiency (SCID) mice engrafted with peripheral blood mononuclear cells (PBMC) from atopic patients.

METHODS

Mice

Female SCID mice (6–7 weeks old) were purchased from CLEA Japan (Tokyo, Japan). Some leaky mice were eliminated by the measurement of serum levels of IgM and IgG as described previously.[7] Non-leaky mice were maintained under specific pathogen-free conditions and had free access to sterilized food and water. All procedures were performed under a laminar flow hood and conducted according to the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Reconstitution of SCID mice with atopic human PBMC

Peripheral blood mononuclear cells were isolated from the heparinized venous blood of atopic patients allergic to house dust mites by density gradient sedimentation, as described previously.[8] Based on preliminary studies using SCID mice inoculated intraperitoneally with 3×10^7 cells suspended in 0.5 mL Hanks' balanced salt solution (HBSS), PBMC from three patients were selected to cause IgE production and were used in this study. The patients' serum IgE levels were 473, 810 and 3740 IU/mL. The donors used for collection of peripheral blood gave informed consent to participate in the study.

Collection of blood and preparation of peritoneal lavage cells from SCID mice

Severe combined immunodeficiency mice reconstituted with atopic human PBMC were bled retro-orbitally at a weeks interval. Serum levels of human IgE and IgG were measured by isotype-specific sandwich radioimmunoassays, as described previously.[9] Peritoneal washings were harvested 0–7 days postengraftment from the mice by injecting 5 mL cold HBSS. Phenotypic analysis showed that approximately 50% of the peritoneal lavage cells was of human origin immediately after PBMC transfer and this ratio gradually decreased afterwards.

Antibodies used for *in vivo* treatment of SCID mice

Mouse antihuman CD154 mAb (IgG1), which inhibits the binding of CD154 to CD40, was obtained from Ancell (Bayport, MN, USA) and purified myeloma mouse IgG1 (control antibody) was from Cappel (West Chester, PA, USA). Either anti-CD154 mAb or control antibody (100 μg/animal per day) was administered intraperitoneally to reconstituted SCID mice for up to 5 days.

Reverse transcription–polymerase chain reaction analysis

Extraction of total cellular RNA, cDNA synthesis by reverse transcription (RT) and polymerase chain reaction (PCR) were performed exactly as described previously.[9] The 5′ sense primers and 3′ antisense primers used for the amplification of mRNA for human IL-4, germline Cε and mature Cε in peritoneal lavage cells from reconstituted SCID mice were the same as described previously.[9,10] Primers pairs specific for human β-actin were obtained from Clontech Laboratories (Palo Alto, CA, USA). For semiquantitative PCR analysis, total cellular RNA, which contains both murine and human RNA, was serially diluted and each dilution was applied to PCR. The PCR products were separated on a 2% agarose gel and stained with ethidium bromide. Polymerase chain reaction blots were quantitated by scanning with a GS-700 imaging densitometer (Bio-Rad Laboratories, Hercules, CA, USA). The β-actin amplification product obtained from each RNA sample (100 ng) was used as an external standard and the amount of each amplification product was evaluated as the ratio of IL-4, germline Cε or mature Cε mRNA : β-actin mRNA × 100.

Flow cytometric analysis

Surface expression of human αβ TCR, CD4, CD8 and CD45 and murine H-2K^d on peritoneal lavage cells from reconstituted SCID mice was analyzed by flow cytometry as described previously.[7] Detection of intracellular human IL-4 was performed by using a commercially available intracellular staining kit (Becton Dickinson, San Jose, CA, USA). Briefly, cells were incubated with fluorescein isothiocyanate (FITC)- or biotin-labeled mouse mAbs to human αβ TCR, CD4, CD8 or CD45, phycoerythrin (PE)-labeled rat mAb to murine H-2K^d or FITC-labeled streptoavidin (in the case of biotin-labeled mAbs) followed by fixation with 4% paraformaldehyde and incubation with permeabilizing solution containing 0.1% saponin. After washing, cells were incubated with a PE-labeled mouse mAb to human IL-4 to stain intracellular IL-4. Isotype-matched control mAbs were used for negative staining. The mAbs to αβ TCR, CD4, CD8 and CD45 were obtained from Becton Dickinson and anti-H-2K^d mAb was from PharMingen (San Diego, CA,

USA). Stained cells were analyzed by a FACScan (Becton Dickinson) using a gate for human CD45$^+$ cells. Fluorescence data were collected by using logarithmic amplification.

