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Oral Nickel Tolerance: Fas Ligand-Expressing Invariant NK T Cells Promote Tolerance Induction by Eliciting Apoptotic Death of Antigen-Carrying, Effete B Cells

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Whereas oral nickel administration to C57BL/6 mice (Ni\textsuperscript{high} mice) renders the animals tolerant to immunization with NiCl\textsubscript{2} combined with H\textsubscript{2}O\textsubscript{2} as adjuvant, as determined by ear-swelling assay, it fails to tolerize Jo18\textsuperscript{−/−} mice, which lack invariant NKT (iNKT) cells. Our previous work also showed that Ni\textsuperscript{high} splenic B cells can adoptively transfer the nickel tolerance to untreated (Ni\textsuperscript{low}) recipients, but not to Jo18\textsuperscript{−/−} recipients. In this study, we report that oral nickel administration increased the nickel content of splenic Ni\textsuperscript{high} B cells and up-regulated their Fas expression while down-regulating expression of bcl-2 and Bcl-x\textsubscript{L}, thus giving rise to an Ag-carrying, apoptosis-prone B cell phenotype. Although oral nickel up-regulated Fas expression on B cells of both wild-type Ni\textsuperscript{high} and Jo18\textsuperscript{−/−} Ni\textsuperscript{high} mice, only the former showed a reduced number of total B cells in spleen when compared with untreated, syngeneic mice, indicating that iNKT cells are involved in B cell homeostasis by eliciting apoptosis of effete B cells. Upon transfer of Ni\textsuperscript{high} B cells, an infectious spread of nickel tolerance ensues, provided the recipients are immunized with NiCl\textsubscript{2}/H\textsubscript{2}O\textsubscript{2}. As a consequence of immunization, Fas ligand-positive (FasL) molecules develop a lymphoproliferative disease associated with untreated, syngeneic mice, indicating that iNKT cells are involved in B cell homeostasis by eliciting apoptosis of effete B cells. The apoptotic Ni\textsuperscript{high} B cells were taken up by splenic dendritic cells, which thereby became tolerogenic for nickel-reactive Ni\textsuperscript{low} T cells. In conclusion, FasL molecules, respectively, leading to the induction of T regulatory (Treg) cells specific for apoptotic cell-associated Ags (3, 4). Death of Antigen-Carrying, Effete B Cells

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In the mouse model of oral nickel tolerance, T cell-depleted spleen cells (termed APCs (14)), which must express CD1d, also proved tolerogenic. These APCs, consisting mainly of B cells, were able to transfer the tolerance from donor mice that were orally treated with NiCl₂ (termed Ni<sup>high</sup> throughout this work) to untreated, syngeneic recipients (termed Ni<sup>low</sup>), provided the latter possessed iNKT cells (14). Although purified splenic B cells from Ni<sup>high</sup> donors could transfer nickel tolerance (17), the question remained whether they needed to interact with iNKT cells in this process. When the inoculum of Ni<sup>high</sup> donor cells consisted of T cells, instead of APCs, iNKT cells were not required for the tolerance transfer. This indicated that the transferred APCs (possibly Ni<sup>high</sup> B cells) and the recipient’s iNKT cells were involved in the induction phase of specific Treg cells and not their effector phase (14). Previously, it has been demonstrated that nickel tolerance, induced by transferred APCs from Ni<sup>high</sup> donors, generated specific Treg cells in the recipient. Paradoxically, this infectious spread of tolerance from the cells of Ni<sup>high</sup> donors to those of Ni<sup>low</sup> recipients required vigorous immunization of the latter, which was achieved through intradermal (i.d.) injection of NiCl₂ combined with H₂O₂ as adjuvant (17).

It is only poorly understood what functional links exist between tolerogenic B cells, iNKT cells, DCs, and Treg cells, and how these cell types interact with one another in the above-mentioned tolerance models (15–18). Therefore, in the present study, we asked whether iNKT cells are involved in the apoptotic death of Ni<sup>high</sup> B cells, leading to the uptake of apoptotic cell fragments by DCs and, thus, induction of tolerogenic DCs and eventually nickel-specific Treg cells.

Materials and Methods

Reagents and Abs

NiCl₂ × 6H₂O (denoted as NiCl₂) and 2,4-dinitrofluorobenzene (DNFB) were purchased from Sigma-Aldrich, and H₂O₂ from E. Merck. Abs were purchased from BD Biosciences, microbeads from Miltenyi Biotec, and Newport Green DCF from Invitrogen Life Technologies.

