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I. Allergy and its control

Production of IL-4 and expression of CD40 ligand by human CD8⁺ T cells

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Background: The role of CD8⁺ T cells in IgE synthesis remains unclear.

Objective: The aim of this study was to investigate IL-4 production and CD40 ligand expression by human CD8⁺ T cells.

Methods: We conducted functional and phenotypic analyses of human T cells in peritoneal washings from severe combined immunodeficiency mice reconstituted with PBMCs from normal and atopic human donors. We also examined the expression of IL-4 and CD40 ligand by CD8⁺ T cells from a patient with adenosine deaminase deficiency who received autologous T cell-directed gene therapy.

Results: Transfer of atopic cells into the mice caused production of IgE and IgG with increased expression of IL-4 and CD40 ligand mRNA. In addition, both intracellular IL-4 and cell surface CD40 ligand were detected in CD8⁺ and in CD4⁺ T cells. CD8⁺ T-cell lines generated from the patient's T cells carrying the adenosine deaminase gene expressed not only IL-4 mRNA and protein but also CD40 ligand mRNA and protein after being stimulated with an anti-CD3 mAb. After anti-CD3 stimulation and paraformaldehyde fixation, CD8⁺ T cells induced IgE synthesis by normal human B cells in the presence of recombinant IL-4.

Conclusion: Taken together, these results demonstrate that IL-4-producing and CD40 ligand-expressing CD8⁺ cells are detectable among human T cells and suggest that such cells may promote IgE production by B cells under some conditions. (*J Allergy Clin Immunol* 1999;103:S405-11.)

Abbreviations used

ADA: Adenosine deaminase
CD40L: CD40 ligand
G3PDH: Glyceraldehyde 3-phosphate dehydrogenase
SCID: Severe combined immunodeficiency

Key words: CD8⁺ T cells, IL-4, CD40 ligand, IgE production, reconstitution, severe combined immunodeficiency mice, T cell-directed gene therapy

The balance between various cytokines produced by T cells plays an important regulatory role in the immune response. Among the various T-cell subpopulations, CD4⁺ T-helper cells are classified in 2 subsets, termed T_{H1} and T_{H2} cells, based on their pattern of cytokine production.^{1,2} T_{H1} cells secrete IL-2, interferon- γ , and lymphotoxin; T_{H2} cells produce IL-4, IL-5, IL-6, IL-10, and IL-13. CD8⁺ cytotoxic T cells are also similarly divided into distinct cytokine-secreting subsets, designated as T_{C1} and T_{C2} cells.³⁻⁷ T_{C1} cells produce T_{H1}-type cytokines that are involved in cell-mediated inflammatory responses; T_{C2} cells secrete T_{H2}-type cytokines that are associated with antibody and allergic responses. Cytokines secreted by these T-cell subsets also influence the differentiation of precursors into T_{H1}/T_{H2} or T_{C1}/T_{C2} cells.^{5,8,9}

Despite the different profiles of cytokine production by subsets of CD4⁺ and CD8⁺ T cells, T_{H1} and T_{H2} cells express high levels of CD40 ligand (CD40L); T_{C1} and T_{C2} cells show only a low level of CD40L expression.⁷ Promotion of B-cell antibody production is largely dependent on the expression of CD40L by T cells, because the interaction of CD40 with CD40L in the presence of cytokines is critical for immunoglobulin class switching.^{10,11} For example, costimulation of human B

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cells with a soluble form of CD40L and either IL-4 or IL-13 induces isotype switching to IgE that leads to mature C ϵ transcription and IgE synthesis. Although our knowledge of the contribution of T_{H2} cells to the regulation of humoral immune responses is extensive, recent studies in animal models have shown that T_{C2} cells, like T_{H2} cells, support B-cell antibody production.⁵⁻⁷ T_{C2}-type CD8⁺ human T cells have also been described in patients with lepromatous leprosy, AIDS, or atopy.¹²⁻¹⁵ However, there is a paucity of information on the induction of IgE synthesis by a subset of CD8⁺ T cells.

