Increased T Cell Autoreactivity in the Absence of CD40-CD40 Ligand Interactions: A Role of CD40 in Regulatory T Cell Development

Atsushi Kumanogoh, Xiaosong Wang, Ihnsook Lee, Chie Watanabe, Masahito Kamanaka, Wei Shi, Kanji Yoshida, Takehito Sato, Sonoko Habu, Misako Itoh, Noriko Sakaguchi, Shimon Sakaguchi and Hitoshi Kikutani

J Immunol 2001; 166:353-360; doi: 10.4049/jimmunol.166.1.353
http://www.jimmunol.org/content/166/1/353

Why The JI?

- Rapid Reviews! 30 days* from submission to initial decision
- No Triage! Every submission reviewed by practicing scientists
- Speedy Publication! 4 weeks from acceptance to publication

*average

References
This article cites 46 articles, 21 of which you can access for free at: http://www.jimmunol.org/content/166/1/353.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at: http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Increased T Cell Autoreactivity in the Absence of CD40-CD40 Ligand Interactions: A Role of CD40 in Regulatory T Cell Development

Atsushi Kumanogoh,* Xiaosong Wang,* Ihnsook Lee,* Chie Watanabe,* Masahito Kamanaka,* Wei Shi,* Kanji Yoshida,* Takehito Sato,* Sonoko Habu,* Misako Itoh,* Noriko Sakaguchi,† Shimon Sakaguchi,‡ and Hitoshi Kikutani‡

Mutations in the CD40 ligand (CD40L) gene lead to X-linked immunodeficiency with hyper-IgM, which is often associated with autoimmune diseases. To determine the contribution of defective CD40-CD40L interactions to T cell autoreactivity, we reconstituted CD40-CD40L interactions by transferring T cells from CD40-deficient mice to syngeneic athymic nude mice and assessed autoimmune development. T cells from CD40-deficient mice triggered autoimmune diseases accompanied with elevations of various autoantibodies, while those from wild-type mice did not. In CD40-deficient mice, the CD25+ CD45RBlow CD4+ population which regulates T cell autoreactivity was markedly reduced. CD40-deficient APCs failed to induce T regulatory cells 1 producing high levels of an inhibitory cytokine, IL-10 in vitro. Furthermore, autoimmune development was inhibited when T cells from CD40-deficient mice were cotransferred with CD45RBlow CD4+ T cells from wild-type mice or with T regulatory cells 1 induced on CD40-expressing APCs. Collectively, our results indicate that CD40-CD40L interactions contribute to negative regulation of T cell autoreactivity and that defective interactions can lead to autoimmunity. The Journal of Immunology, 2001, 166: 353–360.

Immunodeficiency is often associated with autoimmune diseases, including autoimmune hemolytic anemia, thrombocytopenic purpura, Sjögren’s syndrome, systemic lupus erythematosus, thyroiditis, and myasthenia gravis (1, 2). It is also accompanied by autoantibodies against blood cells, Igs, and various tissue Ags. Such paradoxical complications were already noticed in an agammaglobulinemia case study by Bruton in 1952 (3), and it has been enigmatic how impaired immune functions paradoxically lead to increased autoreactivity. Production of autoantibodies and development of autoimmune diseases are also observed in patients with X-linked hyper-IgM syndrome (HIGM), a rare genetic immune disorder due to mutations in the CD40 ligand (CD40L) gene (1, 2, 4–10). These findings suggest that defects of CD40L may evoke autoreactivity. However, the precise pathological mechanisms still remain to be clarified.

