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Infectious Nickel Tolerance: A Reciprocal Interplay of Tolerogenic APCs and T Suppressor Cells That Is Driven by Immunization

Karin Roelofs-Haarhuis,* Xianzhu Wu,* Michael Nowak,* Min Fang,* Suzan Artik,† and Ernst Gleichmann2*

Previously, we reported that tolerance to nickel, induced by oral administration of Ni2+ ions, can be adoptively transferred to naïve mice with only 10^2 splenic T cells. Here we show that 10^2 T cell-depleted spleen cells (i.e., APCs) from orally tolerized donors can also transfer nickel tolerance. This cannot be explained by simple passive transfer of the tolerogen. The APCs from orally tolerized donors displayed a reduced allostimulatory capacity, a tolerogenic phenotype, and an increased expression of CD38 on B cells. In fact, it was B cells among the APCs that carried the thrust of tolerogenicity. Through serial adoptive transfers with tolerized donors displayed a reduced allostimulatory capacity, a tolerogenic phenotype, and an increased expression of CD38 on B cells. In fact, it was B cells among the APCs that carried the thrust of tolerogenicity. Through serial adoptive transfers with Ly5.1+ donors and two successive sets of Ly5.2+ recipients, we demonstrated that nickel tolerance was infectiously spread from donor to host cells. After the transfer of either T cells or APCs from orally tolerized donors, the spread of tolerance to the opposite cell type of the recipients (i.e., APCs and T cells, respectively) required recipient immunization with NiCl2/H2O2. For the spread of tolerance from a given donor cell type, T cell or APC, to the homologous host cell type, the respective opposite cell type in the host was required as intermediate. We conclude that T suppressor cells and tolerogenic APCs induced by oral administration of nickel are part of a positive feedback loop that can enhance and maintain tolerance when activated by Ag associated with a danger signal. Under these conditions, APCs and T suppressor effector cells infectiously spread the tolerance to naïve T cells and APCs, respectively. The Journal of Immunology, 2003, 171: 2863–2872.

A hallmark of peripheral T cell tolerance is its dominance. The mechanisms underlying this phenomenon can be particularly well investigated by adoptively transferring T cells from tolerant donor animals into naïve recipients, which thereafter become unresponsive to immunization with the specific Ag studied. The term “infectious tolerance” has been coined for this phenomenon and denotes the ability of T cells from a tolerant donor to spread the unresponsiveness to Ag-specific T cells in naïve recipients (1). A formal demonstration that the property of specific unresponsiveness, which was acquired by the T suppressor (Ts)3 cells of the tolerized donor, indeed was transmitted to the T cells of recipient mice has come from adoptive transfer experiments that used genetic markers to distinguish donor T cells from host T cells (2–4). One study performed 25 years ago (5) reported that macrophages can act as intermediates in the passage of suppressor signals between T cell subsets, but a more detailed analysis of the mechanisms involved remains incomplete.

More recently, a number of studies performed in vitro indicated that, indeed, APCs can mediate the contagious dissemination of T cell unresponsiveness (6–9). For instance, in one model it was demonstrated that T cells tolerant for peptide A could spread the tolerance to other T cells specific for peptide A in the culture, but only if the latter recognized the peptide on the same APC as the “infectious” T cells (6). In fact, the authors specified a clear-cut sequence of events: after the first set of T cells had acquired tolerance, they were “infectious” in that they rendered the APCs in the culture tolerogenic. In other words, the Ts cells endowed APCs with the capacity to tolerize new sets of T cells added to the culture. Similarly, unresponsive T cell clones, which suppressed proliferation of responsive T cells specific for the same alloantigen, required the presence of dendritic cells (DCs) in the culture for the suppression to be mediated and to become effective (7, 8). There is now general agreement that Ag presentation by DCs, which are not fully competent or mature, fails to sensitize T cells for immunity and induces T cell tolerance instead (Refs. 10–12 and reviewed in Ref. 13). Taken together, these findings indicate that there is a reciprocal interplay of unresponsive, suppressive T cells and tolerogenic APCs that accounts for the spread of and hence the dominance of tolerance detectable in these in vitro systems. It should be noted, however, that some authors reported a model of infectious tolerance in vitro that is not dependent upon APCs, but is based on direct T-T cell interactions (14, 15). With regard to the spread of T cell tolerance in vivo, the question remains whether this is mediated by direct T-T interactions or by a reciprocal interplay of APCs and Ts cells.

We have developed a mouse model of contact hypersensitivity to nickel, one of the most common contact allergens in humans. Our results indicated that intradermal administration of Ni2+ ions, as present in NiCl2 and NiSO4, induces de novo immunization when coadministered with H2O2, a relevant, endogenous adjuvant.

[52x528]Ly5.1
[90x404]the Heinrich Heine University by the Deutsche Forschungsgemeinschaft and Nickel Producers Environmental Research Association (Research Triangle Park, NC). M.F. is supported by a fellowship from the European Postgraduate College in Food Toxicology that was granted to the Heinrich Heine University by the Deutsche Forschungsgemeinschaft.

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3 Abbreviations used in this paper: Ts, T suppressor; DC, dendritic cell; DNFB, 2,4-dinitrofluorobenzene; MHC-II, MHC class II; CD40L, CD40 ligand; ACAID, anterior chamber-associated immune deviation.