We performed 2 independent studies on the generation of T_{C2}-type CD8⁺ human T cells. One study involved severe combined immunodeficiency (SCID) mice reconstituted with human PBMCs to produce human immunoglobulins, including IgE.¹⁶⁻²⁰ CD8⁺ T cells in peritoneal washings from the mice were analyzed for IL-4 production and CD40L expression. The second study used a patient with adenosine deaminase (ADA) deficiency who received autologous T-cell-directed gene therapy.²¹ After the start of treatment, the patient's serum IgE level increased despite a marked and sustained increase in the number of circulating CD8⁺ T cells. Both studies resulted in the detection of human CD8⁺ T cells that produced IL-4 and expressed CD40L.

METHODS

Reconstitution of SCID mice with human PBMC

PBMC were isolated from the heparinized venous blood of normal or atopic donors by density gradient sedimentation and were injected intraperitoneally into CB-17 SCID mice (CLEA, Tokyo, Japan). The normal donors had no history of allergy; the atopic donors had bronchial asthma, allergic rhinitis, or atopic dermatitis. Informed consent was obtained from the donors who were examined in this study. Before the transfer of human PBMC, some "leaky mice" were eliminated by the measurement of serum levels of IgM and IgG. Based on preliminary studies with various numbers of cells, 3×10^7 PBMC were injected into the mice. Reconstituted mice were bled while receiving anesthesia, and the serum levels of human immunoglobulins were determined. Peritoneal lavage cells were also harvested from the mice and were used for phenotypic and functional analyses.

ADA-deficient patient treated by T-cell-directed gene transfer

A boy with ADA deficiency was treated by autologous T-cell-directed gene transfer, as reported by Onodera et al.²¹ Informed consent for participation was obtained from the patient. Gene transduction was performed by retroviral-mediated transfer of the human ADA gene into peripheral blood T cells. The patient received periodic infusion of genetically modified T cells that had been transduced with the ADA gene after stimulation of PBMC with an anti-CD3 mAb and IL-2. Such immunologic reconstitution resulted in normalization of the circulating lymphocyte count, restoration of cellular and humoral immune responses, and increased levels of serum immunoglobulins, although circulating CD8⁺ T cells markedly increased in number. To generate CD8⁺ T cell lines, PBMC of the patient were stimulated with phytohemagglutinin and IL-2, as described in detail elsewhere.¹⁴

For IgE synthesis, PBMC (1×10^6 /mL) were stimulated for 14

days with or without recombinant human IL-4 (200 U/mL, Genzyme Corp, Cambridge, Mass). Net IgE synthesis was determined as described previously.²² The capacity of lined CD8⁺ T cells to induce IgE synthesis was tested as follows: After highly purified B cells were isolated from normal human PBMC by immunomagnetic selection,²³ B cells (5×10^5 /mL) were added to lined CD8⁺ T cells (5×10^5 /mL) that had been stimulated for 20 hours with or without anti-CD3 mAb (100 ng/mL; PharMingen, San Diego, Calif) followed by fixation with 0.5 % paraformaldehyde. These cell mixtures were cultured in the absence or presence of recombinant IL-4 for 14 days. Either a neutralizing mAb directed against human IL-4 (10 μ g/mL; PharMingen) or a recombinant soluble form of human CD40-human IgM (20 μ g/mL; a gift of Dr P. Lane, Basel Institute for Immunology, Basel, Switzerland) was added to some cultures.

Assays for immunoglobulins, soluble CD23, and IL-4

Human IgE, IgG, and soluble CD23 were determined by solid-phase sandwich radioimmunoassays as described previously.^{22,24} Human IL-4 was assayed with a commercially available ELISA kit (BioSource International, Camarillo, Calif) according to the manufacturer's instructions.

Flow cytometric analysis

Cell surface expression of human CD3, CD4, CD8, CD23, CD40, and CD40L was analyzed by flow cytometry as described previously.^{23,24-27} Detection of intracellular human IL-4 was performed with the method of Sander et al,²⁸ with slight modifications. Briefly, cells were incubated with FITC-labeled mouse mAbs to human CD4 or CD8, followed by fixation with 4% paraformaldehyde and incubation with 0.1% saponin containing a phycoerythrin-labeled mouse mAb to human IL-4. Isotype-matched control mAbs were used for negative staining (all mAbs were obtained from PharMingen). The stained cells were analyzed by flow cytometry on a FACScan (Becton Dickinson, Mountain View, Calif).

