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**Research Article**

Adenosine deaminase (*ADA*) gene defects are among the most common causes of SCID. Restoration of purine metabolism and immune functions can be achieved by enzyme replacement therapy, or more effectively by bone marrow transplant or HSC gene therapy (HSC-GT). However, autoimmune complications and autoantibody production, including anti-nuclear antibodies (ANAs), frequently occur in ADA-SCID patients after treatment. To assess whether ADA deficiency affects the establishment of B cell tolerance, we tested the reactivity of recombinant antibodies isolated from single B cells of ADA-SCID patients before and after HSC-GT. We found that before HSC-GT, new emigrant/transitional and mature naive B cells from ADA-SCID patients contained more autoreactive and ANA-expressing clones, indicative of defective central and peripheral B cell tolerance checkpoints. We further observed impaired B cell receptor (BCR) and TLR functions in B cells after ADA inhibition, which may underlie the defects in B cell tolerance. Strikingly, after HSC-GT, ADA-SCID patients displayed quasi-normal early B cell tolerance checkpoints, as evidenced by restored removal of developing autoreactive and ANA-expressing B cells. Hence, ADA plays an essential role in controlling autoreactive B cell counterselection by regulating BCR and TLR functions.



# Defective B cell tolerance in adenosine deaminase deficiency is corrected by gene therapy

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**Adenosine deaminase (*ADA*) gene defects are among the most common causes of SCID. Restoration of purine metabolism and immune functions can be achieved by enzyme replacement therapy, or more effectively by bone marrow transplant or HSC gene therapy (HSC-GT). However, autoimmune complications and autoantibody production, including anti-nuclear antibodies (ANAs), frequently occur in ADA-SCID patients after treatment. To assess whether ADA deficiency affects the establishment of B cell tolerance, we tested the reactivity of recombinant antibodies isolated from single B cells of ADA-SCID patients before and after HSC-GT. We found that before HSC-GT, new emigrant/transitional and mature naive B cells from ADA-SCID patients contained more autoreactive and ANA-expressing clones, indicative of defective central and peripheral B cell tolerance checkpoints. We further observed impaired B cell receptor (BCR) and TLR functions in B cells after ADA inhibition, which may underlie the defects in B cell tolerance. Strikingly, after HSC-GT, ADA-SCID patients displayed quasi-normal early B cell tolerance checkpoints, as evidenced by restored removal of developing autoreactive and ANA-expressing B cells. Hence, ADA plays an essential role in controlling autoreactive B cell counterselection by regulating BCR and TLR functions.**

## Introduction

Genetic defects in the adenosine deaminase (*ADA*) gene are among the most common causes of SCID (1). Immunological defects associated with this disease include impaired T, B, and NK cell development and function, complete absence of cellular and humoral immunity, and recurrent infections (1). The metabolic basis underlying this immune cell deficiency is related to the physiological effect of accumulating adenosine and deoxyadenosine ADA substrates. Enzyme replacement therapy with pegylated bovine ADA (PEG-ADA) results in clinical improvement with about 70%–80% survival, but often incomplete immunological reconstitution. Hence, like in other forms of SCID, bone marrow transplantation (BMT) from an HLA-identical sibling donor is the treatment of choice in ADA-deficient patients, but is available only for a minority of patients (2). Recent trials provided the first demonstration of long-term clinical efficacy of HSC gene therapy (HSC-GT) for ADA-SCID, underlining that HSC-GT has a favorable safety profile and is effective in restoring purine metabolism and immune function (3, 4). Gene therapy with retroviral vector–transduced $CD34^+$ BM cells resulted in sustained engraftment with multilineage differentiation, increased lymphocyte counts, and improved cellular and humoral responses (5, 6).

Although varying degrees of immune reconstitution can be achieved by these treatments, breakdown of tolerance is a major concern in ADA-SCID. Key findings of milder forms of the disease or in patients undergoing PEG-ADA are complications of immune dysregulations, including autoimmunity and allergic manifestations (7–10). Recently, autoimmune manifestations have also been reported in patients receiving BMT (11, 12) and in patients treated with HSC-GT (3). Although autoimmunity is commonly observed in certain B cell and/or T cell immunodeficiencies, it remains unclear to what extent some tolerance mechanisms might be specifically affected in ADA deficiency (13). Few reports are available on the contribution of B cells to the autoimmune manifestations in ADA-SCID. In patients undergoing PEG-ADA treatment, after an initial increase, B cell counts remained low and showed a restricted Ig repertoire (9, 14). However, it is unknown how patients' B cells contribute to autoimmune complications and whether B cell tolerance is established properly in ADA-deficient patients before and after treatment.

Autoreactive B cells generated by random V(D)J Ig gene assembly are normally eliminated during their development by both central and peripheral B cell tolerance checkpoints (15). The central B cell tolerance checkpoint is mostly controlled by intrinsic B cell factors that sense whether B cell receptors (BCRs) recognize self-antigens (16–18). BCR-mediated selection plays a crucial role in controlling B cell survival, and decreased BCR signaling can lead to the inefficient depletion of developing autoreactive B cells (19–22). In addition to their BCRs, B cells also express TLRs, germline-encoded transmembrane receptors that were originally described to bind microbial components, but are also able to recognize self-antigens (23). Human B cells express TLR7 and TLR9 that bind RNA and DNA, respectively, which may be involved in the removal of developing anti-nuclear antibody–expressing (ANA-expressing) B cells (24). Interestingly, both BCR- and TLR-mediated B cell responses seem to be modulated by adenosine receptor signaling and intracellular cAMP, which are both affected by ADA deficiency (25, 26). We therefore assessed B cell tolerance checkpoints in ADA-SCID patients by cloning antibodies expressed by single B cells before and after successful HSC-GT. We found that ADA-SCID patients exhibited central and peripheral B cell tolerance defects that were mostly corrected after gene transfer. In addition, we showed that

**Conflict of interest:** The authors have declared that no conflict of interest exists.
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**Table 1**
ADA-SCID patient characteristics before HSC-GT

| | Patient 1 | Patient 2 | Patient 3 |
|---|---|---|---|
| Ethnicity | Hispanic | Mixed European | Mixed European |
| Sex | Male | Female | Male |
| Mutation in *ADA* | G74V, R282Q | G216R, E319fsX3 | R211H homozygous |
| Prior treatment | Failed haplo-BMT | 1.25 yr PEG-ADA | 5.3 yr PEG-ADA |
| T cell counts (cells/μl) | 138 | 170 | 626 |
| B cell counts (cells/μl) | 0 | 323 | 152 |
| IVIg | Yes | Yes | Yes |
| Serum IgG (g/l) | 8.97 | 9.07 | 10.61 |
| Serum IgA (g/l) | 0.35 | 0.46 | 0.66 |
| Serum IgM (g/l) | 0.07 | 0.76 | 0.57 |

ADA-SCID patients 1–3 correspond to subjects 3, 5, and 6, respectively, as reported in ref. 3.

ADA inhibition altered BCR- and TLR-induced B cell responses, further suggesting the importance of both pathways in the regulation of early B cell tolerance in humans.

## Results

*Improved B cell development in ADA-SCID patients after HSC-GT.* To study B cell tolerance and development before and after HSC-GT, we analyzed 3 ADA-SCID patients that displayed full immune reconstitution after treatment (Tables 1 and 2 and ref. 3). Patient 1 had received haploidentical BMT, which resulted in lack of engraftment, and patients 2 and 3 received PEG-ADA, with inadequate immune reconstitution. HSC-GT treatment was performed as described previously using autologous HSCs transduced with a retroviral vector containing the *ADA* gene after reduced intensity conditioning with busulfan (3). After HSC-GT, all 3 patients showed good engraftment in multiple lineages, including B cells, restoration of ADA enzymatic activity in lymphocytes, and adequate metabolic detoxification (3). This led to improved T cell counts and recovery of immune cell function, such as antibody responses after vaccination (Table 2).

B cell tolerance was investigated around 2 years after HSC-GT, when B cell reconstitution stabilizes in most patients (3). As expected, in patient 1 (who did not receive PEG-ADA before HSC-GT), B cells were virtually absent, whereas in patients 2 and 3 (who underwent PEG-ADA treatment before HSC-GT), B cells were present, but at reduced levels compared with age-matched healthy donor (HD) controls (Table 1, Figure 1, and ref. 27). B cell maturation was severely impaired in all ADA-SCID patients before HSC-GT, as illustrated by the prevalence of $CD19^+CD10^+IgM^{hi}CD27^-$ immature new emigrant/transitional B cells (51%–75%, compared with 5%–20% of B cells in HDs) and the decreased frequencies of $CD19^+CD10^-IgM^+CD27^-$ mature naive B cells (Figure 1A). HSC-GT resulted in improved B cell development in all ADA-SCID patients, as illustrated by decreased new emigrant/transitional B cell and increased mature naive B cell frequencies (7%–17% before HSC-GT, compared with 20%–61% after). Patients 1 and 2 also showed increased production of memory B cells, as previously reported (3), whereas no difference was observed in patient 3, who was evaluated at a shorter time after HSC-GT (Figure 1B). Hence, we concluded that HSC-GT improves the development of B cells in ADA-SCID patients by allowing the progression of new emigrant/transitional B cells into mature naive B cells.

