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Human NKT Cells Direct Differentiation of Myeloid APCs that Regulate T cell Responses via Expression of Programmed Cell Death Ligands

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Abstract

NKT cells are innate lymphocytes that can recognize self or foreign lipids presented by CD1d molecules. NKT cells have been shown to inhibit the development of autoimmunity in murine model systems, however, the pathways by which they foster immune tolerance remain poorly understood. Here we show that autoreactive human NKT cells stimulate monocytes to differentiate into myeloid APCs that have a regulatory phenotype characterized by poor conjugate formation with T cells. The NKT cell instructed myeloid APCs show elevated expression of the inhibitory ligand PD-L2, and blocking PD-L1 and PD-L2 during interactions of the APCs with T cells results in improved cluster formation and significantly increased T cell proliferative responses. The elevated expression of PD-L molecules on NKT-instructed APCs appears to result from exposure to extracellular ATP that is produced during NKT-monocyte interactions, and blocking purinergic signaling during monocyte differentiation results in APCs that form clusters with T cells and stimulate their proliferation. Finally, we show that human monocytes and NKT cells that are injected into immunodeficient mice co-localize together in spleen and liver, and after 3 days *in vivo* in the presence of NKT cells a fraction of the myeloid cells have upregulated markers associated with differentiation into professional APCs. These results suggest that autoreactive human NKT cells may promote tolerance by inducing the differentiation of regulatory myeloid APCs that limit T cell proliferation through expression of PD-L molecules.

1. Introduction

Natural Killer T (NKT) cells comprise a small population of innate T lymphocytes that recognize lipid and glycolipid antigens presented by CD1d, a non-classical antigen presenting molecule that is constitutively expressed by monocytes and other types of myeloid APCs [1, 2]. In addition to recognizing specific foreign glycolipids [3-6], NKT cells can also recognize self lipids such as lyso-phosphatidylcholine (LPC), a lipid mediator

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that is continuously produced at low levels as part of endogenous lipid metabolic cascades and is also produced at greatly increased levels during inflammation [7]. Because of their reactivity to self lipids, NKT cells can become activated even in the absence of foreign microbial challenge [8]. Hence, it is thought that they may carry out important functions during immunologically quiet times as well as during periods of inflammatory immune activation.

CD1d-restricted NKT cells have been shown to exert tolerogenic effects in a variety of murine autoimmune disease models, including type 1 diabetes (e.g. NOD mice), systemic lupus erythematosus (MRL/lpr mice), and multiple sclerosis (EAE) (reviewed in [9]). Notably, it seems that recognition of endogenous antigens is sufficient to activate tolerogenic functions of NKT cells in these models, since simply over-expressing CD1d molecules or increasing the frequency of NKT cells results in protection from autoimmune pathology [10-12]. The tolerogenic effects of NKT cells often appear to involve interactions with myeloid APCs [13], however, the mechanisms by which NKT cells promote peripheral tolerance remain unclear. Additionally, it has been established that autoimmune diseases in human patients are often accompanied by numeric or functional deficiencies in CD1d-restricted NKT cells [14-17], suggesting human NKT cells may also normally help to prevent autoimmunity, although specific tolerogenic pathways mediated by human NKT cells remain poorly characterized. We have previously found that human NKT cells can interact with freshly isolated peripheral blood monocytes to direct their differentiation into cells resembling myeloid DCs [18]. The differentiation effect is mediated by NKT cell secretion of the cytokines GM-CSF and IL-13, and requires access to CD1d molecules on the monocytes but does not require the addition of foreign antigens such as α -GalCer, suggesting that NKT cell recognition of self antigens is sufficient [18]. Remarkably, the myeloid APCs resulting from this process of monocyte “instruction” by NKT cells have a highly non-inflammatory phenotype, as they produce IL-10 but little or no IL-12, do not efficiently activate human peripheral blood T cells to produce IFN- γ , and do not promote antigen-dependent tissue inflammation *in vivo* [19]. Moreover, the NKT-instructed APCs do not efficiently stimulate T cell proliferation, despite expressing high levels of MHC at the cell surface [19]. Thus, our analyses suggest that autoreactive human NKT cells are able to induce the differentiation of regulatory myeloid APCs.