Studies on CD40- or CD40L-deficient mice and analysis of HIGM have established that interactions between CD40 on B cells and CD40L on activated T cells are essential for effective humoral immune responses, including Ag-specific Ab production. Ig class switching, and germinal center formation (11–14). CD40-CD40L interactions play important roles in cellular immunity as well. For example, CD40 is expressed on various APCs (15, 16), thereby CD40-CD40L interactions are critically involved in T cell priming, activation, and differentiation into Th1 cells through the expression of various costimulatory molecules such as B7-1 and B7-2 on APCs and secretion of cytokines such as IL-12 (17–20). Moreover, the prevalence of autoimmune diseases in HIGM patients suggests that CD40-CD40L interactions might be involved in the control of autoreactive lymphocytes. Although CD40- or CD40L-deficient mice appear to be a suitable model to explore autoreactivity in the absence of CD40-CD40L interactions, autoimmune diseases have not been reported either in CD40- or CD40L-deficient mice so far. One of the reasons for this is that coexisting impairments of T cell activation due to the lack of CD40-CD40L interactions (17–20) would conceal autoreactivity and make it difficult to detect autoimmunity in such mice maintained in pathogen-free conditions.

To maintain immunological self-tolerance and avoid autoimmunity, pathogenic self-reactive T cells are clonally deleted or rendered anergic in the thymus and periphery (21–24). T cell-mediated control of self-reactive T cells also plays a significant role in self-tolerance (25). For example, transfer of T cells depleted of CD25+ cells or CD45RBlow cells causes various autoimmune diseases in recipient nude or scid mice, respectively, while CD25+ CD45RBlow CD4+ T cells in chimpanzees can inhibit the development of autoimmunity when cotransferred (26, 27). These observations suggest that the CD25+ CD45RBlow CD4+ T cell subpopulation may be regulatory T cells that suppress autoreactive lymphocytes. Besides such regulatory T cells defined by expression levels of cell surface markers, T cells producing inhibitory cytokines may also act as regulatory T cells. A TGF-β-producing T cell subset, called Th3, has been identified mainly in oral tolerance systems (28). In addition, T regulatory cells 1 (Tr1) subset has been recently identified and been generated in vitro (29). Tr1 cells produce large amounts of IL-10 but small amounts of IFN-γ and IL-4. Upon
stimulation, Tr1 cells immediately produce IL-10, which modulates activation of APCs and their expression of costimulatory molecules (30).

In this report, we demonstrate significant increases in T cell autoreactivity in the absence of CD40-CD40L interactions. We show that autoimmune diseases can be induced by transferring T cells from CD40-deficient BALB/c mice into BALB/c athymic (nu/nu) mice which lack T cells but have B cells and other APCs expressing CD40. Furthermore, we show severe reduction of CD25\(^ {hi} \)CD45RB low CD4\(^ {+} \)T cells in CD40-deficient mice and impaired differentiation into Tr1 cells on CD40-deficient APCs. Our results indicate that defective development of regulatory T cells leads to increased T cell autoreactivity in the absence of CD40-CD40L interactions.

Materials and Methods

Mice
To generate BALB/c CD40-deficient mice, CD40-deficient mice (12) were crossed for more than 10 generations with BALB/c mice purchased from SLC (Shizuoka, Japan). BALB/c nu/nu, C57BL/6, or DBA/2 mice of 6 wk of age were also purchased from SLC. Mice expressing the transgenic OVA (OVA\(_ {323-339} \))-specific αβ TCR on a BALB/c background were produced as previously described (31). All of these mice were maintained in our animal facility and cared for in accordance with institutional guidelines for animal welfare.

Preparation of T cells
Spleen cells were removed from wild-type or CD40-deficient BALB/c mice, C57BL/6, or DBA/2 mice and teased into single-cell suspensions. Erythrocytes were removed by hypotonic lysis. For enrichment of Thy-1\(^ {hi} \), CD45RB high or low CD4\(^ {+} \), or CD62L bright T cells, we isolated each fractions from the spleen cell suspensions using MACS or FACS sorting. The purity of the cells were checked using FACScan (Becton Dickinson, Mountain View, CA). The resulting purity was \( > 95-98\% \) in all experiments.