*Impaired central B cell tolerance in ADA-SCID patients.* ADA deficiency has been previously reported to interfere with TCR and BCR functions by altering the intracellular concentration of cyclic AMP (28, 29). To assess whether the central B cell tolerance checkpoint, which normally removes highly polyreactive and ANA-expressing developing B cells in the bone marrow, is affected by the absence of functional ADA, we cloned antibodies expressed by single new emigrant/transitional B cells from 3 ADA-SCID patients prior to HSC-GT (Table 1) and tested their reactivity by ELISA (15). The reactivities of antibodies expressed by new emigrant/transitional B cells from these ADA-SCID patients were compared with their counterparts in HDs (refs. 15, 24, 30, 31, Figure 2, and Supplemental Tables 1–5; supplemental material available online with this article; doi:10.1172/JCI61788DS1). We found that polyreactive new emigrant/transitional B cells were significantly increased in all 3 ADA-SCID patients compared with HDs (25%–40% of clones, compared with 5%–11%; Figure 2, A and B, and refs. 15, 30, 32, 33). Using indirect immunofluorescence assays with HEp-2 cell–coated slides, we found that the proportion of ANA-expressing clones in new emigrant/transitional B cells from the 3 ADA-SCID patients (representing 20%, 18%, and 27%) was also significantly increased compared with HDs (Figure 2C). These ANAs displayed Ig heavy chain (IgH) complementarity determining regions 3 (CDR3) that contained the highest number of positively charged aas, such as arginines, previously shown to favor anti-DNA autoreactivity (Supplemental Figure 1A, Supplemental Tables 3–6, and refs. 15, 24, 30, 31). ANAs expressed by ADA-SCID B cells showed a large diversity of anti-nuclear staining patterns and could be divided into those that reacted or not with the condensed chromatin material in mitotic cells (Figure 2, C and D). Chromatin-nonreactive ANAs accounted for 14%–18% of new emigrant (ne) B cells of ADA-SCID patients; in contrast, chromatin-reactive ANAs represented 4%–14% of these cells (Figure 2E). The chromatin-nonreactive patterns included speckled patterns, such as neADA2-κ2 and neADA3-κ26, that may recognize nuclear proteins, anti-mitotic spindle clones (neADA1-κ33), and, most often, nucleolar patterns

**Table 2**
ADA-SCID patient characteristics after HSC-GT

| | Patient 1 | Patient 2 | Patient 3 |
|---|---|---|---|
| Age at HSC-GT (yr) | 1 | 1.6 | 5.6 |
| Post–HSC-GT analysis (yr of follow up) | 2 | 2.5 | 1.8 |
| T cell counts (cells/μl) | 1230 | 1498 | 1173 |
| B cell counts (cells/μl) | 251 | 340 | 101 |
| Vector-positive cells $NeoR^+$ in CD19 (%) | 86 | 96 | 78 |
| IVIg | No | No | No |
| Serum IgG (g/l) | 5.98 | 8.65 | 7.13 |
| Serum IgA (g/l) | 0.55 | 1.51 | 0.36 |
| Serum IgM (g/l) | 0.74 | 1.47 | 0.35 |
| Clinical condition | Well | Well | Well |
| Clinical manifestations of autoimmunity | None | None | None |
| Vaccination responses | Positive | Positive | Positive |

ADA-SCID patients 1–3 correspond to subjects 3, 5, and 6, respectively, as reported in ref. 3.

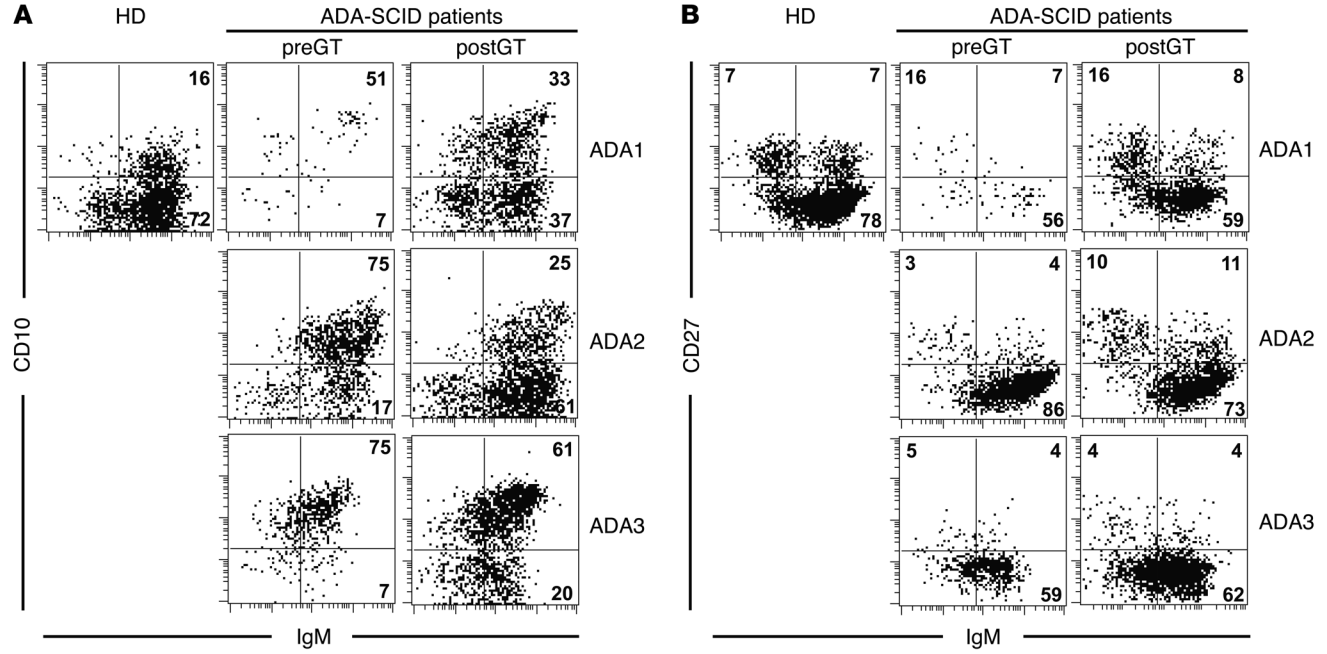


**Figure 1**

*ADA* HSC-GT rescues B cell development in ADA-SCID patients. (**A**) CD10 and IgM expression on gated CD19$^+$CD27$^-$ naive B cells from an age-matched HD and 3 ADA-SCID patients (ADA1–ADA3) before and after HSC-GT (preGT and postGT, respectively). (**B**) CD27 and IgM expression on gated CD19$^+$ B cells from an age-matched HD and 3 ADA-SCID patients before and after HSC-GT.

potentially associated with anti-RNA polymerase I complex antibodies (neADA1-κ9, neADA1-κ45, neADA2-κ209, neADA2-κ225, neADA3-κ2, and neADA3-κ6; Figure 2D and ref. 34). Most of the chromatin-reactive recombinant antibodies displayed diverse "nucleolar-like" clumpy staining, potentially associated with fibrillarin recognition patterns (neADA2-κ30, neADA3-κ38, and neADA3-κ46, Figure 2D and ref. 34). The reactivity of recombinant antibodies from ADA-SCID patients was further analyzed by indirect immunofluorescence on *Crithidia luciliae* (Figure 2, E and F). Antibody recognition of the kinetoplast of *C. luciliae*, an organelle composed of dsDNA, is the most specific assay to identify anti-native dsDNA and is routinely used for the detection of these autoantibodies in SLE patients. Using this assay, we found that new emigrant B cells from ADA-SCID patients were also enriched in kinetoplast-reactive clones, which represented 4%–9% of new emigrant B cells, whereas HDs were virtually devoid of such clones (Figure 2E). Most kinetoplast-reactive antibodies bound both the nucleus and the kinetoplast of *C. luciliae* and were often ANA-reactive clones recognizing chromosomal material (Figure 2F and Supplemental Table 6). Similarly to IRAK-4– and MyD88-deficient patients, who also fail to counterselect ANA-expressing and kinetoplast-reactive clones (24), we found that ANAs and kinetoplast-reactive antibodies expressed by new emigrant/transitional B cells from ADA-SCID patients used κ chains (Figure 2, D and F, and Supplemental Table 6), which indicates that editing using λ chains was not induced during the development of these B cells. The elevated frequency of polyreactive and ANA-expressing clones in new emigrant/transitional B cells from ADA-SCID patients suggests that central B cell tolerance is defective in the absence of functional ADA, whose expression is essential for the early silencing of developing autoreactive B cells in humans. It is worth noting that all 3 patients analyzed prior to HSC-GT showed impaired central B cell tolerance, but the untreated patient 1 displayed the highest percentage of polyreactive and ANA-expressing clones.

