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Regulatory CD8+ T Cells Control Neonatal Tolerance to a Th2-Mediated Autoimmunity1

Anne-Christine Field, Laure Caccavelli, Marie-Françoise Bloch, and Blanche Bellon2

Exposure of newborn animals to a foreign Ag may result in immunological tolerance to that specific Ag, a phenomenon called neonatal tolerance. We have previously reported that neonatal administration to Brown-Norway rats of mercury, a heavy metal toxicant, induces a dominant tolerance, specific for the chemical otherwise responsible for Th2 cell-mediated autoimmune responses in this susceptible strain of rats. Neonatal exposure to Ags can prime immunity, rather than inactivate or delete responses, and sustain regulatory functions effective against autoreactive T cells. Here, we address whether such a tolerant response is due to the generation of regulatory cells. The results suggest that the CD8+ T cell subset is involved in neonatal tolerance to mercuric salt-induced Th2 autoimmune disease. Thus, we demonstrate that in vivo CD8 depletion breaks tolerance following mercury recall in animals under a neonatal tolerance protocol. Furthermore, adoptive cotransfer of splenocytes from naive and tolerant rats as well as transfer of CD8+ T cells from tolerant animals prevent naive syngeneic rats from developing pathologic Th2 immune responses. These observations indicate that CD8+ T cells are endowed with regulatory functions in neonatal tolerance and mediate active suppression. Moreover, neonatal tolerance induced the expansion of CD8+CD45RChigh T cells and the emergence of a high percentage of IFN-γ-synthesizing CD8+ T cells, which probably reflects the implication of regulatory Tc1 cells. Thus, in vivo induction of neonatal tolerance suppresses Th2 autoimmune responses via generation of a CD8+ cell-mediated regulatory response. The Journal of Immunology, 2003, 170: 2508–2515.

Following activation, naive CD8+ T cells may be polarized into two cytotoxic T cell types, called Tc1 and Tc2, according to their functional capacities and their profile of cytokine production (1, 2). CD8+ Tc1 cells produce IL-2, IFN-γ, and TNF-α and promote cellular immune responses and cytotoxic effects. Tc2 cells exert less cytotoxic functions; secrete IL-4, IL-5, IL-10, and IL-13; and help B lymphocytes to produce Abs. The cytokine environment is one of the major factors influencing the differentiation of each of these T cell subpopulations; IL-12 directs the response toward type 1, while IL-4 directs it to type 2. An imbalance between type 1 and type 2 activation can result in immune dysregulations, leading to impaired cell-mediated immunity with an increased incidence of infectious disease or cancer and/or aberrant humoral immunity that may culminate in autoimmune disease.

Chronic exposure to mercuric salts, a still widely distributed environmental and industrial pollutant (3), can lead to the development of systemic lupus erythematosus (SLE)-like autoimmune responses by critically upsetting the balance between Th1/Th2 cells (4, 5). Experimental mercury disease damages multiple organs and causes diverse and variable clinical manifestations similar to SLE, including lymphoproliferation (6), hypergammaglobulinemia (7), autoantibody production (8), and immune globulinemia (7), autoantibody production (8), and immune globulinemia (7). Generation of IgE responses requires switching of B cells to H chain e isotype production, and this process is highly dependent on IL-4 and strongly inhibited by IFN-γ (12, 13). Among the numerous autoantibodies produced in SLE and mercury disease, dsDNA represents a major target (14). The HgCl2-induced predominant Th2 response is followed by an immunosuppressive reaction with inhibitory Th1-type responses (15, 16), while CD8+ T cells are not involved in the initial induction, magnitude, or spontaneous resolution of the mercury disease (17).

Immune tolerance may be mediated by clonal anergy (18), clonal deletion (19), or active suppression (20). In active suppression, regulatory cells have been shown to be capable of transferring tolerance to naive syngeneic recipients (21, 22). Such regulatory cells are thought to play a critical role by modulating the immune response through secretion of cytokines (23). We have previously reported that a strong tolerant state could be induced by neonatal injections of mercuric salts in BN rats and that this neonatal immunological, mercury-specific tolerance was dominant. Indeed, adoptively transferred spleen cells were able to specifically inhibit the development of autoimmunity in vivo, indicating that in this model tolerance was achieved by active suppression (24). Since peripheral tolerance of Th2 cells was found to be crucial in the prevention of immunopathological disorders such as SLE and allergy, we used this neonatal tolerance model to further characterize the cellular population endowed with this protective capacity. Most of the current literature on regulatory T cells has focused on CD4+ T cells (25, 26), while few studies have evaluated the

1 This work was supported by funds from the Institut National de la Santé et de la Recherche Médicale and the Association pour la Recherche sur le Cancer. A.-C.F. is the recipient of a doctoral fellowship from the Association pour la Recherche sur le Cancer, France. B.B. is supported by Centre National de la Recherche Scientifique.