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(16). In contrast, administration of NiCl₂ alone, given orally or i.p., induced a long-lasting tolerance toward nickel, which could be further adoptively transferred into naïve recipients with as few as 10² bulk T cells. The splenic T cells possessing this ability were anergic in the presence of nickel ions and consisted of both CD4⁺ and CD8⁺ T cells. To explain how a minute number of anergic donor T cells could suppress the numerous nickel-reactive T cells in naïve recipients, we postulated that infectious tolerance could be involved (17). In the present investigation, genetic cell markers were used to demonstrate that infectious tolerance is indeed the amplification mechanism accounting for the spread of nickel unresponsiveness in vivo. Nickel-specific Ts cells and tolerogenic APCs were found to interact in this process, but only if they were rescued from their state of inertia by immunization with NiCl₂ in conjunction with H₂O₂. The APCs used were T cell-depleted spleen cells and, among these, B cells carried the thrust of tolerogenicity.

Materials and Methods

Mice
Specific pathogen-free female C57BL/6J (H-2b) mice, which express Ly5.2 (CD45.2), were obtained from Janvier (Le Genest St. Isle, France). Congenic Ly5.1⁺ (CD45.1⁺) C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were kept in accordance with the animal husbandry as described in the previous papers (16, 17). They were 6–8 wk of age at the onset of experiments.

Reagents

NiCl₂·6H₂O (henceforth referred to as NiCl₂) and 2,4-dinitrofluorobenzene (DNFB) were purchased from Sigma-Aldrich (Steinheim, Germany), and hydrogen peroxide (H₂O₂) was obtained from E. Merck (Darmstadt, Germany). Streptavidin-PerCP and Streptavidin-APC were obtained from BD PharMingen (Heidelberg, Germany).

Antibodies

The following anti-mouse mAbs were purchased from BD PharMingen: APC-labeled anti-CD3e (Clone 145-2C11), PerCP-labeled anti-CD4 (Clone RM4-5), PE-labeled anti-CD8β2 (Clone 53-5-6), FITC- and biotin-labeled anti-CD11c (Clone HL3), FITC-labeled anti-CD19 (Clone 1D3), biotin-labeled anti-B220 (Clone B6H12), FITC-labeled anti-CD40 (Clone HM40-3), PE-labeled anti-CD45.1 (Clone A20), FITC-labeled anti-CD45.2 (Clone 104), biotin-labeled anti-CD80 (Clone 16-10A1), biotin-labeled anti-CD86 (Clone GL1), FITC-labeled anti-I-A⁻ (Clone AF6-120.1), PE-labeled anti-I-A⁻/I-E⁻ (Clone M5/114.15.2), and FITC-labeled anti-TCR-β-chain (Clone H57-597). FITC-labeled anti-DEC-205 (Clone NLDC145) was purchased from DPC Biermann (Bad Nauheim, Germany). Magnetically labeled anti-CD11c, anti-CD19, anti-CD8, anti-CD45.1, FITC-labeled anti-CD45.2, PE-labeled anti-CD45.1, and FITC-labeled anti-TCRβ-chain were obtained from Miltenyi Biotec (Bergisch Gladbach, Germany).

Oral tolerance induction

Mice were treated with 10 mM NiCl₂ in the drinking water (17) for at least 4 and to a maximum of 8 wk. Unless mentioned otherwise, they were sacrificed and their spleens were excised immediately after treatment. Age- and sex-matched control mice received tap water without any additional NiCl₂.

Immunization of mice

Mice were immunized as described previously (16, 17). In the case of nickel, they were injected intradermally into both flanks (50 μl each) with either 10 mM NiCl₂ in sterile, pyrogen-free saline (negative control) or 10 mM NiCl₂ in saline containing 1% H₂O₂. For DNFB treatment, mice were immunized by painting 0.5% (w/v) DNFB on the shaved flanks (20 μl each); DNFB was resolved in a 4:1 (v/v) mixture of acetone and olive oil.

Challenge for recall and ear-swelling test

Ten days after immunization, mice were rechallenged by injecting 50 μl of 10 mM NiCl₂ in sterile, pyrogen-free saline into the pinna of each ear or by applying 20 μl of 0.2% DNFB onto each ear. Forty-eight hours after rechallenge with NiCl₂ and 24 h after rechallenge with DNFB, delayed-type hypersensitivity reactions were determined by measuring the increment in ear thickness in comparison with the prechallenge values. To determine the prechallenge values, mice were anesthetized with di-ethyl-ether, whereas for the measurement after challenge, mice were killed by asphyxiation with CO₂. Measurements were performed in a blind fashion (16, 17) using a micrometer gauge (Oditest D 1000 gauge; The Dyer Company, Lancaster, PA). The following results represent the mean ear-swelling response from groups comprising five to six mice and are expressed in units of mm × 10⁻² ± SEM.