*ADA deficiency also alters peripheral B cell tolerance.* Autoreactive new emigrant/transitional B cells that recognize self-antigens in the periphery may be further eliminated at a second B cell tolerance checkpoint before they enter the CD20$^+$CD10$^-$CD21$^+$IgM$^+$CD27$^-$ mature naive B cell compartment (15). The effect of ADA deficiency on this peripheral B cell tolerance checkpoint was assessed by characterizing the reactivity of antibodies expressed by mature naive B cells from ADA-SCID patients using an ELISA to screen for binding to antigens expressed by the HEp-2 cell line (15). Mature naive B cells could be sorted from the 2 ADA-SCID patients who were on PEG-ADA treatment, whereas the untreated patient 1 virtually lacked such a B cell population (Figures 1 and 3). The frequency of HEp-2–reactive mature naive B cells was significantly increased in both ADA-SCID patients compared with HDs (57%–58%, compared with 17%–26%; Figure 3, A and B, Supplemental Tables 7–10, and refs. 15, 21, 22, 30, 33). ADA-SCID mature naive B cells were also enriched in polyreactive clones compared with HDs (Figure 3C). In addition, the frequency of ANA-expressing mature naive B cells was significantly increased in ADA-SCID patients, averaging 35% of clones compared with only 3.7% in HDs (Figure 3, D and F). They were especially enriched in chromatin-nonreactive clones, displaying diverse staining patterns (Figure 3, E and F). Interestingly, polyreactive and ANA-expressing B cell frequencies increased between the ADA-SCID new emigrant/transitional and the mature naive B cell stage, thereby further suggesting a defective peripheral B cell tolerance checkpoint in ADA-SCID patients. We concluded that ADA deficiency affects the removal

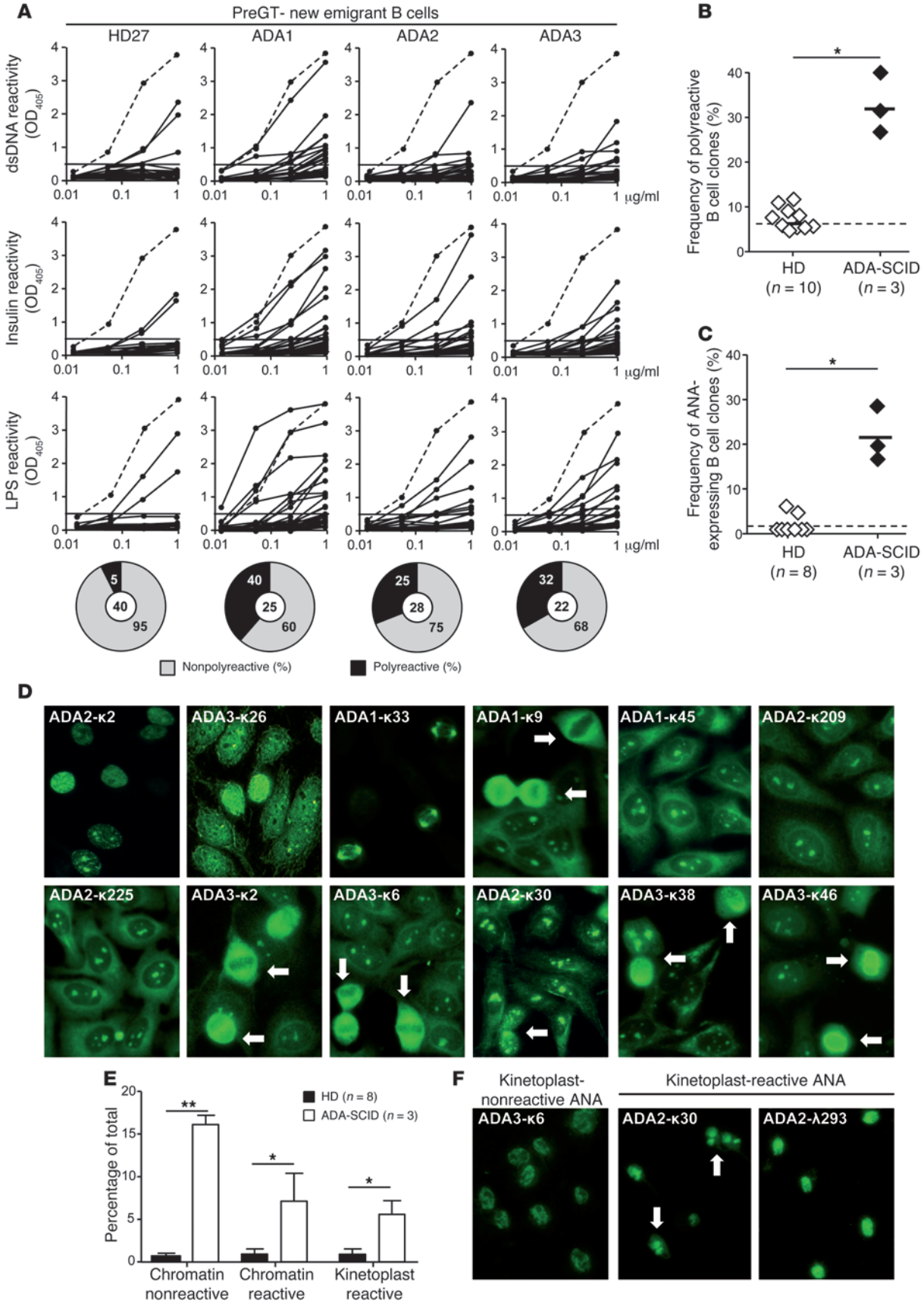
A
PreGT- new emigrant B cells
HD27
ADA1
ADA2
ADA3
dsDNA reactivity (OD405)
Insulin reactivity (OD405)
LPS reactivity (OD405)
0.01
0.1
1
µg/ml
5
40
95
40
25
60
25
28
75
32
22
68
Nonpolyreactive (%)
Polyreactive (%)
B
Frequency of polyreactive B cell clones (%)
HD (n = 10)
ADA-SCID (n = 3)
C
Frequency of ANA-expressing B cell clones (%)
HD (n = 8)
ADA-SCID (n = 3)
D
ADA2-κ2
ADA3-κ26
ADA1-κ33
ADA1-κ9
ADA1-κ45
ADA2-κ209
ADA2-κ225
ADA3-κ2
ADA3-κ6
ADA2-κ30
ADA3-κ38
ADA3-κ46
E
Percentage of total
HD (n = 8)
ADA-SCID (n = 3)
Chromatin nonreactive
Chromatin reactive
Kinetoplast reactive
F
Kinetoplast-nonreactive ANA
Kinetoplast-reactive ANA
ADA3-κ6
ADA2-κ30
ADA2-λ293

**Figure 2**
Defective central B cell tolerance in ADA-SCID patients before HSC-GT. (**A**) Antibodies expressed by new emigrant/transitional ($CD19^+CD10^+IgM^{hi}CD27^-$) B cells from 3 ADA-SCID patients were tested by ELISA for reactivity against dsDNA, insulin, and LPS. HD27 is shown as representative control. Antibodies were considered polyreactive when they recognized all 3 antigens. Dotted lines show ED38-positive control (15, 46). Horizontal lines show cutoff $OD_{405}$ for positive reactivity. The frequencies of polyreactive and nonpolyreactive clones are summarized in the pie charts, with the number of antibodies tested shown in the center. (**B** and **C**) Frequency of (**B**) polyreactive and (**C**) ANA-expressing new emigrant/transitional B cells. Each symbol represents an individual, horizontal bars denote means, and dashed lines show the average frequency in HDs. $^*P \leq 0.05$. (**D**) ANAs expressed by ADA-SCID B cells show various anti-nuclear staining patterns. Arrows denote dividing cells, indicative of negative (ADA3-κ6) or positive (ADA3-κ46) staining for the condensed chromatin material. Original magnification, ×40. (**E**) Increased frequency of chromatin-nonreactive and -reactive clones in ADA-SCID patients. ADA-SCID clones expressed ANAs that recognized the kinetoplast of *C. luciliae*. Values are mean + SEM. $^*P \leq 0.05$; $^{**}P \leq 0.005$. (**F**) ADA-SCID clones expressed ANAs that recognized the kinetoplast of *C. luciliae*. Arrows denote positively stained kinetoplasts and nuclei (ADA2-κ30) or the kinetoplast only (ADA2-λ293) of *C. luciliae*. Original magnification, ×40.

of autoreactive B cells in the periphery and results in the accumulation of large numbers of autoreactive mature naive B cells, often expressing ANAs.

