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*J Immunol* 2002; 168:3747-3754; doi: 10.4049/jimmunol.168.8.3747
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CD154 is transiently expressed by activated T cells and interacts with CD40 on B cells, dendritic cells, macrophages, and monocytes. This costimulatory receptor-ligand couple seems decisive in Ag-driven immune responses but may be differentially involved in type 1 vs type 2 responses. We studied the importance of CD40-CD154 in both responses using the reporter Ag popliteal lymph node assay in which selectively acting drugs generate clearly polarized type 1 (streptozotocin) or type 2 (D-penicillamine, diphenylhydantoin) responses to a constant coinjected Ag in the same mouse strain. Treatment of mice with anti-CD154 reduced characteristic immunological parameters in type 2 responses (B and CD4^+ T cell proliferation, IgG1 and IgE Abs, and IL-4 secretion) and only slightly affected the type 1 response (small decrease in IFN-γ production, influx of CD11c^+ and F4/80^+ cells, and prevention of architectural disruption of the lymph node, but no effect on IgG2a Ab and TNF-α secretion or B and CD4^+ T cell proliferation). The findings indicate that the CD40-CD154 costimulatory interaction is a prerequisite in drug-induced type 2 responses and is only marginally involved in type 1 responses. The observed expression patterns of CD80 and CD86 on different APC (B cells in type 2 and dendritic cells in type 1) may be responsible for this discrepancy. The Journal of Immunology, 2002, 168: 3747–3754.

The interaction between the costimulatory molecules CD40 and CD154 appears to be of pivotal importance to elicit and further Ag-driven immune responses. In transplant models anti-CD154 treatment prolongs allograft survival (1–3), and in models mimicking autoimmune disease, such as systemic lupus nephritis (4, 5), collagen-induced arthritis (6), autoimmune diabetes, insulinitis (7), colitis (8–10), and experimental autoimmune encephalomyelitis (11, 12), anti-CD154 treatment reduced autoantibody production and development of disease. Based on these results, blocking of the CD40-CD154 pathway is considered a promising opportunity to modulate autoimmune disease and adverse (drug-induced) autoimmune-like reactions in man. Studies using CD154-deficient mice or various autoimmune, infection, and transplant models have shown that CD40-mediated costimulation is involved in both type 1 and type 2 responses. However, results concerning the particular requirement for CD40-mediated co-stimulation in both responses are contradictory (7, 13–24).

In conjunction with IL-4, cross-linking of CD40 is able to stimulate B cell survival, proliferation, and maturation into germinal center (GC)^3 cells (13–16). Moreover, in CD154-deficient mice, IgG1 Ab responses (characteristic for type 2 responses in mice) to viruses were severely decreased and no memory B cells were found, whereas IgG2a, IgG2b, and IgG3 Ab-producing cells (representing type 1 responses) were still present (17). This indicates that without CD154 triggering, type 2 mechanisms leading to Th2-type Ab production are disabled while type 1 responses are still operational. Additionally, Kishimoto et al. (18) concluded that CD154 ligation was decisive in an allogeneic Th2 response but had a minor impact on regulation of the allogeneic Th1 response. Finally, CD4^+ T cell priming was not impaired in the absence of CD154 ligation in the Th1-mediated autoimmune encephalitis system (19).

In contrast, only the development into Th1, and not Th2, cells was impaired after in vitro stimulation to OVA peptide when using OVA-specific T cells from CD154-deficient mice (20). Blocking of CD154 in NOD/shi mice also impaired the development of islet-reactive Th1 cells (7). Moreover, the CD40-CD154 interaction was demonstrated to be crucial for in vivo priming of Th1 cells by IL-12-producing APC in SJL/J mice (21), and supplementary expression of CD154 directs the response to respiratory syncytial virus toward a type 1 phenotype (22). At last, anti-CD154 Ab was found to induce tolerance to 2,4-dinitrofluorobenzene-induced contact hypersensitivity, a typical type 1 phenomenon (23), but CD154-deficient mice showed normal delayed-type hypersensitivity responses to OVA (24).

