

# Differential Requirement for CD28/CTLA-4-CD80/CD86 Interactions in Drug-Induced Type 1 and Type 2 Immune Responses to Trinitrophenyl-Ovalbumin

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The use of mAbs to abrogate costimulatory interactions has attracted much attention with regard to prevention and modulation of adverse (auto)immune-like reactions. However, the role of costimulatory molecules and possible therapeutic use of Ab-treatment in drug-induced immunostimulation is poorly elucidated. In the present studies, we show that CD28/CTLA-4-CD80/CD86 costimulatory interactions differently regulate drug-induced type 1 and type 2 responses to an identical bystander Ag, TNP-OVA, in BALB/c mice using the reporter Ag popliteal lymph node assay. The antirheumatic drug D-Penicillamine, which may induce lupus-like side-effects, stimulated type 2 responses against TNP-OVA, characterized by the production of IL-4 and TNP-specific IgG1 and IgE. These responses were abrogated in CD80/CD86-deficient mice and in wild-type mice that were treated with anti-CD80 and anti-CD86, or CTLA-4-Ig. Anti-CTLA-4 intensively enhanced the D-Penicillamine-induced effects. In contrast, the type 1 response (IFN- $\gamma$ , TNF- $\alpha$ , IgG2a) to TNP-OVA induced by the diabetogen streptozotocin still developed in the absence of CD80/CD86 costimulatory signaling. In addition, it was demonstrated that coadministration of anti-CD80 and anti-CD86 mAbs slightly enhanced streptozotocin-induced type 1 responses, whereas the CTLA-4-Ig fusion protein completely abrogated this response. In conclusion, different drugs may stimulate distinct types of immune responses against an identical bystander Ag, which are completely dependent on (type 2) or independent of (type 1) the CD28/CTLA-4-CD80/CD86 pathway. Importantly, the effects of treatment with anti-CD80/CD86 mAbs and CTLA-4-Ig may be considerably different in responses induced by distinct drugs. *The Journal of Immunology*, 2005, 175: 3707–3714.

Immune-mediated drug hypersensitivity reactions (IDHR)<sup>2</sup> comprise 6–10% of all adverse drug reactions with both allergic and autoimmune-like phenomena (1), attending considerable problems for patients and physicians. Recently, it has been suggested (2–5) that costimulatory interactions, which are decisive in sensitization of Ag-specific T cells by APCs (6, 7), are also important for the elicitation of drug-induced idiosyncratic immune responses. To date, the functional importance of specific costimulatory molecules in development of IDHR is unclear.

For this reason, we have previously studied the role of costimulation in drug-induced adverse immune reactions, particularly with respect to type 1 (Th1) and type 2 (Th2) responses. These responses were directed against an identical bystander Ag that was injected in a nonsensitizing dose together with different drugs that either stimulate type 1 or a type 2 response to that Ag. The type 1 response was induced by an injection of the antineoplastic drug streptozotocin (STZ; known to provoke diabetes (8)) and was char-

acterized by increased levels of IFN- $\gamma$ , IgG2a, and numbers of CD8<sup>+</sup> T cells and macrophages. The antirheumatic drug D-Penicillamine (D-Pen; known to cause lupus-like symptoms in humans (9)) was used as a model autoimmunogenic chemical, inducing type 2 responses, such as increased levels of IL-4, IgG1, and IgE, and formation of germinal centers. It was shown that type 2 phenomena were completely dependent on CD154, whereas type 1 responses were still operational despite an interruption of CD40-CD154 interactions in vivo (10). D-Pen and STZ were also shown to induce distinct, drug-specific, and time-dependent changes in the expression of regulatory molecules (CTLA-4) on T cells and costimulatory molecules (such as CD40, CD80, and CD86) on different APC (43). Type 2 responses to D-Pen were characterized by an increase in CD86-expressing dendritic cells (DC) as well as B cells and a complete absence of CD80 and CTLA-4 expression. However, in STZ-induced type 1 responses, expression of CTLA-4 was increased and both CD80 and CD86 were expressed exclusively on DC.

From these observations, we hypothesized that the differential regulation of the CD28/CTLA-4-CD80/CD86 pathway, in relation to different dominating APC and effector cells, might be decisive in the elicitation of drug-induced type 1 vs type 2 immune responses. Such ambiguity in the functionality of costimulatory pathways was previously observed in different models for autoimmune disease showing that abrogation of CD28-CD80/CD86 interactions inhibits type 1 (11) or type 2 phenomena (12). Furthermore, anti-CD80 mAbs reduced disease severity in experimental autoimmune encephalomyelitis, whereas anti-CD86 exacerbated this disease (13), and opposite effects of these Abs were observed for the development of diabetes in nonobese diabetic mice (14).

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<sup>2</sup> Abbreviations used in this paper: IDHR, immune-mediated drug hypersensitivity reaction; ASC, Ab-secreting cell; PLN, popliteal lymph node; PLNA, PLN assay; CY, CyChrome; D-Pen, D-Penicillamine; STZ, streptozotocin; WT, wild type.

In the present study, the importance of the costimulatory pathway CD28/CTLA-4-CD80/CD86 was investigated in characteristic type 1 and type 2 drug-induced immune responses to an identical bystander Ag in CD80/CD86- and CD28/CTLA-4-deficient knockout (KO) mice and in wild-type (WT) mice that were treated with blocking mAb (anti-CD80, anti-CD86, anti-CTLA-4) or CTLA-4-Ig fusion protein. The popliteal lymph node assay (PLNA) (15–17), using TNP-OVA as bystander Ag to facilitate the read out of the responses, was shown to be extremely suitable for this purpose (10). Using this assay, we have previously shown that immunostimulating chemicals may differently dictate the type of response to the bystander Ag, TNP-OVA, and that the use of this bystander Ag facilitates the assessment of immunomodulating effects of a specific treatment in drug-induced responses (10, 18). This enables the study of the immunomodulatory effects of different treatments using an identical specific readout parameter in different types of responses without the need to know the (auto)specificity of the immune response.

Here, we provide further evidence that the CD28/CTLA-4-CD80/CD86 pathway is differently regulated in STZ-induced type 1 vs D-Pen-induced type 2 immune responses. The present findings may have important implications for the use of selective costimulatory blockades to modulate adverse (drug-induced) autoimmune-like reactions and autoimmune diseases in man.

## Materials and Methods

### Mice

Female, specific pathogen-free WT BALB/c mice (6–12 wk old) were obtained from Harlan, and CD80/CD86- and CD28/CTLA-4-deficient mice on BALB/c background were provided by Drs. M. Oosterwegel and F. Hofhuis (Department of Immunology, University Medical Center, Utrecht, The Netherlands). Animals were housed according to The Association for Assessment of Laboratory Animal Care. Mice were allowed to settle for a week and were maintained under barrier conditions in filter-topped Macrolon cages with wood chip bedding, at a mean temperature of  $23 \pm 2^\circ\text{C}$ , 50–55% relative humidity, and 12 h light/dark cycle. Drinking water and standard laboratory food pellets were provided ad libitum. Mice were randomly assigned to specific treatment. The experiments were conducted according to the guidelines of the animal experiments committee of the Faculty of Veterinary Medicine, Utrecht University (Utrecht, The Netherlands).