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3 Abbreviations used in this paper: SLE, systemic lupus erythematosus; BN, Brown-Norway; AU, arbitrary units.

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importance of CD8⁺ regulatory cells and their potential to suppress Th function (27, 28).

In the present study we demonstrate the presence of regulatory CD8⁺ T cells in neonatally tolerized BN rats. Indeed, in vivo depletion of CD8⁺ cells broke tolerance to autoimmune humoral responses in animals under a neonatal tolerance protocol, and adoptive transfer of these CD8⁺ T cells into naive syngeneic rats transferred tolerance to the mercury disease. Assessment of the functional phenotype of CD8⁺ T cells, CD8⁺ CD45RCαb (29), and the presence of IFN-γ-synthesizing CD8⁺ T cells suggest that Tc1 regulatory cells are generated in the course of neonatal tolerance and suppress Th2 responses. These results demonstrate that in vivo neonatal priming with mercury protects against Th2-mediated SLE-like immune responses induced by this chemical via generation of a CD8⁺ T cell regulatory response.

Materials and Methods

Animal model

Brown-Norway (BN) rats from CERJ (Genest St. Isle, France) were bred in our own animal facilities. Animals were weaned at 3 wk of age and were cared for and handled according to the guidelines of the institutional animal care committee. Neonates and 2- to 6-mo-old male rats were used in the following experiments.

Neonatal tolerance protocol

To induce neonatal tolerance to mercury disease, 24-h-old BN rats received s.c. injections of HgCl₂ (Prolabo, Briare, France) three times a week for 2 wk at a dose of 100 μg/100 g body weight as previously described (24). Control rats did not receive any neonatal injection.

Induction of mercury disease

BN rats (8–16 wk of age), either under a neonatal tolerance protocol or not, were s.c. injected on day 0 with HgCl₂ and then three times a week at a dose of 50 μg/100 g body weight for a period of 4 wk.

Depletion of CD8⁺ cells

In vivo depletion of CD8⁺ cells was achieved by an i.p. injection of 0.5 mg of OX8 mAb (mouse mAb anti-rat CD8α) on days −3, −1, 7, 14, 21, and 28. Control rats were i.p. injected with the same dose of OX21 mAb (mouse mAb anti-human C3b inactivator) as an isotype-matched control in the same schedule. The efficiency of CD8 depletion was determined on days 0 and 28 by flow cytometry (FACScan; BD Biosciences, Pont de Claix, France).

Isolation of CD8⁺ and CD4⁺ T cells

Ex vivo purification of CD8⁺ spleen T cells was achieved with rat CD8 affinity immunocolumns (Cytovax Biotech; Tebu, Le Perray en Yvelines, France) by negative selection. Briefly, the columns were incubated with polyclonal goat anti-rat IgG and goat anti-mouse IgG Abs. Meanwhile suspensions of spleen cells were incubated on ice with W3/25 mAb (mouse mAb anti-rat CD4). The columns were loaded with these cell suspensions, and the CD8⁺ T cells were eluted. The purity of the cells was assessed by flow cytometry. Ex vivo purification of CD4⁺ T cells was achieved by negative selection from spleen cells using magnetic beads (Dynal, Compiègne, France). Splenocytes were incubated with mouse mAbs anti-rat κ and λ light chains (MARK-1 and MARL-15; Lo-Imex, Brussels, Belgium). After washing and incubation under agitation with goat anti-mouse IgG-coupled magnetic beads, T cells were isolated by magnetic depletion. For further purification of CD4⁺ T cells, a similar technique was applied, with the addition of OX8 mAb. The collected cells that were not retained by magnetic particle concentrator were purified CD4⁺ T cells.

Cell transfer

Donor rats were 2- to 4-mo-old tolerant BN rats, and recipients were slightly 113Cs gamma-irradiated (200 rad) naive BN rats. 8–12 wk of age. Recipient rats were i.v. injected with either 40 × 10⁶ CD4⁺ or 10 × 10⁶ CD8⁺-purified splenocytes or i.v. coincubated with 50 × 10⁶ splenocytes from tolerant donor rats mixed with 50 × 10⁶ splenocytes from naive BN rats. Recipient rats reconstituted with 100 × 10⁶ splenocytes from naive BN rats served as diseased controls. Twenty-four hours after irradiation and adoptive transfer, recipient rats were exposed to 50 μg/100 g body weight of HgCl₂ three times a week as previously described (24).