In investigating what factors caused the regulatory phenotype of the NKT-instructed myeloid APCs, we found that their failure to produce IL-12 was mainly due to exposure to IL-6 that is produced during the interaction of NKT cells with monocytes [19]. However, IL-6 exposure did not seem to cause a defect in the ability of myeloid APCs to stimulate T cell proliferation. Additionally, the T cell proliferation defect did not seem to be due to IL-10 production by the NKT-instructed APCs [19]. Thus, critical unanswered questions about the NKT-instructed APCs include identifying the factors produced during interactions between monocytes and NKT cells that cause the proliferation-regulating phenotype of the resulting APCs, and understanding the mechanisms by which these APCs regulate T cell responses. As peripheral blood monocytes are one of the most abundant and accessible types of human cells that can serve as a professional APC precursor population, and NKT cells are an innate lymphocyte population that can be clinically manipulated, insight into these questions is likely to prove useful for human immunotherapeutic applications.

2. Materials and Methods

2.1. Isolation of monocytes

Human peripheral blood mononuclear cells (PBMCs) were purified from fresh blood obtained from healthy adult donors using ficoll paque density gradient centrifugation (GE Health Sciences). Monocytes were isolated by magnetic sorting using anti-CD14 beads

(Mitenyi Biotech). The purity of the resulting monocytes, as assessed by flow cytometric analysis, was typically greater than 98%. Analyses involving human samples were approved by the University of Wisconsin Minimal Risk IRB, and written informed consent was obtained from all blood donors.

2.2. NKT cells

Human NKT cell clones were generated and maintained as described previously [18]. Briefly, NKT cells were cultured in RPMI 1640 medium supplemented with 2mM L-glutamine, 100µg/ml each of penicillin and streptomycin, 10% fetal bovine serum and 5% bovine calf serum (Hyclone), 5% human AB serum (Atlanta Biologicals), 400 U/ml recombinant human IL-2 (Chiron), and were stimulated periodically by the addition of irradiated allogeneic PBMCs, and 250 ng/ml PHA (Sigma-Aldrich).

2.3. Monocyte differentiation

Monocyte differentiation was carried out in RPMI 1640 culture medium supplemented with 2 mM L-glutamine, 100 µg/ml penicillin and streptomycin, and 10% fetal bovine serum. Monocytes alone were placed in the lower wells of trans-well plates, and a 2:1 mixture of NKT cells and monocytes were placed in the trans-well insert. After 8 hours, the trans-well insert was removed, and the monocytes in the lower well were cultured in the remaining culture medium for an additional 64 hours to allow differentiation to occur. In experiments designed to block purinergic signaling, 30 µM Suramin (Acros Organics) was added to the medium at the beginning of the culture protocol. In experiments designed to test the role of NKT cell contact, monocytes were co-cultured for 72 hours at a 1:2 ratio with NKT cells, then the NKT cells were removed by magnetic sorting using antibodies against receptors specifically expressed on the NKT cells (e.g. CD3 and CD4). Cytokine-induced APCs were generated by culturing monocytes for 72 hours in medium containing 300 U/ml recombinant human GM-CSF (Berlex Labs) and 200 U/ml recombinant human IL-4 (Peprotech). All APCs were treated with 250 ng/ml of *Salmonella typhimurium* LPS for 8 hours prior to use in experiments.

2.4. T cell proliferation

T cells were isolated from human PBMCs as described previously [19], and labeled with 2.5 µM CFSE (Invitrogen). The T cells were cultured at a 50:1 ratio with allogeneic APCs, and T cell proliferation was assessed on day 7 by flow cytometric analysis. The percentage of live T cells that had undergone cell division was determined by gating on DAPI-negative CD3⁺ cells, and assessing the fraction that showed diminished CFSE fluorescence intensity. Where indicated, inhibitors of arginase (Nor-NOHA, from Calbiochem) or inducible nitric oxide synthase (1400W, from Calbiochem) were added to the APC and T cell co-culture. For blocking experiments, T cells and APCs were co-incubated in medium containing neutralizing antibodies against PD-L1 and PD-L2 (clones 29E.2A3 and MIH18 or 24F.10C12), or isotype-matched negative control antibodies, at 10 µg/ml each.