Reverse transcriptase-polymerase chain reaction

Extraction of total cellular RNA, conversion from mRNA to complementary DNA by reverse transcriptase, and performance of the PCR were performed as described previously.^{23,25,27} The 5' sense primers and 3' antisense primers used for the amplification of mRNA for human IL-4, CD40L, mature C ϵ , glyceraldehyde 3-phosphate dehydrogenase (G3PDH), and β -actin were the same as described previously.^{23,25} The amplified products were visualized on 2% agarose gel in buffer containing ethidium bromide.

Dot blot analysis

Dot blot analysis of human CD23a and CD23b mRNA was performed according to the method described previously.²⁹ Briefly, after extraction of total cellular RNA, 10 μ g RNA was serially diluted, and each dilution was applied with suction to a spot. Membranes hybridized with a 32p-labeled CD23a or CD23b probe were exposed to high performance autoradiography film (Amersham, Life Science, Buckinghamshire, UK).

RESULTS

IgE production by SCID mice reconstituted with human PBMC

SCID mice received PBMC from 5 normal donors with serum IgE levels less than 50 IU/mL and from 7

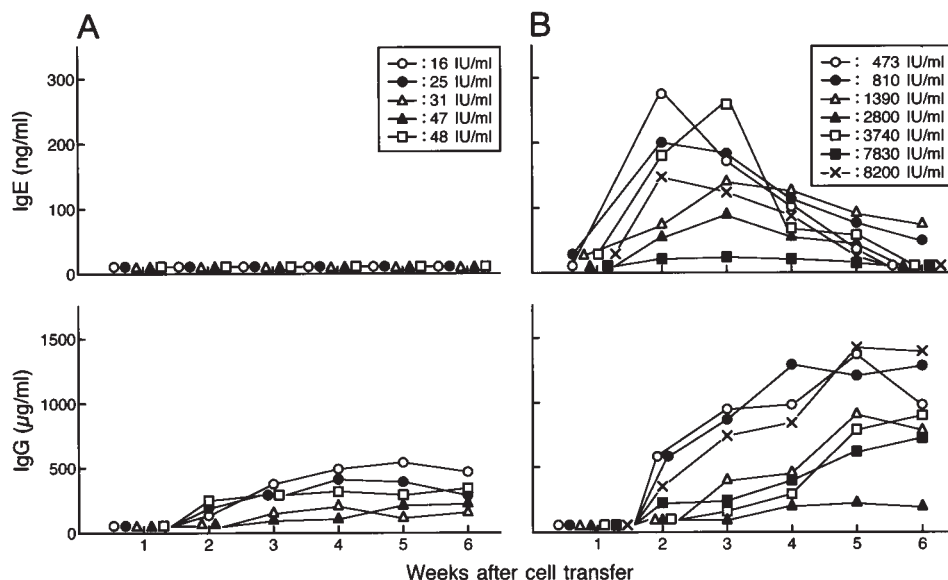


FIG 1. Human IgE and IgG levels in SCID mice engrafted with PBMC from normal donors (A) and from atopic patients (B). Reconstituted mice were bled at the indicated times, and serum levels of human IgE and IgG were measured by isotype-specific radioimmunoassays.