These contradictory data may be the consequence of comparing studies with different experimental set-ups: transplantation models, spontaneous or induced autoimmune diseases, or the use of transgenic mice, in which responses are all under extensive control by diverse regulatory mechanisms, which complicates the interpretation of the importance of CD40-CD154 in the sensitization phase in type 1 vs type 2 responses. Therefore, we intended to study the involvement of CD154 in type 1 vs type 2 immune responses in a relatively simple model using a predefined Ag in a wild-type strain of mice, the reporter Ag popliteal lymph node assay (RA-PLNA).
The PLNA, originally developed to investigate immunomodulating properties of autoimmunogenic chemicals, was extended by the use of a reporter Ag (trinitrophenyl (TNP)-OVA) to allow detection of dichotomous immune responses elicited by chemicals. Previously, we have shown that the response to TNP-OVA parallels the type of response expected to occur in the case of the xenobiotic itself (25). Notably, the response to TNP-OVA in the presence of the lupus-inducing chemicals HgCl₂ and diphenylhydantoin (DPh; anti-epileptic drug) is type 2 (IgG1 Ab-secreting cells (ASC), GC formation, influx of IL-4-producing CD4⁺ cells) (26), whereas the response to the same Ag elicited by the anti-neoplastic diabetogenic chemical streptozotocin (STZ) is type 1 (IgG2a ASC, influx of IFN-γ-producing CD8⁺ cells and macrophages) (27).

In the present study the RA-PLNA was used to investigate the role of CD154 in both type 1 and type 2 responses induced by STZ and DPH or D-penicillamine (D-Pen; anti-rheumatic drug), respectively, by simultaneously treating drug-exposed mice with a CD154 antagonist. Our findings clearly show that type 1 and type 2 immune responses are differently regulated by CD40-CD154 interactions and support the hypothesis that the dichotomy in immune responses is at least partially dependent on different responsiveness to costimulatory molecules provided by different APC.

Materials and Methods

Mice

Specific pathogen-free female BALB/c mice (6–12 wk old) were obtained from the Utrecht University breeding facility (Gemeenschappelijk Dier Laboratorium, Utrecht, The Netherlands) and randomly assigned to specific treatment. Mice were allowed to settle for 1 wk and were maintained under barrier conditions in filter-topped Macrolon cages with wood chips bedding, at a mean temperature of 23 ± 2°C, 50–55% relative humidity, and a 12-h light/dark cycle. Drinking water and standard laboratory food pellets were provided ad libitum. The experiments were conducted according to the guidelines of the animal experiments committee of the Veterinary Faculty of Utrecht University.

Chemicals and reagents

Chemicals were obtained from Sigma-Aldrich ( Zwijndrecht, The Netherlands) unless stated otherwise. Saline (0.9%; B. Braun Melsungen, Melsumg, Germany); citrate buffer (0.1 M citric acid and 0.1 M Na₂HPO₄, pH 6) were used to dilute test chemicals. TNP-OVA and blocking agent TNP-BSA were prepared as previously described by Albers et al. (26). Imidol-P membranes (Imobilon PVDF Transfer; Millipore, Etten-Leur, The Netherlands) were coated overnight with PBS/0.05% Tween/TNP-BSA (10 µg/ml) and blocked for 1 h with PBS/Tween/1% BSA. These membranes were clamped in spot blocks (made in-house), and 5 × 10⁶ cells were centrifuged on the membranes and incubated for 4 h at 37°C. Membranes were removed from the spot blocks, washed with PBS and PBS/Tween, and incubated overnight at 4°C with AP-conjugated Abs in PBS/Tween/casein (1:500). Membranes were washed and para-nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate toluidine salt reagent to accomplish color development of TNP-specific Ab spots. These spots were counted by two independent observers using a stereomicroscope.

Cell culture and cytokine measurement

Cell suspensions (1 × 10⁶ cells in 100 µl complete RPMI 1640 from Life Technologies supplemented with 10% FCS, 50 mM L-ME, and 200 mM L-glutamin) were incubated with 50 µl Con A (15 µg/ml), LPS (6 µg/ml) or medium in 96-well plates (Highbond 3590; Costar, Cambridge, MA) overnight at 37°C in 5% CO₂. After centrifugation for 10 min at 1000 rpm, supernatant was collected and stored at −70°C until analysis. IFN-γ and IL-4 were determined by sandwich ELISA. Plates were coated overnight at 4°C with 1 µg/ml rat-anti-mouse IFN-γ or 1 µg/ml rat-anti-mouse IL-4 in 0.05 M carbonate buffer (pH 9.6), washed with PBS/Tween, and blocked with PBS/Tween/casein for 4 h at room temperature. IFN-γ and IL-4 standards (100 µl) were added in several dilutions and incubated overnight at 4°C. After washing, plates were incubated with 0.25 µg/ml biotinylated rat anti-mouse IFN-γ or IL-4 conjugate diluted in PBS/Tween/casein for 1 h at room temperature. Plates were washed again and incubated with streptavidin-HP (0.3 µg/ml) diluted in PBS/Tween/casein for 45 min at room temperature.

For TNF-α ELISA, 96-well plates were coated with 1.25 µg/ml rat anti-mouse TNF-α in PBS (100 µl, overnight) and after washing with PBS/Tween were blocked with PBS/0.5% BSA for 2 h at room temperature. Plates were washed, and TNF-α standards and samples were added in several dilutions. Immediately, biotinylated anti-TNF-α diluted in PBS/Tween/0.5% BSA was added and incubated for 2 h at room temperature. Plates were washed and incubated with streptavidin (0.3 µg/ml) diluted in PBS/Tween/0.5% BSA for 45 min at room temperature.