### Chemicals, reagents, and mAbs

Chemicals were obtained from Sigma-Aldrich unless stated otherwise. D-Pen and STZ were diluted in saline (0.9%; B. Braun) just before injection. Cells producing anti-CTLA-4 (HB-304; 4F10), anti-CD80 (HB-301; 16-10A1), and anti-CD86 (HB-253; GL1) were obtained from American Type Culture Collection, and Abs were purified using thiophilic agarose (SeaKem) under aseptic conditions. Murine CTLA-4-IgG fusion protein was prepared as previously described (19) and kindly provided by Dr. A. van Oosterhout (Department of Pharmacology and Pathophysiology, Faculty of Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands). Appropriate isotypes for each mAb (hamster IgG and rat IgG2a) were purchased from BD Pharmingen. Human IgG (ICN Biomedicals) was used as a control for CTLA-4-IgG fusion protein. All Abs and fusion proteins were subjected to the *Limulus* amebocyte lysate assay to determine endotoxin levels. All samples contained  $<6$  ng endotoxin per injection. TNP-OVA and TNP-BSA were prepared as previously described (17). Alkaline phosphatase-conjugated goat anti-mouse human-adsorbed IgG1, IgG2a, and IgM; and alkaline phosphatase-conjugated rat anti-mouse IgE were obtained from Southern Biotechnology Associates. To visualize TNP-specific Ab-secreting cells (ASC) in the ELISPOT assay, fresh stock solutions of para-NBT and 5-bromo-4-chloro-3-indolylphosphate toluidine salt in dimethylformamide (BDH Laboratory Supplies) were prepared and diluted in Tris buffer (100 mM Trizma base, 100 mM NaCl, 5 mM  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , pH 9.5). For cytokine measurements, IL-4 and IFN- $\gamma$  capture and detecting Abs were obtained from BD Pharmingen, TNF- $\alpha$  ELISA was purchased from BioSource, casein from BDH, and streptavidin-conjugated HRP from Sanquin. BD Pharmingen was the supplier of FcBlock (CD16/CD32, Fc $\gamma$ III/IIIR, 2.4G2), CD3 $\epsilon$  PE-CyChrome (CY) (145-2C11), CD4 FITC (RM4-5), CD8a PE (53-6.7), CD19 PE (1D3), CD11c FITC (HL3),

CD80 (16-10A1), CD86 (GL1), CD54 (3E2), streptavidin CY, and rat anti-mouse MHC-II Biotin (NIMR-4). F4/80 PE was obtained from Caltag Laboratories. Medium that was used for in vitro restimulation was complete RPMI 1640 with Glutamax-I (Invitrogen Life Technologies) supplemented with 10% FBS (ICN Pharmaceuticals) and 2% penicillin-streptomycin.

### RA-PLNA and Ab treatment

Mice were injected s.c. into the hind footpad with a freshly prepared dilution of 1 mg of D-Pen or STZ (quantity was immunostimulatory in the PLNA in previous experiments (10, 17)) together with a predefined sub-sensitizing dose (10  $\mu\text{g}$ ) of TNP-OVA diluted in saline in a total volume of 50  $\mu\text{l}$ . Mice were treated i.p. on days 0, 2, and 4 with 100  $\mu\text{g}$  of anti-CTLA-4, 100  $\mu\text{g}$  of anti-CD80, 100  $\mu\text{g}$  of anti-CD86, or 100  $\mu\text{g}$  of anti-CD80 plus 100  $\mu\text{g}$  of anti-CD86. CTLA-4-Ig was administered i.p. on days 0 and 2 in a dose of 200  $\mu\text{g}$ . Appropriate isotype controls had no effect on measured parameters in control or drug-exposed groups (data not shown). Mice were sacrificed after 7 days and the popliteal lymph node (PLN) was excised and separated from adherent fatty tissue. PLN were isolated in ice-cold complete RPMI and single-cell suspensions were prepared, washed ( $150 \times g$ ,  $4^\circ\text{C}$ ), resuspended in 1 ml of complete RPMI, counted using a Coulter counter (Coulter Electronics) and adjusted to  $1 \times 10^6$  cells/ml for ELISPOT and flow cytometry and to  $2.5 \times 10^6$  cells/ml for ex vivo restimulation. For immunohistology, PLN were snap-frozen in liquid nitrogen and stored at  $-20^\circ\text{C}$  until preparation of sections.

### ELISPOT assay

The ELISPOT assay was performed based on the operating procedure described by Schielen et al. (20). Immobilon-P membranes (Immobilon PVDF Transfer; Millipore) were coated overnight at  $4^\circ\text{C}$  with TNP-BSA (10  $\mu\text{g}/\text{ml}$ ) in PBS-Tween 20 (0.05%) and blocked for 1 h at room temperature with BSA (1%) in PBS-Tween 20. Membranes were washed and clamped in spot blocks (made in-house), and  $5 \times 10^5$  cells were centrifuged onto the membranes and incubated for 4 h at  $37^\circ\text{C}$ . Membranes were vigorously washed with PBS and PBS-Tween 20 to remove cells and debris and incubated overnight at  $4^\circ\text{C}$  with alkaline phosphatase-conjugated Abs (anti-IgM, -IgG1, -IgG2a, or -IgE) diluted in PBS-Tween 20 buffer. Membranes were washed and incubated with NBT/5-bromo-4-chloro-3-indolylphosphate toluidine salt reagent to visualize development of TNP-specific Ab spots. Spots were counted by two independent observers using a stereomicroscope.

### Flow cytometry

Cell subtypes and expression of costimulatory molecules were analyzed using flow cytometry. Cells (100  $\mu\text{l}$ ;  $1 \times 10^6/\text{ml}$ ) were centrifuged in 96-well plates, resuspended in PBS/0.05% BSA/0.1%  $\text{NaN}_3$ /3% normal mouse serum, and incubated with 50  $\mu\text{l}$  of FcBlock for 30 min at  $4^\circ\text{C}$ . Cells were washed and triple-stained by incubation with FITC-, PE-, and CY-conjugated mAbs (30 min in darkness,  $4^\circ\text{C}$ ). The following combinations of Abs were used: CD4/CD8/CD3, CD11c/F4/80/MHC class II, and CD80, CD86, CD54 in combination with CD19/MHC class II. Samples incubated with biotin-conjugated mAb were washed and incubated with streptavidin-CY in the same way. After final washes, cells were resuspended in formalin (0.1%) and stored until analysis on a FACScan with standard FACSFlow using CellQuest software (BD Biosciences). Cells expressing CD11c, F4/80, CD19, CD80, CD86, or CD54 were checked for MHC class II-expression to assure the Ag-presenting capability.

### Immunohistology

Cryostat sections (7  $\mu\text{m}$ ) were fixed in acetone and incubated with predetermined dilutions of Naphthol AS-BI phosphate and red-violet LB salt in acetate buffer for 30 min at  $37^\circ\text{C}$  to visualize macrophages. Sections were counterstained with Mayer's acid hematoxylin.