2.5. Statistical analyses

All functional experiments were performed in a paired format (e.g. NKT-instructed APCs compared to cytokine-induced APCs prepared from the same monocytes, or NKT-instructed APCs treated with a blocking antibody compared to those treated with an isotype control antibody), and therefore in all cases P values were calculated using a one-tailed paired *t*-test.

2.6 Flow cytometric analysis of T cells

T cells isolated from human PBMC were cultured with the indicated allogeneic APCs for the indicated lengths of time. The cells were then stained with fluorescently labeled

antibodies against T cell specific markers (e.g. CD3, CD4), and with antibodies against the antigen of interest (e.g. CD69, PD-1; clones FN50 and EH12.2H7 from Biolegend, respectively), or isotype matched negative control antibodies. For analysis of FoxP3 expression, the cells were fixed and permeabilized using Cytofix/Cytoperm reagents (BD Biosciences) then stained with an antibody against FoxP3 (Clone 206D, Biolegend) or an isotype matched negative control antibody. Flow cytometric data were collected on a Becton Dickinson LSRII flow cytometer, and analysis was performed using Flowjo software (TreeStar Inc.).

2.7. Conjugation and microscopy

APCs were labeled with Vybrant DiD dye (Invitrogen) and allogeneic T cells were labeled with CFSE (Invitrogen). For short term conjugation assays, APCs were mixed with a 1:1 ratio of allogeneic T cells, centrifuged to initiate contact, and incubated for the indicated times at 37°C. The cells were then re-suspended by vigorous vortexing, and analyzed by flow cytometry. For the “zero” time point, contact was initiated by centrifugation, but the cells were immediately vortexed without a 37°C incubation. For microscopic analysis of the formation of multi-cell clusters, APCs and allogeneic T cells labeled with fluorescent dyes as described above were co-cultured at a 1:1 ratio for 24 hours at 37 °C with 5% CO₂ in a flat-bottom 96 well plate. Microscopic analysis was performed on an Axiovert 100 fluorescent microscope at 20x magnification (Zeiss). Analysis of fluorescent images was performed using Image J software (NIH).

2.8 Analysis of APC PD-L expression

APCs were generated as described in section 2.3, and stained with fluorescently labeled antibodies against PD-L1 (clone 29E.2A3) or PD-L2 (clone 24F.10C12), or isotype matched negative control antibodies.

2.9. Calcium flux

Monocytes were labeled with the calcium indicator dyes Fluo-4 and Fura-Red (Invitrogen) and analyzed by flow cytometry. For each sample, flow cytometric data were first collected on unstimulated cells to provide a baseline to set the threshold, then culture supernatants from NKT cells alone, or a 2:1 ratio of NKT cells and monocytes, were added to the monocyte suspension, and flow cytometric data were acquired for the following 250 seconds. Where indicated, apyrase enzyme (Sigma Aldrich) was added to culture supernatants 10 minutes prior to using them for stimulation of monocytes. The flow cytometric data were analyzed using FlowJo software (TreeStar Inc.).

2.10. ATP measurement

NKT cells alone, or a 2:1 ratio of NKT cells and monocytes, were incubated in culture medium for 8 hours at 37 °C with 5% CO₂. Culture supernatants were centrifuged then filtered through a 0.2 µm membrane to remove any cells. The supernatants were then analyzed for the presence of ATP using the Kinase-Glo Luminescent Kinase Assay kit (Promega), according to the manufacturer's protocol.

2.11. Co-localization of human NKT cells and monocytes after transfer into immunodeficient mice

Purified human peripheral blood monocytes and an autoreactive human NKT cell clone (10 × 10⁶ cells of each) were intravenously injected into NOD/*PRKDC*^{SCID}/*IL-2Rγ*^{-/-} mice (Jackson Laboratories). At varying time-points post-injection (e.g. 3 hrs, 24hrs, 60 hrs) the animals were sacrificed and the spleens and livers harvested.