Sorting of T cells and APCs for adoptive transfer studies

For the transfer of T cells, single-cell suspensions of erythrocyte-free spleen cells, which contained 30–35% T cells, 60–65% B cells, and 1–2% DCs, were passed through nylon wool columns, followed by the depletion of CD11c⁺, CD19⁺, and MHC-II⁺ cells using a magnetic cell sorter (autoMACS, Miltenyi Biotec). For the transfer of APCs, single-cell suspensions of erythrocyte-free spleen cells were depleted of CD4⁺, CD8⁺, and CD90⁺ T cells using the autoMACS. Hereafter, these cells, containing 90–95% CD19⁺ MHC-II⁺ B cells and 1–3% CD11c⁺ MHC-II⁺ DCs, will be referred to as APCs. The purity of the resulting T cell and APC fractions were then clarified by FACs analysis and were found to be contaminated with <0.5% CD19⁺ MHC-II⁺ or CD11c⁺ MHC-II⁺ B cells and <0.5% CD4⁺CD3⁺ or CD8⁺CD3⁺ T cells, respectively. In the serial transfer assays using T cells and APCs, an additional depletion of Ly5.1⁺ (CD45.1⁺) cells was performed between the first set of recipients (i.e., the second donors) and the second set of recipients. After this depletion, the fraction of cells required for transfer contained <0.1% Ly5.1⁺ cells. For the transfer of B cells, single-cell suspensions of erythrocyte-free spleen cells were depleted of CD4⁺, CD8⁺, CD3⁺, and CD11c⁺ cells, using the FACScalibur (BD Biosciences, Heidelberg, Germany). The transferred B cell fractions, containing 90–95% CD19⁺ MHC-II⁺ B cells, were contaminated with <0.5% CD4⁺ T cells (CD4⁺CD3⁻) and CD8⁺ T cells (CD8⁻CD3⁻), but they contained no DCs (CD11c⁺ MHC-II⁺ B cells).

In vitro treatment of APCs before the adoptive transfer

To assess the activities of APCs, separated donor APCs were subjected to various in vitro treatments before transfer. 1) Aliquots of APCs (6.6 × 10⁶ cells/ml) from orally tolerized donors either were killed by three cycles of freezing (−196°C) and thawing (37°C) or were irradiated with 2000 rad. 2) For the transfer of fixed APCs from orally tolerized mice, APCs were fixed with 2% paraformaldehyde (2 × 10⁶ cells/ml, 20 min at room temperature), washed, and diluted with sterile, pyrogen-free PBS, and then were used for adoptive transfer (6.6 × 10⁷ cells/ml). 3) Further aliquots of APCs, obtained from both orally tolerized donors and naive donors, were incubated in vitro with 1 μg/ml LPS (10⁶ cells/ml, 18 h at 37°C), washed, and diluted with sterile, pyrogen-free PBS, and then were used for adoptive transfer (6.6 × 10⁷ cells/ml). For control, APCs from naive donors were incubated in vitro with RPMI 1640 medium containing 75 μM NiCl₂ (10⁶ cells/ml, 18 h at 37°C), washed, and diluted with sterile, pyrogen-free PBS, and then were used for adoptive transfer (6.6 × 10⁷ cells/ml). RPMI 1640 medium was supplemented with 10% FCS, 2 mM glutamine, 1 mM sodium pyruvate, essential and nonessential amino acids, 10 U/ml penicillin/streptomycin, and 50 μM 2-ME.

Adaptive cell transfers

Cell suspensions, containing the type and the number of cells indicated, were diluted in sterile, pyrogen-free PBS and injected i.v. into recipient mice (150 μl). One day later, mice were immunized against NiCl₂ or the control compounds indicated. Ten days thereafter, the recipients were rechallenged at the ears, and 48 h later (or 24 h in the case of DNFB), their ear-swelling response was measured. An exception here was that out of Ly5.2⁺ recipients in the experiments on infectious tolerance: these animals were not rechallenged after the immunization.

Allostimulation in the MLR

In the MLR in vitro, the proliferative responses of BALB/c T cells to APCs from C57BL/6J mice were studied. On day 0, single-cell suspensions of erythrocyte-free spleen cells were prepared in DMEM containing 10% FCS. APC and T cell fractions were obtained by the depletion of CD90⁺ and MHC-II⁺ cells, respectively, using MACS. The obtained fractions were tested for purity by FACs analysis: the sorted APCs and T cells were found to be contaminated with <3% T cells and <1% APCs, respectively. Cells were resuspended in complete medium, i.e., DMEM supplemented with 10% FCS, 2 mM glutamine, 4.5 g/L glucose, 1 mM sodium pyruvate, 10 U/ml penicillin/streptomycin, and 50 μg/ml 2-ME. Irradiated APCs (2000 rad) from both naive and nickel-tolerant C57BL/6 mice were pipetted into
96-well round-bottom plates (10^5 cells/well) in quadruplicates and cultured in either complete medium (100 µl/well) or in complete medium containing 75 µM NiCl2. Irradiated APCs from BALB/c mice served as syngeneic controls. On day 1, freshly isolated T cells (10^5 cells/well) were added with or without 75 µM NiCl2 (to give a final volume of 200 µl/well). On day 5, [3H]thymidine (0.5 µCi/well) was added for the last 16 h. On day 6, cells were harvested using the Inotech Sample Harvesting System (Inotech, Dot-tikon, Switzerland) and were assessed for thymidine incorporation in a liquid scintillation counter (1450 MicroBeta TriLux Liquid Scintillation and Luminescence Counter; Wallac Oy, Turku, Finland). Results are expressed as stimulation indices + SD (stimulation indices = mean cpm of BALB/c T cells stimulated by C57BL/6 APCs/mean cpm of BALB/c T cells stimulated by BALB/c APCs).