Mice and oral tolerance induction

Specific pathogen-free C57BL/6 mice (wild-type (WT) mice) were obtained from Elevage Janvier. B6.MRL-Traf1<sup>−/−</sup> (referred to as Traf-<sup>−/−</sup>) and B6.Snp.Fas<sup>−/−</sup> (referred to as Fas-<sup>−/−</sup>) mice were purchased from The Jackson Laboratory. Joa18<sup>−/−</sup> (referred to as Joa18<sup>−/−</sup>) mice were created at Chiba University (Chiba, Japan) and backcrossed nine times with C57BL/6 mice (21); they were a gift from Dr. S. Balk (Beth Israel Deaconess Medical Center, Harvard University, Boston, MA). All animals received standard rodent diet (V-1324-703; Ssniff). Female mice were used at the time of study (14), referred to as J<sup>gld</sup>/H9251<sup>−/−</sup> (referred to as J<sup>gld</sup>), J<sup>gld</sup>/H9251<sup>−/−</sup>>, and J<sup>gld</sup>/H11001<sup>−/−</sup> mice were purchased from The Jackson Laboratory. J<sup>gld</sup>/H9251<sup>−/−</sup> mice were performed, as described (17), with modifications. Ni<sup>low</sup> mice were injected i.v. into the tail vein of recipient mice. First recipient mice of Ni<sup>low</sup> B cells (shown in Fig. 6) comprised groups of two mice each; all groups of recipient mice used for measurement of ear-swelling reactions were comprised of four to five mice. One day later, mice were challenged i.d., as described above. Ten days later, secondary reactions were elicited by i.d. injections at the ears, and 48 h thereafter their ear-swelling response was measured.

Adoptive cell transfers

Before transfer, isolated cell populations were washed twice in pyrogen-free PBS. Cell suspensions (150 µL), containing the indicated cell numbers, were injected i.v. into the tail vein of recipient mice. First recipient mice of Ni<sup>low</sup> B cells (shown in Fig. 6) comprised groups of two mice each; all groups of recipient mice used for measurement of ear-swelling reactions were comprised of four to five mice. One day later, mice were challenged as described above. Ten days later, secondary reactions were elicited by i.d. injections at the ears, and 48 h thereafter their ear-swelling response was measured.

Flow cytometry

For FACS analysis of B cells and DCs, respectively, FcRs were blocked by using purified anti-CD16/32 mAb. Apoptotic cells were analyzed by double staining with 7-aminoactinomycin D (Molecular Probes) and streptavidin-phycoerythrin. Early apoptotic cells were gated using purified anti-CD16/32 mAb. Apoptotic cells were characterized as annexin V<sup>+</sup> 7-aminoactinomycin D<sup>−</sup>, using groups of at least four mice. Flow cytometric analyses were performed on a FACS Calibur and analyzed with CellQuest software (BD Biosciences).

Challenge for recall and mouse ear-swelling test

Ten days after immunization, mice were challenged for recall by injecting 50 µL of 10 mM NiCl₂ in sterile, pyrogen-free saline into the pinnae of both ears or by applying 50 µL of 0.2% DNFB (w/v) onto both ears. Forty-eight hours later, delayed-type hypersensitivity was determined by measuring the increment in ear thickness compared with prechallenged value. For determination of prechallenge values, mice were anesthetized with CO₂; for measurement after elicitation, the mice were killed by asphyxiation with CO₂. Measurements were performed using a micrometer (Ditest D 1000 gauge; Kroepelin) and in a blind manner.

Cell separations

Splenic B cells were isolated from erythrocyte-free single-cell suspensions by depleting Thy-1.2<sup>−/−</sup>, CD11b<sup>−/−</sup>, and CD11c<sup>−/−</sup> cells using magnetic cell sorting (autoMACS; Miltenyi Biotec), thus enriching CD19<sup>+</sup> B cells to a purity of >95%. For separation of splenic T cells, CD19<sup>−/−</sup>, CD11b<sup>−/−</sup>, and CD11c<sup>−/−</sup> cells were depleted magnetically, yielding a purity of >90%, as determined by flow cytometry (FACCSCalibur; BD Biosciences). DCs were enriched, as described by Steinman et al. (24), with modifications. Briefly, spleen cell homogenates were digested with collagenase D (1 mg/ml) and Dnase I (1 µg/ml) for 1 h at 37°C, then washed and centrifuged in dense BSA. From the interphase cells, DCs (CD11c<sup>+</sup> MHC-II<sup>+</sup>) were then sorted using the FACS Calibur to a purity of >95%.