For flow cytometric analysis, spleens were homogenized by pressing the tissue through a 70 μ M strainer, then red blood cells were lysed using ACK lysis buffer, and the remaining cells were washed with PBS and used for analysis. For recovery of cells from the liver, the tissue was fragmented, then pressed through a 70 μ M strainer, and incubated in PBS containing 2 μ g/ml collagenase B (Roche Applied Science) for 45 minutes at 37 °C. The liver cells were then washed, suspended in 44% percoll solution and centrifuged at 400g for 20 minutes. Red blood cells in the cell pellet were lysed using ACK lysis buffer, and the remaining cells were washed in PBS and stained for flow cytometric analysis with fluorescently labeled antibodies against human CD45, human CD3, and human CD14.

For histological analyses spleen and liver tissues were formalin-fixed and paraffin-embedded, then sectioned and mounted onto slides. The sections were deparaffinized, rehydrated, and antigen retrieval was performed by incubation in citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0) for 20 min at 98°C. Endogenous peroxidases were quenched using 0.3% hydrogen peroxidase solution, and nonspecific labeling was blocked with 5% goat serum. The sections were labeled with an anti-human CD45 antibody (Biocare Medical), then detected using Super Sensitive Polymer-Horseradish Peroxidase Immunohistochemistry second step reagents (BioGenex Inc), followed by diaminobenzidine (Vector Laboratories Inc.) chromogen development, and counterstaining with hematoxylin (Sigma).

2.12. APC differentiation in vivo

Freshly isolated human peripheral blood monocytes and purified autologous T cells (10×10^6 cells of each) were intravenously injected into NOD/*PRKDC^{SCID}*/IL-2R $\gamma^{-/-}$ mice (Jackson Laboratories) with or without an autoreactive human NKT cell clone (10×10^6 cells). Sixty hours post-injection the animals were sacrificed and the spleens and livers harvested. Cells were prepared for flow cytometric analysis as described in section 2.11, and stained with antibodies against the following human antigens: CD45 (clone HI30), CD3 (clone OKT3), CD11b (clone ICRF-44), CD11c (clone 3.9), CD40 (clone HB14), CD80 (clone 2D10), CD86 (clone IT2.2). Negative control antibodies were: murine IgG1 (clone MOPC-21), IgG2a (clone UPC10) murine IgG2b (clone MPC-11).

3. Results

3.1. NKT-instructed APCs are refractory to conjugation with T cells

In our previous analyses we have found that soluble factors produced during a brief (4-8 hour) interaction between human NKT cells and freshly isolated peripheral blood monocytes are sufficient to cause the monocytes to differentiate into DC-like cells that have regulatory properties [18, 19]. There appears to be little clonal variation among individual NKT cells in this effect on monocytes, since all of the NKT cell clones tested (including both CD4⁺ and CD4⁻ ones), as well as a short-term polyclonal human NKT cell line, caused the differentiation of myeloid APCs showing a similar regulatory phenotype [19]. Additionally, consistent with the almost complete lack of allelic polymorphism at the human CD1d locus, we have found that human NKT cells produce similar effects on autologous and allogeneic monocytes. To investigate the properties of APCs that differentiate in response to factors produced by NKT cells without contamination from the NKT cells, we use a trans-well culture system in which freshly purified monocytes alone are placed in the lower well, and monocytes and NKT cells are placed in the trans-well insert. After 8 hours the trans-well insert is removed, and the monocytes in the lower well are allowed to differentiate for an additional 64 hours. The resulting APCs are then removed and used to stimulate purified peripheral blood T cells. NKT-instructed APCs of this type show a reduced capacity to

stimulate allogeneic T cell proliferation compared to APCs differentiated by exposure of monocytes to recombinant cytokines such as GM-CSF and IL-4 (Figure 1A).