**Immune flow cytometry**

For phenotyping of the APCs, single-cell suspensions of erythrocyte-free spleen cells were prepared in PBS containing 0.02% NaN3 and were preincubated with anti-CD16/CD32 mAb (Fc block). B cells were stained with anti-CD19 and either anti-CD40 or anti-CD38, whereas T cells were stained with anti-CD3, anti-CD4, and anti-CD38. DCs were identified using anti-CD11c and anti-MHC-II; in addition, they were stained with anti-CD40, anti-CD80, or anti-CD86 mAb. Intracellular staining for DEC-205 (CD205) was performed as previously described (18). Briefly, cells were fixed in PBS containing 2% paraformaldehyde and preincubated with anti-CD16/CD32 mAb. Permeabilization was performed in PBS containing 1% BSA and 0.5% saponin. Incubation with the DEC-205 mAb was conducted in the presence of BSA and saponin. Flow cytometry was performed using the FACSCalibur, and the results were analyzed with CellQuest software.

To investigate the expression of costimulatory molecules on APCs in the draining lymph nodes after nickel treatment, mice were injected intradermally into both flanks (50 µl each) with pyrogen-free saline, 10 mM NiCl2, 10 mM NiCl3, containing 1% H2O2, or 1% H2O2. Single-cell suspensions of the draining axillary lymph nodes were prepared in PBS and preincubated with anti-CD16/CD32 mAb. To characterize DCs, the lymph node cells were stained with anti-CD11c and anti-MHC-II, whereas for the characterization of B cells, anti-CD19 and anti-MHC-II were used. Subsequently, both subpopulations were stained with either anti-CD80 or anti-CD86 mAb. Flow cytometry was then performed, and the expression of CD80 and CD86 was calculated as the mean fluorescence intensity on the DCs and B cells, respectively.

**Statistical analysis**

Statistical significance of results was determined by ANOVA followed by Newman-Keuls test.

**Results**

**APCs from mice that were orally tolerized toward nickel display a tolerogenic phenotype**

With regard to the overall cellular composition of splenocytes, immune flow cytometry showed no significant differences between orally tolerized and untreated control mice. However, phenotypic differences were detected among the APCs. Splenic DCs from nickel-tolerant mice exhibited not only an increase in the expression of DEC-205 (CD205) (Fig. 1A), but they also displayed a profound decrease in CD40 (Fig. 1B). This decrease in CD40 was even more apparent in the B cell population (Fig. 1C). These alterations point to an immature, potentially tolerogenic phenotype of the APC, albeit these cells did not show any alterations in the expression of CD28, CD80, CD86, and MHC-II (data not shown). Furthermore, although the CD38 expression on the B cells of orally tolerized mice was markedly increased (Fig. 1D), it remained unchanged on the T cells derived from the same animals (Fig. 1E).

**APCs from mice that were orally tolerized toward nickel show a reduced allostimulatory capacity**

The above described tolerogenic phenotype displayed on the APCs from orally tolerized mice was mirrored by their reduced allostimulatory capacity in the MLR in vitro, where these cells were used as allostimulators. Irradiated APCs from nickel-tolerant C57BL/6 mice (H-2b) were cocultured with splenic T cells from untreated BALB/c mice (H-2d). The proliferative responses of these T cells were then tested in the presence or absence of NiCl2. In comparison with the APCs from untreated C57BL/6 mice (Fig. 2, bars 1 and 2), the APCs from nickel-tolerant mice (Fig. 2, bars 3 and 4) showed a significantly reduced allostimulatory capacity, regardless of whether NiCl2 was present.
APCs from orally tolerized donors are able to transfer nickel tolerance to naive recipient mice

Our previous studies demonstrated that as few as 10^2 bulk T cells from the spleens of nickel-tolerant donors were able to adoptively transfer the tolerance to naive syngeneic recipients (17). Here we show that APCs also can transfer nickel tolerance. As shown in Fig. 3A, bars 1-3, the transfer of 10^4, 10^3, and even as few as 10^2 APCs from nickel-tolerant donors succeeded in transferring nickel tolerance to naive syngeneic recipients. The ear-swelling response in the recipients that received tolerogenic APCs was as low as the background ear-swelling previously observed in mice that did not receive a cell transfer and were not immunized, but only were challenged with NiCl_2 (17). Moreover, these ear-swelling values correlated with those of mice that received between 10^2 and 10^4 splenic T cells from nickel-tolerant donors, followed by immunization with NiCl_2/H_2O_2 and challenge with NiCl_2 (17). However, the transfer of only 10^3 APCs from nickel-tolerant donors or of 10^4 APCs from untreated donors (Fig. 3A, bars 4 and 5) failed to transfer nickel tolerance to naive syngeneic recipients (17). Here we show that the APCs conferred unresponsiveness to nickel, but not to DNFB. Consistent with this, we showed previously that the mice orally tolerized to nickel failed to be generally immunosuppressed (17), even though this might be suggested by the tolerogenic phenotype (Fig. 1) and the reduced allostimulatory capacity (Fig. 2) of the APCs of these animals.