### Cell culture and cytokine measurement

Cell suspensions (150  $\mu\text{l}$  of  $2.5 \times 10^6$  cells/ml) were incubated overnight at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$ , with 50  $\mu\text{l}$  of medium, Con A (5  $\mu\text{g}/\text{ml}$ ), or LPS (2  $\mu\text{g}/\text{ml}$ ) in 96-well culture plates (Costar). After centrifugation for 10 min at  $150 \times g$ , supernatant was collected and stored at  $-20^\circ\text{C}$  until analysis. IFN- $\gamma$  and IL-4 were determined by sandwich ELISA. Highbond plates (Costar 3590) were coated overnight at  $4^\circ\text{C}$  with 1  $\mu\text{g}/\text{ml}$  rat anti-mouse IFN- $\gamma$  or 1  $\mu\text{g}/\text{ml}$  rat anti-mouse IL-4 in 0.05 M carbonate buffer (pH 9.6), washed with PBS-Tween 20, and blocked with PBS-Tween 20/casein for 4 h at room temperature. Samples, IL-4 and IFN- $\gamma$  standards were added in several dilutions and incubated overnight at  $4^\circ\text{C}$ . After washing, samples were incubated with 0.25  $\mu\text{g}/\text{ml}$  rat anti-mouse IFN- $\gamma$  or IL-4 conjugate diluted in PBS-Tween 20/casein for 1 h at room temperature. Plates were

washed and incubated with streptavidin-HRP (0.3  $\mu$ g/ml) diluted in PBS-Tween 20/casein for 45 min at room temperature. After the final washes, 3,3',5,5'-tetramethylbenzidine-substrate (0.1 mg/ml) was added and the color reaction was stopped after 10 min with 2 M  $H_2SO_4$ . Absorbance was measured at 450 nm. TNF- $\alpha$  ELISA was performed in accordance with the manufacturer's instructions.

### Statistics

Data are presented as the group mean  $\pm$  SEM. Statistical analyses were performed using independent-samples *t* test procedure. Multiple comparisons 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

### Drug-induced type 1 vs type 2 responses are differently affected in CD80/CD86- and CD28-CTLA-4-deficient mice

In previous experiments in WT mice, STZ and D-Pen were clearly characterized as type 1- and type 2-inducing drugs based on the formation of TNP-specific ASC isotypes and the production of cytokines (IL-4, IFN- $\gamma$ , and TNF- $\alpha$ ) (10).

Present results show that, in contrast to WT mice, CD80/CD86- and CD28/CTLA-4-deficient mice did not produce TNP-specific IgM and IgG1 ASC in response to D-Pen (Fig. 1A). After exposure to STZ, CD28/CTLA-4-deficient mice did not induce the formation of TNP-specific ASC, whereas CD80/CD86-deficient mice produced significantly increased TNP-specific IgG2a and IgM ASC numbers.

Immunohistochemical staining of PLN sections showed that STZ enhanced the influx of macrophages in all three strains of mice (Fig. 1B), which was confirmed by increased proportions of F4/80<sup>+</sup> cells as determined with flow cytometry (Table I). This increase was slightly lower in CD28/CTLA-4-deficient mice than in WT mice. In addition, exposure to STZ increased CD8<sup>+</sup> T cells and CD11c<sup>+</sup> cells in all strains (Table I). Similar results were found for production of IFN- $\gamma$  in supernatants of PLN cell cultures restimulated with Con A in vitro (data not shown).

### Effect of anti-CD80, anti-CD86, CTLA-4-Ig, or anti-CTLA-4 on Ab secretion and cytokine production in drug-induced type 1 vs type 2 responses in WT BALB/c mice

The dependence of D-Pen-stimulated type 2 responses on costimulatory molecules was in line with previous observations (10). However, the apparent lack of importance of CD80 and CD86 in case of STZ-induced immunostimulation is remarkable with regard to previous experiments showing that STZ clearly increased the expression of CD80 and CD86 in WT mice (43). In addition, the dispensability for CD80/CD86 but requirement for their functional ligands, CD28/CTLA-4, to produce TNP-specific IgG2a Abs in STZ-induced responses reveals an inconsistency that requires clarification.

To obtain more insight into the significance of CD80 vs CD86 and of CTLA-4 in STZ-induced immune responses in WT animals, CD80, CD86, and CTLA-4 were blocked with mAbs (anti-CD80 and/or anti-CD86, or anti-CTLA-4), or with the CD80/CD86 binding CTLA-4-Ig fusion protein. In addition, a set of drug-treated animals was treated with an anti-CTLA-4 mAb. For comparison, similar treatments were performed with D-Pen-stimulated mice. The responses in drug-treated mice that were additionally injected (i.p.) with isotypes of the administered mAbs or fusion protein were comparable to those in drug-treated mice that received PBS alone.

In D-Pen-exposed mice, anti-CD86 and particularly the combination of anti-CD80 and anti-CD86, or CTLA-4-Ig, reduced the formation of TNP-specific IgM and IgG1 ASC (Fig. 2) and IL-4 production (Fig. 3). In this type 2 response, the effect of CD80 was mainly supplementary to CD86 because anti-CD80 alone had no effect on IgM and IgG1 ASC numbers and had only a minor effect on IL-4 production. Treatment with anti-CTLA-4 profoundly increased the numbers of TNP-specific IgE ASC and IL-4 production, but did not alter D-Pen-induced production of IgM and IgG1. The production of IFN- $\gamma$  in D-Pen-induced responses, which was 700 times as low as in the case of STZ, was only significantly

**FIGURE 1.** TNP-specific ASC numbers and macrophages staining in PLN from WT, CD80/CD86-deficient (KO1), and CD28/CTLA-4-deficient mice (KO2). PLN were isolated 7 days after s.c. injection in the footpad with either 10  $\mu$ g of TNP-OVA alone (Control) or in combination with 1 mg of D-Pen or STZ. A, IgM, IgG1, and IgG2a ASC were measured by TNP-specific ELISPOT. Bars show representative data of two experiments with four to six mice per group. Levels are expressed as the group mean  $\pm$  SEM. \*, Significant (*p* < 0.05) differences compared with controls. B, Cryostat PLN sections (7  $\mu$ m) from WT BALB/c, CD80/CD86-deficient (KO1) and CD28/CTLA-4-deficient (KO2) mice stained for the presence of macrophages (Naphthol AS-BI phosphate and red-violet LB salt). STZ exposure caused an elevated number of macrophages in both WT and KO (stained dark).