Serum IgE concentration

For measurement of the total IgE concentration in sera, a standard sandwich ELISA was applied. Microtiter plates coated with rat IgE (Lo-Imex) overnight at 4°C. Rat serum duplicate samples were incubated at 1/100 to 1/5000 dilutions, and bound IgE was labeled with MARK1-HRP (Lo-Imex). HRP activity was revealed by adding 3,3’,5,5’-tetramethylbenzidine (Sigma-Aldrich, St. Quentin Fallavier, France); the reaction was stopped with 2 N H₂SO₄, and absorbance was determined at 450 nm. Results were expressed as micrograms per milliliter with reference to a standard pool of HgCl₂-injected BN rat sera containing known amounts of rat IgE.

Serum autoantibody determination

Microtiter plates coated overnight with laminin (5 μg/ml) or DNA (10 μg/ml; Sigma-Aldrich) were saturated. Rat serum duplicate samples were incubated at 1/100 dilution, and bound Ig anti-laminin or anti-DNA was labeled with MARK-1-HRP. Bound HRP activity was revealed as described above. Results were expressed as arbitrary units (AU) representing the percentage of maximum binding activity of a standard pool of HgCl₂-injected BN rat sera.

Detection of cell surface Ags

One million spleen cells were first stained with biotin- or PE-conjugated mAbs for 30 min at 4°C. Cells were then stained with FITC-conjugated mAbs, and biotinylated Abs were revealed, adding streptavidin-PE (BD Biosciences), for 30 min at 4°C. After washing, cells were fixed in PBS containing 1% formaldehyde. Fluorescence was detected using a FACSscan and was analyzed using CellQuest software (BD Biosciences). Cells were counted in a tight electronic gate (R1) set on the lymphocyte cluster on the forward and side scatter plot. The R1-gated cells were analyzed for CD8 or CD4 expression vs CD134 or MHC II. Murine isotypic controls were used as a control for nonspecific binding.

Detection of intracellular cytokines

Measurement of cytokine production was performed by combined surface and intracellular staining with mAbs and subsequent three-color flow cytometric analysis. For detection of cytokine responses, spleen cells were stimulated with PMA (50 ng/ml; Sigma-Aldrich) and ionomycin (1 μg/ml; Calbiochem, San Diego, CA) for 6 h at 37°C in 5% CO₂. Cytokine secretion was inhibited by treatment with 10 μg/ml brefeldin A during the last 2 h of incubation. Stimulated cells were harvested, washed, and stained using FITC-conjugated mouse mAb anti-CD3 (1F4) and PerCP-conjugated anti-CD8 (OX8) or CyChrome-conjugated anti-CD4 (OX35; all from BD Pharmingen, San Diego, CA). Double-labeled cells were fixed and then permeabilized by washing twice in saponin medium (Sigma-Aldrich). Intracellular staining was performed by incubating cells with PE-conjugated anti-rat cytotoxic mAb; anti-IL-2 (BioSource), anti-IFN-γ, and anti-TNF-α (both from BD Pharmingen). Cells were washed twice in saponin medium and were resuspended in PBS for flow cytometry acquisition. Cells were counted in a tight electronic gate set on the lymphocyte cluster on the forward and side scatter plot. For analysis, a tight light scatter region (R1) was drawn around the major lymphocyte population. CD3-positive lymphocytes were identified on a R1 bivariate dot plot where side scatter vs green fluorescence was displayed. An R2 gate was set around the CD3-positive cells. The R2-gated cells were analyzed for CD8 or CD4 expression vs cytokines. Isotype-matched, irrelevant Abs served as controls for intracellular and cell surface staining.

Statistical analysis

Data were analyzed using StatView 4.1.0 software (Abacus Concepts, Berkeley, CA); statistical comparisons between the different groups of rats were conducted by ANOVA with one-way ANOVA and Fischer test as post-hoc procedures. Differences were considered statistically significant at the 5% level of confidence.

Results

Effects of neonatal tolerance protocol on Th2 responses

We first assessed the efficiency of the neonatal tolerance protocol. Naive BN rats exposed to mercury developed typical Th2-mediated autoimmune manifestations, including a dramatic increase in serum IgE (550-fold increase) associated with the production of
autoantibodies to laminin (12-fold increase) and to DNA (5-fold increase; Fig. 1). These potential pathogenic responses were followed by a suppressive response that has been shown to involve inhibitory Th1 cells (15, 16). In contrast, in animals neonatally exposed to HgCl2 and rechallenged as adults, these immune dysregulations were either completely abrogated or profoundly attenuated (Fig. 1). Thus, as expected from our previous study (24), chronic neonatal administration of mercuric salts conferred tolerance to Th2-mediated autoimmune responses.