*ADA deficiency alters TLR7 and TLR9 functions*. The increased frequency of ANA-expressing B cells in ADA-SCID patients was reminiscent of IRAK-4– and MyD88-deficient patients, who displayed similar altered autoreactive B cell counterselection defects (24). IRAK-4/MyD88 complexes mediate the functions of TLR7 and TLR9, which bind nucleic acid–containing antigens and play an essential role in the removal of developing ANA clones (24). To determine whether ADA deficiency affects TLR7 and TLR9 function, we tested B cell responses after TLR7 and TLR9 triggering in the presence of the ADA inhibitor erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA; Figure 4 and Supplemental Figures 3–5). ADA inhibition prevented SYK and BTK phosphorylation after TLR7 and TLR9 stimulation, similar to IRAK-1/4 inhibition (Supplemental Figure 3). In contrast, ADA and IRAK-1/4 inhibition had little or no effect on SYK, BTK, and PLCγ2 phosphorylation after BCR triggering (Supplemental Figure 3). However, further downstream, BCR signaling was altered by ADA inhibition, as evidenced by decreased ERK phosphorylation in mature naive and memory B cells (Supplemental Figure 4). The effects of ADA inhibition were also tested in in vitro culture assays; naive B cells from HDs were stimulated for 2 days by TLR7, TLR9, or their BCRs. The induction of TACI, CD86, CD19, and CD25 after TLR and BCR stimulation was inhibited by EHNA in a dose-dependent manner, as assessed by the percentage of B cells expressing such markers or by their MFI, tested by flow cytometry (Figure 4 and Supplemental Figure 5). These defects were not the result of a global inability of naive B cells to get activated, because they upregulated CD69 cell surface expression after TLR7, TLR9, or BCR stimulation in the presence of ADA inhibitor, although the intensity of the induction was reduced (Supplemental Figure 5, A and C). We concluded that the absence of functional ADA in developing B cells is likely to interfere with TLR7, TLR9, and BCR functions (35), thereby altering the removal of autoreactive B cells in ADA-SCID patients.

*Adenosine receptor stimulation alters TLR7 and TLR9 function*. To further investigate how elevated adenosine serum concentrations, characteristic of ADA deficiency, impinge on TLR responses, we mimicked continuous adenosine receptor stimulation by adding 2-chloroadenosine (cAdo), an ADA-resistant analog of adenosine (36), as an adenosine receptor agonist to stimulate naive B cells (Figure 5 and Supplemental Figure 6). Similar to the activation defects observed after ADA inhibition, increased cAdo concentrations interfered with the induction of TACI, CD19, and CD25 after TLR7, TLR9, and BCR stimulation (Figure 5 and Supplemental Figure 6). In contrast to ADA inhibition by EHNA, upregulation of CD86 did not seem to be inhibited by cAdo (Figure 5). However, CD69 upregulation after TLR7, TLR9, and BCR stimulation was independent of cAdo concentration, as it was of EHNA-induced ADA inhibition (Supplemental Figure 6, A and C), suggesting further that adenosine receptor pathways are not involved in the regulation of CD69 expression. We concluded that elevated agonist binding and stimulation of adenosine receptors can directly alter TLR7 and TLR9 function.

*ADA-SCID HSC-GT corrects central B cell tolerance defects*. In order to further explore ADA-dependent B cell tolerance defects, we analyzed the reactivity of antibodies expressed by B cells from the same 3 ADA-SCID patients after successful HSC-GT with retroviral vector–transduced $CD34^+$ BM cells (ref. 3 and Table 2). All patients were clinically well and did not experience serious adverse events related to HSC-GT. They showed a reduced rate of severe infections and hospitalization days after HSC-GT. Patients stopped i.v. Ig therapy (IVIg) and showed protective antibody responses after vaccination. They did not receive any immunomodulatory therapies and showed no clinical autoimmune manifestations. We found that IgH CDR3s from post–HSC-GT new emigrant/transitional B cells were significantly purged from containing positively charged residues, a feature that favors antibody self-reactivity (Supplemental Figure 1B, Supplemental Tables 11–13, and refs. 15, 37), which suggests that *ADA* gene transfer in HSCs and their progeny may reset central B cell tolerance in ADA-SCID patients. In line with this hypothesis, the frequency of polyreactive new emigrant/transitional B cells significantly decreased in all 3 patients, although remaining slightly elevated compared with HDs (Figure 6, A and B). The correction of the central B cell tolerance defects by HSC-GT was further evidenced by the complete removal of ANA-expressing clones in new emigrant/transitional B cell compartment of ADA-SCID patients after HSC-GT: ANA-expressing new emigrant/transitional B cells represented 1% after HSC-GT versus 23% prior, similar to the 1.6% in HDs (Figure 6C). We confirmed this finding in indirect immunofluorescence assays; virtually all self-reactive clones recognized cytoplasmic structures and did not react with nuclear antigens (Figure 6D). We concluded that reintroduction of functional ADA in ADA-deficient developing B cells allows for central counterselection of developing autoreactive B cells, especially those expressing ANAs.

*ADA-SCID HSC-GT also restores peripheral B cell tolerance defects*. The effects of HSC-GT were also assessed in the mature naive B cell compartment. We found that gene correction in ADA-SCID developing B cells restored peripheral B cell tolerance, as evidenced by the normal frequencies of HEp-2–reactive mature naive B cells in ADA-SCID patients after HSC-GT compared with HDs (Figure 7, A and B, and Supplemental Tables 14–16). Indeed, HEp-2–reactive mature B cells represented 58% prior to HSC-GT and significantly dropped to only 22% after therapy (Figure 7B). A similar trend was observed in the frequency of polyreactive mature naive B cells, which dropped from 49% to 25%, but remained slightly elevated compared with HDs (Figure 7C and Supplemental Figure 2B). ANA-expressing clones were