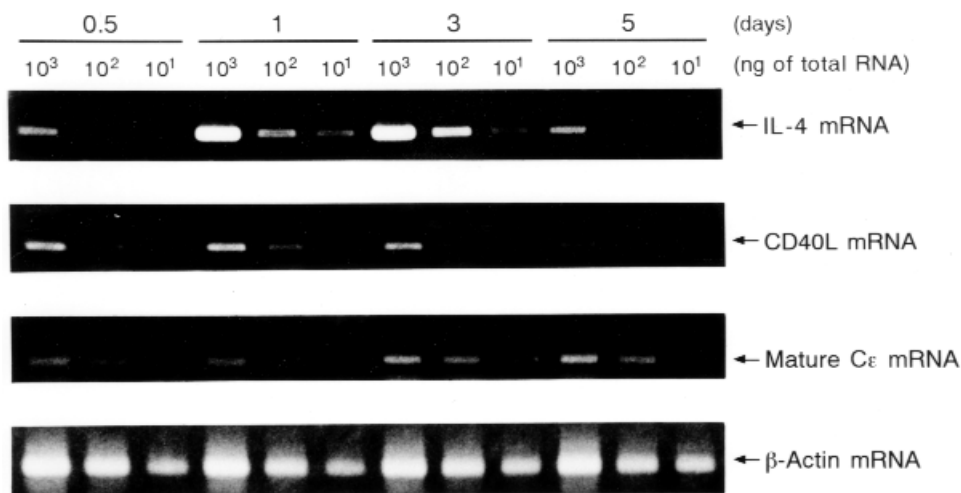


FIG 2. Expression of mRNA for human IL-4, CD40L, and mature C ϵ by peritoneal lavage cells of SCID mice engrafted with atopic human PBMC. Peritoneal lavage cells were recovered from reconstituted mice at the indicated times. After extraction and serial dilution of total cellular RNA, mRNAs for human IL-4, CD40L, mature C ϵ , and β -actin were amplified by the reverse transcriptase-PCR.

atopic patients with elevated serum IgE levels that ranged from 473 to 8200 IU/mL. After PBMC transfer, serum levels of human IgE and IgG were measured in the mice. PBMC from all the normal donors produced IgG in the mice, but no IgE was detectable (Fig 1, A). In contrast, engrafted PBMC from all atopic patients secreted both IgE and IgG (Fig 1, B). IgE production in SCID mice was detectable as early as 1 week after cell transfer, reached a maximum at 2 to 3 weeks, and declined thereafter. However, no correlation was observed between the peak serum IgE level in the mice and the donor's serum IgE level. These results demonstrated that SCID mice show

an IgE response when reconstituted with atopic human PBMC, which confirmed previous studies.^{17,20}

Functional and phenotypic analysis of peritoneal lavage cells from SCID mice reconstituted with human PBMC

Using peritoneal lavage cells from SCID mice engrafted with normal or atopic human PBMC, we studied the expression of mRNA for human IL-4, CD40L, and mature C ϵ . Reconstitution with atopic human PBMC led to increased expression of these 3 transcripts, as deter-