RESULTS

SCID mice engrafted with atopic human PBMC were treated intraperitoneally for 5 days with anti-CD154 mAb or with phosphate-buffered saline (PBS) or with an isotype-matched control antibody. The serum levels of human IgE and IgG were then determined in the mice at a weeks interval over a 4 week period. As shown in Fig. 1, the IgE levels of control mice treated with PBS started to increase as early as 1 week after PBMC transfer, reached a maximum at 2 weeks and declined thereafter, while the IgG concentrations increased gradually after the graft. Treatment with anti-CD154, but not with control antibody, abrogated both IgE and IgG production, which was expected because the CD154–CD40 interaction in the presence of cytokines is required for the induction of isotype switching to IgE or IgG.[3,4]

To evaluate the effects of anti-CD154 treatment on the expression of mRNA for human IL-4, germline Cε and mature Cε, peritoneal lavage cells were harvested 0.5, 1, 3 and 5 days postengraftment from mice treated with control antibody or with anti-CD154 for up to 5 days and were analyzed by semiquantitative RT-PCR (Fig. 2). Table 1 summarizes the results obtained in three separate experiments using mice engrafted with three different PBMC. In control mice, IL-4 mRNA expression rose rapidly and reached peak levels between 1 and 3 days after PBMC transfer and both germline and mature Cε transcripts gradually accumulated afterwards. In mice treated with anti-CD154, IL-4 and germline Cε mRNA levels were significantly increased 5.1-fold (1 day after cell transfer) and 10.2-fold (3 days after the graft), respectively, compared with those of the controls. Nevertheless, mature Cε transcripts were hardly detectable, even after 3–5 days treatment with anti-CD154.

On the basis of the observation that anti-CD154 treatment led to increased expression of IL-4 mRNA, we further examined intracellular expression of IL-4 by human T cells in peritoneal washings from mice treated with control antibody or with anti-CD154 for up to 5 days. To determine the kinetics of human IL-4 synthesis in PBMC-injected mice, peritoneal lavage cells were recovered 1, 3, 5 and 7 days postengraftment from mice and were analyzed by flow cytometry using mAbs to human IL-4 and T cell surface markers. Figure 3 shows a representative time-course of human IL-4 synthesis in control and anti-CD154-treated mice. The peak of intracellular IL-4 synthesis in human αβ T cells from control mice was observed between 3 and 5 days after PBMC transfer. Analysis of human CD4$^+$ and CD8$^+$ cell subsets gave