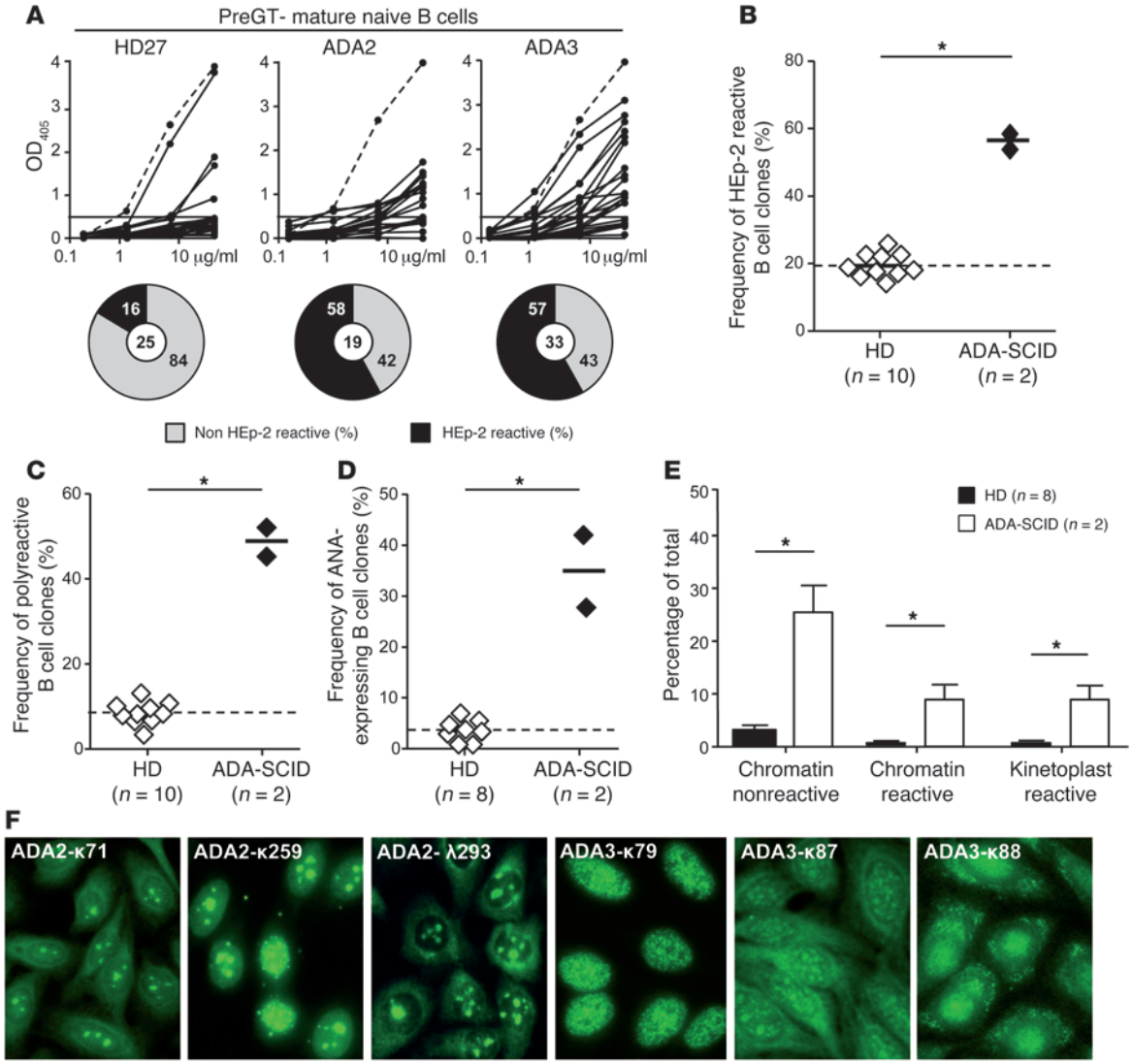


**Figure 3**

Defective peripheral B cell tolerance checkpoint in ADA-SCID patients before HSC-GT. (**A**) Increased frequency of HEp-2–reactive antibodies in ADA-SCID mature naive ($CD19^+CD10^-IgM^+CD27^-$) B cells. Antibodies from mature naive B cells from 2 ADA-SCID patients and HD27 were tested by ELISA for reactivity with HEp-2 cell lysate. Dotted lines show ED38-positive control. Horizontal lines show cutoff $OD_{405}$ for positive reactivity. The frequencies of HEp-2–reactive and non–HEp-2–reactive clones are summarized in the pie charts, with the number of antibodies tested shown in the center. (**B**–**D**) Increased frequency of (**B**) HEp-2–reactive, (**C**) polyreactive (tested against dsDNA, insulin, and LPS), and (**D**) ANA-expressing clones of mature naive B cells of ADA-SCID patients compared with HDs. Each symbol represents an individual, horizontal bars denote means, and dashed lines show the average frequency in HDs. *$P \leq 0.05$. (**E**) Higher frequencies of chromatin-nonreactive, chromatin-reactive, and kinetoplast-reactive clones in ADA-SCID patients than HDs. Values are mean + SEM. *$P \leq 0.05$. (**F**) ANAs from ADA-SCID mature naive B cells show various anti-nuclear staining patterns. 3 examples of ANA-expressing mature naive B cells are shown for each patient. Original magnification, ×40.

properly purged from the mature naive B cell compartment and only represented 4% after HSC-GT, compared with 35% prior (Figure 7, D and E). The HEp-2 staining patterns of self-reactive antibodies expressed by mature naive B cells confirmed that most antibodies recognized cytoplasmic structures (Figure 7E). Importantly, the rare ANA-expressing clones in mature naive as well as new emigrant/transitional B cells after HSC-GT were not gene corrected, as evidenced by the absence of vector-derived neomycin resistance (NeoR) gene transcripts in these B cells (Supplemental Figure 7 and Supplemental Table 6). In agreement with these findings, ADA-SCID patients after HSC-GT showed no or low-titer serum ANAs, whereas all 3 patients displayed high serum ANA titers prior to HSC-GT (Figure 8). No other serum autoantibodies were detectable after HSC-GT (Supplemental Table 17). We concluded that HSC-GT restores early B cell tolerance checkpoints in ADA-SCID patients and prevents the emergence of ANA-expressing B cells in the periphery as well as the secretion of autoreactive antibodies in the serum.

## Discussion

We report herein that ADA-SCID patients showed defects in the establishment of B cell tolerance, which were mostly corrected after HSC-GT. Gene-corrected ADA-deficient B cells overcame the

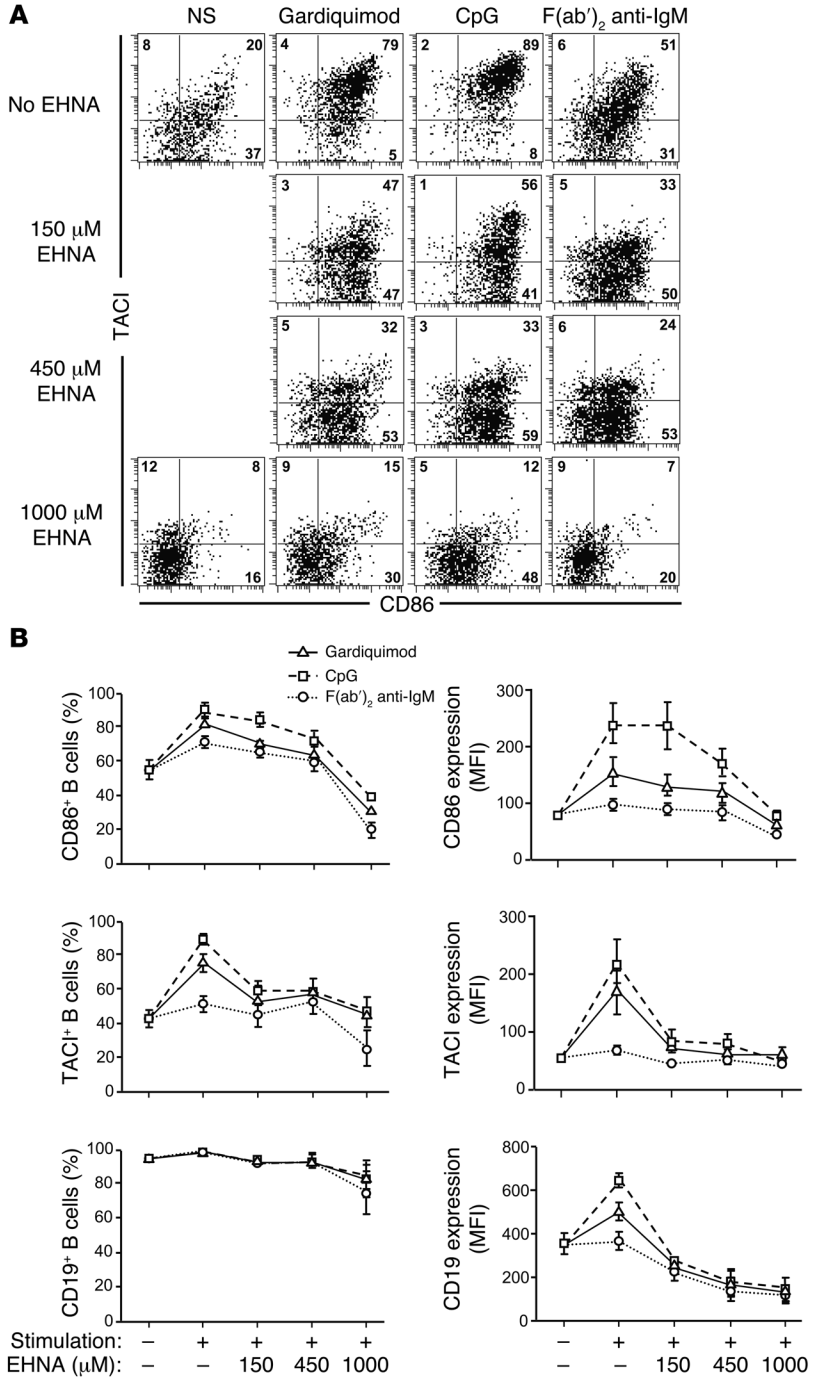


**Figure 4**

Defective TLR-induced upregulation of B cell activation markers after ADA inhibition. (**A**) CD86 and TACI expression on naive B cells from HDs after inhibition of ADA with increasing concentrations of EHNA and no stimulation (NS) or in vitro stimulation with the TLR7 agonist Gardiquimod, the TLR9 agonist CpG, or F(ab′)$_2$ anti-IgM for 2 days. (**B**) Frequency and MFI for CD86, TACI, and CD19. Data are representative of 4 independent experiments ± SEM.

initial block at the new emigrant/transitional B cell stage to progress to the mature naive B cell compartment. In addition, HSC-GT restored the counterselection of developing autoreactive B cells, causing a drastic reduction in the frequency of ANA-expressing clones. Hence, we conclude that ADA plays an essential role in the establishment of early B cell tolerance and the removal of developing autoreactive B cells in humans.

Developing autoreactive B cells are normally removed at 2 distinct checkpoints, first in the bone marrow and then in the periphery. The regulation of the central B cell tolerance checkpoint is mainly controlled by intrinsic B cell molecules that mediate the signaling of BCRs and TLRs potentially triggered by self-antigens at the immature B cell stage (16–18). Alterations of BCR signaling thresholds, such as in B cells from X-linked agammaglobulinemia patients, who carry mutations in their *BTK* gene, result in a defective central B cell tolerance checkpoint and the release of autoreactive B cells in the periphery (22). Similarly, a *PTPN22* allele encoding an R620W variant known to decrease BCR signaling also interferes with the removal of developing autoreactive B cells in humans (21). In addition, we demonstrated that IRAK-4/MyD88, which are known to mediate most TLR functions (24), played an essential role in the regulation of central tolerance. All ADA-SCID patients showed central B cell tolerance checkpoint defects before HSC-GT, as illustrated by increased frequencies of polyreactive B cells in their new emigrant/transitional B cell compartment, which suggests that ADA impinges on BCR and/or TLR signaling. Consistent with this hypothesis, adenosine receptor signaling and intracellular cAMP have been reported to modulate BCR and TLR functions (25, 26, 29, 35). Indeed, adenosine blocks NF-κB activation in murine B cells stimulated through BCRs, as well as TLR4 activation by LPS (26, 29). This mechanism has also been hypothesized to contribute to B cell dysfunction and defective B cell proliferation and activation observed in ADA-deficient mice (35, 38). In agreement with these reports, we demonstrated that the inhibition of ADA enzymatic activity in normal donor cells in vitro decreased TLR signaling in human B cells and, to a lesser extent, BCR signaling involved in the regulation of central B cell tolerance (21, 22, 39, 40). In addition, naive B cells incubated with ADA inhibitor or adenosine receptor agonist failed to properly respond to TLR7, TLR9, and BCR stimulation, further underlining the inability of these receptors to function in the absence of functional ADA. We conclude that developing immature ADA-deficient B cells are not likely to generate proper BCR and TLR signaling, resulting in a failure to induce B cell tolerance mechanisms and in the release of polyreactive B cells in the periphery.