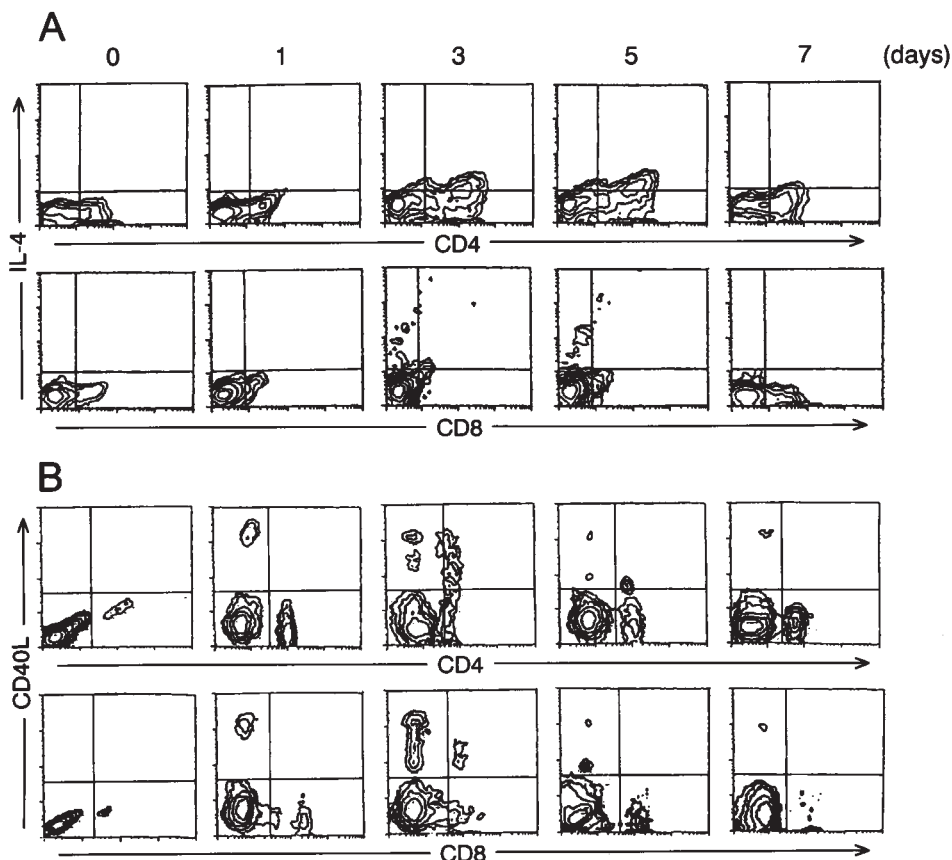


FIG 3. Intracellular expression of IL-4 (**A**) and surface expression of CD40L (**B**) by human CD4⁺ and CD8⁺ T cells from SCID mice engrafted with atopic human PBMC. Peritoneal lavage cells were recovered from reconstituted mice at the indicated times. **A**, Cells incubated with FITC-labeled anti-human CD4 or CD8 mAbs were fixed in paraformaldehyde, permeabilized with saponin, and then stained with a PE-labeled anti-human IL-4 mAb. **B**, Cells incubated with FITC-labeled anti-human CD4 or CD8 mAbs were stained with a phycoerythrin-labeled anti-human CD40L mAb. In each experiment, the stained cells were analyzed by flow cytometry.

mined by semiquantitative reverse transcriptase-PCR analysis (Fig 2). The peak of IL-4 and CD40L mRNA expression was observed between 1 and 3 days after the cell transfer, and mature Cε mRNA gradually accumulated afterwards. Flow cytometric analysis showed that both CD4⁺ T cells and CD8⁺ T cells were positive for both intracellular IL-4 and surface CD40L (Fig 3, A and B). In mice repopulated with normal human PBMC, however, only CD40L mRNA expression was detectable, and it was found on CD4⁺ rather than CD8⁺ T cells (data not shown). These results indicate that only reconstitution with atopic human PBMC yielded CD8⁺ T cells that were primed to produce IL-4 and express CD40L like CD4⁺ T cells.

Functional analysis of PBMC from an ADA-deficient patient treated by T-cell-directed gene transfer

PBMC from an ADA-deficient patient were stimulated with anti-CD3 mAb and IL-2, followed by transduction with the ADA gene. This *in vitro* stimulation

expanded CD8⁺ rather than CD4⁺ T cells (Fig 4, A). After commencement of the trial with gene-corrected T cells, the number of circulating CD8⁺ T cells increased markedly in this patient, accounting for about 90% of all CD3⁺ T cells after several infusions (Fig 4, B). Despite the inversion of the CD4/CD8 ratio, such *in vivo* immunologic reconstitution led to the elevation of serum IgE levels, which reached about 900 IU/mL. Increased expression of CD23a and CD23b mRNA was also detected by dot blot analysis of fresh PBMC from the patient (Fig 5), and a similar result was obtained when atopic human PBMCs were used. Furthermore, recombinant IL-4 upregulated the surface expression of CD23 and CD40 on B cells of the patient (data not shown). The patient's PBMC spontaneously secreted detectable amounts of IgE (0.5 ± 0.1 ng/mL; mean $\pm$ SEM of triplicate cultures) and responded to recombinant IL-4 by IgE production (7.4 ± 1.4 ng/mL, triplicate cultures) and soluble CD23 release (6.1 ± 0.4 ng/mL, triplicate cultures). These results demonstrate that T-cell-directed gene therapy for an ADA-deficient patient resulted in the elevation of serum IgE levels, despite the dramatic inversion of the CD4/CD8

ratio, and suggest that the patient's B cells may be functionally mature with respect to IgE production.