We had previously found that this defect in the induction of allogeneic T cell proliferation did not appear to be due to IL-10 secretion by the NKT-instructed APCs [19], and therefore we investigated whether other suppressive pathways might be responsible. Myeloid APCs have been found to suppress T cell responses by producing immunoregulatory enzymes such as arginase, inducible nitric oxide synthase (iNOS), or indoleamine 2, 3-dioxygenase (IDO), or alternatively by inducing increased frequencies of FoxP3⁺ regulatory T cells (T_{reg}s). However, we found that NKT-instructed and cytokine-induced APCs produced similar levels of arginase (Figure 1B), and chemical inhibitors of arginase, iNOS, and IDO failed to promote T cell proliferation in response to NKT-instructed APCs (Figure 1C and 1D, and data not shown), suggesting that the regulatory effects of the NKT-instructed APCs are not due to these enzymes. Similarly, comparable frequencies of FoxP3⁺ T cells arose after exposure to cytokine-induced or NKT-instructed APCs (Figure 1E), suggesting that the lack of T cell proliferation was not due to the induction of higher numbers of T_{reg} cells in response to NKT-instructed APCs.

To investigate further, we tested expression of the early activation marker CD69 on T cells after exposure to cytokine-induced or NKT-instructed APCs. CD69 was rapidly upregulated on T cells in response to both types of APCs, however, compared to the cytokine-induced APCs, CD69 upregulation brought about by the NKT-instructed APCs was not as efficient and not well sustained (Figure 2A). This finding suggested that there was an early defect in T cell activation by the NKT-instructed APCs. We next compared the ability of the NKT-instructed and cytokine-induced APCs to form tight conjugates with T cells. APCs and allogeneic peripheral blood T cells were fluorescently labeled in different colors, briefly centrifuged to initiate contact, and incubated together at 37 °C for varying lengths of time. The cells were then vortexed vigorously to disrupt loosely associated cells, and analyzed by flow cytometry. Whereas the frequency of T cells that were tightly bound to the cytokine-induced APCs increased over the first 10 minutes of contact time, reaching a plateau of about 30% of the total T cells by 20-30 minutes, the fraction of T cells tightly bound to the NKT-instructed APCs showed no increase from the starting time, remaining under 10% of the total T cells (Figure 2B). Moreover, after 24 hours of co-culture, T cells and cytokine-induced APCs were incorporated into multi-cellular clusters measuring 100 μm in diameter or more, whereas there was little or no evidence of T cells in clusters with NKT-induced APCs (Figure 2C, and Figure S1). Thus, NKT-induced APCs appeared refractory to establishing stable conjugates with T cells, yet exposure to these APCs altered T cell expression of CD69, suggesting that the APCs did indeed deliver a signal to the T cells.

3.2. NKT cells induce myeloid APC upregulation of programmed cell death ligands (PD-Ls)

As APC expression of inhibitory B7 family members such as PD-L1 and PD-L2 has been associated with resistance to conjugation with T cells, we analyzed the expression of these molecules on the NKT-instructed APCs. Compared to the cytokine-induced APCs, the NKT-instructed APCs typically had similar or somewhat lower cell surface expression of PD-L1 but consistently showed markedly higher levels of PD-L2 (Figure 3A). Addition of a blocking antibody against PD-L1 to co-cultures of allogeneic T cells and NKT-instructed APCs produced significantly increased T cell proliferation, whereas the effect of an anti-PD-L2 blocking antibody produced slight increases in T cell proliferation that did not rise to the level of statistical significance (Figure 3B). Inclusion of antibodies against both PD-L1 and PD-L2 typically produced a greater enhancement of T cell proliferation (Figure 3B). The addition of anti-PD-L1 and anti-PD-L2 antibodies also resulted in the incorporation of T cells into multicellular clusters with APCs within 24 hours (Figure 3C). Together, these results suggest that expression of PD-L molecules on the NKT-instructed APCs prevents T

cells from establishing tight conjugates with the APCs and therefore prevents the sustained activation that is required to induce T cell proliferation. We also noted that the frequency of T cells expressing programmed cell death-1 (PD-1, the ligand for PD-L1 and PD-L2), became up-regulated within 8 hours of exposure to NKT-instructed APCs and lasted for at least 96 hours (Figure 3D). Thus, the regulatory phenotype of the NKT-instructed APCs appears to be related to their expression of inhibitory B7 family proteins.