Nickel loading in vitro

Splenic B cells were cultured overnight in complete medium containing 75 µM NiCl₂ and then washed twice in sterile, pyrogen-free PBS (Sigma-Aldrich).

Apopotis induction in vitro

B cell apoptosis was induced by γ-irradiation with 6 Gy in a 137Cs source (gammacell 2000; Munksgaard).

Adaptive cell transfers

For immunization to nickel, mice received i.d. injections into both flanks of NiCl₂/H₂O₂ 18 h later. After another 24 h, 10<sup>5</sup> splenic DCs of these first recipients were prepared and injected i.v. into the tail vein of recipient mice. First recipient mice of Ni<sup>low</sup> B cells were depleted magnetically, yielding a purity of >90%, as determined by flow cytometry (FACCSCalibur; BD Biosciences). All groups of recipient mice used for measurement of ear-swelling reactions were comprised of four to five mice. One day later, mice were challenged i.d., as described above. Ten days later, secondary reactions were elicited by i.d. injections at the ears, and 48 h thereafter their ear-swelling response was measured.

Flow cytometry

For FACS analysis of B cells and DCs, respectively, FcRs were blocked by using purified anti-CD16/32 mAb. Apoptotic cells were analyzed by double staining with 7-aminoactinomycin D (Molecular Probes) and biotin-labeled annexin V, using streptavidin-allophycocyanin. Early apoptotic cells were characterized as annexin V<sup>+</sup> 7-aminoactinomycin D<sup>−</sup>, using groups of at least four mice. Flow cytometric analyses were performed on a FACS Calibur and analyzed with CellQuest software (BD Biosciences).

Nickel content of B cells

The nickel content of Ni<sup>low</sup> B cells was assessed by using the dye Newport Green DCF from Invitrogen Life Technologies.

RNA isolation and RT-PCR

RNA was isolated using TRizol (Invitrogen Life Technologies). Reverse-transcriptase reactions were conducted using 1 µg of RNA in a total volume of 40 µl using mouse mammary tumor virus-reverse transcriptase (Invitrogen Life Technologies). Real-time PCR were performed using the Quantitect SYBR Green PCR Kit (Qiagen) and conducted in a LightCycler (Roche Applied Science).
Statistical analysis

Statistical significances of results were determined by one-way ANOVA, followed by the Newman-Keuls test or Student’s t test (flow cytometry data), performed with GraphPad Prism (GraphPad) or Microsoft Excel (Microsoft).

Results

In WT Ni<sub>high</sub> mice, B cells are numerically decreased and prone to apoptosis

Continuous oral treatment of C57BL/6 mice with 10 mM NiCl<sub>2</sub> induces profound immune tolerance to nickel without signs of overt toxicity (22). This notwithstanding, we noted a significant reduction in spleen weight of WT Ni<sub>high</sub> mice compared with WT Ni<sub>low</sub> mice. However, no reduction in spleen weight was detectable in Jα18<sup>−/−</sup> Ni<sub>high</sub> mice lacking iNKT cells (Fig. 1A, left panel). Flow cytometric analysis of splenocytes showed that the reduction in spleen weight observed in WT Ni<sub>high</sub> mice was due to a significant decrease in the average number of CD19<sup>+</sup> B cells, whereas no such decrease was observed in Jα18<sup>−/−</sup> Ni<sub>high</sub> mice (Fig. 1A, right panel). T cells and DCs were not affected in WT Ni<sub>high</sub> mice and Jα18<sup>−/−</sup> Ni<sub>high</sub> mice (data not shown).