Functional analysis of CD8⁺ T-cell lines from the ADA-deficient patient

Several CD8⁺ T-cell lines were generated from the PBMC of the ADA-deficient patient. After selection of T-cell lines expressing the transduced ADA gene, their capacity to express IL-4 and CD40L was examined. When stimulated with anti-CD3 mAb, lined CD8⁺ T cells synthesized both IL-4 mRNA and CD40L mRNA (data not shown). In addition, 10⁶ CD8⁺ T cells produced more than 100 pg of immunoreactive IL-4 and approximately 15% of the cells expressed surface CD40L. This CD8⁺ T-cell line–derived IL-4 had the capacity to upregulate the surface expression of CD23 on normal human B cells (data not shown). It was also found that fixed, stimulated CD8⁺ T cells could induce IgE synthesis (3.7 ± 0.6 ng/mL, triplicate cultures) in normal human B cells in the presence of recombinant IL-4. This IgE production was completely abrogated by a neutralizing mAb directed against IL-4 and by a recombinant soluble form of CD40. These results demonstrated that CD8⁺ T-cell lines derived from peripheral T cells carrying the inserted ADA gene produced biologically active IL-4 and expressed functional CD40L after activation.

DISCUSSION

It has recently been shown in both animal and human models that CD8⁺ T cells with a T_{H2} phenotype are capable of providing help for B-cell responses.^{5-7,13-15} The present studies were initiated to detect a subset of CD8⁺ human T cells that secrete IL-4 and express CD40L on activation. To this end, we analyzed the functional profile of CD8⁺ T cells from SCID mice reconstituted with human PBMC and from an ADA-deficient patient treated by autologous T-cell–directed gene transfer. Although these 2 systems are quite different, our data indicate that in situations where *in vivo* synthesis of IgE is inducible, some CD8⁺ T cells can acquire the capacity to produce IL-4 and to express CD40L.

It is well known that a simple transfer of human PBMC into SCID mice leads to the synthesis of human immunoglobulins without the requirement for additional stimulation.¹⁶⁻²⁰ Using such an animal model, we first examined the capacity of PBMC from normal and atopic donors to experience the development of human IgE synthesis. In mice reconstituted with atopic human PBMC, there was a relatively high level of IgE synthesis, although normal human PBMC failed to produce IgE. These results suggest that, unlike normal human PBMC, atopic human PBMC have a large population of T cells primed to produce IL-4 or IL-13. This suggestion is supported by reports describing that human IgE formation in SCID mice was dependent on IL-4 production and its interaction with functional L-4 receptors.^{19,20,30} Thus the SCID mouse model is useful for the detection of human T-cell subpopulations that express IL-4 or CD40L in an

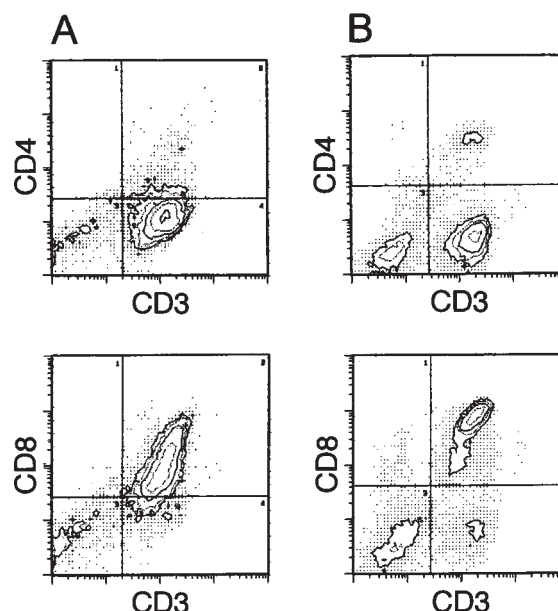


FIG 4. Cell surface phenotype of T cells transduced with the ADA gene (**A**) and circulating T cells from an ADA-deficient patient treated by T cell–directed gene transfer (**B**). Cells were stained with a FITC-labeled anti-CD3 mAb and phycoerythrin-labeled anti-CD4 or anti-CD8 mAbs and were analyzed by flow cytometry.