After the final washes, TMB substrate (0.1 mg/ml) was added, and the color reaction was stopped after 10 min with 2 M H₂SO₄. Absorbance was measured at 450 nm using an ELISA reader ELX800 (Bio-Tek Instruments, Winooski, VT).

Flow cytometry

For flow cytometric analysis, 1 × 10⁶ cells in PBS/BSA were centrifuged, resuspended, and incubated with predetermined dilutions of FITC-, PE-, and CY- conjugated mAbs in 96-well plates (30 min in darkness at 4°C). Samples incubated with biotin-conjugated mAbs were once again centrifuged and incubated with streptavidin-CY in the same way. Cells were washed, resuspended, stored in formalin (0.1%), and analyzed within 18 h. Samples were analyzed on a FACSScan with standard FACScanFlow using CellQuest software (BD Biosciences, Franklin Lakes, NJ).

Immunohistochemistry

Cryostat sections (6 µm) were fixed in acetone and incubated with predetermined dilutions of rat anti-mouse B220 mAb in PBS/1% BSA at room

RA-PLNA

Naive mice were injected s.c. into the right hind footpad with 50 µl of a freshly prepared mixture of the drug together with a sub sensitizing dose (10 µg) of TNP-OVA. Drugs were injected in quantities that were immuno stimulatory in the PLNA in previous experiments (25): 1 mg D-Pen or 2 mg DPh dissolved in saline or 1 mg STZ diluted in citrate buffer (0.1 M, pH 4.0). Treatment of saline or vehicle groups, and the NO-generating capacity in vivo. Control groups treated with TNP-OVA in either saline or citrate buffer were not different when comparing cell counts, TNP-specific Ab numbers or expression of costimulatory molecules. Mice were treated i.p. with 300 µg anti-CD154. Seven days after drug injection, mice were killed by cervical dislocation, blood was drawn by orbital puncture, and the popliteal lymph node (PLN) was excised and separated from adherent fatty tissue. Purified B220⁺/IgM⁺ BSA, a representative of B220⁺/IgM⁺ cells, was prepared, washed (1000 rpm at 4°C), resuspended in 1 ml PBS/1% BSA, counted using a Coulter counter (Coulter Electronics, Luton, U.K.) and adjusted to 1 × 10⁶ cells/ml. For immunohistochemistry PLNs were snap-frozen in liquid nitrogen and stored at −70°C until use.

ELISPOT assay

The ELISPOT assay was performed based on the operating procedure described by Schielen et al. (28). Immobilized P membranes (Immobilon PVDF Transfer; Millipore, Millipore, Etten-Leur, The Netherlands) were coated overnight with PBS/0.05% Tween/TNP-BSA (10 µg/ml) and blocked for 1 h with PBS/Tween/1% BSA. These membranes were clamped in spot blocks (made in-house), and 5 × 10⁶ cells were centrifuged on the membranes and incubated for 4 h at 37°C. Membranes were removed from the spot blocks, washed with PBS and PBS/Tween, and incubated overnight at 4°C with AP-conjugated Abs in PBS/Tween/casein (1:500). Membranes were washed and para-nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate toluidine salt reagent to accomplish color development of TNP-specific Ab spots. These spots were counted by two independent observers using a stereomicroscope.

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temperature for 1 h. After three washes with PBS/Tween, sections were incubated (at room temperature for 1 h) with polyclonal peroxidase-conjugated rabbit anti-rat Ig in PBS/10% normal mouse serum. After washes with PBS/Tween and acetate buffer, peroxidase-based staining was performed with 3-aminio-9-ethylcarbazole or diaminobenzidine, and counterstaining was performed with hematoxylin.

Statistics
Values deviating >2 SD from group means were considered outliers and were not included in statistical analyses. Preceding analyses absolute cell numbers were log-normally transformed. Multiple comparison of group means were analyzed using one-way ANOVA with Bonferroni as post-hoc test. A value of \( p < 0.05 \) was considered statistically significant.

Results
Distinct type 1 or type 2 immune responses to TNP-OVA in BALB/c mice induced by type 1 or type 2 priming drugs using RA-PLNA

The definitions of type 1 and type 2 responses are based on differentiation of Th cells in vitro and Th2 cells, which are characterized by their pattern of cytokine secretion (29) that determines the IgG isotype that is secreted by B cells. In mice, IFN-γ and IgG2a are generally used as type 1 prototypes, whereas IL-4 and IgG1 indicate a type 2 response (30). Although the Th1/Th2 paradigm is probably an oversimplification, as cells and mediators of one type regulate the characteristic features of the other type, we use these prototypic parameters to differentiate type 1 from type 2 drug-induced responses to TNP-OVA.