In vitro treatment of the APCs from orally tolerized donors abrogates their tolerogenicity

We then asked whether in vitro treatment of the tolerogenic APCs before transfer would interfere with their tolerogenicity. This was indeed found to be the case because killing, irradiation, or fixation could abrogate the tolerogenicity of the APCs (Fig. 3B, bars 3–5). Furthermore, in accordance with other authors (13), we found that the immature phenotype of the APCs (Fig. 1) was needed for their tolerogenic function in vivo. If the APCs from tolerant donors were activated with LPS, and thus lost their immature phenotype (Refs. 19 and 20 and data not shown), they also lost their tolerogenic activity (Fig. 3B, bar 6). Hence, for the successful transfer of nickel tolerance, the APCs must be intact cells that have been neither activated nor inactivated or killed.

In vitro nickel loading of APCs from naive donors fails to render them tolerogenic

The adoptive transfer data presented above clearly indicated that the APCs from orally tolerized donors were tolerogenic. In contrast, the transfer of 10^3 APCs from naive donors, which had been pulsed in vitro with a concentration of NiCl_2 known to activate nickel-specific T cells (16, 17), failed to tolerize the recipients (Fig. 3B, bar 8). Thus, the transfer of tolerance with APCs from orally tolerized donors was not due to simple passive transfer of nickel ions to the recipients. Consistent with this, APCs from naive mice incubated with NiCl_2 in vitro also failed to be hypostimulatory in the allo-MLR (Fig. 2). These findings indicate that the APCs of the orally tolerized donors possessed a tolerogenic property that was not present in the nickel-loaded APCs of untreated mice.

The tolerogenic capacity of APCs from orally tolerized mice is transient

Both APCs and T cells from orally tolerized mice were able to transfer nickel tolerance when the respective donors were sacrificed after a treatment-free interval of 1 wk after oral tolerance induction (Fig. 4, bars 1 and 4). Moreover, consistent with our previous findings (17), nickel tolerance could be transferred with bulk T cells as late as 20 wk after the termination of oral NiCl_2 treatment (Fig. 4, bar 5). Obviously, T cells with a long-lasting suppressive capacity, specific for nickel, were formed during the 4-wk period of oral tolerance induction. In contrast with T cells,
the APCs from these mice had completely lost their capacity to transfer tolerance after a treatment-free interval of 20 wk (Fig. 4, bar 2). The biological half-life of nickel ions is so short that, in men, 50% of the nickel ions in the body are eliminated within 2–3 days (21). Hence, it is likely that after a treatment-free interval of 20 wk, the concentration of nickel ions in the orally tolerized donors was too low to induce new tolerogenic APCs. In all likelihood, the original tolerogenic APCs that had been induced by the 4-wk period of oral nickel treatment were lost because of their short lifetime. The reduction of allostimulatory capacity of the APCs from orally treated mice (Fig. 2) also was a transient phenomenon that disappeared if the oral NiCl2 treatment was followed by a treatment-free interval of 20 wk (data not shown). Thus, although the APCs in the orally tolerized mice were found to be an essential element in nickel tolerance, the T cells of these animals are the responsible factor for the long-term maintenance of tolerance.

Among the APCs from orally tolerized donors, it is the B cells that primarily carry the tolerogenicity

The 10^5 splenic APCs from orally tolerized donors, which were found to transfer nickel tolerance (Fig. 3), consisted of mostly (90–95%) B cells and only a few (1–3%) DCs. Therefore, we asked whether B cells alone were able to transfer the tolerance. Indeed, we were able to transfer the tolerance with 10^5 purified B cells that were devoid of DCs (Fig. 5, bar 1). Because the purified B cell fraction contained maximal 0.5% of T cells, at most five T cells could have been transferred alongside the purified B cells. We have previously shown that this T cell number is too low for successful transfer of tolerance (17).

Demonstration of infectious tolerance: both APCs and T cells are involved

To investigate the role of infectious tolerance in our model, serial adoptive cell transfers were performed. These involved orally tolerized mice as primary cell donors, a first set of recipient mice that in turn became the secondary cell donors, and, finally, a second set of recipient mice that were assayed for tolerance induction after immunization with NiCl2/H2O2 and rechallenge at the ears. To be able to distinguish the cells from the primary donors from those of the recipients, congenic C57BL/6 mice, which express Ly5.1 on the surface of all lympho-hematopoietic cells, were used as the primary donors, instead of the Ly5.2<sup>+</sup> wild-type mice. As shown in Fig. 6, 10^4 T cells or APCs from orally tolerized or untreated Ly5.1<sup>+</sup> primary donors were transferred to the first set of Ly5.2<sup>+</sup> recipients, which were immunized with NiCl2/H2O2 1 day later. After a further 10 days, APCs or T cells were isolated from the first recipients and depleted of any contaminating Ly5.1<sup>+</sup> cells remaining from the primary donors. These cells (10^4/mouse) were then transferred into the second set of Ly5.2<sup>+</sup> recipients. If the latter received T cells or APCs from those first recipients, which themselves had been tolerized by injection of the opposite cell type (APCs and T cells, respectively) originating from the orally tolerized Ly5.1<sup>+</sup> donors, they were unresponsive to immunization (Fig. 6, bars 1 and 4). Consequently, Ly5.1<sup>+</sup> cells derived from the primary donors possessed the ability to infectious spread nickel tolerance to the first recipients (and prospective secondary donors), indicating that indeed infectious tolerance operates in orally induced nickel tolerance. Both T cells and APCs from the Ly5.1<sup>+</sup> primary donors were able to initiate this cascade mechanism of tolerance in vivo. Interestingly, however, although the tolerance was successfully transferred by T cells of the primary donors to APCs of the first recipients and vice versa by APCs of the primary donors to T cells of the first recipients, there was no direct tolerance transfer either from donor T cells to host T cells or from donor APCs to host APCs (Fig. 6, bars 3 and 6).