in vivo environment. Next, we studied human IL-4, CD40L, and mature C ϵ mRNA expression in peritoneal lavage cells from SCID mice engrafted with atopic or normal human PBMC. Reconstitution with atopic human PBMC led to increased expression of mRNA for IL-4, CD40L, and mature C ϵ . Flow cytometric analysis showed that PBMC transfer induced CD4⁺ and CD8⁺ T cells expressing both intracellular IL-4 and surface CD40L. Although we found no significant differences between CD4⁺ and CD8⁺ T cells with respect to the kinetics of IL-4 or CD40L expression, it has been reported that CD40L expression by allospecific CD8⁺ T cells stimulated with anti-CD3 mAb is very slow compared with that by CD4⁺ T cells.⁷ This contrasts with our observation that, in SCID mice repopulated with atopic human PBMC, the kinetics of surface CD40L expression were about the same for CD4⁺ and CD8⁺ T cells. Although the reason for these discrepancies is not clear, one can speculate that optimal conditions for induction of CD40L expression by CD4⁺ or CD8⁺ T cells might differ between the *in vitro* and *in vivo* settings. On the other hand, when SCID mice were repopulated with normal human PBMC, expression of mRNA for CD40L, but not for IL-4 or mature C ϵ , was detectable; and surface CD40L was expressed on CD4⁺ rather than CD8⁺ T cells. This is in keeping with a previous report that cloned CD8⁺ T cells without the capacity to secrete IL-4 failed to express CD40L.⁶ These findings suggest that the immunologic functions of normal and atopic human CD8⁺ T cells may differ as a result of their capacity to produce IL-4 and to express CD40L. Although CD8⁺ T cells and CD4⁺ T cells could be primed to produce IL-4

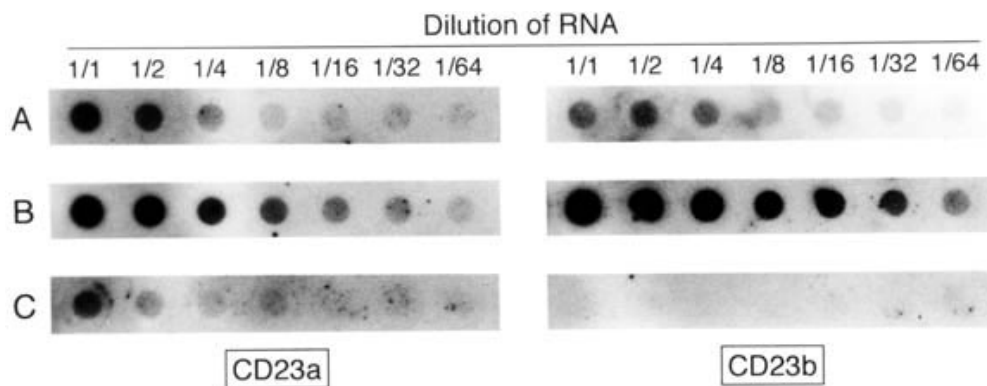


FIG 5. Dot-blot analysis of CD23a and CD23b mRNA expression by PBMC from an ADA-deficient patient treated by T-cell-directed gene transfer (**A**), an atopic patient (**B**), and a normal donor (**C**). After extraction of total cellular RNA from freshly isolated PBMC, RNA was serially diluted, and each dilution was applied with suction to a spot.

and to express CD40L in SCID mice reconstituted with atopic human PBMC, other cells (such as $\gamma\delta$ T cells and high-affinity IgE receptor-bearing cells) were also positive for both IL-4 and CD40L (unpublished observation). It is therefore likely that several types of cells producing IL-4 and expressing CD40L contribute to IgE production in SCID mice.