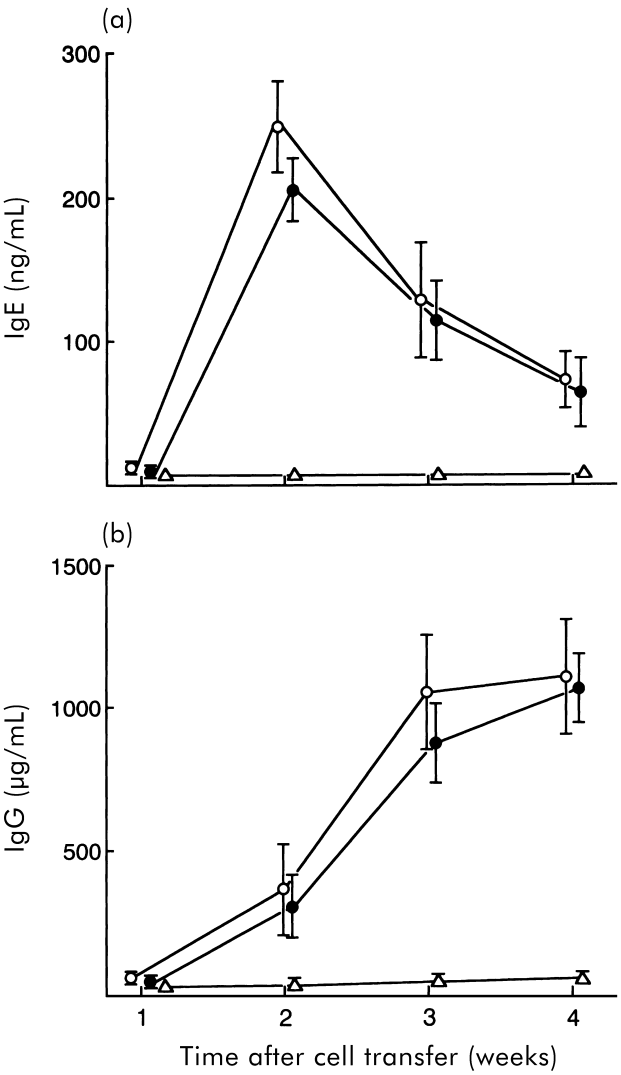

Fig. 1 Effects of anti-CD154 monoclonal antibody (mAb) on human (a) IgE and (b) IgG production in severe combined immunodeficiency mice engrafted with peripheral blood mononuclear cells (PBMC) from an atopic patient. Engrafted mice were treated for 5 days with phosphate-buffered saline (○), control antibody (●) or anti-CD154 mAb (△). After being bled at the indicated times, serum levels of human IgE and IgG were measured. Results are the mean ± SEM of five mice. Similar results were obtained when PBMC from two other atopic patients were used.