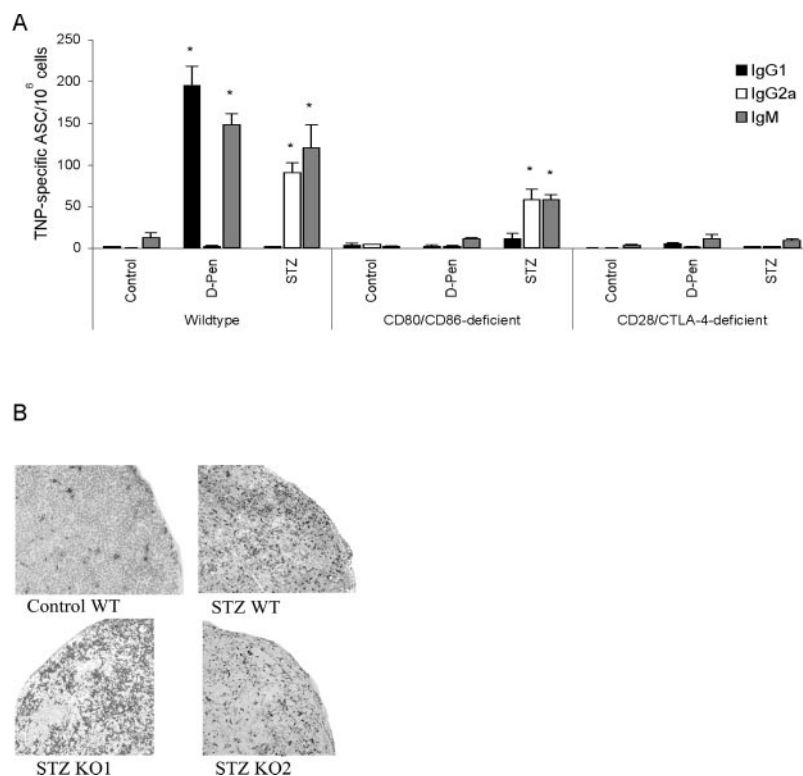


Table I. *Cell subtypes of PLN in WT, CD80/CD86- (KO1), and CD28/CTLA-4-deficient (KO2) mice*

| Strain | Treatment | Total Cells<br>(*1 × 10 <sup>6</sup> ) | CD4 <sup>+</sup> T Cells                                | CD8 <sup>+</sup> T Cells                                | B Cells                                               | CD11c <sup>+</sup>                                      | Macrophages                                           |
|--------|-----------|----------------------------------------|---------------------------------------------------------|---------------------------------------------------------|-------------------------------------------------------|---------------------------------------------------------|-------------------------------------------------------|
| WT     | Control   | (1.61 ± 0.61)                          | 52.6 ± 0.9 <sup>a</sup><br>(0.85 ± 0.35)                | 18.6 ± 0.3<br>(0.30 ± 0.06)                             | 26.1 ± 0.8<br>(0.44 ± 0.04)                           | 1.3 ± 0.1<br>(0.02 ± 0.01)                              | 1.4 ± 0.1<br>(0.02 ± 0.01)                            |
|        | STZ       | (4.38 ± 2.16) <sup>b</sup>             | 39.4 ± 3.8 <sup>b</sup><br>(1.60 ± 0.60)                | 23.4 ± 0.5 <sup>b</sup><br>(1.09 ± 0.10) <sup>b</sup>   | 26.5 ± 2.7 <sup>b</sup><br>(1.24 ± 0.07) <sup>b</sup> | 13.9 ± 0.4 <sup>b</sup><br>(0.53 ± 0.03) <sup>b</sup>   | 12.7 ± 1.5 <sup>b</sup><br>(0.50 ± 0.08) <sup>b</sup> |
| KO1    | Control   | (1.60 ± 0.65)                          | 54.4 ± 3.5<br>(0.95 ± 0.24)                             | 15.4 ± 0.7<br>(0.26 ± 0.06) <sup>c</sup>                | 23.9 ± 1.2<br>(0.31 ± 0.06) <sup>c</sup>              | 1.4 ± 0.3<br>(0.03 ± 0.01)                              | 1.7 ± 0.4<br>(0.03 ± 0.01)                            |
|        | STZ       | (4.11 ± 0.66) <sup>P</sup>             | 18.5 ± 1.7 <sup>b,c</sup><br>(0.77 ± 0.15) <sup>c</sup> | 33.6 ± 3.7 <sup>b,c</sup><br>(1.30 ± 0.16) <sup>b</sup> | 34.5 ± 1.7 <sup>b</sup><br>(1.44 ± 0.19)              | 21.1 ± 1.6 <sup>b,c</sup><br>(0.88 ± 0.18) <sup>b</sup> | 9.1 ± 1.1 <sup>b</sup><br>(0.37 ± 0.10) <sup>b</sup>  |
| KO2    | Control   | (0.85 ± 0.25)                          | 42.9 ± 0.7 <sup>c</sup><br>(0.38 ± 0.07) <sup>c</sup>   | 12.8 ± 0.5 <sup>c</sup><br>(0.10 ± 0.02) <sup>c</sup>   | 39.7 ± 0.8 <sup>c</sup><br>(0.34 ± 0.11) <sup>c</sup> | 1.2 ± 0.4<br>(0.01 ± 0.01)                              | 1.7 ± 0.5<br>(0.02 ± 0.01)                            |
|        | STZ       | (2.25 ± 0.22) <sup>b</sup>             | 40.5 ± 5.0<br>(0.78 ± 0.10) <sup>b</sup>                | 24.1 ± 1.6 <sup>b</sup><br>(0.45 ± 0.09) <sup>b,c</sup> | 30.6 ± 4.7<br>(0.72 ± 0.09) <sup>b,c</sup>            | 13.4 ± 2.6 <sup>b</sup><br>(0.25 ± 0.05) <sup>b,c</sup> | 6.7 ± 2.6<br>(0.13 ± 0.03) <sup>b,c</sup>             |

<sup>a</sup> Levels are expressed as mean percentage and absolute numbers (\*1 × 10<sup>6</sup>; between parentheses) ± SEM.

<sup>b</sup> Statistically significant (*p* < 0.05) difference with strain control.

<sup>c</sup> Statistically significant difference with WT group.

inhibited by the combination of anti-CD80 and anti-CD86 or by CTLA-4-Ig.

In concordance with findings in CD80/CD86-deficient mice, the STZ-induced type 1 responses were still operational in the presence of anti-CD80 and anti-CD86 mAbs. Remarkably, STZ-induced IgM and IgG2a ASC numbers (Fig. 2), as well as the production of IFN-γ and particularly TNF-α (Fig. 3) were even further increased by simultaneous treatment with anti-CD80 and anti-CD86. These parameters were not significantly affected by treatment with anti-CD80 (with the exception of TNF-α) or anti-CTLA-4 alone, whereas anti-CD86 had a significant stimulatory effect on IgM and TNF-α production. The production of IL-4 was inhibited by anti-CD86 alone or in combination with anti-CD80, or by CTLA-4-Ig, whereas IL-4 levels were increased by anti-CTLA-4. In contrast to the results that were obtained with the combined treatment of anti-CD80 and anti-CD86, CTLA-4-Ig totally abrogated the production of TNP-specific Abs and of IFN-γ and TNF-α.

#### *Effect of anti-CD80, anti-CD86, CTLA-4-Ig, or anti-CTLA-4 on shifts in cell subtypes in drug-induced type 1 vs type 2 responses*

Previously, we have shown (10) that, in the present assay, STZ activates CD11c<sup>+</sup> DC and CD8<sup>+</sup> T cells and that D-Pen causes activation of B cells. In the present study, macrophages and CD8<sup>+</sup> T cells were also stimulated in both KO strains. Therefore, we also assessed the effect of mAb treatment on drug-induced shifts in T and B lymphocytes, macrophages, and DC in WT mice.