ADA is a purine catabolic enzyme that triggers the deamination of adenosine and deoxyadenosine and subsequently causes the formation of inosine and deoxyinosine. Accumulation of intracellular adenosine and deoxyadenosine in lymphocytes impairs immune function because these 2 ADA substrates are preferentially converted to toxic compounds such as deoxyadenosine triphosphate.³¹ Therefore a genetic defect of ADA results in SCID characterized by severe T-cell dysfunction, agammaglobulinemia, and recurrent infection. Several studies have shown that ADA gene therapy successfully restores immune function in patients with ADA deficiency.³²⁻³⁵ Onodera et al²¹ also succeeded with gene therapy for an ADA-deficient patient. This patient received periodic infusion of genetically modified autologous T cells that had been transduced with the ADA gene after stimulation with anti-CD3 mAb and IL-2. Such in vitro stimulation expanded CD8⁺ rather than CD4⁺ T cells, thereby resulting in a favorable balance for CD8⁺ T cell gene transduction. We found that CD8⁺ T cells purified from T cells transduced with the ADA gene had a T_{H0}-like profile of cytokine production (unpublished observation). After the trial was started with gene-corrected T cells, circulating CD8⁺ T cells progressively increased in the patient and accounted for about 90% of all CD3⁺ T cells after several infusions. This dramatic inversion of the CD4/CD8 ratio was caused by an increase of the absolute CD8⁺ T-cell count and may have resulted from preferential proliferation of CD8⁺ T cells during culture and transduction. Interestingly, the serum IgE level also increased in the course of gene therapy. There was concurrent spontaneous production of IgE by unstimulated PBMC and increased expression of CD23a and CD23b

mRNA by fresh PBMC. Because CD23b can be induced in B cells and monocytes by IL-4,³⁶ the B cells of the patient were evidently activated in vivo, at least through hyperproduction of endogenous IL-4.

The data described led us to examine the possibility that some CD8⁺ T cells carrying the inserted ADA gene might contribute to IgE production by B cells. Functional analysis of peripheral lymphocytes from the patient treated by T-cell-directed gene transfer yielded 2 interesting findings. The first was that the PBMC responded to recombinant IL-4 by IgE production. This suggests that the patient's B cells were functionally mature with respect to IgE isotype switching. However, because the patient received enzyme replacement with polyethylene glycol-modified bovine ADA before and after gene therapy,²¹ it cannot be ruled out that B cells expressing exogenous ADA have responded by IgE production. The second finding was that CD8⁺ T-cell lines derived from peripheral T cells carrying the inserted ADA gene expressed both IL-4 and CD40L after anti-CD3 mAb stimulation. Furthermore, T-cell line-derived IL-4 was biologically active, because it upregulated the surface expression of CD23 on normal human B cells. Also, when stimulated with anti-CD3 mAb followed by fixation, lined CD8⁺ T cells could induce IgE synthesis by normal human B cells in the presence of recombinant IL-4. This contact-dependent induction of IgE was completely abrogated by a recombinant soluble form of CD40, indicating that CD40L expressed on activated CD8⁺ T cells was functional. These findings suggest that gene-corrected CD8⁺ T cells contribute to in vivo production of IgE and that immunologic reconstitution with genetically modified T cells (particularly CD8⁺ T cells) may prompt the generation of T_{H2} cells. Because both IL-4 and IL-13 are sometimes involved in CD8⁺ T cell-mediated IgE production,^{13-15,37} the development of T_{H2}-type CD8⁺ T cells is not always specific to T-cell-directed gene therapy.

In summary, IL-4-producing and CD40L-expressing CD8⁺ human T cells were detected in SCID mice repopulated with PBMC from atopic patients and in an ADA-

deficient patient treated by autologous T-cell-directed gene transfer. Although these 2 systems are special cases, our results demonstrate that T_{C2}-type CD8⁺ T cells arise in the course of immunologic reconstitution associated with hyperproduction of IgE. In recent years, evidence supporting the concept that T_{C2} cells, like T_{H2} cells, provide help for B cells in IgE production continues to mount. This subset of CD8⁺ T cells may therefore be 1 of the important targets for achieving the downregulation of IgE synthesis by B cells. Further characterization of T_{C2} cells is necessary to better understand the mechanisms regulating IgE responses.

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