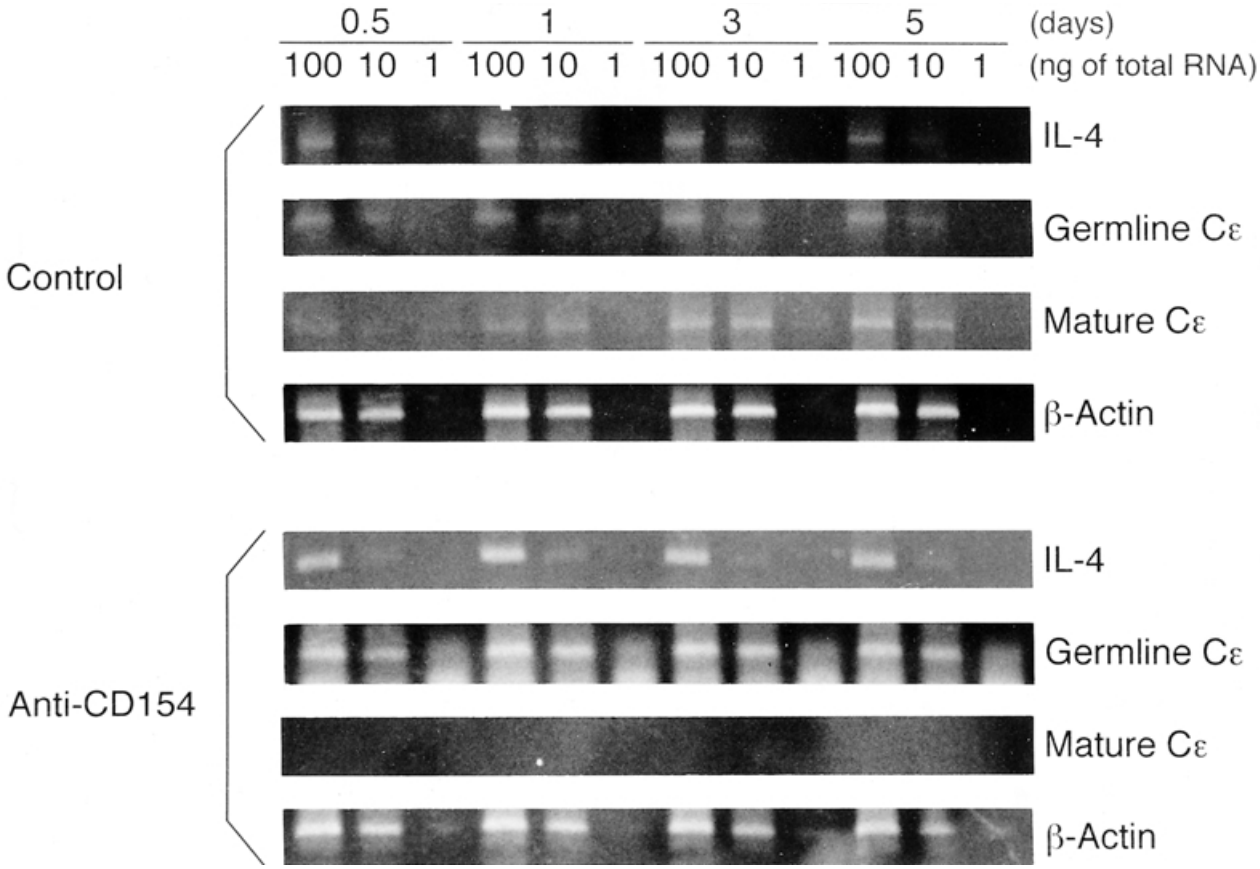

Fig. 2 Effects of anti-CD154 monoclonal antibody (mAb) on the expression of mRNA for human interleukin (IL)-4, germline Cε and mature Cε in severe combined immunodeficiency mice engrafted with atopic human peripheral blood mononuclear cells (PBMC). Peritoneal lavage cells were harvested at the indicated times from mice treated with control antibody or with anti-CD154 mAb for up to 5 days. After extraction and serial dilution of total cellular RNA, the expression of mRNA for human IL-4, germline Cε, mature Cε and β-actin was analyzed by semiquantitative reverse transcription–polymerase chain reaction.

Table 1 Effects of anti-CD154 monoclonal antibody on the expression of mRNA for human interleukin-4, germline Cε and mature Cε in severe combined immunodeficiency mice engrafted with atopic human peripheral blood mononuclear cells

Treatment	mRNA	Days after cell transfer			
		0.5	1	3	5
Control antibody	IL-4	3.8 ± 1.6	8.2 ± 2.4	9.6 ± 2.7	6.2 ± 2.3
	Germline Cε	4.5 ± 1.2	6.1 ± 2.8	10.5 ± 3.2	24.0 ± 5.2
	Mature Cε	1.2 ± 0.3	9.5 ± 2.1	18.8 ± 3.4	25.7 ± 4.9
Anti-CD154 mAb	IL-4	15.3 ± 2.5*	42.0 ± 6.6**	31.2 ± 4.7**	24.8 ± 5.9*
	Germline Cε	23.9 ± 4.1**	51.9 ± 9.8**	107.6 ± 8.2**	115.1 ± 12.4**
	Mature Cε	< 0.1**	< 0.1***	< 0.1***	< 0.1***

Peritoneal lavage cells were harvested 0.5–5 days postengraftment from mice treated with control antibody or with anti-CD154 monoclonal antibody (mAb) for up to 5 days. After extraction of total cellular RNA, mRNA for human interleukin (IL)-4, germline Cε and mature Cε were amplified by reverse transcription–polymerase chain reaction (RT-PCR) and the PCR products were quantified by a densitometer. The amount of each amplification product was evaluated as the ratio of IL-4 or Cε mRNA : β-actin mRNA × 100.

Results are expressed as the mean ± SEM of three separate experiments, each performed in triplicate. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with control antibody treated mice (Mann–Whitney *U*-test).