**Effects of in vivo treatment with anti-CD8 mAb on tolerant animals**

Chronic exposure to HgCl2 not only induces immune disorders that spontaneously resolve, but also renders the animals resistant to rechallenge with mercury, with an involvement of CD8+ T cells in this phenomenon (30). To determine the effect of CD8+ cells on tolerant humoral responses, BN rats were treated with anti-CD8 mAb and administered HgCl2. The group of animals that received an irrelevant isotype control (OX21, IgG1 mAb) showed no significant difference from nondepleted rats (data not shown). We confirmed by flow cytometric analysis that spleen and lymph nodes of anti-CD8 mAb-treated animals were almost completely depleted of CD8+ cells at all time points analyzed (data not shown). The total serum IgE concentration in the sera was significantly higher in tolerant rats following anti-CD8 mAb treatment compared with nondepleted tolerant animals (Fig. 2). To a lesser extent, total anti-laminin and anti-DNA Abs were increased in CD8+ -depleted tolerant rats (Fig. 2). Administration of anti-CD8 mAb triggered the development of mercury disease, as illustrated by these three hallmarks of the disease. The highest response of IgE was seen in diseased rats, confirming the induction of mercury disease. Removal of CD8+ cells from naive rats resulted in a mild decrease in the total serum IgE concentration during HgCl2 exposure, but did not affect serum autoantibody levels (Fig. 2). Thus, in vivo CD8 depletion enhanced Ab and autoantibody production in tolerant animals during mercury recall, indicating that neonatal tolerance is broken, and that CD8+ cells, most likely through a suppressive activity, are responsible for the induction of tolerance in this model.

**Transfer of CD8+ regulatory T cells**

We have previously shown that in vivo transfer of spleen cells from tolerant animals exposed to mercury during the neonatal stage protected syngeneic naive rats against mercury disease, thus emphasizing an active suppressive mechanism (24). To assess the presence of regulatory T cells in the spleen of tolerant animals, we cotransferred splenocytes from naive and tolerant animals to naive syngeneic irradiated recipients the day before the initiation of chronic administration of mercuric salts. The production of IgE (Fig. 3A) and autoantibodies (Fig. 3, B and C) in animals receiving...
FIGURE 3. Transfer of tolerance by CD8\(^+\) regulatory T cells. Levels of total serum Igs were monitored from day 0 until day 28 by specific ELISA. On day 0, spleen cells from naive and/or tolerant rats were harvested, pooled, or purified as indicated in Materials and Methods. Purified subsets or total splenocytes were i.v. injected into naive syngeneic animals. Naive rats reconstituted with naive spleen cells ( □ ) represent positive controls for mercury disease. Tolerant rats were rechallenged with HgCl\(_2\) ( □ ) for control of tolerance. From day 1 post-transfer, tolerant and recipient animals were administered with mercuric salts (three times a week). At each time point of kinetics, the data obtained were averaged from 6–15 rats. The maximal value reached by the averaged data defined the peak value. Peak values are expressed as the mean ± SEM for 6–15 rats/experimental group from five separate experiments. *, p < 0.05; **, p < 0.001; ***, p < 0.0001.

To examine the splenic T cell responses in rats in a neonatal tolerance protocol and further characterize the regulatory CD8\(^+\)/H11001 T cells present in the spleen of neonatally treated animals and mediate active suppression.

A mixture of splenic cells from naive and tolerant rats was significantly decreased compared with that in naive animals reconstituted with naive splenocytes that developed mercury disease. In these cotransfected animals, neither maximal serum IgE concentration nor maximal circulating autoantibody titers were significantly different compared with those in tolerant BN rats (Fig. 3, A–C). These data suggest that regulatory cells are present in the spleen of neonatally treated animals and mediate active suppression.

To demonstrate the involvement of a particular subpopulation of T cells in this regulatory process, we transfected purified splenic CD8\(^+\) or CD4\(^+\) T cells from tolerant animals into naive syngeneic irradiated recipients the day before the initiation of chronic administration of mercuric salts. As shown in Fig. 3, prevention of mercury disease was achieved with a single injection of CD8\(^+\) T cells from tolerant animals. In CD8-transfused animals, the maximal concentration of serum IgE was significantly decreased (Fig. 3A), and peak levels of anti-laminin and anti-DNA Abs were significantly decreased compared with those in tolerant BN rats (Fig. 3, B–C). All these data suggested that regulatory cells are present in the spleen of neonatally treated animals and mediate active suppression.