The D-Pen-induced increases in B cell percentages and concomitant decreases in CD4<sup>+</sup> and CD8<sup>+</sup> T cell proportions were prevented by anti-CD86 and particularly by the combination of anti-CD80/CD86, or by CTLA-4-Ig (Table II). Anti-CTLA-4 further enhanced the D-Pen-induced increases in B cell and CD11c<sup>+</sup> cell proportions and reduction in CD8<sup>+</sup> T cells. Additionally, anti-CTLA-4 significantly increased the expression of CD86 and CD54 on B cells (data not shown).

In case of STZ, anti-CD80 and/or anti-CD86 further decreased CD4<sup>+</sup> T cell percentages and increased B cell and macrophage proportions, but did not change CD8<sup>+</sup> T or CD11c<sup>+</sup> cell proportions. Again, CTLA-4-Ig prevented any shift in PLN cell proportions when mice were exposed to STZ. Anti-CTLA-4 did not affect STZ-induced shifts in lymphocytes and DC or expressions of CD80, CD86, and CD54.

#### *CTLA-4-Ig requires signaling via CD80 or CD86 to inhibit STZ-induced responses*

Present results show that CTLA-4-Ig blocks the STZ-induced responses, whereas anti-CD80 and anti-CD86 seem to stimulate these responses. To test whether the inhibitory effect of CTLA-4-Ig is restricted to ligation of the protein to CD80 and CD86, CD80/CD86-deficient mice were exposed to STZ and were additionally treated with CTLA-4-Ig.

In contrast to the observations in WT STZ-exposed mice, CTLA-4-Ig did not inhibit the formation of TNP-specific ASC (Fig. 4A), the increases in the numbers of CD8<sup>+</sup> cells and macrophages (Fig. 4B), or the production of IFN-γ (Fig. 4C).

## Discussion

The interference with costimulatory interactions has been recognized as a tool to modulate autoimmune reactions and may also be of interest to prevent or treat IDHR. However, the effectiveness of such treatments appears to be dependent on the type of the induced immune responses (11–14). In the present studies, we show that CD28/CTLA-4-CD80/CD86 costimulatory interactions differently regulate drug-induced type 1 and type 2 responses to an identical bystander Ag in the same mouse strain. Type 2 responses were completely dependent on, and intensively regulated by this costimulatory pathway, whereas type 1 responses still developed in the absence of CD80/CD86 costimulatory signaling. In addition, it is demonstrated that anti-CD80/anti-CD86 mAb and CTLA-4-Ig fusion protein have a totally different effect on STZ-induced type 1 responses.

Our data obtained with mAb and KO mice indicate that CD80 and CD86 are indispensable for the development of type 2 responses after exposure to D-Pen. We have previously shown that the antirheumatic drug D-Pen rapidly stimulates the expression of CD40 and CD86, but not CD80, on APC (43). Here we show that CD86 is indeed the most effective B7 molecule in these responses, but also that anti-CD80 substantially intensifies the inhibitory effect of anti-CD86. This modulatory effect of CD80 on CD86-signaling is in agreement with a number of studies indicating that CD80 is functionally linked to CD86 (21–24).

In addition, our data show that anti-CTLA-4 profoundly increased D-Pen-induced production of IL-4 and IgE, confirming that CTLA-4 strongly regulates the development of Th2 cells (25, 26). Regulation of D-Pen-induced type 2 responses by CTLA-4 is

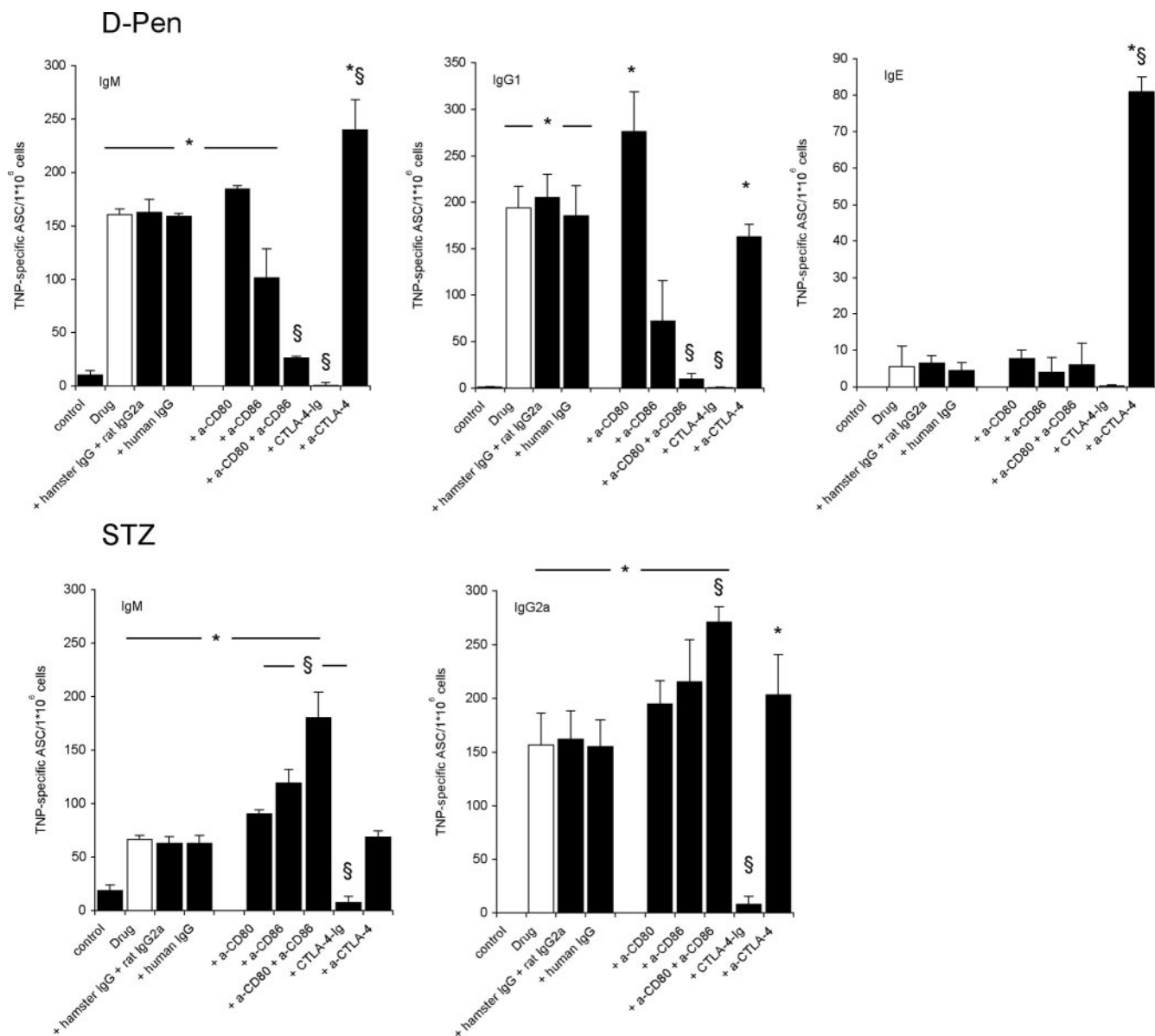


**FIGURE 2.** Effect of anti (a)-CD80, anti-CD86, CTLA-4-Ig, or anti-CTLA-4 on TNP-specific IgM, IgG1, IgE, and IgG2a ASC numbers of WT BALB/c. Mice were s.c. exposed in the hind footpad with either 10  $\mu$ g of TNP-OVA alone or in combination with 1 mg of D-Pen or STZ. Indicated groups received blocking anti-CD80, anti-CD86, a combination of both mAbs, anti-CTLA-4, or CTLA-4-Ig fusion protein. Treatment of mice with the appropriate Ab isotype controls had no effect on drug-induced responses. After 7 days, cells were isolated and ASC were detected by ELISPOT. Levels are expressed as group means  $\pm$  SEM of four to six mice per group. \*, Significant differences ( $p < 0.05$ ) from control. §, Significant differences from the drug-exposed mice without mAb treatment.