The results shown in Fig. 6 demonstrate that the successful tolerance transfer from the primary Ly5.1<sup>+</sup> donors to the second set of Ly5.2<sup>+</sup> recipients involved infectious tolerance. We ruled out the possibility that the transfer or tolerance was due to a contamination through residual Ly5.1<sup>+</sup> cells derived from the tolerant primary donors, because immunofluorescent staining showed that, among the cells isolated from the first set of Ly5.2<sup>+</sup> recipients, there was a maximum of 0.1% contaminating Ly5.1<sup>+</sup> cells. Thus, along with the 10^4 cells obtained from the first recipients and transferred to each mouse in the second set of Ly5.2<sup>+</sup> recipients, a
primary markers of costimulation, we determined the expression of CD80 and CD86 on APCs, that is, DCs (as gated in Fig. 7A) and B cells, in the draining axillary lymph nodes after immunization of naive mice at the flanks. Indeed, the expression of CD80 and CD86 on DCs (Fig. 7, C and D) as well as B cells (Fig. 7, E and F) was higher after the injection of NiCl₂/H₂O₂ or H₂O₂ alone than after an injection of NiCl₂ alone or saline. These results confirmed our concept that NiCl₂ alone induces only signal 1 (neoantigens) on
d
maximum of 10⁴ cells derived from the Ly5.1⁺ primary donors could have been cotransferred. This small contamination was negligible because neither 10⁴ T cells (17) nor 10⁴ APCs (Fig. 3, bar 4) from orally tolerized donors were able to transfer the tolerance. Another reason why this small contamination was negligible came from the observation that the second set of recipients failed to be tolerized when they received 10⁴ T cells from those first Ly5.2⁺ recipients, which themselves were rendered tolerant by the injection of T cells from the primary Ly5.1⁺ donors (Fig. 6, bar 6). Although the first recipients (and thus the secondary donors) taken for this T cell → T cell transfer were identical with those used for the T cell → APC transfer, only the APCs isolated from the first recipients were found to tolerate the second set of recipients (Fig. 6, bar 3 vs bar 4). These tolerogenic APCs, however, contained the same percentage (≤0.1%) of contaminating Ly5.1⁺ cells as the ineffective T cell fraction isolated from the same animals.

Immunization is needed for the infectious spread of tolerance in recipient mice

Next, we asked whether both signal 1 and signal 2 were required for the infectious spread of tolerance from donor T cells to host APCs and vice versa. Previously, we proposed that Ni²⁺ ions, unlike other sensitizing metal ions (22–25), would be unable to up-regulate the expression of costimulatory molecules, whereas the administration of NiCl₂ together with H₂O₂ would induce both signal 1 and signal 2 (17). Here, the validity of this hypothesis has been confirmed. Focusing on CD80 and CD86 as recognized pri-

FIGURE 6. Infectious tolerance is the amplification mechanism accounting for the transfer of nickel tolerance in vivo; to be effective, T cells and APCs must cooperate in this process. Prospective Ly5.1⁺ donor mice were orally tolerized to nickel or were left untreated, as indicated. Thereafter, T cells or APCs from the Ly5.1⁺ primary donors were transferred (10⁴ cells per recipient) to a first set of Ly5.2⁺ recipients. The latter were injected with NiCl₂/H₂O₂ within 24 h after transfer. On day 11, T cells and APCs from these first recipients were isolated and depleted of donor Ly5.1⁺ cells and subsequently were transferred (10⁴ cells per recipient) into a second set of Ly5.2⁺ recipients. As control groups for nickel tolerance and allergy (bottom), 10⁴ nylon wool-enriched T cells from tolerant or naive Ly5.2⁺ donors were transferred into groups of Ly5.2⁺ recipients. As control groups for nickel tolerance and allergy (bottom), 10⁴ nylon wool-enriched T cells from tolerant or naive Ly5.2⁺ donors were transferred into groups of Ly5.2⁺ recipients, as indicated. All recipients were immunized and rechallenged, and the ear-swelling responses were determined in accordance with the standard protocol. The displayed data represent the mean ear-swelling response + SEM from groups of five mice each. Results represent one of two experiments, which yielded comparable results.

FIGURE 7. Contrary to immunization with NiCl₂/H₂O₂, an injection of NiCl₂ alone fails to up-regulate CD80 and CD86 on the APCs in the draining lymph nodes. Groups of three mice were injected into both flanks with NaCl, NiCl₂ alone, NiCl₂/H₂O₂, or H₂O₂ alone, as indicated. After 1, 2, 3, and 4 days, the draining axillary lymph nodes were prepared, and their cells were stained for surface MHC-II, CD11c, and CD80 or CD86. DCs were gated as CD11c⁺ MHC-II high ) are displayed in A and B, respectively, whereas the kinetics of CD80 and CD86 expression on B cells (CD19⁺ MHC-II high ) are displayed in E and F, respectively. Results represent one of three experiments, which yielded comparable results.
APCs, whereas NiCl₂ in conjunction with H₂O₂ induces both signal 1 and signal 2 (costimulation) and H₂O₂ alone induces only signal 2.