Phenotypic analysis of CD8\(^+\) regulatory T cells

To examine the splenic T cell responses in rats in a neonatal tolerance protocol and further characterize the regulatory CD8\(^+\) T cells, we performed a kinetics analysis of total T lymphocyte subsets and activated cells before and upon rechallenge with chemical HgCl\(_2\). As described above, naive BN rats developed typical mercury disease during exposure to mercury, whereas tolerant BN rats did not (Fig. 1). Before the administration of mercury, lymphocyte subsets from naive and tolerant animals differed in their distribution. Indeed, CD8\(^+\) and CD4\(^+\) subsets increased by 54 and 23%, respectively, in the spleen of tolerant animals compared with naive rats (Fig. 4A and Table I). Activation-associated cell surface Ags, such as IL-2R (CD25) and the MHC class II (RT1.B) Ag, are present on activated rat T lymphocytes. In tolerant animals both CD8\(^+\) and CD4\(^+\) subsets exhibited a similar activated pattern consisting of significant increases in CD25 and MHC II expression, as illustrated in Fig. 4C and Table I. Thus, the tolerance protocol modifies the representation of splenic lymphocyte subsets and their expression of activation markers.

After administration of mercury, we assessed the functional phenotype of CD8\(^+\) regulatory lymphocytes. Cells were stained with mAbs against CD8 and CD45RC to determine the percentage of the Tc1 subset. The expression of the high m.w. isoform CD45RC defines type 1 subpopulations of CD8\(^+\) or CD4\(^+\) T cells. The fraction of potentially regulatory CD8\(^+\) cells with a Tc1 phenotype and, in parallel, the CD8\(^+\)CD25\(^+\) subset rapidly and significantly increased after the first three injections of mercuric salts (day 7; Fig. 4, B and C). Indeed, there was a strong positive correlation between the CD8\(^+\) CD45RC\(^{hi}\) and CD8\(^+\)CD25\(^+\) subsets in animals during the neonatal protocol (data not shown). In contrast, the percentage of CD8\(^+\) T cells highly expressing CD45RC obtained from diseased animals moderately decreased until day 21, with an up-regulation on day 28 at the time of spontaneous regulation of mercury disease (Fig. 4B). No difference was observed in the percentage of CD8\(^+\)CD25\(^+\) double-positive cells from diseased animals over time (Fig. 4C). For appropriate effector function, T and B lymphocytes use costimulatory signals generated via accessory molecules such as CD134, a member of the TNF receptor family involved in T cell-dependent Ab production. Rechallenge with HgCl\(_2\) markedly decreased the percentage of CD134\(^+\) cells within the CD8\(^+\) T cell population from rats in the neonatal tolerance protocol. The percentages of CD8\(^+\)CD134\(^+\) as well as CD8\(^+\) MHC II\(^+\) double-positive cells from tolerant and diseased rats were significantly different, as illustrated in Fig. 4C. All these data...
suggest that CD8+ regulatory T cells transferring neonatal tolerance are expanding and acquire a Tc1 phenotype associated with CD25 expression upon recall with mercury.

Phenotypic analysis of CD4+ T cells from animals in a neonatal tolerance protocol indicated that neither CD4+ T cells expressing CD25 nor CD4+ cells expressing a Th1 phenotype were expanded upon rechallenge with HgCl₂. Although the percentage of CD4+ T cells expressing CD134 was slightly increased, the percentage of activated CD4+ MHC II+ double-positive T cells was significantly diminished upon mercuric salts recall. At variance, in diseased rats CD4+CD25+ T cells expanded, but CD4+ T cells expressing a Th1 phenotype did not, during the course of HgCl₂ administration. The percentage of activated CD4+ T cells from diseased animals evidenced by the expression of either CD134 or MHC class II Ag was significantly increased upon priming with mercury (Table I). Thus, animals that received mercuric salts as neonates mounted a characteristic Tc1 response as adults, and not a Th1 response, typified by a CD8+ CD45RC-high phenotype.

Intracellular analysis of type 1 cytokine synthesis by CD8+ regulatory T cells
One of the possible mechanisms by which CD8+ regulatory T cells may control tolerance to mercury disease is through the production of inhibitory cytokines. The intracellular contents of IFN-γ, IL-2, and TNF-α were analyzed by flow cytometry in splenocytes obtained from tolerant and diseased animals at different time points.

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** Regulatory CD8+ T cells display a type 1 phenotype. The CD8+ population, the CD8+ Tc1 subset, and activated CD8+ spleen cells were phenotyped. All animals, either under the neonatal tolerance protocol or not, were challenged with HgCl₂ starting from day 1 until day 28 (three times a week). Cells were stained with FITC-conjugated anti-CD8 mAb (A) and with PE-conjugated anti-CD45RC (B) or anti-CD25, anti-CD134, or anti-MHC II (C) mAbs. Three rats were used per experimental group and per day. Data are expressed as the mean percentage of positive cells ± SEM. *, p < 0.05; **, p < 0.001; ***, p < 0.0001 (comparing tolerant and naive/diseased animals). †, p < 0.05 (comparing prechallenge and challenge conditions).