Titers of serum Abs
IgG anti-DNA at 12 wk after the transfer were determined by ELISA on sera diluted to 1:100, as previously described (27). Calf thymus DNA (Sigma, St. Louis, MO) was purified with a Sepa Gene kit (Nippon Gene, Toyama, Japan), and dsDNA was obtained by treating the DNA with S1 nuclease (Sigma). ssDNA was derived by boiling the DNA solution for 15 min followed by immediate immersion on ice. Wells of microtiter plates were coated with 5 \( \mu g/ml \) of either dsDNA or ssDNA. Similarly, serially diluted serum samples and control serum derived from MRL/lpr mice at 4 mo of age were incubated, followed by addition of AP-labeled anti-mouse IgG Abs (Southern Biotechnology Associates, Birmingham, AL). We defined serum titers of MRL/lpr mice at 4 mo of age as 1000 U/ml. Serum titers of autoantibodies specific for gastritic parietal cells were also assessed by ELISA as previously described (32). For detection of anti-CD40 Abs, wells of microtiter plates were coated with 5 \( \mu g/ml \) mouse CD40-human IgG Fc fusion protein (kindly provided by Dr. D. Gray (33)). Then diluted serum samples were incubated, followed by addition of AP-labeled anti-IgG Abs (Southern Biotechnology Associates).

FIGURE 1. Titers of autoantibodies in nu/nu mice inoculated with T cells from CD40-deficient or wild-type mice. Thy-1\(^ {+} \) cells were separated from splenocytes of CD40-deficient or wild-type mice from the same litter using the MACS system. A total of 1 \( \times 10^7 \) cells of the separated Thy-1\(^ {+} \) cells was transferred to BALB/c nu/nu mice. Titters of anti-ssDNA autoantibodies (A), anti-dsDNA autoantibodies (B), and anti-parietal cell autoantibodies (C) were determined by ELISA 12 wk after the transfer. Vertical bars indicate mean ± SD from 12 mice in each group.

FIGURE 2. Development of autoimmune diseases in the recipient nu/nu mice. Autoimmune diseases developed in the recipient nu/nu mice inoculated with T cells from CD40-deficient mice (A–F) but not from wild-type mice (G–L) (hematoxylin and eosin) 12 wk after the transfer. A and G, Gastric mucosa (original magnification, \( \times 250 \)); B and H, thyroid (original magnification, \( \times 500 \)); C and I, sialoadenoid (original magnification, \( \times 250 \)); D and J, ovary (original magnification, \( \times 100 \)); E and K, adrenal gland (original magnification, \( \times 500 \)); and F and L, pancreatic islet (original magnification, \( \times 500 \)).
CD40-deficient or normal BALB/c mice were injected with splenocytes (1 × 10^7 cells/mouse) of CD40-deficient BALB/c, normal BALB/c, C57BL/6, or DBA/2 mice i.v. One week after priming, CD4 T cells were purified from the primed mice and were stimulated with irradiated (3000 rad) splenocytes from CD40-deficient mice, normal BALB/c, C57BL/6, or DBA/2 mice for 3 and 5 days at a ratio of 1:5. For proliferation assays, cells were pulsed with 2 μCi of [3H]thymidine for the last 12 h. For cytokine production, culture supernatants from 3- or 5-day cultures were harvested and the levels of IFN-γ and IL-10 were measured by ELISA kit (R&D Systems, Minneapolis, MN).

**Induction and in vivo transfer of Tr1 cells**

CD42\textsuperscript{bright} CD4+ T cells from OVA-TCR-transgenic BALB/c mice were sorted out by FACS and 1 × 10^6 cells of the isolated T cells were cultured with OVA peptide (323–339; 0.6 μM) and irradiated splenic APCs (5 × 10^6 cells) in the presence of IL-10 (100 U/ml) (PharMingen, San Diego, CA) in 24-well plates as previously described (29). The stimulation, under the same conditions, was repeated weekly for 3 consecutive weeks. For analysis of the effect of Tr1 cells on autoimmunity, the recipient nu/nu mice were injected i.v. with 100 μl of PBS containing 2 × 10^6 Tr1 differentiated cells with 5 × 10^6 cells of T cells from CD40-deficient mice. The recipient mice were orally treated with OVA (100 ng/ml) as previously described (29).