To test whether signal 1 and a danger signal (26) were needed for the spread of tolerance, APCs from the Ly5.1⁺ primary donors were transferred to Ly5.2⁺ recipients. Ten days after priming with NiCl₂/H₂O₂, NiCl₂ alone, or H₂O₂ alone, as indicated in Fig. 8A, T cells of the first Ly5.2⁺ recipients were depleted of Ly5.1⁺ cells and transferred to a second set of Ly5.2⁺ recipients. The latter recipients were then immunized and tested for tolerance to nickel. Only when the first set of recipients was immunized with NiCl₂/H₂O₂ could the transferred Ly5.1⁺ APCs succeed in infecting their host T cells so that these cells could in turn transfer the tolerance to the second set of Ly5.2⁺ recipients (Fig. 8A, bar 1). Hence, for the infectious spread of tolerance from Ly5.1⁺ APCs to Ly5.2⁺ T cells, both signal 1 and a danger signal were needed. The same was true for the infectious spread of tolerance from Ly5.1⁺ donor T cells to Ly5.2⁺ APCs (Fig. 8B, bar 1). Although signal 1 can be clearly defined as nickel-induced neoantigens, the relevant molecules induced by the danger signal remain unresolved. Whether this is indeed CD80/CD86 or some other costimulatory or coinhibitory molecule(s) requires further investigation.

Tolerance can spread from Ly5.1⁺ T cells to Ly5.2⁺ T cells and from Ly5.1⁺ APCs to Ly5.2⁺ APCs, if the Ly5.2⁺ recipients are immunized twice

When cells from the orally tolerized Ly5.1⁺ donors were transferred into the first recipients that were immunized once after the transfer, the tolerance failed to spread from donor T cells to host T cells or from donor APCs to host APCs (Fig. 6, bars 3 and 6). In view of the finding that complete immunization was needed for each spread of tolerance from one cell type to the opposite one (Fig. 8A and B), we hypothesized that the one-time immunization protocol used for the experiment shown in Fig. 6 would not have given sufficient immunizing efficacy and/or time to first engage the respective opposite cell type into the tolerance spread and, eventually, reach the homologous cell type of the host. If this is true, this would mean that for the spread of tolerance from a given donor cell type to the homologous cell type in the recipients, the latter would require a double immunization: the first time to induce the spread of tolerance from the respective donor cell type to the opposite host cell type and the second time to allow the subsequent spread from the first host cell type to the second one. Consequently, the second cell type would be homologous with that of the primary Ly5.1⁺ donor cell type. To test this hypothesis, we performed serial transfer experiments in which the first set of recipients were immunized with NiCl₂/H₂O₂ twice, the first time within 24 h after transfer and the second time on day 11. With the second cell transfer, which was performed on day 21, we could show that the recipients of Ly5.1⁺ Ts cells indeed possessed tolerant Ly5.2⁺ T cells if they had been immunized twice (Fig. 9, bar 2). Corresponding experiments performed with APCs showed the spread of tolerance from donor APCs to host APCs (Fig. 9, bar 5). We conclude that both the long-lived Ts cells and the APCs (Fig. 4) from orally tolerized donors can indeed spread the tolerance to the same cell type in the recipients, but only if they encounter the tolerogen, NiCl₂, in conjunction with a danger signal, in our case H₂O₂, more than once.

Discussion

In the present study, we first focused on the characterization of the APCs from orally tolerized mice and then studied the two-way interactions between Ts cells and APCs in the infectious tolerance pathway in vivo. Compared with splenocytes of untreated control mice, the APCs from animals orally tolerized to nickel exhibited an increase in DEC-205 expression by DCs, an increase in CD38⁺ on B cells, and a striking decrease in the expression of CD40 on DCs and in particular on B cells. This tolerogenic phenotype of DCs and B cells in the spleens of orally tolerized mice conforms not only with the remarkable efficiency of their APCs to adoptively transfer the tolerance, but also with their reduced allostimulatory capacity. DEC-205 is mainly expressed by CD8α⁺ DCs in the
could prevent the T cell priming toward nickel. Conceivably, the administration of NiCl₂ via the oral route corroborates this effect. Once the nickel-specific Ts cells were induced through nickel exposure without costimulation, activation of these Ts cells by injection of NiCl₂ and H₂O₂ might have prompted them to inhibit the up-regulation of costimulatory signals (7, 8, 37) or induce inhibitory signals (38) on APCs. Consistent with this, it has been noted recently that the injection of NiCl₂/H₂O₂ into orally tolerant mice failed to up-regulate CD80/CD86 expression on the APCs in the draining lymph nodes (M.F., unpublished results). The induction of oral tolerance toward nickel also resulted in a notable up-regulation of CD38 expression on splenic B cells. CD38 is widely expressed on a variety of different cell types, including B cells and T cells (39). The ligation of CD38 induces Ca²⁺ influxes and has been shown to enhance cell proliferation (39, 40). Although the functional significance of the enhanced CD38 expression by B cells of orally tolerantized mice is unknown, this observation is consistent with the other findings reported here, which all point to an active contribution by B cells to initiate and transfer tolerance toward nickel. A CD38⁺ T cell population with suppressive properties has been reported (41), but in our model an alteration in CD38 expression by T cells could not be detected.