3.3 A role for secreted ATP in inducing the regulatory APC phenotype

We have previously found that soluble factors produced by NKT cells have a dominant effect on the phenotype of the APCs, since the regulatory phenotype of the NKT-instructed APCs cannot be overcome by the addition of GM-CSF and IL-4 during monocyte differentiation, whereas monocytes that are exposed to recombinant GM-CSF and IL-4 alone acquire a strongly pro-inflammatory phenotype [19]. This suggests that the regulatory phenotype of the NKT-instructed APCs is not due to insufficient stimulation during differentiation, but rather to the presence of one or more factors that alter the properties of the resulting APCs.

Therefore, we explored whether there is a signaling difference in monocytes treated with soluble NKT cell factors compared to those treated with recombinant GM-CSF and IL-4. Fresh peripheral blood monocytes that were exposed to supernatants from NKT cells cultured with monocytes showed a marked cytoplasmic calcium flux, whereas exposure to GM-CSF and IL-4 induced little or no detectable response (Figure 4A). The calcium flux induced by NKT+monocyte culture supernatants consistently had a biphasic appearance resembling that induced by extracellular ATP, which stimulates intracellular calcium flux both through G-protein coupled receptors and ion channel receptors [20]. Based on this similarity, we investigated further and found that supernatants from NKT cells cultured with monocytes contained significantly elevated levels of ATP compared to those of NKT cells alone (Figure 4B), and treating the NKT+monocyte supernatant with the enzyme apyrase, which breaks down ATP and ADP, resulted in a reduction in the ability of the supernatant to induce monocyte calcium flux (Figure 4C). Importantly, whereas monocytes cultured alone showed substantial cell death, cell death was markedly reduced when the monocytes were cultured in the presence of soluble factors produced by NKT cells (Figure 4D). Therefore, the induction of monocyte calcium flux did not correlate with cell death. Thus, the interaction between NKT cells and monocytes appears to result in the release of ATP into the culture supernatant without inducing substantial cell death.

Supernatants from monocytes cultured alone caused little or no detectable calcium flux in fresh monocytes (Figure 4A), suggesting that NKT cells are required for the generation of the soluble factor that induces monocyte calcium flux. However, while supernatants from NKT cells stimulated with an anti-CD3 antibody induced some calcium flux in fresh monocytes, the flux induced by these supernatants was consistently less than that induced by supernatants from co-cultures of NKT cells with monocytes (Figure 4E). This suggested that both NKT cells and monocytes might contribute to the secreted factors that induce monocyte calcium flux. To investigate this further, we performed blocking experiments. NKT cells were cultured with monocytes in the presence of antibodies against CD1d or CD40L, or with an isotype-matched negative control antibody. Blocking CD1d during co-incubation of NKT cells with monocytes resulted in culture supernatants that induced approximately 3-fold less monocyte calcium flux than supernatants from NKT cells and monocytes co-incubated in the presence of an isotype-matched control antibody, while blocking CD40L led to about a 2-fold reduction (Figure 4F). Together, these results suggest that both the activation of NKT cells by CD1d molecules on monocytes, as well as the activation of monocytes by CD40L on NKT cells, result in the secretion of ATP that induces monocyte calcium flux.

To further investigate, we tested the effects of suramin, a drug that is an antagonist of cellular purine receptors. NKT-instructed APCs that were generated in the presence of suramin showed markedly reduced expression of PD-L1 and PD-L2 (Figure 5A). Moreover, addition of suramin during the monocyte differentiation stage resulted in NKT-instructed APCs that formed large clusters with allogeneic T cells (Figure 5B), and that showed a significantly enhanced ability to stimulate T cell proliferation compared to NKT-instructed APCs that were generated in the absence of suramin (Figure 5C). These results support the possibility that ATP secreted during the interaction of NKT cells with monocytes is involved in inducing the distinctive conjugation-resistant phenotype of the NKT-instructed APCs.