As revealed by analysis of Fas expression on B cells, Ni<sub>low</sub> Jα18<sup>−/−</sup> mice exhibited an increased fraction of Fas<sup>+</sup> splenic B cells (Fig. 1B, right panel) compared with Ni<sub>low</sub> WT mice (Fig. 1B, left panel). Additionally, in both WT and Jα18<sup>−/−</sup> mice, we observed an increased frequency of Fas<sup>+</sup> B cells upon oral administration of NiCl<sub>2</sub>, and, again this frequency was higher in Jα18<sup>−/−</sup> mice (Fig. 1B, right panel) than in WT mice (Fig. 1B, left panel). These observations prompted us further to investigate the susceptibility to apoptosis induction in splenic B cells of Ni<sub>high</sub> mice. Indeed, compared with the results from WT Ni<sub>low</sub> mice, WT Ni<sub>high</sub> B cells showed an increased mRNA expression of the proapoptotic surface protein Fas (Fig. 1C, left panel), consistently with a strong reduction in mRNA expression of the antiapoptotic proteins bcl-2 and Bcl-x<sub>L</sub> (Fig. 1C, center and right panels). When comparing the uptake of nickel ions in B cells from Ni<sub>low</sub> and Ni<sub>high</sub> mice, indeed, Ni<sub>high</sub> B cells contained about three times as many nickel ions as Ni<sub>low</sub> B cells (Fig. 1D). This conforms to recent results to be reported in another study, in which the nickel concentration in spleen was measured by atomic absorption spectroscopy (X. Wu, K. Roelofs-Haarhuis, J. Zhang, M. Nowak, L. Layland, M. Fang, E. Iermann, and E. Gleichmann, submitted for publication). Apparently, the proapoptotic alterations seen in Ni<sub>high</sub> B cells were a direct consequence of the increased nickel concentrations of these cells.

**Fas-defective mice are resistant to oral tolerance induction toward nickel**

To analyze whether Fas or FasL molecules were, in fact, required for the induction of oral nickel tolerance, WT and Fas-defective Fas<sup>−/−</sup> mice were subjected to the oral tolerance treatment or remained untreated. Mice were then immunized with either NiCl<sub>2</sub> alone or NiCl<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>, challenged with NiCl<sub>2</sub> for elicitation, and tested for nickel hypersensitivity vs tolerance. As expected, immunization of WT Ni<sub>low</sub> mice with NiCl<sub>2</sub> alone induced back-ground ear swelling, whereas immunization with NiCl<sub>2</sub>/H<sub>2</sub>O<sub>2</sub> induced nickel hypersensitivity (Fig. 2, bar 1 vs bar 2). Immunization of Fas<sup>−/−</sup> Ni<sub>low</sub> mice with NiCl<sub>2</sub> alone, however, induced a marked ear-swelling response, comparable to that induced by NiCl<sub>2</sub>/H<sub>2</sub>O<sub>2</sub> (Fig. 2, bar 3 vs bar 4). This discrepancy between WT Ni<sub>low</sub> and Fas<sup>−/−</sup> Ni<sub>low</sub> mice may be due to differences in the expression of co stimulatory molecules CD80 and CD86.
CD86 on DCs of draining lymph nodes. In WT Nihigh mice, immunization with NiCl2/H2O2 up-regulates the expression of these molecules, whereas immunization with NiCl2 alone fails to do so (17). Because Fas-lpr mice possess an a priori stimulated immune system with increased expression of CD80 and CD86 (26), they promptly responded with hypersensitivity to immunization with NiCl2 alone. Nickel hypersensitivity was significantly reduced in WT Nihigh mice when compared with WT Nilow mice (Fig. 2, bar 5 vs bar 2), confirming previous results (22). Notably, however, Fas-lpr Nihigh mice failed to show a reduced response to nickel (Fig. 2, bar 7). Comparable results were obtained using Fasl-defective Fas-gld mice (data not shown). Apparently, the induction of oral nickel tolerance involves Fas/FasL-mediated apoptotic cell death.

Nihigh B cells need to express Fas to acquire tolerogenicity

The tolerance of Nihigh mice can be adaptively transferred not only through T cells, but also by B cells (17). Because induction of nickel tolerance requires intact Fas and FasL molecules (Fig. 2, and results not shown), we asked whether the B cells of Nihigh mice need to express functional Fas to be effectively tolerogenic upon transfer to Nilow recipients. Prospective WT and Fas-lpr donor mice were orally treated with NiCl2 or remained untreated. Splenic B cells from these donors were then transferred into Nihigh recipients, which were subsequently immunized with NiCl2 alone or NiCl2/H2O2, rechallenged with NiCl2, and tested for induction of nickel tolerance. As shown before (17), the transfer of B cells from WT Nihigh donors, but not Nilow donors, conferred nickel tolerance to Nihigh recipients (Fig. 3, bar 4 vs bar 3). In contrast, transfer of B cells from Fas-lpr Nihigh donors failed to do so (Fig. 3, bar 6), demonstrating that Nihigh B cells require the expression of functional Fas to induce tolerance in Nihigh recipients.