### Table I. Analysis of phenotypic changes in CD4+ T cells

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Prior to HgCl₂</th>
<th>Upon HgCl₂</th>
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<tr>
<td></td>
<td>Tolerant (%)</td>
<td>Naive (%)</td>
</tr>
<tr>
<td>CD4*</td>
<td>49.7 ± 1.9</td>
<td>40.4 ± 1.5</td>
</tr>
<tr>
<td>CD4*CD45RC-high</td>
<td>25.3 ± 1.1</td>
<td>22.4 ± 1.9</td>
</tr>
<tr>
<td>CD4*CD25+</td>
<td>47.9 ± 2.7</td>
<td>25.3 ± 2.2</td>
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<tr>
<td>CD4<em>CD134</em></td>
<td>10.2 ± 1.6</td>
<td>7.4 ± 0.5</td>
</tr>
<tr>
<td>CD4*MHC II+</td>
<td>40.5 ± 0.8</td>
<td>26.6 ± 2.1</td>
</tr>
</tbody>
</table>

*The CD4+ population, CD4+ Th1 subset, and activated CD4+ spleen cells were phenotyped. Results are the percentage of cells and are presented as the mean ± SEM from three rats per day and per experimental group. The p values comparing phenotypes in tolerant and naive/diseased animals are given.

*Peak values. At each time point of kinetics, the data obtained were averaged from three rats. The maximal value reached by the averaged data defined the peak value.

† p < 0.001, prechallenge vs challenge.

* p < 0.0001, prechallenge vs challenge.

†† p < 0.05, prechallenge vs challenge.
of HgCl₂ administration. Bivariant dot plots of lymphocytes derived from the spleen after short ex vivo stimulation and triple staining are illustrated in Fig. 5A. In pre- and challenge conditions we found that IFN-γ producers were significantly more represented in CD3⁺CD8⁺ subsets from tolerant animals compared with those in naive and diseased rats (Fig. 5). Importantly, the percentage of double-positive CD8⁺IFN-γ⁺ T cells in animals in the neonatal tolerance protocol was stable during sensitization with mercuric salts. CD8⁺ T lymphocytes derived from the spleen compartment of tolerant and diseased animals differ in their ability to synthesize IFN-γ (Fig. 5B). Examination of the IL-2 response of CD8⁺ T cells showed that before HgCl₂ there was an increased percentage of these double-positive cells in tolerant compared with naive rats. Upon recall with HgCl₂, significantly higher percentages of CD8⁺IL-2⁺ cells were observed in tolerant rats regardless of the day-point of the kinetics. No significant differences between tolerant and diseased animals were observed in CD8⁺TNF-α⁺ double-positive cells at any time (Fig. 5B). Overall, these results indicate that neonatal priming of BN rats with HgCl₂ led to a preferential expansion of CD8⁺ T cells synthesizing type 1 cytokines.

The production of type 1 cytokines by the CD3⁺CD4⁺ cell subset in tolerant and diseased rats under pre- and challenge conditions is summarized in Table II. IFN-γ, IL-2, and TNF-α were

Table II. Analysis of intracellular synthesis of type 1 cytokines in CD4⁺ T cells

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Prior to HgCl₂ (%)</th>
<th>Upon HgCl₂ (%)</th>
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<tr>
<td>CD3⁺IFN-γ⁺</td>
<td>25.8 ± 6.9</td>
<td>18.1 ± 0.2</td>
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<td>CD3⁺IL-2⁺</td>
<td>42.1 ± 0.7</td>
<td>29.0 ± 1.4</td>
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<tr>
<td>CD3⁺TNF-α⁺</td>
<td>10.1 ± 1.2</td>
<td>4.0 ± 1.1</td>
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</table>

The synthesis of IL-2, IFN-γ, and TNF-α was analyzed by calculating the percentage of cytokine-positive cells within the CD3⁺CD4⁺ T cells from spleens of tolerant and naive/diseased animals. Results are percentage of cytokine-producing cells and are presented as the mean ± SEM from three rats per day and per experimental group. The p values comparing cytokine-positive cells in tolerant and naive/diseased animals are given.