Our present observations confirm earlier results described by Albers and colleagues (25, 26) showing that coinjection of TNP-OVA with DPH or D-Pen elicits type 2 responses, whereas exposure to STZ stimulates a type 1 response. Responses evoked by DPH and D-Pen are characterized by elevated levels of TNF-specific IgM, IgG1, and IgE ASC numbers (Fig. 1A). In this study we additionally show that IL-4 levels were increased after in vitro restimulation of PLN cells with Con A, while IFN-γ and TNF-α levels were not different from control values (Fig. 1B), indicating that Th2 cells activated B cells to proliferate and secrete TNF-specific Abs.

In contrast, immune responses evoked by STZ were characterized by type 1 phenomena. Despite the fact that B cells do not typically mediate this response, there is a significant increase in IgM and IgG2a ASC (Fig. 1A) indicative of Th1 cell-dependent B cell priming. Indeed, extremely high levels of IFN-γ and TNF-α, but not IL-4, confirmed Th1 involvement (Fig. 1B).

We further identified the type of response by characterizing the cell types present in the draining lymph node. All chemicals significantly increased PLN cellularity (Table I), but the compositions of various cell types present in the PLN as well as the PLN architecture were completely different between type 1 and type 2 responses. CD4+ and CD8+ T cells increase in concert after D-Pen and DPH injection (Table I), but T:B cell ratios are significantly decreased compared with controls due to extensive proliferation of B (CD19+) cells. Immunohistochemical examination of the draining PLN showed GC in lymph nodes from animals injected with D-Pen, indicating B cell maturation and differentiation into memory cells (Fig. 2B). STZ exposure increased absolute numbers of CD4+ T and CD8+ T and B cells (Table I). The response was characterized by a strong influx of CD8+ T cells as the Th1 balance changed in favor of Te and the T:B cell ratio was significantly increased compared with controls. Interestingly, a rather disturbed structure of the lymph node was observed, showing B cells scattered through the entire PLN tissue, and no GC were present (Fig. 2E). Numbers of CD11c+ and F4/80+ cells were increased in STZ-mediated responses. These data prove that RA-PLNA using adjuvating drugs provides a model to study both type 1 and type 2 immune parameters in response to a well-defined Ag in the same mouse strain. We used this experimental set-up to study the role of CD154 in both types of response.

Effect of anti-CD154 treatment on drug-induced type 1 and type 2 responses

To study the role of CD40-CD154 ligation in drug-induced type 1 and type 2 responses, mice were treated i.p. with anti-CD154 mAb. Anti-CD154 treatment decreased total PLN cell numbers in both D-Pen- and DPH-induced responses (inhibition, 63 and 54%, respectively), but in type 2 responses primed by D-Pen and DPH injection (Table I), but T:B cell ratios are significantly decreased compared with controls due to extensive proliferation of B (CD19+) cells. Immunohistochemical examination of the draining PLN showed GC in lymph nodes from animals injected with D-Pen, indicating B cell maturation and differentiation into memory cells (Fig. 2B). STZ exposure increased absolute numbers of CD4+ T and CD8+ T and B cells (Table I). The response was characterized by a strong influx of CD8+ T cells as the Th1 balance changed in favor of Te and the T:B cell ratio was significantly increased compared with controls. Interestingly, a rather disturbed structure of the lymph node was observed, showing B cells scattered through the entire PLN tissue, and no GC were present (Fig. 2E). Numbers of CD11c+ and F4/80+ cells were increased in STZ-mediated responses. These data prove that RA-PLNA using adjuvating drugs provides a model to study both type 1 and type 2 immune parameters in response to a well-defined Ag in the same mouse strain. We used this experimental set-up to study the role of CD154 in both types of response.

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In the type 1 response, on the other hand, secretion of TNF-
completely inhibited by treatment with anti-CD154 mAb (Fig. 3). In the type 1 response anti-CD154 did not affect the proliferation of B and CD4 T cells, but relative numbers of CD8 T cells were slightly lower compared with those in mice without anti-CD154 treatment. Interestingly, fewer CD11c and F4/80 cells were present in the PLN of anti-CD154-treated mice compared with drug-exposed mice that were not injected with anti-CD154. Additionally, anti-CD154 prevented the architectural disruption of the PLN in STZ-exposed mice (Fig. 2). Furthermore, IL-4 secretion, which was induced by D-Pen, was completely inhibited by treatment with anti-CD154 mAb (Fig. 3). In the type 1 response, on the other hand, secretion of TNF-α was not inhibited, and IFN-γ was only slightly inhibited by anti-CD154. Consistently, TNP-specific Ab secretion was inhibited by anti-CD154 in type 2, and not in type 1, drug-induced responses. Anti-CD154 decreased TNP-specific IgM ASC numbers (per 1 × 10⁶ cells) with >80% in the case of type 2 responses (Fig. 4). The numbers of IgG1 and IgE ASC (per 1 × 10⁶ cells) were also significantly reduced in the case of DPH (87 and 84%, respectively) and D-Pen (84% reduction in IgG1 and no detectable IgE; Fig. 5). In responses to STZ no changes were observed in TNP-specific IgM and IgG2a ASC numbers after CD154 treatment.