The preapoptotic state of Nihigh B cells is enhanced by NiCl2/H2O2 immunization

Previously, our laboratory reported that upon adoptive transfer of Nihigh B cells to Nilow recipients, immunization of the latter with NiCl2/H2O2 was required for the infectious spread of tolerance to nickel-reactive T cells in Nihigh recipients. Paradoxically, to be set into motion, this tolerance spread required posttransfer immunization of recipients with NiCl2 and the adjuvant H2O2 (17). Because immunization with NiCl2/H2O2 up-regulated mRNA expression of the proapoptotic protein bax in Nihigh B cells (Fig. 4), we examined whether posttransfer recipient immunization with NiCl2/H2O2 would drive Nihigh donor B cells into apoptosis. Applying annexin V expression as readout parameter, indeed, there was a significant increase of Nihigh B donor cells undergoing apoptosis in the recipients immunized with NiCl2/H2O2 (Fig. 5, B and D, right panel) compared with the fraction of NiLow donor B cells dying from apoptosis under these conditions (Fig. 5, A and C, right panel). Moreover, in general, the percentage of apoptotic donor B cells in the recipient spleen (Fig. 5, C and D) was higher than in draining lymph nodes (Fig. 5, A and B). Two conclusions can be drawn from the results shown in Figs. 4 and 5. First, only Nihigh B cells up-regulated the proapoptotic protein bax in response to immunization with NiCl2/H2O2, and second, significantly more B cells from Nihigh than from Nilow donors underwent apoptosis in recipient mice after posttransfer NiCl2/H2O2 immunization.

DCs from recipients of Nihigh B cells can induce nickel tolerance

To test whether adoptive transfer of Nihigh B cells, followed by the apoptosis-enhancing recipient immunization with NiCl2/H2O2, could generate DCs that were tolerogenic for nickel, we performed a serial adoptive cell transfer experiment, in which we sequentially used two sets of Nihigh recipient mice. Adoptive transfer of 10^7 Nihigh, but not Nilow B cells, followed by recipient immunization, generated DCs tolerogenic for nickel, but not DNFβ, as determined by DC transfer from the first to the second set of Nilow recipients (Fig. 6). For the following two reasons we can rule out the possibility that the tolerogenic effect induced by the second set of transferred cells (i.e., 10^5 DCs) was due to a contamination within DCs by residual B cells of the first transfer. First, as shown in Fig. 5, the majority of Nihigh B cells undergo apoptotic death within 24 h after the posttransfer recipient immunization. Second, a spleen from a Nihigh recipient mouse contains ~10^9 cells (data not shown), and of these, 46.7% are B cells (Fig. 1A). In the unlikely event that donor Nihigh B cells from the primary donor

**FIGURE 3.** Fas expression by Nihigh B cells is required for their tolerogenicity. Ear-swelling responses of B cell recipients of Fas-lpr and WT mice after immunization and challenge. Prospective Fas-lpr and WT donor mice were orally treated with NiCl2 (Nihigh) or left untreated (Nilow). Splenic B cells, were then adoptively transferred into Nilow recipients (10^5 cells/mouse). After immunization and challenge, their ear-swelling responses were determined. Results shown represent one of three experiments yielding comparable results.

**FIGURE 4.** Immunization with NiCl2/H2O2 enhances the preapoptotic state of Nihigh B cells. Expression of bax mRNA by splenic B cells of Nihigh and Nilow mice was determined at 24 h after indicated immunizations. Expression levels were calculated as relative expression levels compared with nonimmunized Nilow B cells. Results shown represent one of three experiments yielding comparable results.
would remain intact and replace recipient B cells in the spleen, the 10^7 B cells that were initially transferred from the primary donors would still only account for 21.4% of total splenic B cells in the first set of recipient mice. Even in the improbable case that all of the 5% non-DCs contaminating the 10^4 cells used in the second transfer consisted of intact Ni^{high} B cells, they were still fewer than 10^6 cells, and thus would have fallen below the number of Ni^{high} B cells required for transfer nickel tolerance (17).