Prior to HgCl₂

<table>
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<tr>
<th>Cytokine</th>
<th>Tolerant (%)</th>
<th>Naive (%)</th>
<th>p value</th>
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<tr>
<td>CD3⁺IFN-γ⁺</td>
<td>25.8 ± 6.9</td>
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<td>CD3⁺TNF-α⁺</td>
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<td>4.1 ± 0.2</td>
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Upon HgCl₂

<table>
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<th>Cytokine</th>
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<th>Diseased (%)</th>
<th>p value</th>
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<tr>
<td>CD3⁺IFN-γ⁺</td>
<td>18.1 ± 0.2</td>
<td>11.7 ± 0.9</td>
<td>NS</td>
</tr>
<tr>
<td>CD3⁺IL-2⁺</td>
<td>29.0 ± 1.4</td>
<td>24.0 ± 1.4</td>
<td>NS</td>
</tr>
<tr>
<td>CD3⁺TNF-α⁺</td>
<td>4.0 ± 1.1</td>
<td>2.2 ± 0.2</td>
<td>0.05</td>
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</table>
detected after short ex vivo stimulation in animals in the neonatal tolerance protocol before HgCl2 recall. At this stage, naive rats showed significantly different profiles with 5-, 2-, and 2.5-fold decreased percentages of double-positive CD4+IFN-γ−, CD4+IL-2+, and CD4+TNF-α+ T cells, respectively, compared with tolerant animals. In contrast, rechallenging rats under neonatal tolerance protocol with mercuric salts decreased the percentage of IFN-γ− and of IL-2-expressing CD4+ T cells as well as the percentage of CD4+TNF-α+ double-positive cells. Interestingly, on day 14 the percentage of CD4+ T cells producing type 1 cytokines was not significantly different in tolerant rats compared with animals that developed Th2 mercury disease (Table II). Thus, animals that received mercuric salts as neonates manifested a characteristic Tc1 response, and not a Th1 response, against mercury recall, typified by IFN-γ synthesis.

Discussion

Exposure of newborn animals to a foreign Ag may result in immunological tolerance to that specific Ag. In recent years it has become clear that neonatal exposure to Ag can prime immunity, rather than inactivate or delete responses (31), and sustain regulatory functions effective against autoreactive T cells (32, 33). Depending on the dose and timing of the antigenic challenge, tolerance has been reported to result in clonal deletion (19), anergy (18), or active suppression (20). Several studies have provided evidence that regulatory mechanisms play a dominant role in neonatal tolerance (34, 35). Most of the autoimmune diseases suppressed by an adequate tolerance protocol are mediated by pathogenic Th1 cells. To the best of our knowledge, tolerance to a Th2-mediated autoimmune disease with neonatal sensitization with a chemical has not been documented by any other group.

In the experiments described here we have examined whether the generation of regulatory cells plays a role in neonatal tolerance to a model of Th2-mediated autoimmunity. We describe CD8+ T cells that exhibit regulatory function and are produced under the neonatal tolerance protocol to Th2-mediated autoimmunity disease induced by chronic exposure to mercuric salts. In vivo depletion of these CD8+ regulatory T cells enhances Abs production and breaks neonatal tolerance upon mercury recall. In addition, adoptive transfer of splenic CD8+ regulatory T cells from animals in the neonatal tolerance protocol protects naive syngeneic recipients against mercury disease. The high percentage of CD8+ T cells synthesizing IFN-γ, with expansion of cells of the CD8+CD45RChigh phenotype, strongly suggests that regulatory CD8+ T cells are Tc1 cells.

Exposure of newborn BN rats to mercuric salts results in a specific tolerance to the chemical otherwise responsible for the development of a Th2 autoimmune disease. BN rats neonatally challenged with mercuric salts do not develop mercury disease when neonates or upon mercury recall later in life as adults. This neonatal mercury-specific tolerance is dominant, since adoptively transferred spleen cells are able to inhibit the development of mercury-induced autoimmunity (24). In vivo depletion of CD8+ T cells was designed to explore the role of these cells in neonatal tolerance mediated by active suppression. Although depletion in CD8+ T cells by anti-CD8 mAb treatment broke neonatally induced tolerance, Th2 response for IgE production was not completely restored. This might be due to incomplete depletion of CD8+ cells by in vivo administration of anti-CD8 mAb. Furthermore, in addition to CD8+ T cells, CD8− NK cells might be depleted by anti-CD8 mAb treatment, and their possible involvement in regulating the Th2 response cannot be excluded. Indeed, along with CD8+ T cells, CD8− NK cells produce IFN-γ and can alter CD4+ Th2 cell responses. On the other hand, recent studies have shown that removal of CD8+ T cells before immunization may inhibit the IgE response, suggesting that these cells may help to initiate the IgE response in addition to suppressing ongoing IgE production (36).