**Table I. Absolute and relative numbers of cell types characterized by flow cytometry and ratios of CD4+:CD8- cells and CD4- and CD8+:CD19+ cells**

<table>
<thead>
<tr>
<th>Group</th>
<th>Total Cell Number (×10⁶)</th>
<th>CD4⁺ (×10⁶)</th>
<th>CD8⁻ (×10⁶)</th>
<th>CD19⁺ (×10⁶)</th>
<th>CD11c⁺ (×10⁶)</th>
<th>F4/80⁺ (×10⁶)</th>
<th>Th/Te</th>
<th>T:B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.34 ± 0.16</td>
<td>61 ± 14 (52 ± 1)</td>
<td>32 ± 3 (21 ± 1)</td>
<td>56 ± 10 (28 ± 2)</td>
<td>9 ± 3 (2 ± 0)</td>
<td>8 ± 2 (4 ± 0)</td>
<td>2.5 ± 0.2</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>D-Pen</td>
<td>11.38 ± 1.06b</td>
<td>337 ± 54 (28 ± 1)b</td>
<td>158 ± 26 (14 ± 1)b</td>
<td>471 ± 59 (55 ± 3)b</td>
<td>20 ± 4 (4 ± 0)b</td>
<td>15 ± 4 (5 ± 1)</td>
<td>2.1 ± 0.1</td>
<td>0.8 ± 0.2b</td>
</tr>
<tr>
<td>D-Pen anti-CD154</td>
<td>4.91 ± 0.61c</td>
<td>231 ± 27 (36 ± 1)c</td>
<td>104 ± 11 (18 ± 0)c</td>
<td>200 ± 21 (40 ± 3)c</td>
<td>20 ± 3 (3 ± 0)</td>
<td>16 ± 0 (4 ± 1)</td>
<td>2.0 ± 0.1c</td>
<td>1.4 ± 0.2c</td>
</tr>
<tr>
<td>DPH anti-CD154</td>
<td>9.86 ± 1.95c</td>
<td>383 ± 47 (32 ± 2)c</td>
<td>146 ± 17 (13 ± 1)c</td>
<td>430 ± 71 (51 ± 1)c</td>
<td>17 ± 3 (2 ± 0)</td>
<td>ND</td>
<td>2.4 ± 0.1</td>
<td>0.9 ± 0.1c</td>
</tr>
<tr>
<td>DPH anti-CD154</td>
<td>4.52 ± 0.15c</td>
<td>135 ± 22 (40 ± 3)c</td>
<td>86 ± 13 (19 ± 1)c</td>
<td>265 ± 9 (40 ± 2)c</td>
<td>6 ± 2 (1 ± 0)</td>
<td>ND</td>
<td>2.1 ± 0.1</td>
<td>1.6 ± 0.2c</td>
</tr>
<tr>
<td>STZ anti-CD154</td>
<td>4.78 ± 0.55b</td>
<td>144 ± 7 (29 ± 2)b</td>
<td>174 ± 6 (34 ± 2)b</td>
<td>102 ± 8 (20 ± 1)b</td>
<td>63 ± 6 (12 ± 3)b</td>
<td>63 ± 9 (14 ± 2)</td>
<td>0.8 ± 0.1b</td>
<td>3.1 ± 0.3b</td>
</tr>
<tr>
<td>STZ, anti-CD154</td>
<td>5.61 ± 0.56b</td>
<td>140 ± 7 (30 ± 2)b</td>
<td>166 ± 16 (27 ± 1)b</td>
<td>97 ± 9 (21 ± 2)b</td>
<td>38 ± 9 (7 ± 2)</td>
<td>23 ± 5 (7 ± 1)</td>
<td>0.9 ± 0.1b</td>
<td>3.2 ± 0.3b</td>
</tr>
</tbody>
</table>

* Levels are expressed as group means ± SEM. Numbers in parentheses represent percentages. Boldface indicates differences between drug-exposed anti-CD154-treated and non-anti-CD154-treated animals. Data for each condition are derived from two separate experiments with four mice per group.