Appearance of FasL-expressing NKT cells in the draining lymph nodes and spleen is required for tolerance transfer by Ni^{high} B cells

Previously, our group demonstrated that the presence of iNKT cells in recipients and recipient immunization with NiCl_2/H_2O_2 were required for the tolerance transfer by T cell-depleted spleen cells from Ni^{high} donors (14). In view of the pivotal role of Fas-mediated apoptosis in the tolerance transfer by B cells (Fig. 2, bar 7), we tested whether, apart from their IL-4 and IL-10 production (14), iNKT cells might contribute to nickel tolerance by apoptosis induction of donor B cells, using their expression of FasL. Because recipient injection with NiCl_2/H_2O_2 is necessary for the spread of tolerance from Ni^{high} donor cells to Ni^{low} recipient cells (17), we asked whether this immunization up-regulates FasL on recipient iNKT cells, which would enable them to elicit apoptosis in the strongly Fas^- Ni^{high} B cells shown above (Fig. 1, B and C, left panels).

For this purpose, we determined both the total number of NKT cells and the number of FasL^+ NKT cells in the draining axillary and inguinal lymph node and spleen of Ni^{low} mice following i.d. injection at the flanks of either NiCl_2/H_2O_2, NiCl_2 alone, or saline. Under physiological conditions, i.e., in the absence of any injection, the numbers of NKT cells in both lymph nodes and spleen were relatively low (Fig. 7, B and D). However, 24 h after injection of either NiCl_2/H_2O_2, NiCl_2 alone, or saline, the absolute number of NKT cells in both the draining lymph node and spleen was increased. By 48 h, the numbers of NKT cells had declined to the baseline level seen in untreated mice or below (Fig. 7, B and D).

Similarly, in the absence of any injection, the numbers of NKT cells expressing FasL on their surface were relatively low (Fig. 7, C and E), but at 24 h after injection of either NiCl_2/H_2O_2, NiCl_2 alone, or saline, again, the number of FasL^+ NKT cells in the draining lymph node and, even more pronounced, the spleen was increased (Fig. 7, C and E). Again, this effect was irrespective of the type of substance injected. In both the draining lymph nodes and spleen, the appearance of FasL^+ NKT cells peaked on day 1 after injection and returned to preinjection values by day 2 (Fig. 7, C and E).

In contrast to the draining axillary and inguinal lymph nodes, no changes in NKT cellularity were observed in the distal poplitical lymph nodes (Fig. 7F). The increase in and the kinetics of the number of FasL^+ NKT cells (Fig. 7, C and E) showed a very similar pattern to that of the total number of NKT cells in the draining lymph node and spleen (Fig. 7, B and D). This suggests that the increase in the number of FasL^+ NKT cells was due to an influx of NKT cells into these lymphoid organs, rather than up-regulation of FasL on resident NKT cells.

iNKT cells contribute to tolerance induction by expression of FasL

Being aware that iNKT cells are required for the tolerance transfer by Ni^{high} B cells (14), we analyzed whether iNKT cells need to express FasL to mediate the tolerance transfer. For this purpose, iNKT-deficient Jα18^-/- mice, instead of Ni^{low} mice, were used as recipients and injected with either Ni^{low} or Ni^{high} B cells, as indicated (Fig. 8A). Two groups receiving Ni^{high} B cells were coincubated with spleen cells from either untreated Ni^{low} or untreated, FasL-defective Fas-gld mice, serving as a source of iNKT cells (Fig. 8A, bars 3 and 4). Confirming and extending the results previously obtained after the transfer of Ni^{high} APCs (i.e., T cell-depleted spleen cells) to Jα18^-/- recipients (14), in this study we show that in the absence of iNKT cells, the Ni^{high} B cells failed to transfer nickel tolerance (Fig. 8A, bar 2), but were able to do so when the recipients were reconstituted by cotransfer of Ni^{low} (i.e., WT) spleen cells as a source of iNKT cells (Fig. 8A, bar 3). However, when the iNKT cell-reconstituting splenocytes for cotransfer were provided by FasL-defective Fas-gld mice, the ear-swelling...
response of J/H9251/H18/H11002 recipients of NH high B cells failed to be reduced (Fig. 8A, bar 4). Hence, iNKT cells were involved in the tolerance transfer through their expression of FasL, which can elicit apoptotic death of FasN/high B cells.