Central B cell tolerance was further disrupted in all ADA-SCID patients before HSC-GT, because new emigrant/transitional B cells often expressed ANAs. We previously reported high frequencies of ANA-expressing new emigrant/transitional B cells

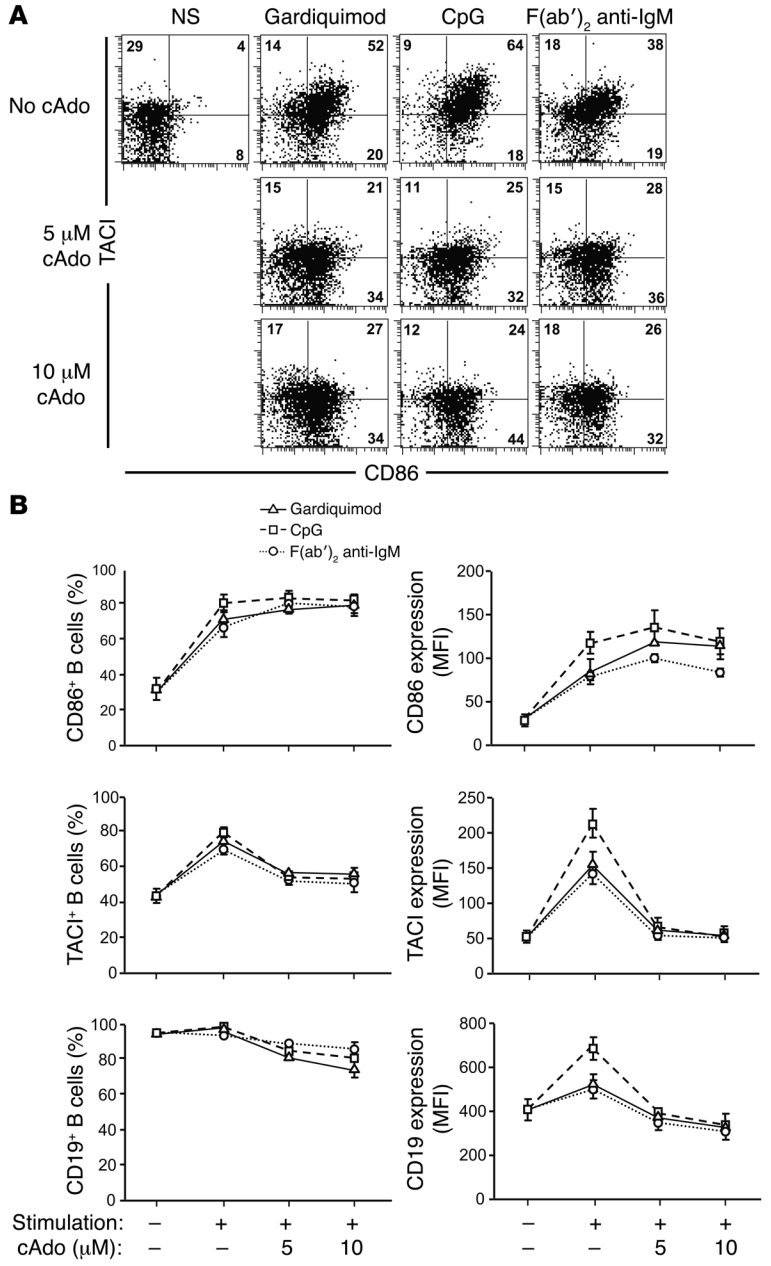


**Figure 5**
Defective TLR-induced upregulation of B cell activation markers after stimulation in the presence of cAdo. (**A**) CD86 and TACI expression on naive B cells from HDs exposed to increasing concentrations of cAdo and stimulated or not in vitro with Gardiquimod, CpG, or $F(ab')_2$ anti-IgM for 2 days. (**B**) Frequency and MFI for CD86, TACI, and CD19. Data are representative of 6 independent experiments ± SEM.

in IRAK-4– and MyD88-deficient patients (24). Sensing nucleic acid–containing immune complexes relies on the coengagement of endosomal members of the TLR family, TLR7 and TLR9 (23), which are possible receptor candidates for the removal of ANA-expressing clones. In line with this hypothesis, we found that stimulations through TLR7 and TLR9 were significantly dependent on proper ADA enzymatic activity and adenosine receptor signaling.

In contrast to central tolerance, the peripheral B cell tolerance checkpoint seems to rely on extrinsic cellular and molecular regulators, such as Tregs and serum BAFF concentration (30, 41). The increased frequency of autoreactive B cells in the mature naive compartment of ADA-SCID patients before HSC-GT compared with HDs is suggestive of peripheral B cell tolerance mechanism defects in ADA deficiency. In addition, the frequency of both polyreactive and ANA-expressing clones worsened between the new emigrant/transitional and mature naive B cell stages. Interestingly, we previously found that Treg functions are defective in PEG-ADA–treated ADA-SCID patients and corrected after HSC-GT, and may therefore contribute to the impairment in the autoreactive B cell counterselection in the periphery (42). Moreover, self-reactive B cells that escape deletion compete with non–self-reactive B cells for survival signals, such as BAFF. This key factor modulating the survival of peripheral B cells is elevated under lymphopenic conditions, such as SCID, and may therefore also contribute to the abnormal accumulation of autoreactive and ANA-expressing B cells, as suggested by mouse models and hyper-IgM patients (41, 43).

Both PEG-ADA and HSC-GT improve B cell numbers in ADA-SCID patients to variable degrees (3, 44). In the 3 HSC-GT–treated patients analyzed, we observed an