**Anti-CD154-induced inhibition of type 2 response to TNP-OVA elicited by D-Pen is dose dependent**

Coinjection of TNP-OVA and D-Pen together with i.p. treatment with 0, 75, 150, 300, or 500 μg anti-CD154 resulted in a dose-dependent inhibition of the immune response indicated by cellularity of PLN (data not shown) and the presence of TNP-specific IgM and IgG1 ASC (Fig. 6). Mice treated with either 300 or 500 μg anti-CD154 (separate experiments) showed significantly reduced total PLN cell numbers compared with drug-exposed mice that did not receive anti-CD154. ELISPOT data showed that TNP-specific IgG1 and IgM ASC numbers were significantly reduced after treatment with 300 and 500 μg anti-CD154 compared with those in groups that received a lower dose. IgG1 ASC numbers were comparable after 300 or 500 μg anti-CD154 treatment, whereas numbers of IgM ASC were slightly lower after treatment with 500 μg anti-CD154 compared with 300 μg. In an additional experiment it was tested whether a 300-μg dose of anti-CD154 on 2 consecutive days (days 0 and 1) could inhibit these parameters more effectively, but the results showed no differences between treatments. A specific ELISA was performed to assess the amount of anti-CD154 mAb circulating in the body in time, and anti-CD154 levels were still detectable in sera 7 days after injection (data not shown).

**Increased expression of costimulatory molecules on different cell types in type 1 vs type 2 responses**

Others have already shown that CD40-CD154 ligation is an important stimulus for dendritic cells (DC) and B cells to up-regulate the expression of the surface molecules CD80, CD86, and CD54 (31). Therefore, we set out to characterize expression of these molecules in our model on both B and non-B cells, as shown in Fig. 7. These graphs show that in type 2 responses relative numbers of CD80+ cells were unchanged, whereas percentages of CD86+ cells were significantly increased, but only when analyses were performed for B cells separately. Relative numbers of CD45-expressing cells were all significantly increased compared with controls. In the response to D-Pen, B cells expressed 57, 68, and 94% of the total expression of CD80, CD86, and CD54, respectively. In DPH-elicited responses these numbers were even higher: 88, 61, and 94%, respectively, indicating that B cells are efficient in Ag presentation and providing costimulation in type 2 conditions.
On the contrary, B cells expressed only 23, 9, and 50% of the total expression of CD80, CD86, and CD54, respectively, in response to STZ. All markers were significantly increased when analyzing all PLN cells, but when looking specifically at B cells in the type 1 response a significant increment was found for CD80 (both relative and absolute) and CD54 (only absolute numbers; data not shown), but not for CD86. These data indicate that B cells are not the prominent APC in this STZ-induced type 1 response, but that presumably DC and/or macrophages provide the required costimulation.

Modulation of CD80, CD86, and CD54 expression by anti-CD154 in drug-induced type 1 vs type 2 responses

Fig. 7 indicates that the expressions of CD86 costimulatory molecules were differentially regulated in type 2 responses. Anti-CD154 inhibited CD86 expression to control levels in response to D-Pen. In the type 1 response anti-CD154 inhibited CD86 expression, but CD80 expression remained unaffected. Notably, the inhibition of cells expressing CD86 was only significant for B cells in type 2 responses, but CD86-expressing non-B cells were decreased in the type 1 response. Anti-CD154 treatment had a diverting impact on the expression of CD54, as numbers decreased significantly in type 2 responses, but there was no change in expression in the type 1 response.

Discussion

Although the roles of CD40 and CD154 have been thoroughly investigated, the involvement of this receptor-ligand couple in relation to activation of Th1 vs Th2 cells has not been elucidated as yet. In this study we show that CD154 is differently involved in selective drug-induced type 2 vs type 1 immune responses directed to the same Ag (TNP-OVA) in the same mouse strain (BALB/c). In the case of the type 2 priming drugs D-Pen and DPH, anti-CD154 inhibits IL-4 secretion and disables CD4+ T cell, CD8+ T cell, and primarily B cell proliferation, GC formation, and the production of high-affinity TNP-specific IgM and Th2-related isoatypes (IgG1 and IgE). The inhibition of total cell and TNP-specific ASC counts was dependent on the dose of anti-CD154, which is in agreement with the finding that the abundance of CD154 in activated T cells limits the rate and magnitude of the Ab response to T-dependent Ags (32).

In contrast, blocking of CD154 in the case of the type 1 immune response elicited by STZ did not affect the proliferation of B and CD4+ T cells, TNF-α secretion, and TNP-specific IgM and IgG2a.

FIGURE 4. TNP-specific IgM ASC numbers. Cells were isolated from PLN after injection of TNP-OVA in saline (control) alone or together with D-Pen, DPH, or STZ in the hind footpad of BALB/c mice. Shown are the group mean ± SEM of mice without (■) and with (□) additional i.p. anti-CD154 treatment. Levels from all drug-exposed mice are significantly higher than control values. *, Significant differences (p < 0.05) between group means with and without anti-CD154 treatment after injection of a specific chemical. The data presented for each condition are derived from two separate experiments with four mice per group.