Apoptosis induction in NH high B cells before transfer replaces iNKT cells for tolerance induction

Next, we tested whether the apoptosis-inducing function of FasL+iNKT cells could be replaced by inducing apoptosis of NH high B cells before transfer. Therefore, NH high B cells were γ-irradiated in vitro and transferred into iNKT-deficient J/H9251/H18/H11002 mice, which then were immunized, rechallenged, and tested for tolerance. Transfer of nonirradiated NH high B cells into iNKT-deficient J/H9251/H18/H11002 mice failed to mediate the tolerance. This failure could be corrected by cotransfer of Ni low spleen cells as a source of iNKT cells (Fig. 8B, bars 2 and 3). However, even in the absence of iNKT cells, NH high B cells were able to transfer the tolerance when they were irradiated and, hence, rendered apoptotic before transfer (Fig. 8B, bar 4).

Discussion

Interaction of Fas with its natural ligand FasL contributes to the induction and maintenance of immune tolerance (3, 6, 7). However, it was not resolved by these studies whether Fas/FasL-mediated apoptosis operated in the induction or effector phase of Treg cells and which cell types required expression of which molecules for tolerance to be established. In the present study, we show that Fas+ B cells loaded with Ag have superb tolerogenic potential and that the FasL+ cell type that elicits their apoptotic death is the iNKT cell.
Our results indicate that nickel ions exert two different functions in the model of oral nickel tolerance: apart from generating specific neo-Ags, they exert proapoptotic effects and, hence, open up a pathway to tolerance induction. Therefore, three different explanations, which are not mutually exclusive, could account for the tolerogenicity of Ni\textsuperscript{high} B cells. First, due to the higher nickel content of Ni\textsuperscript{high} B cells, the number of nickel-induced neo-Ags present in these cells probably exceeded that in Ni\textsuperscript{low} B cells. This would correlate to findings that, in general, higher Ag doses induce more profound immune tolerance than lower doses (27). Second, nickel ions per se could be tolerogenic because they induce proapoptotic events (28). Consistent with this, we observed an emi-

npt apoptotic susceptibility of Ni\textsuperscript{high} B cells, as manifested by down-regulation of bcl-2 and Bcl-x\textsubscript{L} and up-regulation of Fas and bax. The enhanced Fas expression by Ni\textsuperscript{high} B cells was shown to be due to excess of nickel ions, and not to lack of iNKT cells that delete Fas\textsuperscript{+} cells. Consequently, upon transfer and recipient immunization, Ni\textsuperscript{high} B cells rapidly underwent apoptosis, which at last put their tolerogenic potential into effect. In contrast, Ni\textsuperscript{low} B cells were less susceptible to apoptosis and, consequently, failed to be tolerogenic. Third, the tolerogenicity of Ni\textsuperscript{high} B cells might also be accounted for by their lower CD40 expression compared with that of Nilow B cells (17). It is known that ligation of CD40 might use their FasL molecules to induce apoptosis of Fas\textsuperscript{+} Ni\textsuperscript{high} B cells. Indeed, in cotransfer experiments with Ni\textsuperscript{high} B cells (Fig. 8A), we found that the FasL-defective splenocytes of Fas\textsuperscript{-/−} mice, unlike those of Ni\textsuperscript{low} mice, were unable to compensate for the iNKT cell deficiency of J\textsuperscript{18−/−} recipients of tolerogenic Ni\textsuperscript{high} B cells. Admittedly, we cannot rule out the possibility that iNKT cells transferred from Fas\textsuperscript{-/−} mice have other defects besides their FasL mutation. This is unlikely, however, in view of our finding that Ni\textsuperscript{high} B cells, which were driven into apoptosis by γ-irradiation ex vivo, proved able to induce tolerance in the absence of iNKT cells (Fig. 8B), thus bypassing their function.

NKT cells are known to exert effects through cytokine secretion (13) or cytotoxic mechanisms, including FasL-Fas interaction (37). With regard to tolerance induction, the implication in most studies was that iNKT cells operate solely through cytokine secretion. Only two studies suggested that they might promote tolerance by other mechanisms, namely α-GalCer-induced cytotoxicity (38) or elicitation of apoptotic death of activated T cells (39). That B cells interact with iNKT cells in the process of tolerance induction was first realized in the ACAID model (40), in which Ag-transporting F4/80\textsuperscript{+} APCs and splenic marginal zone B cells cooperate in a CD1d-dependent manner with iNKT cells (20), the latter then acting by secretion of IL-10 (19). Likewise, for successful transfer of nickel tolerance, Ni\textsuperscript{high} B cells must express CD1d on their surface (14), suggesting that they activate iNKT cells before these elicit B cell apoptosis.