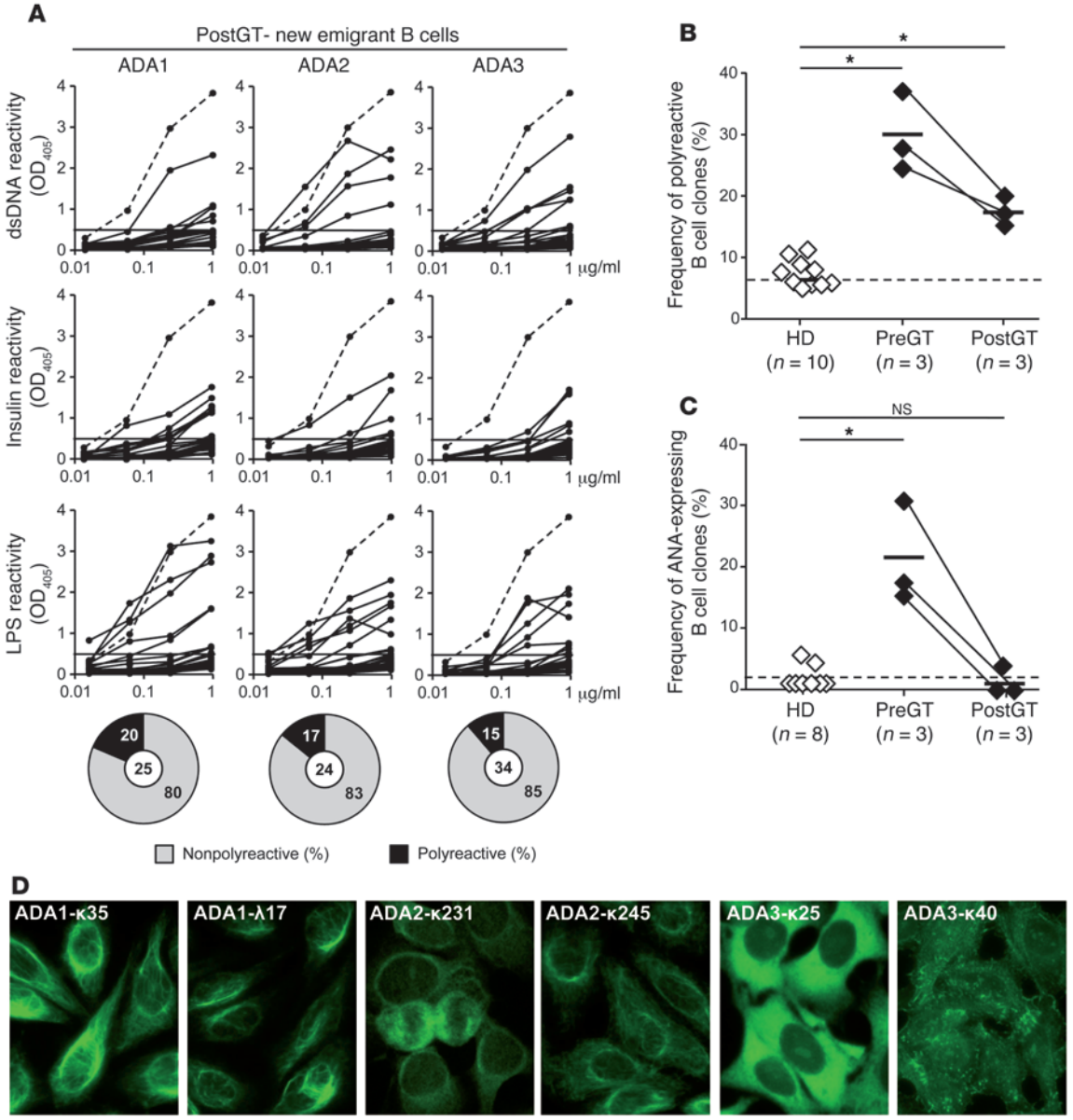


**Figure 6**

*ADA* HSC-GT corrects central B cell tolerance in ADA-SCID patients. (**A**) Antibodies expressed by new emigrant/transitional B cells from ADA-SCID patients were tested by ELISA for polyreactivity against dsDNA, insulin, and LPS. Dotted lines show ED38-positive control. Horizontal lines show cutoff $OD_{405}$ for positive reactivity. The frequencies of polyreactive and nonpolyreactive clones are summarized in the pie charts, with the number of antibodies tested indicated in the center. (**B**) Polyreactive new emigrant/transitional B cell frequency decreased in ADA-SCID patients after HSC-GT, but remained significantly elevated compared with that in HDs. (**C**) ADA HSC-GT completely restored the central removal of ANA-expressing developing B cells. ANA-expressing new emigrant/transitional B cell frequency in ADA-SCID patients after HSC-GT was significantly decreased compared with pretherapy frequencies, comparable to those in HDs. (**B** and **C**) Each symbol represents an individual, horizontal bars denote means, and dashed lines show the average frequency in HDs. $^*P \leq 0.05$. (**D**) Antibodies expressed from ADA-SCID new emigrant/transitional B cells showed various cytoplasmic staining patterns and were devoid of nuclear reactivity. 2 examples for anticytoplasmic new emigrant B cells are shown for each patient. Original magnification, ×40.

increased progression of new emigrant/transitional B cells to the mature naive B cell stage compared with ADA-SCID patients on PEG-ADA. The elimination of ADA substrates, which cause apoptosis in lymphocytes, results in increased B cell counts (35), potentially by improving BCR signaling, which plays an essential role in B cell survival (20). Similar to our previous findings in T cells showing corrected TCR signaling and apoptosis after HSC-GT (28), our present findings suggested that intrinsic ADA enzymatic activity may be more effective than serum detoxification by PEG-ADA. Moreover, our data showed that enzyme replacement therapy did not seem to correct the counterselection of developing autoreactive B cells. In contrast, after HSC-GT, polyreactive and ANA-expressing developing B cells were counterselected during bone marrow B cell development, indicative of

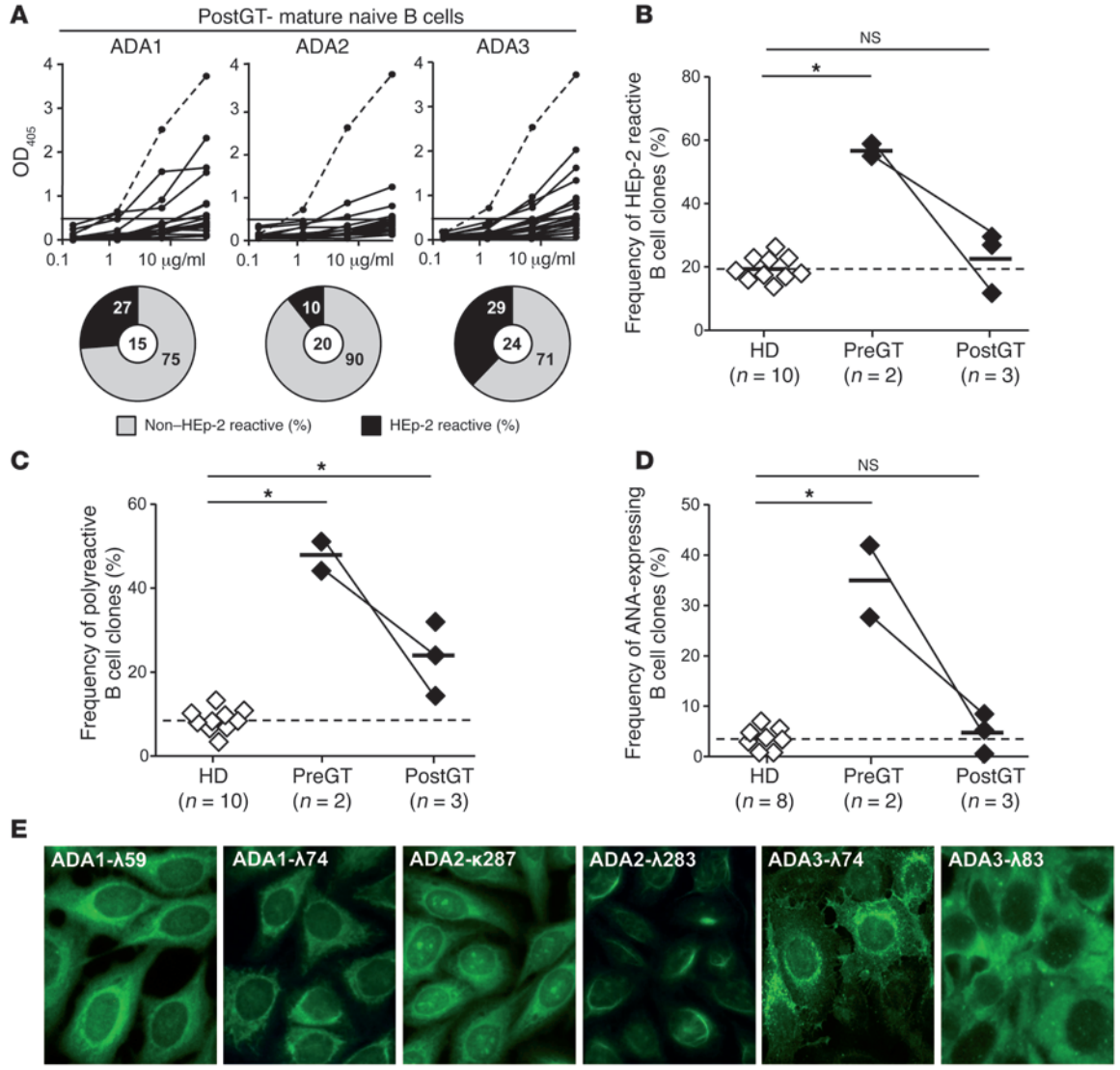


**Figure 7**

*ADA* HSC-GT also restores the peripheral B cell tolerance checkpoint in ADA-SCID patients. (**A**) Antibodies from mature naive B cells from 3 ADA-SCID patients were tested by ELISA for reactivity with HEp-2 cell lysate. Dotted lines show ED38-positive control. Horizontal lines show cutoff $OD_{405}$ for positive reactivity. The frequencies of HEp-2–reactive and non–HEp-2–reactive clones are summarized in the pie charts, with the number of antibodies tested shown in the center. (**B**) The frequency of HEp-2–reactive clones in mature naive B cells of ADA-SCID patients was corrected after HSC-GT and comparable to HDs. (**C**) The frequencies of polyreactive (tested against dsDNA, insulin, and LPS) clones in mature naive B cells of post–HSC-GT ADA-SCID patients were significantly reduced, but remained higher than those in HDs. (**D**) The removal of ANA-expressing mature naive B cells was completely restored by HSC-GT. (**B**–**D**) Each symbol represents an individual, horizontal bars denote means, and dashed lines show the average frequency in HDs. *$P \leq 0.05$. (**E**) Antibodies expressed by post–HSC-GT ADA-SCID mature naive B cells showed various cytoplasmic staining patterns and did not recognize nuclear structures. 2 examples for anti-cytoplasmic new emigrant B cells are shown for each patient. Original magnification, ×40.