FIGURE 5. TNP-specific IgG1, IgG2a, and IgE ASC numbers. Cells were isolated from PLN after injection of TNP-OVA in saline (control) alone or together with D-Pen, DPH, or STZ in the hind footpad of BALB/c mice. Levels are expressed as the group mean ± SEM. All IgG1 levels of D-Pen- and DPH-exposed groups are significantly higher than control values. D-Pen, D-Pen/anti-CD154-, and DPH-treated groups also show higher IgE levels than controls. *, Significant differences (p < 0.05) between group means with and without anti-CD154 treatment after injection of a specific chemical. The data presented for each condition are derived from two separate experiments with four mice per group.
production. Anti-CD154 only slightly affected the observed relative increase in the number of CD8⁺ T cells and IFN-γ production and inhibited the influx of F4/80⁺ and CD11c⁺ cells into the PLN. Together these results show that CD40-CD154 interaction is crucial in type 2 responses and is only marginally important in type 1 responses elicited by drugs with opposing adjuvant potential.

CD40 engagement is an important stimulus for DC and B cells to up-regulate the expression of the costimulatory molecules CD80 and CD86 (15, 33–35), and, vice versa, ligation of the CD80/86 ligand CD28 on T cells increases the expression of CD154 (36). Activation through these costimulatory molecules together with CD40-mediated up-regulation of cytokine receptors (37) and secretion of proinflammatory cytokines (13, 31) are required for DC and B cells to become efficient APC and to activate T cells (18, 38). In the present study CD86 and CD54 expressions were enhanced in both type 1 and type 2 circumstances, but, interestingly, in elicitation of type 2 responses these molecules appeared particularly on B cells, whereas in the STZ-induced type 1 response expression was found predominantly on non-B cells, which appeared to be CD11c⁺ and F4/80⁺. CD80 expression, on the other hand, was increased only in the type 1 response and exclusively on non-B cells. We further show that in anti-CD154-treated mice the expression of CD86 on B cells is inhibited and not different from that in controls in the case of DPH and on non-B cells after STZ exposure. However, the numbers of CD86-expressing B cells in D-Pen-exposed animals are still elevated compared with control values after CD154 treatment. Surprisingly, anti-CD154 had no effect on CD80 expression induced by STZ.

From this study important conclusions can be drawn concerning the involvement of CD80 and CD86 and the cells that function as APC in drug-induced type 1 vs type 2 responses. First, DC and/or macrophages serve as the most efficient APC in type 1 responses, whereas this Ag presentation function is taken over by B cells in type 2 responses. Second, CD86 is not as crucial in all type 2 responses. Third, CD80 expression on non-B cells appears only important in the type 1 response. Finally, this CD80 expression is not affected by anti-CD154 and thus is independent of CD40 interaction with its ligand. From the latter we infer that CD80 signaling may circumvent the need for CD40 triggering in STZ-treated animals.

These conclusions support the idea that CD80 is important in activating Th1 cells (39, 40), whereas CD86 preferentially induces Th2 responses (41–43). Rather, our results suggest that type 2 responses arise upon CD86 ligation in the absence of CD80, whereas the expressions of both molecules prime for type 1 responses. However, evidence accumulates that many more features, such as involvement of regulatory genes, effects of different APC and Ag, location and duration of exposure, and costimulatory expression pattern in several stages of the response, determine the actual balance between type 1 and type 2 immune responses (44). We are currently looking into the kinetics of CD80 and CD86 up-regulation and expression of other costimulatory molecules on specified APC to investigate their importance in drug-induced sensitization.

In line with the multifactorial aspect of immune response polarization, we show that anti-CD154 treatment affects some Th1 parameters more than others; i.e., anti-CD154 did not disturb (IgG2a and TNF-α production or B and CD4⁺ T cell proliferation)

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**FIGURE 6.** Percentage of TNP-specific ASC in PLN referred to PLN of D-Pen-exposed mice without anti-CD154 treatment. Mice received 1 mg D-Pen in the hind footpad and were treated i.p. with various doses of anti-CD154 (0, 75, 150, 300, or 500 µg). *, Different from all other groups except the 500-µg group; †, different from all other groups except the 300-µg group (p < 0.05). Data are from one experiment with five mice per group.