The results of the present study, obtained from adoptive transfer experiments with Ni\textsuperscript{high} B cells, are first that iNKT cells must induce FasL-mediated apoptosis of preapoptotic B cells to make B cell tolerogenicity effective. Therefore, the lack of iNKT cells in J\textsuperscript{18−/−} mice, which failed to become tolerant upon transfer of Ni\textsuperscript{high} B cells, could be bypassed if Ni\textsuperscript{high} B cells were rendered apoptotic (i.e., γ-irradiated) before transfer, indicating that elicitation of apoptosis of Ni\textsuperscript{high} B cells is the main tolerogenic function of iNKT cells in our model.

This conclusion does not necessarily contradict the previous report from our laboratory (14) that production of IL-4 and IL-10 by iNKT cells is required for the tolerance transfer by nonirradiated Ni\textsuperscript{high} APCs (i.e., T cell-depleted spleen cells). Our observations that γ-irradiated, apoptotic Ni\textsuperscript{high} B cells are able to induce tolerance in the absence of FasL-expressing (Fig. 8) and IL-4- and
IL-10-producing (14), iNKT cells might suggest that these cytokines act upstream of apoptosis induction. This view is supported by the fact that iNKT cell-derived IL-4 in an autocrine fashion up-regulates the FasL expression on the producing cells (41). Hence, the tolerance-promoting activity of iNKT cells may be due to an autocrine action of IL-4 that promotes FasL-mediated cytotoxicity, which is bypassed by rendering Ni\textsuperscript{high} B cells apoptotic by γ-irradiation. There is no information indicating a tolerogenic activity of IL-10 upstream of B cell apoptosis. Interestingly, Gao et al. (42) showed that apoptosis of murine spleen cells prompts the cells to synthesize and secrete IL-10, regardless whether apoptosis was induced by Fas-FasL interaction or γ-irradiation. Supposing that this mechanism operates in γ-irradiated Ni\textsuperscript{high} B cells as well, it could account for their tolerogenicity in Jα18\textsuperscript{−/−} recipients.

The present study is the first to show that iNKT cells use expression of FasL for elicitation of apoptotic cell death leading to tolerance induction. This characterizes iNKT cells as cells to assist in maintaining tolerance toward the self Ags carried by apoptotic cells, in our case the nickel-induced quasi-self Ags carried by Ni\textsuperscript{high} B cells. Furthermore, our finding of an increased fraction of Fas\textsuperscript{+} B cells in the spleens of untreated (Ni\textsuperscript{low}) Jα18\textsuperscript{−/−} mice suggests that a physiological role of iNKT cells is the elicitation of FasL-mediated apoptosis in effete B cells. Apparently, this is a continuous process in WT Ni\textsuperscript{low} mice, which requires neither oral nickel nor immunization with NiCl\textsubscript{2}/H\textsubscript{2}O\textsubscript{2}.

Following the uptake of apoptotic bodies, DCs present and cross-present phagocytosed Ags to T cells. This process, which has begun to be elucidated for autologous cell types other than B cells (4), is known to result in T cell tolerance, rather than autoimmunity. The uptake by DCs of apoptotic B cells that contain nickel-induced neo-Ags follows this tolerogenic pathway. Indeed, we observed that DCs incorporated apoptotic bodies of Ni\textsuperscript{high} B cells (data not shown). The functional consequence of this uptake was the induction of tolerogenic DCs in recipients of Ni\textsuperscript{high} B cells, as demonstrated by sequential adoptive cell transfers. Those DCs apparently presented antigenic material carried by the apoptotic cells, in our case the nickel ions present in Ni\textsuperscript{high} B cells, in tolerogenic fashion to T cells of the second set of Ni\textsuperscript{high} recipients. This finding is consistent with data reported by Ferguson et al. (3). They transferred DCs from recipients of haptenated, apoptotic splenocytes to a second set of naïve recipients and found that the DCs had transduced the tolerogenic signal so that eventually CD8\textsuperscript{+} Treg cells were induced. The existence of an iNKT cell-DC axis for tolerance induction proposed in the present work is supported by a recent paper of Chen et al. (43); albeit these authors did not invoke apoptosis of Ag-carrying cells as the initial tolerogenic signal. In summary, the present study demonstrated that iNKT cell-mediated apoptosis of Ag-carrying, effete B cells conditioned DCs such that they conveyed tolerogenic signals to nickel-reactive T cells (Fig. 9).

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**References**