restoration of early B cell tolerance checkpoints in these patients. Intracellular gene correction of ADA-deficient B cells by retroviral HSC-GT may lead to superior detoxification of ADA substrates and restoration of BCR and TLR signaling thresholds and function, thereby securing central B cell tolerance. In agreement with this hypothesis, we found that most of the rare B cell clones expressing ANAs after HSC-GT were not gene corrected, further suggesting that extrinsic detoxification, such as in PEG-ADA–treated patients, is not sufficient to ensure proper autoreactive B cell counterselection. In addition, *ADA* gene transfer into HSCs was able to correct peripheral B cell tolerance and the counterselection of ANA-expressing B cell clones. This may be the consequence of the restoration of suppressive Treg function by HSC-GT (42), further underlining the importance of Tregs for effective peripheral B cell tolerance. However, the coexistence of noncorrected autoreactive and ANA-expressing B cells and gene-corrected functional T cell help may explain why some ADA-SCID patients develop autoimmune manifestations after HSC-GT (3). Additionally, partial ADA correction resulting in low enzymatic activity may mimic late-onset patients, which typically display a

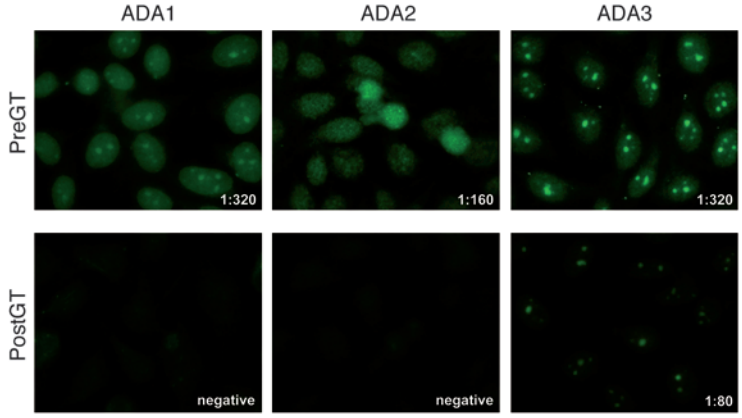


**Figure 8**
Serum ANAs in ADA-SCID patients are decreased after HSC-GT. All 3 studied ADA-SCID patients showed high titers of serum ANAs before HSC-GT. Serum ANAs were no longer detectable in 2 ADA-SCID patients after HSC-GT, whereas the third patient showed dull staining at 1:80 dilution, considered a low titer or borderline positive. Serum ANA titers are indicated for each sample. Original magnification, ×40.

higher prevalence of autoimmune manifestations (8, 45). Modification of the preparatory regimen or increased gene transfer efficiency by more robust approaches, such as lentiviral vectors, may further improve HSC-GT outcome (46).

In summary, we demonstrated that ADA played an essential role in the establishment of early B cell tolerance in humans and that retroviral gene correction of *ADA* restored central and peripheral B cell tolerance in ADA-SCID patients.

## Methods

*ADA-SCID patients and HDs*. Research was conducted on biological specimens from ADA-SCID patients and HDs. HSC-GT for ADA-SCID was performed as described previously (3). HD27 is a 25-year-old Asian female, and HD28 is a 50-year-old male of mixed European descent. None of the studied subjects was under immunomodulatory therapy at the time of analysis. None of the HDs and ADA-SCID patients were carriers of the *PTPN22* risk allele, which is reported to interfere with the removal of developing autoreactive B cells (21).

*Cell staining and sorting*. Peripheral B cells were purified from the blood of patients and HDs by positive selection using CD20-magnetic beads (Miltenyi). Enriched B cells were stained with FITC anti-human CD27, PE anti-human CD10, anti-human IgM biotin revealed using streptavidin–PE-Cy7, and allophycocyanin (APC) anti-human CD21, all from BD Biosciences — Pharmingen. Single $CD19^+CD10^+IgM^{hi}CD27^-$ new emigrant/transitional and $CD19^+CD10^-IgM^+CD27^-$ peripheral mature naive B cells from patients and HDs were sorted on a FACSVantage (BD) into 96-well PCR plates containing 4 μl lysis solution (0.5× PBS containing 10 mM DTT, 8 U RNAsin [Promega], and 0.4 U 5′-3′ RNase Inhibitor [Eppendorf]) and immediately frozen on dry ice.

*cDNA, RT-PCR, antibody production, and purification*. RNA from single cells was reverse-transcribed in the original 96-well plate in 12.5-μl reactions containing 100 U Superscript II RT (Gibco, Invitrogen) for 45 minutes at 42°C. RT-PCR reactions, primer sequences, cloning strategy, expression vectors, antibody expression, and purification were as described previously (15, 47). Ig sequences were analyzed by Ig BLAST comparison with GenBank. IgH CDR3 was defined as the interval between the conserved arginine/lysine at position 94 in the $V_H$ framework 3 and the conserved tryptophan at position 103 in $J_H$ segments.

*NeoR RT-PCR*. The following primers were used to detect NeoR gene transcripts in cDNA from single B cells after HSC-GT by nested PCRs performed at 62°C: first PCR, sense NeoR1 CGCTTCAGTGACAACGTCGAG and antisense NeoR1 CGCTTCAGTGACAACGTCGAG; second PCR, sense NeoR2 GGCTATGACTGGGCACAACAG and antisense NeoR2 GTCGTGGCCAGCCACGATAG.

*ELISAs and immunofluorescence assays*. Antibody concentrations and reactivity were as described previously (15, 47). Highly polyreactive ED38 was used as positive control in HEp-2 reactivity and polyreactivity ELISAs (15, 47). Antibodies were considered polyreactive when they recognized all 3 analyzed antigens: dsDNA, insulin, and LPS. For indirect immunofluorescence assays, HEp-2 cell–coated slides (Bion Enterprises Ltd.) and *C. luciliae*–coated slides (Antibodies Inc.) were incubated in a moist chamber at room temperature with purified recombinant antibodies at 50–100 μg/ml or diluted serum samples according to the manufacturer's instructions. FITC-conjugated goat anti-human IgG was used as detection reagent.

*B cell activation*. Naive B cells were plated at 150,000–200,000 cells/well in a 96-well plate in RPMI 1% serum and 2.5 μg/ml polyclonal $F(ab')_2$ rabbit anti-human IgM (Jackson Immunoresearch), 0.25 μg/ml CpG (TLR9 agonist; Invitrogen) or 0.5 μg/ml Gardiquimod (TLR7 agonist; InvivoGen) for 48 hours. ADA inhibition with EHNA is a commonly used method to mimic ADA deficiency in vitro (48). EHNA (Sigma-Aldrich) was resolved in PBS and used at 150, 450, and 1,000 μM. Adenosine receptor was triggered by exposure to cAdo, an ADA-resistant analog of adenosine, which is widely used as an adenosine receptor agonist (36). cAdo (Sigma-Aldrich) was used at 5 and 10 μM. Flow cytometry analysis was performed using anti-CD25–FITC, CD86-APC (Biolegend), CD80-FITC, TACI-PE, CD69-PE, CD19-PECy7, CD95/Fas-APC (BD Pharmingen).

For intracellular phosphospecific flow cytometric analysis, PBMCs ($1.5 \times 10^6$) were preincubated at 37°C in a $CO_2$ incubator for 1.5 hours in 200 μl medium (RPMI plus 1% FBS) with or without ADA inhibitor (1,000 μM EHNA) or IRAK-1/4 inhibitor (1,000 nM; EMD Chemicals). PBMCs were then stimulated for 15 minutes with 10 μg/ml polyclonal $F(ab')_2$ rabbit anti-human IgM (Jackson Immunoresearch), CpG (InvivoGen), or Gardiquimod (InvivoGen). Cell fixation and permeabilization were performed in 1.5% paraformaldehyde for 10 minutes at room temperature followed by resuspension in 80% ice-cold methanol for 30 minutes. Cells were then washed twice with 1× PBS plus 0.5% BSA and stained with Syk–Alexa Fluor 488 (pY348) or PLCγc2–Alexa Fluor 488 (pY759), Btk–Alexa Fluor 647 (pY551) or ERK1/2–Alexa Fluor 647 (pT202/pY204), and CD27-PE and CD19-PECy7 (BD Biosciences — Pharmingen) for 20 minutes at room temperature. Cells were analyzed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences — Pharmingen).

*Statistics*. Statistical analysis was performed using GraphPad Prism (version 5.0; GraphPad). Comparisons between HDs and patients were assessed using the Mann-Whitney test. FACS data were analyzed for statistical significance by unpaired 2-tailed Student's *t* tests. For all tests, α was set equal to 0.05; a *P* value of 0.05 or less was considered significant.

*Study approval*. Permission from HDs or patient's parents/guardians was obtained for all studies, according to HSR Institutional Ethical Committee–approved protocols. Patients treated with HSC-GT were enrolled in gene therapy protocols (ClinicalTrials.gov ID NCT00598481 and NCT00599781) approved by the HSR Ethical Committee and by the Italian Istituto Superiore di Sanità. The protocol for research studies on biological material from patients with primary immunodeficiency (TIGET 02), including ADA-SCID, was approved by the HSR Institutional Ethical Committee.

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