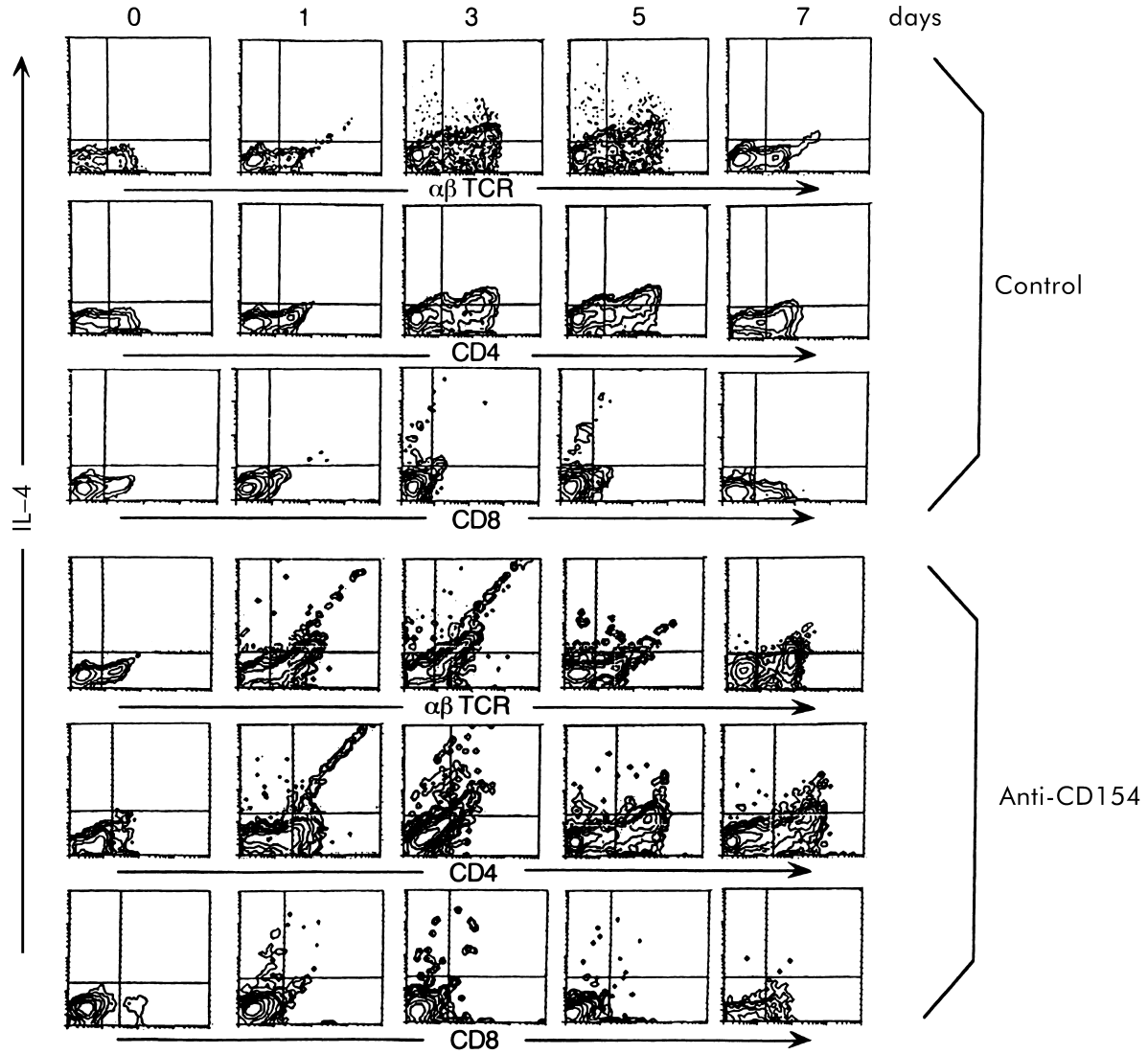

Fig. 3 Effects of anti-CD154 monoclonal antibody (mAb) on the intracellular expression of human interleukin (IL)-4 in severe combined immunodeficiency mice engrafted with atopic human peripheral blood mononuclear cells (PBMC). Peritoneal lavage cells were harvested at the indicated times from mice treated with control antibody or with anti-CD154 mAb for up to 5 days. Cells were incubated with fluorescein isothiocyanate-labeled antihuman CD4 or CD8 mAbs, fixed in paraformaldehyde, permeabilized with saponin, and then stained with a phycoerythrin (PE)-labeled antihuman IL-4 mAb. In each experiment, background staining was obtained by incubating cells with isotype-matched control mAbs. Stained cells were analyzed by flow cytometry.

similar results. In mice treated with anti-CD154, IL-4 synthesis by human αβ T cells was rapid compared with the controls and reached a maximum between 1 and 3 days after PBMC transfer. In addition, fluorescence intensity for intracellular IL-4 was markedly enhanced by anti-CD154 treatment. The effect was observed in human $CD4^+$ as well as in $CD8^+$ T cells, although it was more evident in $CD4^+$ T cells.