**FIGURE 7.** Expression of costimulatory molecules. Expression of CD80, CD86, and CD54 on day 7 after coinjection of TNP-OVA together with D-Pen, DPH, or STZ in the hind footpad of BALB/c mice. □, Costimulatory expression on all cells present in PLN; ■, expression on B cells only. Levels are expressed as the percentage ± SEM. *, Significant (p < 0.05) differences compared with control levels; †, significant differences in costimulatory molecule-expressing B cells in drug-exposed groups without anti-CD154 and drug-exposed groups with anti-CD154; §, significant differences in costimulatory molecule-expressing non-B cells in drug-exposed groups without anti-CD154 and drug-exposed groups with anti-CD154. The data presented for each condition are derived from two separate experiments with four mice per group.
or only slightly disturbed (CD8+ T cells and IFN-γ production) effector functions induced by Th1 cells. The independence of IgG2a production for the CD40-CD154 interaction is also demonstrated in CD154-deficient mice (17), but in the same as well as other studies (17, 45) CTL function of CD8+ T cells was found to be unaffected. Our results together with the fact that at least a subgroup of CD8+ T cells expresses CD154 (13) indicate that CD8+ T cells, at least for some functions, can be affected by anti-CD154.

Compared with the slight effects on CD8+ T cell activation and IFN-γ production, the observed profound reduction of STZ-induced migration of CD11c+ and F4/80+ cells into the lymph node and the recovery of the disturbed PLN morphology by interference with the CD40-CD154 interaction are remarkable. In previous studies (27) we have demonstrated that this disturbed PLN morphology is accompanied by an increase in apoptotic cells, possibly initiated by macrophage excretion products (enzymes, NO). These observations are in agreement with data from CD154-deficient mice showing that Langerhans cells fail to migrate to the draining lymph node (46) and with studies demonstrating that anti-CD154 treatment prevents the secretion of tissue-damaging chemokines and reactive oxygen species, such as NO (47). It can be concluded that the CD40-CD154 couple strongly determines macrophage influx as well as excretion of tissue-damaging products, and that the macrophage activation by STZ is largely independent of Th1 cells. However, part of this macrophage-stimulating effect of the costimulatory receptor-ligand couple might be mediated via CD8+ T cells, as we observed that STZ stimulated these cells to produce large quantities of IFN-γ (this study combined with Ref. 27), being a chemoattractant for macrophages. In agreement with this, anti-CD154 slightly inhibited STZ-induced IFN-γ excretion by 27% and may thus contribute to the inhibition of macrophage function by anti-CD154.

Thus, our results give support to the following concept for dependence of drug-induced type 1 vs type 2 responses on costimulation. Type 2 responses are initiated by professional APC (48), probably DC and macrophages (marginal influx, Table I), but, conceivably, Ag presentation is soon taken over by B cells. This Ag presentation by B cells strongly depends on CD40-CD154 interaction (49). Once activated to become effective APC, these B cells may stimulate Th2 cells that, in turn, trigger the same or other B cells to produce IgG1 and IgE. Type 1 responses also start with professional APC activation, which expresses both CD80 and CD86, but in this situation DC remain the APC throughout the response. We show that CD86, but not CD80, expression on macrophage-like DC and the destructive machinery of these cells are also sensitive to anti-CD154 treatment. On the other hand, Th1 cell-dependent effector functions (CD8+ T cell proliferation and IgG2a) seem less dependent on CD40-CD154 and may somehow be mediated by CD80 ligation. We cannot exclude that CD86 has an influence on the destructive macrophage activity or IFN-γ production by CD8+ T cells.

In summary, our data show that the CD40-CD154 costimulatory interaction is highly important in drug-induced type 2 responses and only marginally involved in the drug-induced type 1 response. We confirm that the PLNA, using a reporter Ag together with adjuvating drugs, provides a suitable model to study specific immune parameters and mechanisms in prototype primary type 1 and type 2 responses. This model has the advantage that these selectively acting drugs condition the immunostimulatory microenvironment in such a way that opposing immune responses arise against the same Ag and on the same genetic background. This allows detection of immune modulation using simple read-out parameters shortly after exposure, thereby minimizing the involvement of complex regulatory mechanisms. However, as different drugs are used to facilitate a specific type of response, we cannot exclude that the specific characteristics of the chemical may be responsible for our observations. In our current experiments we are studying the CD40-CD154 interaction in type 1 and type 2 responses elicited by the same chemical using mice with different H2 haplotypes.

Besides basic immunological mechanistic information, the present findings provide important new insights into the adjuvant activity of allergenic or autoimmunogenic drugs and may help to define early activation molecules as predictive parameters for adverse immune effects of newly developed drugs exerting their effect via either a type 1 or type 2 mechanism.

Acknowledgments
We thank Tanox Pharma B.V. for the gift of anti-CD154 mAb. We also acknowledge Prof. Willem Seinen (Institute for Risk Assessment Sciences, Utrecht University, Utrecht, The Netherlands) for critical reading of the manuscript.

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