Like the nickel-specific, anergic Ts cells from orally tolerantized donor mice (17), APCs also proved capable of transferring nickel tolerance and, here too with as few as 10² cells. This transfer of tolerance by APCs from orally tolerantized donors was not due to a simple passive transfer of nickel, but involved intact cells that actively contributed to the induction of tolerance in the recipients. Why was it so relatively easy to demonstrate the tolerogenic function of APCs in orally induced tolerance to nickel? Nickel ions can be considered as haptons that differ qualitatively and quantitatively from conventional Ag: they distribute ubiquitously within the body (17), do not need to be processed, and do not have to enter into peptide competition for MHC binding sites because most nickel ion-induced neoantigens result from exogenous attachment of the metal ions to MHC molecules and those self peptides that are presented anyway (16, 42). Theoretically, each nickel ion could form a neoantigen, so that virtually all APCs from orally tolerantized mice would carry those neoantigens. Therefore, both the number of tolerogenic APCs and the density of nickel ion-induced neoantigens carried by them are probably much higher if compared with the APCs in those tolerant hosts that presented more common Ags, such as foreign peptides. The unusually low numbers, not only of bulk T cells but also of APCs, which we found capable of transferring specific unresponsiveness, accentuate the enormous infectivity and hence the dominance of T cell tolerance toward nickel. We are aware of only one other investigation in which a comparably small number of tolerogenic APCs sufficed to induce tolerance. In that experiment, an injection of only 20 peritoneal exudate cells, which were treated in vitro with Ag and TGF-β, induced tolerance in the recipient mice (43). Interestingly, these cells also showed a reduced CD40 expression (44).

Among the donor APC fraction, primarily B cells were found to carry the tolerogenicity. Previous studies have demonstrated that B cells from tolerant donor mice can indeed induce Ts cells upon adoptive transfer (45, 46). For instance, in the anterior chamber-associated immune deviation (ACAID) model, B cells were found to become tolerogenic through contact with tolerogenic macrophages. Even though those B cells were unable to directly suppress the development of delayed-type hypersensitivity, they were capable of inducing specific Ts cells (45). For this to occur, B cells were required to present the Ag, which they had acquired from the tolerogenic macrophages, in the context of Qa-1 (45). Qa-1 is an MHC class I b molecule known to guide the suppressive activity of

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**FIGURE 9.** Infectious tolerance spread from a given donor cell type. T cell or APC, to the homologous cell type in the recipients requires a double immunization of recipient mice. Prospective Ly5.1⁺ donor mice were orally tolerantized to nickel or were left untreated, as indicated. Thereafter, T cells or APCs from these primary Ly5.1⁺ donors were transferred (10⁴ cells per recipient) into a first set of Ly5.2⁺ recipients. The first set of recipients was immunized (with NiCl₂/H₂O₂) twice on days 1 and 11. On day 21, T cells and APCs from the first recipients were isolated and depleted of Ly5.1⁺ donor cells, before they were transferred (10⁵ cells per recipient) into a second set of Ly5.2⁺ recipients. As control groups for tolerance and allergy (bottom), 10⁴ nylon wool-enriched T cells from tolerant or naive Ly5.2⁺ recipients were transferred into groups of Ly5.2⁺ recipients, as depicted. All recipients were immunized and rechallenged, and the ear-swelling responses were determined. The data shown represent the mean ear-swelling response ± SEM from groups of five mice each. Results represent one of two experiments, which yielded comparable results.
human CD4+CD25+ regulatory T cells required preactivation (provided in an Ag-nonspecific manner) to enable them to spread the tolerance through cell-cell contact to activated conventional T cells present in the same culture. However, in contrast with those in vitro studies, for the different Ag-reactive T cells to meet in vivo and specifically suppress or be suppressed, APCs are apparently needed to act as bridges and, as discussed before, also as mediators of suppression.

Taken together, our results demonstrate that infectious tolerance in vivo involves a reciprocal interplay of specific Ts cells and tolerogenic APCs that is driven by immunization. With regard to the consequences of immunization, however, we noted a striking difference between the induction phase of T cell suppression (which can be defined as the time period of oral nickel treatment) and the effector phase (defined here as the time period after adoptive cell transfer and the subsequent immunization of the recipients). Before or early on in the induction phase, Ag administration together with enhanced costimulation, as inducible by H2O2, would obviate the tolerization. The opposite effect is achieved when Ag and a source of “danger” (26), such as H2O2, intrude into an immune system that harbors a few anergic Ts cells or tolerogenic DCs and B cells: in the effector phase of suppression, immunization with NiCl2/H2O2 led to a dramatic spread of tolerance. Thus, once Ts cells and tolerogenic APCs were induced by oral administration of nickel, the tolerance proved to be self-enhancing and self-maintaining when the Ts cells and tolerogenic APCs were exposed to Ag in the presence of danger. Under these conditions, tolerogenic APCs and the Ts effector cells engaged naïve T cells and normal APCs, respectively, into the tolerization process so that unresponsiveness prevailed.

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