**Flow cytometry and Abs**

Single-cell suspensions were prepared from wild-type BALB/c or CD40-deficient BALB/c spleens at 6 wk of age. After purification of CD4+ cells using the MACS system, flow cytometric analysis was performed using FACScan. Abs used for staining were biotinylated anti-CD25 (7D4), and PE-conjugated streptavidin (PharMingen). Analysis of intracellular cytokines by flow cytometry was performed using Golgi-plug kits (PharMingen). Briefly, cells (10^5 cells/ml) were activated with immobilized anti-CD3 and anti-CD28 mAbs (PharMingen) for 6 h. Golgi-plug (PharMingen), containing brefeldin A was added for the last 4 h of culture. Then cells were incubated with the following mAbs (PharMingen): PE-conjugated anti-IL-10 (JES-16E3), FITC-conjugated anti-IL-4 (BVD-1D11), and FITC-conjugated anti-IFN-γ (XMG1.2). Samples were analyzed on FACScan.

**Results**

T cells from CD40-deficient mice elicit histologically and serologically evident autoimmune diseases when transferred to syngenic nu/nu mice

To determine whether pathogenic self-reactive T cells have developed in CD40-deficient mice, we transferred T cells from CD40-deficient BALB/c or normal BALB/c mice into BALB/c nu/nu mice, thereby reconstituting CD40-CD40L interactions between CD40L on T cells derived from CD40-deficient mice and CD40 on B cells and APCs derived from the recipient nu/nu mice. Twelve weeks after cell transfer, significant titers of autoantibodies specific for ssDNA, dsDNA, or gastric parietal cell Ags were observed in the nu/nu mice inoculated with T cells from CD40-deficient mice. To quantify CD40-specific Abs, serum samples of recipient mice 12 wk after the transfer of T cells from CD40-deficient (●) or wild-type mice (○) from the same litter were added to microtiter plates coated with mouse CD40-human IgG Fc fusion proteins, and bound Abs were detected using ELISA. B, T cells from CD40-deficient mice did not respond to CD40-positive cells. CD40^+/+ BALB/c, and CD40^−/- BALB/c mice were primed with splenocytes of CD40^+/+ (●), CD40^+/+ (●) BALB/c mice, C57BL/6 (●), or H-2d-matched DBA/2 (○) mice. One week after priming, CD4+ T cells from the primed mice were cultured for 3 or 5 days in the presence of irradiated splenocytes from the mice of the strain used for each priming. For proliferation assays, cells were pulsed with [3H]thymidine for the last 12 h. For the production of IFN-γ and IL-4 assays, the culture supernatants of the 3- and 5-day cultures were harvested and measured by ELISA. As positive controls, T cells from either CD40^+/+ or CD40^−/- BALB/c mice exhibited marked MLR responses against splenocytes of C57BL/6 or DBA/2 mice.

**Table 1. Induction of autoimmune diseases by T cell transfer**

<table>
<thead>
<tr>
<th>Inoculated Cells</th>
<th>Total No. of Mice</th>
<th>No. of Mice with Autoimmune Disease*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Gastritis</td>
</tr>
<tr>
<td>CD40^−/-</td>
<td>12</td>
<td>11 (91.7%)^b</td>
</tr>
<tr>
<td>CD40^+/+</td>
<td>12</td>
<td>0</td>
</tr>
</tbody>
</table>

* Number of mice with histologically evident autoimmune diseases.

b Incidence.