mediated by a low expression of these molecules (43). This can be explained by the formation of stable multimeric complexes consisting of one CTLA-4 homodimer bound to two B7 molecules (27). In addition, CD80 (28), but not CD86, (29) has two binding sites for CTLA-4, enabling cross ligation. Moreover, CTLA-4 has a much higher avidity for B7 molecules than CD28 (30, 31).

Apparently, CTLA-4 is capable of efficiently regulating drug-induced type 2 immune responses, which may contribute to the understanding of drug allergic responses in man. Notably, type 2 immune responses, including increases in IgE levels have been associated with adverse drug-induced clinical phenomena (32) and moreover, polymorphisms of CTLA-4 have been linked to the intensity of allergic (33, 34) and autoimmune manifestations (35). Altogether, people with particular CTLA-4 genotypes may be genetically predisposed for the development of IDHR-associated clinical effects.

These findings together with our previous data showing the requirement of CD154 (10), suggest that D-Pen-conditioned stimulation of type 2 responses to TNP-OVA depends on classical APC-Th2 interactions that are intensively regulated by costimulation for its effects.

Intriguingly, our experiments with STZ revealed a totally different involvement for CD28/CTLA-4-CD80/CD86 costimulatory interactions in type 1 responses to TNP-OVA. Data from KO mice showed that STZ-induced activation of CD8<sup>+</sup> cells, macrophages, and production of IFN- $\gamma$  is independent of CD80/CD86 and CD28/CTLA-4, whereas the formation of ASC was only present in CD80/CD86-deficient mice and not in CD28/CTLA-4 KO. This dispensability for costimulation is remarkable as CD80, CD86, and CTLA-4 are all readily up-regulated after STZ exposure (43). Moreover, data obtained with WT mice that were treated with mAb show that anti-CD80/CD86 that were efficient in inhibiting

**FIGURE 3.** Cytokine concentrations in supernatant of in vitro restimulated PLN cells. WT BALB/c mice were injected s.c. in the hind footpad with either 10  $\mu$ g of TNP-OVA alone or in combination with 1 mg of D-Pen or STZ. Indicated groups received blocking anti (a)-CD80, anti-CD86, a combination of both mAbs, anti-CTLA-4, or CTLA-4-Ig fusion protein. Treatment of mice with the appropriate Ab isotypes had no effect on drug-induced responses. After 7 days, mice were sacrificed and PLN cells were cultured with Con A (IFN- $\gamma$  and IL-4) or LPS (TNF- $\alpha$ ). Levels are expressed as the group mean  $\pm$  SEM of four to six mice per group. \*, Significant differences ( $p < 0.05$ ) from control. §, Significant differences from the drug-exposed mice without mAb treatment.