The presence of regulatory cells can be demonstrated by in vivo cell transfer (37, 38). Cotransfer of spleen cells from naive and tolerant animals into syngeneic naive rats was efficient, as shown by impaired mercury-induced autoimmune responses. Given that regulatory T cells exist in the spleen of animals neonatally exposed to mercury, the distinction between CD4− and CD8−-mediated effects appears critical. Our data obtained from subsequent transfer experiments demonstrate that CD8−, but not CD4−, T cells transfer tolerance and mediate protection against mercury disease and therefore indicate that CD8− regulatory T cells are generated under this neonatal tolerance protocol. Although most of the current literature on regulatory T cells has focused on CD4+ T cells (CD4+CD25+ Treg, Th3, and Tr1), most of the autoimmune diseases suppressed by regulatory CD4+ T cells are mediated by pathogenic Th1 cells. Nevertheless, the role of CD4+CD25+ T cells in a Th2 cell-mediated model of allergic inflammation was recently defined. CD4+CD25+ T cells were found to modulate the Th1/Th2 balance toward Th2 cells and thus to up-regulate Th2-mediated allergic inflammation (39).

The high m.w. isoform CD45RC defines the type 1 subset of CD8+ or CD4+ T cells (29). Upon recall with mercury of tolerant animals, a significant up-regulation of CD8+CD45RCloph− T cells caused this population to represent 60% of the CD8+ subset. This finding in the context of the tolerizing protocol suggests that the regulatory cells are CD8− Tc1 cells. IL-2Rα-chain (CD25) expression to date is one of the most used markers for identification of regulatory CD4+ T cells (26, 40), although CD25 is an imperfect marker that is expressed on every activated T cell (41). Interestingly, CD25 can also be expressed on CD8+ T cells. In our study the fraction of CD8+ T cells expressing CD25 rapidly increased after the first three injections of mercuric salts recall, suggesting that CD8− regulatory cells might be CD8−CD25+ phenotyped. Moreover, the strong positive correlation between the CD8−CD45RCloph and CD8−CD25+ subsets obtained from animals in the neonatal tolerance protocol is in favor of CD8+ regulatory cells expressing both Tc1 and CD25 phenotypes. This subset of T cells that may exert regulatory effects represents ~5% of the total splenic cells. Animals in the neonatal tolerance protocol showed a decrease in CD8−CD134+ T cells and no significant change in CD4+CD134+ T cells, clearly in contrast with the observed increase in the percentage of CD134+ T cells in diseased rats. The TNF receptor family member CD134 is a cell surface receptor mainly involved in T cell-dependent Ab production. A strong increase in the expression of CD134 on CD4+ T cells from BN rats exhibiting mercury disease has also been described (42). Recent studies have reported enhanced expression of CD134 on human CD8+ and CD4+ T cells in SLE (43) and in chronic graft-vs-host disease (44). The expression of CD134 may directly contribute to activation of type 2 cells. As observed for CD134 Ag, an important decrease in MHC II molecule expression was noticed on CD8+ and CD4+ T cells of tolerant rats upon recall with mercuric salts, in contrast to the increase observed in diseased animals. MHC class II Ags directly participate in mercury-induced cell activation, and overexpression of MHC II molecules has been considered an important issue in the occurrence of autoimmunity (45). Thus, the regulatory CD8+ Tc1-like cell population expressing CD25 may inhibit the proliferation of activated CD134+ and MHC class II+ T cells.

The mechanism by which regulatory CD8+ T cells may control tolerance is still unclear, although regulatory activity may be mediated by soluble factors (46). For instance, CD8+ T cells are
capable of producing large amounts of IFN-γ, which, in turn, can counter-regulate Th2 responses (47). In agreement with the putative Tc1 phenotype of the regulatory cells, we detected intracellular IFN-γ synthesis in 65% of splenic CD8+ T cells in rats injected as neonates with mercuric salts and rechallenged as adults. This finding suggests that Th2 to Th1 immune deviation is involved in this phenomenon of neonatal tolerance. However, Th1 cells were not expanded in tolerant animals upon mercury recall. Moreover, the Th1 subset, while producing IFN-γ, did not transfer tolerance into naive syngeneic recipients as CD8+ cells did. In another set of experiments, assessment of the production of IL-10 or TGF-β by spleen cells indicated that rats in the neonatal toler-ance protocol did not produce significant amounts of either of these regulatory cytokines (data not shown). Moreover, we have studied the effects of in vivo treatment with anti-TGF-β mAb on tolerant animals. Our findings showed that TGF-β is not involved in this experimental setting (data not shown). Together these data favor our conclusion that immune deviation is unlikely to be a mechanism involved in the neonatal tolerance controlled by regulatory CD8+ T cells.

The involvement of CD8+CD25+ Tc1-like regulatory cells in protection against Th2-mediated SLE-like responses is in agreement with the suppressive effects conferred to CD8+ T cells. Generation of Tc1 regulatory cells may have important clinical implications for the development of more effective therapies for autoimmunity, transplantation-associated disease, and allergic manifestations.

References