Histological analysis revealed severe gastritis with loss and damage of the parietal cells and chief cells and marked infiltration of mononuclear cells into the gastric mucosa (Fig. 2A). In addition, histologically evident thyroiditis and sialoadenitis developed in 40–50% of mice (Fig. 2, B and C; Table 1). Some (~25%) recipients also...
CD41-deficient wild-type mice (Fig. 2, G–L) deficient mice. CD45RB low T cells were included in CD45RB low T cells as previously described (27).

developed oophoritis, adrenalitis, or insulitis (Fig. 2, D–F). No autoimmunity developed in the nu/nu mice inoculated with T cells from wild-type mice (Fig. 2, G–L). Furthermore, the autoimmunity could be induced by CD4+ T cells alone but not CD8+ T cells from CD40-deficient mice (data not shown), indicating that CD4+ T cells are key effector cells in the autoimmunity.

These autoimmunities cannot be attributed to a graft-versus-host (GVH) reaction which might be elicited by the responses of T cells from CD40-deficient mice to CD40 molecules in the recipient nu/nu mice, since there were no differences in the titers of anti-CD40 Abs between the nu/nu mice inoculated with T cells from either CD40-deficient or wild-type mice (Fig. 3A). In addition, to determine whether or not T cells from CD40-deficient mice respond to CD40 molecules, we primed CD40-deficient BALB/c mice with splenocytes from CD40-positive BALB/c, CD40-deficient BALB/c, C57BL/6, or DBA/2 mice. One week later, we examined MLR of T cells from the primed mice against each priming lymphocyte (Fig. 3B). T cells from CD40-deficient mice that were primed with BALB/c CD40-positive cells neither proliferate nor produce IFN-γ in response to BALB/c CD40-positive cells, indicating that CD40-deficient T cells did not show MLR against CD40-positive cells. However, they reacted to H-2d-matched or -mismatched allogenic splenocytes of DBA/2 or C57BL/6 mice. Taken together, these results suggest that a T cell response against CD40 molecules did not take place in the present experimental system.

The number of CD25+ CD45RB low CD4+ T cells is significantly reduced in CD40-deficient mice

It has been shown that autoimmune diseases similar to those produced in our experiments can be induced by simply transferring CD25+ cell- or CD45RB low cell-depleted T cell suspensions from normal mice into recipient nu/nu mice (25, 27, 34). This autoimmune induction can be prevented by cotransfer of the eliminated population, suggesting that the CD25+ or CD45RB low T cell subpopulation may contain regulatory T cells capable of controlling autoreactive T cells (25, 27, 34, 35). The CD25+ CD45RB low CD4+ T cell subpopulation was significantly reduced in CD40-deficient mice compared with wild-type mice (Fig. 4), although the lymphocytes from the former did not display any abnormality in the phenotype of other surface markers, as previously described (data not shown) (12). The CD25+ CD4+ T cells were included in CD45RB low CD4+ T cell populations (data not shown) (27). To determine whether or not the reduction of such subpopulations is responsible for the increased autoreactivity, CD45RB low CD4+ T cells were purified from wild-type mice and mixed with T cells from CD40-deficient mice at a physiological ratio (1:5), and then transferred into nu/nu mice. Cotransfer of the CD45RB low CD4+ T cells from wild-type mice almost completely suppressed elevations in the titers of autoantibodies, including anti-ssDNA, antidsDNA, anti-parietal cell autoantibodies, and histologically evident gastritis development driven by T cells from CD40-deficient mice (Fig. 5).

There is a possibility that the CD45RB high T cell population of CD40-deficient mice may contain more aggressive autoreactive T cells. To test this possibility, CD45RB high T cells were purified from wild-type mice or CD40-deficient mice and then transferred into nu/nu mice, respectively. As shown in Fig. 6, we could not...
detect any significant differences in the ability of CD45RB<sup>high</sup> T cells to induce autoimmunity between wild-type and CD40-deficient mice. Collectively, CD40-CD40L interactions appear to be required for generating regulatory T cells that negatively regulate T cell autoreactivity.