We have previously shown that GM-CSF and IL-13 are the main factors produced by NKT cells that induce monocytes to differentiate into cells resembling myeloid DCs [18]. However, we have found that the APCs resulting from monocytes that were exposed to the mixture of factors produced by NKT cells consistently stimulate lower T cell proliferative responses than those generated by culturing monocytes with recombinant GM-CSF and IL-13 alone (Figure S2). To further investigate the roles of IL-6 and ATP in producing this regulatory phenotype of the NKT-instructed APCs, we performed reconstitution experiments in which freshly isolated monocytes were incubated for 72 hours in medium containing GM-CSF and IL-13 in the presence or absence of IL-6 and ATP, and the resulting APCs were analyzed for expression of PD-L1 and PD-L2. This analysis revealed that exposure to IL-6 during monocyte differentiation led to slightly enhanced PD-L1 expression but had only minimal impact on PD-L2 levels (Figure 5D). The addition of ATP to GM-CSF and IL-13 produced somewhat elevated expression of PD-L2, and adding both ATP and IL-6 resulted in greatly up-regulated expression levels (Figure 5D). Thus, the presence of extracellular ATP during differentiation induced by GM-CSF and IL-13 appears to particularly increase PD-L2 expression on the resulting APCs.

3.4. NKT cell-mediated induction of monocyte differentiation in vivo

A key question about the human monocyte differentiation pathway identified here is whether it occurs *in vivo*. We have observed that although only soluble factors are required for the induction of monocyte differentiation into DC-like cells, monocytes that are co-cultured together with NKT cells in a format that allows for cell contact acquire a similar APC phenotype (Figure 6A, and [18]). To confirm that myeloid APCs that differentiate through contact with NKT cells have similar regulatory properties as those that differentiate through exposure to cytokines alone, NKT cells were co-cultured with freshly isolated peripheral blood monocytes for 3 days, then the NKT cells were removed by magnetic sorting, resulting in preparations of myeloid APCs that were routinely more than 95% pure. Purified APCs from the contact co-culture that were matured by exposure to LPS failed to efficiently induce proliferation of allogeneic T cells, similar to NKT-instructed APCs that were exposed only to secreted factors (Figure 6B). Thus, contact-dependent stimulation of monocytes by NKT cells (e.g. via CD40L-CD40 interactions) does not prevent the induction of the regulatory phenotype.

We next investigated the ability of human NKT cells and monocytes to co-localize *in vivo*. Purified human monocytes and were injected together with human NKT cells into highly immunodeficient mice (NOD/PRKDC^{SCID}/IL-2R γ ^{-/-}) that lack murine T cells and B cells. After varying amounts of time, the mice were sacrificed and spleens and livers were harvested and analyzed for the presence of human cells. We were able to detect the presence of NKT cells and monocytes in spleen and liver samples by flow cytometric analysis at time points ranging from 3-60 hours post-injection (Figure 6C, and data not shown). Histological analyses revealed that the human cells were initially dispersed throughout the murine tissues, but at later time-points some of the human cells appeared to be localized together in

clusters (Figure 6D), suggesting that the human cells are able to find each other and make contact in an *in vivo* environment.

To investigate whether human NKT cells are able to interact functionally with monocytes in the presence of other human T cells, monocytes were co-injected with purified autologous peripheral blood T cells, in the presence or absence of an autoreactive human NKT cell clone. Murine spleen and liver samples were harvested at 60 hours post-injection and analyzed by flow cytometry for human myeloid cells expressing markers associated with differentiation into professional APCs. In the presence of the human NKT cells a fraction of the myeloid cells showed elevated expression of CD11c with upregulated CD40, CD86, and CD80, whereas this did not occur when the monocytes were co-injected with autologous T cells in the absence of added NKT cells (Figure 6E). These results suggest that interactions with the NKT cells caused a fraction of the monocytes to undergo activation or differentiation, providing support for the possibility that NKT cells can induce monocyte differentiation into APCs *in vivo*.