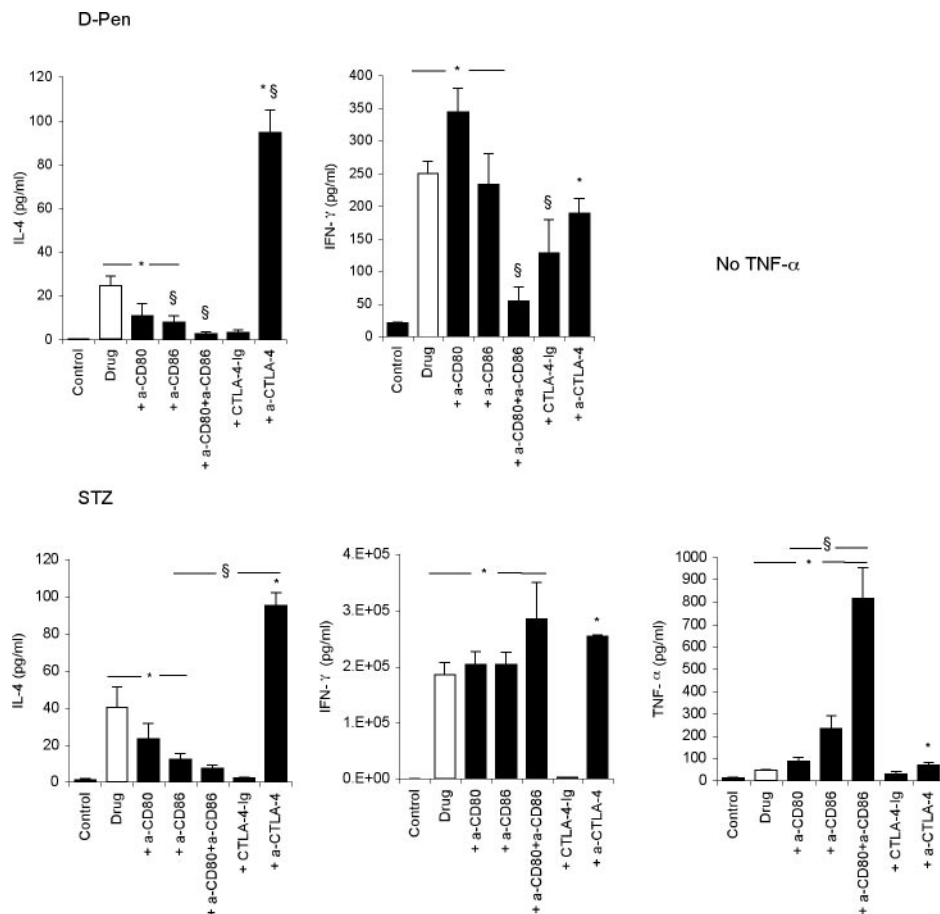


Table II. Cell subtypes of PLN

| Treatment               | Total Cells<br>(*1 × 10 <sup>6</sup> ) | CD4 <sup>+</sup> T Cells                                | CD8 <sup>+</sup> T Cells                                | B Cells                                                   | DC                                                        | Macrophages                                              |
|-------------------------|----------------------------------------|---------------------------------------------------------|---------------------------------------------------------|-----------------------------------------------------------|-----------------------------------------------------------|----------------------------------------------------------|
| Control                 | (1.63 ± 0.21)                          | 47.3 ± 0.8 <sup>a</sup><br>(0.82 ± 0.17)                | 16.6 ± 0.4<br>(0.27 ± 0.06)                             | 29.4 ± 0.7<br>(0.48 ± 0.12)                               | 3.4 ± 0.4<br>(0.05 ± 0.01)                                | 3.1 ± 0.3<br>(0.05 ± 0.01)                               |
| D-Pen                   | (12.66 ± 0.91) <sup>b</sup>            | 33.3 ± 2.3 <sup>b</sup><br>(4.17 ± 0.22) <sup>b</sup>   | 10.8 ± 1.0 <sup>b</sup><br>(1.34 ± 0.06) <sup>b</sup>   | 50.9 ± 3.6 <sup>b</sup><br>(6.51 ± 0.89) <sup>b</sup>     | 5.6 ± 0.5 <sup>b</sup><br>(0.71 ± 0.09) <sup>b</sup>      | 5.5 ± 0.4 <sup>b</sup><br>(0.70 ± 0.09) <sup>b</sup>     |
| + Anti-CD80             | (8.74 ± 1.52) <sup>b</sup>             | 32.6 ± 3.3 <sup>b</sup><br>(2.95 ± 0.64)                | 10.2 ± 1.3 <sup>b</sup><br>(0.91 ± 0.20)                | 51.0 ± 3.8 <sup>b</sup><br>(4.40 ± 0.77) <sup>b</sup>     | 6.0 ± 1.1<br>(0.57 ± 0.18) <sup>b</sup>                   | 7.2 ± 1.1<br>(0.65 ± 0.15) <sup>b</sup>                  |
| + Anti-CD86             | (9.17 ± 1.48) <sup>b</sup>             | 40.0 ± 1.2 <sup>b,c</sup><br>(3.68 ± 0.65) <sup>b</sup> | 13.6 ± 0.3<br>(1.25 ± 0.23) <sup>b</sup>                | 40.6 ± 1.3 <sup>b,c</sup><br>(3.71 ± 0.54) <sup>b,c</sup> | 5.4 ± 0.9<br>(0.54 ± 0.18)                                | 3.8 ± 0.7<br>(0.38 ± 0.13) <sup>c</sup>                  |
| + Anti-CD80 + anti-CD86 | (5.67 ± 1.25) <sup>b,c</sup>           | 44.9 ± 2.0 <sup>c</sup><br>(2.50 ± 0.49) <sup>c</sup>   | 16.0 ± 0.5<br>(0.90 ± 0.19)                             | 31.7 ± 3.9<br>(1.84 ± 0.55) <sup>c</sup>                  | 4.6 ± 0.3<br>(0.27 ± 0.07) <sup>c</sup>                   | 3.6 ± 0.3<br>(0.20 ± 0.04) <sup>c</sup>                  |
| + CTLA-4-Ig             | (4.69 ± 1.56)                          | 46.0 ± 2.2 <sup>c</sup><br>(2.49 ± 0.77)                | 16.0 ± 1.6 <sup>c</sup><br>(0.86 ± 0.24)                | 31.7 ± 2.1 <sup>c</sup><br>(1.76 ± 0.75) <sup>c</sup>     | 4.1 ± 1.9<br>(0.14 ± 0.07) <sup>c</sup>                   | 1.6 ± 0.5 <sup>c</sup><br>(0.09 ± 0.03) <sup>c</sup>     |
| + Anti-CTLA-4           | (15.69 ± 1.73) <sup>b</sup>            | 31.1 ± 1.2 <sup>b</sup><br>(4.93 ± 0.67) <sup>b</sup>   | 7.7 ± 0.3 <sup>b</sup><br>(1.23 ± 0.16) <sup>b</sup>    | 55.2 ± 1.8 <sup>b</sup><br>(8.60 ± 0.82) <sup>b</sup>     | 11.1 ± 0.7 <sup>b,c</sup><br>(1.73 ± 0.21) <sup>b,c</sup> | 9.5 ± 0.5 <sup>b,c</sup><br>(1.49 ± 0.17) <sup>b,c</sup> |
| STZ                     | (4.52 ± 0.89) <sup>b</sup>             | 39.9 ± 1.0 <sup>b</sup><br>(1.96 ± 0.44)                | 30.0 ± 2.6 <sup>b</sup><br>(1.30 ± 0.18) <sup>b</sup>   | 24.7 ± 2.0<br>(1.16 ± 0.28)                               | 21.4 ± 4.2 <sup>b</sup><br>(0.88 ± 0.12) <sup>b</sup>     | 13.9 ± 2.5 <sup>b</sup><br>(0.57 ± 0.06) <sup>b</sup>    |
| + Anti-CD80             | (7.52 ± 1.65) <sup>b</sup>             | 34.3 ± 3.2<br>(2.67 ± 0.84)                             | 26.0 ± 0.6 <sup>b</sup><br>(1.91 ± 0.40) <sup>b</sup>   | 32.0 ± 1.8 <sup>b</sup><br>(2.30 ± 0.41) <sup>b</sup>     | 16.4 ± 1.7 <sup>b</sup><br>(1.14 ± 0.14) <sup>b</sup>     | 10.3 ± 0.7 <sup>b</sup><br>(0.73 ± 0.11) <sup>b</sup>    |
| + Anti-CD86             | (8.47 ± 1.28) <sup>b</sup>             | 24.8 ± 2.7 <sup>b,c</sup><br>(2.13 ± 0.42)              | 27.8 ± 1.0 <sup>b</sup><br>(2.37 ± 0.42) <sup>b</sup>   | 38.2 ± 1.7 <sup>b,c</sup><br>(3.21 ± 0.43) <sup>b,c</sup> | 18.7 ± 1.4 <sup>b</sup><br>(1.59 ± 0.30) <sup>b</sup>     | 11.2 ± 0.8 <sup>b</sup><br>(0.95 ± 0.17) <sup>b</sup>    |
| + Anti-CD80 + anti-CD86 | (12.04 ± 1.53) <sup>b</sup>            | 29.7 ± 6.0 <sup>b</sup><br>(3.36 ± 0.37) <sup>b</sup>   | 24.7 ± 2.9 <sup>b</sup><br>(2.89 ± 0.35) <sup>b,c</sup> | 35.4 ± 5.2<br>(4.49 ± 1.07) <sup>b,c</sup>                | 17.8 ± 1.4 <sup>b</sup><br>(1.77 ± 0.59) <sup>b,c</sup>   | 13.3 ± 2.6 <sup>b</sup><br>(1.30 ± 0.47) <sup>b,c</sup>  |
| + CTLA4-Ig              | (4.98 ± 0.87) <sup>b</sup>             | 52.1 ± 6.9 <sup>c</sup><br>(2.66 ± 0.64)                | 17.6 ± 1.3 <sup>c</sup><br>(0.91 ± 0.27)                | 23.7 ± 7.0<br>(1.25 ± 0.53)                               | 2.4 ± 0.5 <sup>c</sup><br>(0.23 ± 0.10) <sup>c</sup>      | 2.8 ± 0.5 <sup>c</sup><br>(0.27 ± 0.40) <sup>c</sup>     |
| + Anti-CTLA-4           | (4.12 ± 0.49) <sup>b</sup>             | 31.1 ± 2.8 <sup>b</sup><br>(1.28 ± 0.17)                | 32.1 ± 1.1 <sup>b</sup><br>(1.31 ± 0.13) <sup>b</sup>   | 26.4 ± 3.0<br>(1.12 ± 0.24)                               | 29.5 ± 1.8 <sup>b</sup><br>(1.19 ± 0.09) <sup>b</sup>     | 20.4 ± 1.3 <sup>b</sup><br>(0.83 ± 0.08) <sup>b,c</sup>  |

<sup>a</sup> Levels are expressed as mean percentage and absolute numbers (\*1 × 10<sup>6</sup>; between parentheses) ± SEM.