Discussion

In the present study, we investigated the effect of anti-CD154 administration on human IL-4 synthesis and on IgE production in SCID mice engrafted with atopic human PBMC in order to assess some of the immunologic mechanisms involved in the CD154–CD40 interaction. We found that anti-CD154 treatment of engrafted mice

greatly enhanced expression of IL-4 mRNA and protein in T cells, thereby contributing to upregulation of germline Cε transcription in B cells. In contrast, mature Cε transcription and IgE production were abrogated by the treatment, in accordance with expectations. These results confirm, in part, some of the previously published data[3–6,11,12] and demonstrate that blocking the CD154–CD40 interaction with anti-CD154 differentially regulates IL-4 synthesis in T cells and IgE production in B cells.

The enhancement of IL-4 synthesis observed in T cells from anti-CD154-treated mice is in keeping with a previous study describing that IL-4 synthesis in $CD4^+$ T cells was enhanced by triggering of CD154 in conjunction with ligation of CD3 and CD28.[6] However, in this *in vitro* study[6] it was not examined whether costimulation of CD154 on $CD8^+$ T cells could enhance IL-4 synthesis. Intracellular staining of IL-4 synthesis in our *in vivo* experiments revealed that upregulation of IL-4 synthesis by anti-CD154 treatment was inducible in $CD4^+$ and $CD8^+$ T cells. The enhanced induction of IL-4 synthesis in these two T cell subsets necessarily requires their surface expression of CD154. In this context, we reported that CD154 expression was found on human $CD4^+$ as well as on $CD8^+$ T cells after reconstitution of SCID mice with atopic human PBMC.[7] To our knowledge, CD154-mediated enhancement of IL-4 synthesis in $CD8^+$ T cells has not been previously demonstrated both *in vitro* and *in vivo*. Although CD154 engagement has been shown to play an accessory role in the regulation of $CD4^+$ T cell function and cytokine production,[5,6,11,12] our data showed that $CD8^+$ T cells, like $CD4^+$ T cells, can be primed to enhance IL-4 synthesis through triggering of CD154. These findings indicate that CD154 ligation plays an important role in the regulation of IL-4 synthesis in T cells, including $CD4^+$ and $CD8^+$ subsets.

We also demonstrated that germline Cε transcription was markedly enhanced in anti-CD154-treated mice. This could be most likely due to the enhanced IL-4 synthesis that resulted from antibody ligation of CD154 on $CD4^+$ and $CD8^+$ T cell subsets. Actually, enhancement of IL-4 synthesis by anti-CD154 treatment correlated well with upregulation of germline Cε transcription. Because both IL-4 and IL-13 contribute to the induction of germline Cε transcripts, the possibility of the participation of IL-13 in the regulation of germline Cε transcription cannot be ruled out. In our SCID mouse model, the peak of IL-4 mRNA expression was observed between 1 and 3 days after PBMC transfer, whereas IL-13 mRNA started to accumulate at 5 days after the graft (data not shown). Interestingly, the upregulation of germline Cε transcription in anti-CD154-treated mice could be seen before IL-13 mRNA induction, which suggested that IL-4, but not IL-13, was a contributing factor to increased germline Cε transcription. However, it remains to be determined whether CD154 ligation regulates IL-13 synthesis in $CD4^+$ and in $CD8^+$ T cells.

In summary, we have shown that anti-CD154 treatment of atopic human PBMC-injected SCID mice upregulates germline Cε transcription in B cells by potentiating IL-4 synthesis in $CD4^+$ and $CD8^+$ T cell subsets, despite inevitable abrogation of mature Cε transcription and IgE production. Thus, blocking the CD154–CD40 interaction with anti-CD154 allows reciprocal regulation of germline and mature Cε transcription. While numerous studies have shown that ligation of CD40 on B cells is critical for immunoglobulin class switching,[13–15] the major finding in the present study is that *in vivo* triggering of CD154 on T cells functions as a costimulus for IL-4 synthesis in $CD4^+$ T cells as well as in $CD8^+$ T cells. Our data support the notion that CD154–CD40 interactions are critically involved in the regulation of T and B cell function.

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