**CD40-deficient APCs cannot induce a regulatory T cell subset, Tr1**

A recent study established a regulatory T cell subset, Tr1, which produces high levels of an inhibitory cytokine, IL-10, but not IFN-γ or IL-4 (29). Tr1 cells were induced in vitro by Ag stimulation in the presence of IL-10 and inhibited CD45RB<sup>high</sup> cell-induced colitis. To determine the ability of CD40-deficient APCs to induce Tr1, CD62L<sup>bright</sup> CD4<sup>+</sup> naive T cells of OVA-specific TCR-transgenic mice were stimulated with an OVA peptide plus CD40-deficient or wild-type APCs in the presence of IL-10. After 3 wk of consecutive stimulation, T cells were harvested, restimulated with anti-CD3 and anti-CD28 Abs, and stained for detection of intracellular cytokines. Fig. 7 shows that wild-type APCs could predominantly induce the Tr1 subset characterized by high levels of IL-10 and low levels of IFN-γ or IL-4, as previously described (29). On the other hand, instead of the Tr1 subset, Th2-like cells secreting high levels of IL-4 and IL-10 predominantly developed on CD40-deficient APCs. Even 1 wk after stimulation, CD40-deficient APCs induced a significant number of Th2 cells (data not shown). This preferential induction of Th2 cells on CD40-deficient APCs is in accord with the previous observations that CD40- or CD40L-deficient mice mount Th2 responses instead of Th1 responses because of a lack of IL-12 production by APCs (18, 19). Cytokine secretion of Tr1-differentiated cells on the wild-type APCs showed a typical Tr1-type cytokine profile (IL-10, 1080 ± 110 pg/ml; IL-4, <50 pg/ml; IFN-γ, <8 pg/ml; and TGF-β, 660 ± 110 pg/ml). In contrast, Th2-differentiated cells on CD40-negative APCs showed a Th2-type cytokine profile (IL-10, 520 ± 100 pg/ml; IL-4, 1280 ± 125 pg/ml; IFN-γ, <8 pg/ml; and TGF-β, 768 ± 100 pg/ml).

To determine whether or not in vitro-induced Tr1 cells can suppress autoimmunity elicited by T cells from CD40-deficient mice, we cotransferred OVA-specific Tr1 cells and T cells from CD40-deficient mice into nu/nu mice, respectively. Titers of anti-ssDNA autoantibodies (A), anti-dsDNA autoantibodies (B), and anti-parietal cell autoantibodies (C) were determined by ELISA 12 wk after the transfer. All of recipient mice developed gastritis.

**Discussion**

The paradoxical complications of autoimmune diseases in immuno-deficiency have been a classical enigma (1–3). By employing the nu/nu mice transfer system, we showed here that T cell autoreactivity is significantly increased in the absence of CD40-CD40L interactions. For the following reasons, abnormal T cell autoreactivity which elicits autoimmune diseases in the recipient nu/nu mice could be attributed to severe impairments in the generation of regulatory T cells due to the absence of CD40-CD40L interactions. First, CD40-deficient mice have few CD25<sup>+</sup> CD45RB<sup>low</sup> CD4<sup>+</sup> T cells to induce autoimmunity between wild-type and CD40-deficient mice. Collectively, CD40-CD40L interactions appear to be required for generating regulatory T cells that negatively regulate T cell autoreactivity.

![FIGURE 6. The pathogenic activity of CD45RB<sup>high</sup> T cells of wild-type or CD40-deficient mice. CD45RB<sup>high</sup> T cells (2 × 10<sup>6</sup> cells/mouse) sorted out from wild-type or CD40-deficient mice were transferred into nu/nu mice, respectively. Titers of anti-ssDNA autoantibodies (A), anti-dsDNA autoantibodies (B), and anti-parietal cell autoantibodies (C) were determined by ELISA 12 wk after the transfer. All of recipient mice developed gastritis.](http://www.jimmunol.org/)