<sup>b</sup> Statistically significant ( $p < 0.05$ ) difference with controls.

<sup>c</sup> Statistically significant difference with drug-treated group.

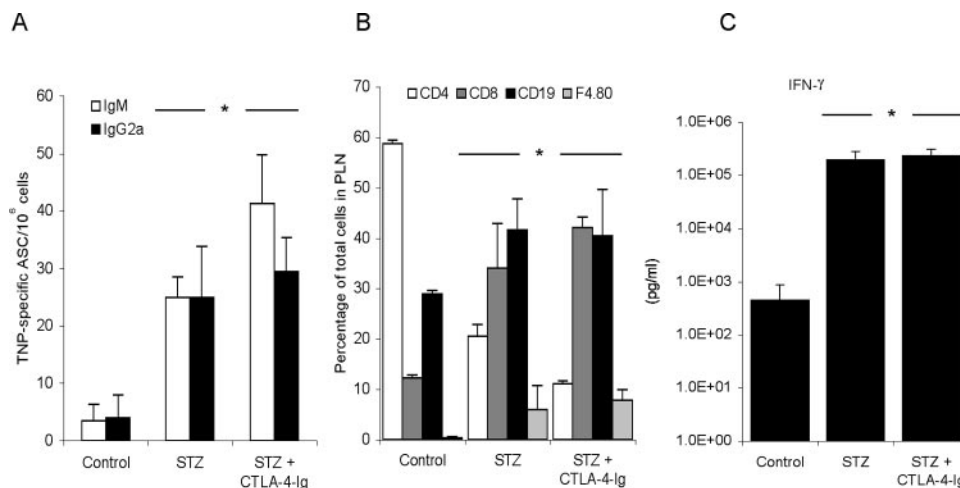


**FIGURE 4.** STZ-induced immune responses in WT and CD80-CD86-deficient mice after treatment with CTLA-4-Ig. WT BALB/c and CD80/CD86-deficient mice were injected s.c. in the hind footpad with either 10  $\mu$ g of TNP-OVA alone or in combination with 1 mg of STZ. Indicated groups received 200  $\mu$ g of CTLA-4-Ig fusion protein. After 7 days, mice were sacrificed and PLN cells were analyzed for TNP-specific ASC formation, cytokine production, and secretion of IFN- $\gamma$  and IL-4 (in Con A-restimulated cells) or TNF- $\alpha$  (in LPS-restimulated cells). Levels are expressed as the group mean  $\pm$  SEM of five mice per group. \*, Significant differences ( $p < 0.05$ ) from control. Note that IFN- $\gamma$  levels are expressed on a log scale.

D-Pen-induced type 2 phenomena, further stimulated STZ-induced type 1 immune responses (IgG2a, IFN- $\gamma$ , and TNF- $\alpha$  production).

Together, these data suggest that STZ-induced responses do not require stimulation via CD80 or CD86, but that these molecules may be involved in down-regulating the response. Such a down-regulatory effect of these molecules has been observed previously in murine models of autoimmune disease (13, 14, 36), but in those studies, the expression of CTLA-4 and functionality of CTLA-4 ligation was not addressed. Using the RA-PLNA, STZ was indeed shown to profoundly increase the expression of CTLA-4 (43). As such, mAbs to CD80 and CD86 may hinder the binding of CTLA-4 to the respective molecules. Since CD86, and even more so CD80, bind with a much higher affinity to CTLA-4 than to CD28 (37), anti-CD80, and/or anti-CD86 might abrogate CTLA-4-mediated inhibitory signals and thus further stimulate the STZ-induced response. In agreement, anti-CTLA-4 slightly, but not significantly, increased type 1 phenomena (IgG2a, IFN- $\gamma$ , macrophages).

CTLA-4-Ig had a strong inhibitory effect on all STZ-induced responses, and that effect was clearly dependent on the presence of CD80 and CD86 (Fig. 4). These results are in contrast with those obtained with blocking mAb and may be explained by interactions of CTLA-4-Ig with B7, which has previously been shown to induce the catabolism of tryptophan through the activation of IDO in macrophages and DC (38, 39). Depletion of tryptophan and increase in proapoptotic kynurenines inhibit the activation of T cells, especially Th1 cells, and B cells (38).

Among the drug-induced immune responses, the antineoplastic and experimental diabetogen STZ is a clear exception because unlike most drugs it induces a type 1 response, characterized by a strong activation of macrophages and CD8<sup>+</sup> T cells. STZ-induced stimulation of immune responses is less dependent on costimulation through the CD28/CTLA-4-CD80/CD86 pathway, possibly due to its strong proinflammatory and macrophage- and CD8<sup>+</sup> T cell-stimulating effects. Cytokines and chemokines produced by these cells may influence Ab responses without the need for additional costimulatory signals. In this context, it is interesting to note that naive CD8<sup>+</sup> T cells require costimulation only when the level of Ag is low (40).

STZ induced the formation of TNP-specific ASC in CD80/CD86-deficient mice, whereas CD28/CTLA-4-deficient mice

showed all characteristics of type 1 responses except the formation of ASC. The reason for this is unknown. Previously, it has been hypothesized that there may be a third, yet unidentified costimulatory molecule, which may be capable of binding to CD28/CTLA-4 but is different from CD80 and CD86 (41, 42). So far, we were unable to show the existence of an alternative molecule in the case of STZ-induced immune responses. In addition, CTLA-4-Ig fails to modulate STZ-induced responses in CD80/CD86-deficient mice, which does not support this hypothesis.

In conclusion, this study indicates the importance of costimulatory interactions in drug-induced adverse immune responses. The immune responses created by D-Pen exposure are totally dependent on the CD28/CTLA-4-CD80/CD86 pathway and results suggest that CD86 might suffice to sustain a type 2 response leaving only an additional role for CD80. Because most drug hypersensitivity responses have characteristics of type 2 immune responses (high IgE, nonorgan-specific lupus-like symptoms (9)), costimulation may be a general immunoregulatory process in these adverse drug reactions. Thus, present findings may have therapeutic implications for treatment of serious drug hypersensitivity responses in man. In case of STZ, it can be inferred that CD80 and CD86 are not important for the activation of immune responses, but that they may play a regulatory role to control nonproductive type 1 responses induced by STZ. The surprising immunological mechanisms of these responses may contribute to the understanding of immune responses in animal models of STZ-induced diabetes. Increased knowledge on the modulatory effects of costimulatory molecules in different types of immune responses may eventually help to predict, prevent, or treat drug-induced allergies and autoimmunity.

## Disclosures

The authors have no financial conflict of interest.

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