![FIGURE 7. Intracellular cytokine analysis of Tr1 cells. CD62L<sup>bright</sup> CD4<sup>+</sup> naive T cells from BALB/c mice transgenic for OVA-TCR were cultured with OVA (0.6 μM) plus CD40-deficient or wild-type irradiated (3300 rad) splenic APCs in the presence of IL-10 (100 U/ml). The stimulation was repeated weekly for 3 consecutive wk. Cells were collected, washed, and restimulated with anti-CD3 (10 μg/ml) and anti-CD28 mAb (10 μg/ml) with brefeldin A. The cells were fixed and stained for detection of intracellular cytokines using FACScan. The purity of CD4<sup>+</sup> remained <99% after 3 wk of culture. The isotype-matched controls (x-axis; rat IgG2a, y-axis; rat IgG1 plus rat IgG2b) were also shown. A representative result of five independent experiments is shown.](http://www.jimmunol.org/)
CD40L interactions are required for generation of regulatory T cells from CD40-deficient mice to expand rapidly, to differentiate into the Tr1 subset that emerged from 1-wk in vitro culture without IL-10 (data not shown). Development of Tr1-like cells required at least 3 wk on CD40-positive APCs in the presence of IL-10, as reported previously. Moreover, Tr1 cells proliferate poorly presumably due to the lack of CD40-CD40L interactions affects thymic deletion of T cells reactive to several endogenous Ags (38). However, there was no significant abnormality in deletion of T cells expressing Vβ11 and Vβ12 TCR reactive to endogenous Mls in CD40-deficient BALB/c mice. T cells expressing Vβ8 TCR were also eliminated in CD40-deficient mice when staphylococcal enterotoxin B was systemically injected (our unpublished results). A recent study has shown that CD40 deficiency only affects negative selection of T cells reactive to weakly stimulating superantigens presented by B cells but not to strongly stimulating Ags (39). As for peripheral anergy, it has been shown to require adequate activation of autoreactive T cells (40).

The Tr1 subset has been functionally characterized by their secretion of high levels of IL-10. Although there is substantial data that regulatory CD25⁺CD45RB⁺⁺CD4⁺ T cells control other T cells by a cognate cellular interaction on APCs (25, 36, 37), the relationship between the CD25⁺CD45RB⁺⁺CD4⁺ T cell subpopulation and the Tr1 population remains to be determined. The Tr1 subset can be induced by in vitro stimulation with APCs and specific Ags in the presence of IL-10 and can inhibit CD45RBhighCD4⁺ T cell-induced autoimmunity (29). Unlike Th1 or Th2 cells that emerged from 1 wk in vitro culture without IL-10 (data not shown), development of Tr1-like cells required at least 3 wk on CD40-positive APCs in the presence of IL-10, as reported previously. Moreover, Tr1 cells proliferate poorly presumably due to their IL-10 production (29). This delayed induction and poor proliferative capacity of Tr1 cells may allow autoreactive T cells from CD40-deficient mice to expand rapidly, to differentiate into the Tr1 subset that emerged from 1-wk in vitro culture without IL-10 (data not shown), development of Tr1-like cells required at least 3 wk on CD40-positive APCs in the presence of IL-10, as reported previously. Moreover, Tr1 cells proliferate poorly presumably due to their IL-10 production (29). This delayed induction and poor proliferative capacity of Tr1 cells may allow autoreactive T cells from CD40-deficient mice to expand rapidly, to differentiate into the Th2 effector cells, and thereby to cause autoimmune responses in nu/nu recipients. Thus, our results indicate that CD40-CD40L interactions are required for generation of regulatory T cells. However, a very small number of CD25⁺CD45RB⁺⁺CD4⁺ T cells could be detected in CD40-deficient mice. At present, it is unclear whether or not these residual CD25⁺CD45RB⁺⁺CD4⁺ T cell subpopulations in CD40-deficient mice contain functional regulatory T cells because of difficulty in enrichment of enough numbers of these populations from CD40-deficient mice for the in vivo functional analysis. Further studies will be necessary to address this question.

In addition to defects in generating regulatory T cells presented here, there are two other possible mechanisms that may underlie the ability of T cells from CD40-deficient mice to induce autoimmunity. First, autoimmune diseases observed here may be due to responses of T cells which are not tolerant to the CD40 molecule. However, there were no differences in the titers of anti-CD40 Abs between the nu/nu mice inoculated with T cells from either CD40-deficient or wild-type mice T cells. In addition, T cells from CD40-deficient mice failed to react with CD40-positive cells even when in vivo primed (Fig. 3). These results suggest that T cell responses against CD40 molecules did not take place in our experiments. Second, impaired clonal deletion and peripheral anergy might be the cause of autoimmunity. A previous report has shown that the lack of CD40-CD40L interactions affects thymic deletion of T cells reactive to several endogenous Ags (38). However, there was no significant abnormality in deletion of T cells expressing Vβ11 and Vβ12 TCR reactive to endogenous Mls in CD40-deficient BALB/c mice. T cells expressing Vβ8 TCR were also eliminated in CD40-deficient mice when staphylococcal enterotoxin B was systemically injected (our unpublished results). A recent study has shown that CD40 deficiency only affects negative selection of T cells reactive to weakly stimulating superantigens presented by B cells but not to strongly stimulating Ags (39). As for peripheral anergy, it has been shown to require adequate activation of autoreactive T cells (40). Autoreactive T cells may not undergo activation before clonal anergy in CD40-deficient mice because of impaired CD40-CD40L interactions, resulting in accumulated nontolerized T cells. However, severity and pathological features of autoimmune diseases induced by unfractionated T cells or CD45RBhigh T cells of CD40-deficient mice were very similar to those of diseases induced by CD45RBhigh T cells from normal BALB/c mice (Fig. 6) (27, 34). Thus, autoimmune diseases induced by T cells of CD40-deficient mice may not be further modified or enhanced by GVH reactions against CD40 molecules or by enhanced production of autoreactive T cells due to impaired clonal deletion or anergy.

Patients with HIGM not only produce various autoantibodies, but also succumb to various autoimmune diseases (1, 2). Thus, CD40- and CD40L-deficient mice may be expected to be susceptible to autoimmune diseases. However, autoimmune diseases have not been detected in CD40-deficient mice possibly because activation of autoreactive T cells is severely affected in CD40- or CD40L-deficient mice (41–43). In CD40-deficient mice, therefore, reactive T cells may fail to differentiate into pathogenic effector cells. HIGM patients frequently suffer from persistent infections by various kinds of pathogens, some of which might stimulate T cells without CD40-CD40L interactions, resulting in accumulated autoreactive T cell responses. In fact, several pathogens such as...
lymphocytic choriomeningitis virus and murine CMV have been reported to induce fully functional CD4+ effector T cells without CD40-CD40L interactions (44, 45), suggesting that the lack of CD40-CD40L interactions in T cell activation might be compensated by other stimuli. Since CD40- or CD40L-deficient mice maintained in pathogen-free conditions cannot be stimulated by these pathogens, it may be difficult to trigger autoimmunity in these mice. Alternatively, production of high-affinity IgG autoantibodies may be necessary for full-blown autoimmune diseases mediated by T cells. For instance, CD40-dependent Ab production has been shown to be involved in tissue-specific infiltration of autoreactive T cells in the autoimmune arthritis model (46). Thus, defective Ab production might also conceal T cell-mediated autoimmune diseases in CD40-deficient mice.

In conclusion, we have presented evidence that the lack of CD40-CD40L interactions can cause increased autoreactivity despite immunodeficiency. The relationship between immunodeficiency and autoimmunity has been a paradoxical phenomenon. It is difficult to treat patients of immunodeficiency accompanied by autoimmune diseases because immunosuppressive drugs must be used despite immunodeficiency (1). The present study will provide clues for understanding the pathogenesis of autoimmune complications in immunodeficiency and contribute to developing better treatments.

Acknowledgments

We gratefully acknowledge Dr. T. Yoshimura for advice concerning intracellular cytokine staining. We also thank K. Kubota for excellent secretarial assistance.

References