4. Discussion

It is now well established that NKT cells function to promote immunological tolerance in a variety of contexts, including delaying the onset of autoimmune diseases [10-12], promoting acceptance of allogeneic tissue transplants [21-25], contributing to burn injury induced immune suppression [26-28], and enhancing tolerance to specific antigens that are introduced orally or at immunologically privileged sites [29, 30]. However, there has been little understanding of how NKT cells endogenously promote immunological tolerance. Here we provide novel evidence that the induction of myeloid APCs that have regulatory effects on other T cells comprises one pathway of NKT cell tolerogenic activity.

Interestingly, it has recently been reported that human FoxP3⁺ T_{reg} cells promote the upregulation of PD-L1 on myeloid DCs, leading to a tolerogenic effect on human T cells in an acute GVHD model [31]. Thus, the results presented here suggest that there may be parallels between the tolerance mechanisms employed by human NKT cells T_{reg} cells. Notably, in our system the NKT-instructed myeloid APCs express both PD-L1 and PD-L2, with expression levels of PD-L2 appearing more markedly elevated compared to cytokine-induced APCs. Anti-PD-L1 did generally have a stronger effect than anti-PD-L2 blocking antibodies in regards to reversing the functional defect in T cell proliferation, but a combination of both antibodies gave the greatest effects, suggesting that both inhibitory ligands contribute to the regulatory properties of the NKT-instructed APCs. Additionally, we have previously noted that APCs that differentiate in response to factors produced by autoreactively activated NKT cells tend to express somewhat lower levels of co-stimulatory ligands such as CD80 and CD86 than their cytokine-induced counterparts [19], and thus it is likely that reduced co-stimulatory ligand expression also accounts for a part of the failure of NKT-instructed APCs to efficiently stimulate T cell proliferation. Therefore, it is not yet clear whether myeloid APCs that have been conditioned by human T_{reg} cells are phenotypically or functionally distinct from the NKT-instructed APCs described here, and it will be of great interest to determine whether these two pathways of APC instruction lead to different effects on downstream T cell responses.

Another question of great interest is the mechanism by which regulatory T cells are able to induce the generation of regulatory APCs. Our results suggest that extracellular ATP is produced during monocyte interactions with NKT cells and signaling of this ATP into monocytes via purinergic receptors is important for the induction of the APC regulatory phenotype. Although extracellular purines are often thought to arise mainly through cell death or damage to tissues, recent studies have shown that monocytes constitutively secrete

low levels of ATP, and stimulation with LPS or other TLR ligands increases their ATP secretion [32, 33]. Additionally, TCR stimulation has recently been found to trigger a rapid release of ATP by T cells [34]. Thus, the extracellular ATP we detect from co-cultures of monocytes with NKT cells could come from the NKT cells or from the monocytes themselves. Extracellular ATP produced in this manner could provide either autocrine or paracrine signals to the monocytes through purine receptors, since human monocytes have been shown to express P2Y₁, P2Y₂, P2Y₄, P2Y₁₁, and P2Y₁₃ receptors, as well as P2X₁, P2X₄, and P2X₇ receptors [20]. The P2X receptors function as plasma membrane ion channels [35], while the P2Y receptors are G-protein-coupled receptors [36]. Extracellular ATP can therefore induce cytoplasmic calcium flux in monocytes by binding to P2X receptors and causing them to admit calcium from the extracellular environment, and also by stimulating P2Y receptors that cause the activation of protein kinase C and consequent generation of cytoplasmic inositol tri-phosphate which induces the release of calcium stored in the endoplasmic reticulum. The biphasic calcium flux responses we observe in monocytes exposed to culture supernatants from NKT cells incubated with monocytes are consistent with sequential activation of both types of purinergic receptor, suggesting that the signaling induced is complex and likely involves more than one pathway.

We have previously determined that IL-6 secreted during the interaction of NKT cells with monocytes also contributes to the regulatory phenotype of the resulting APCs [19]. It is thus interesting to note that the presence of extracellular ATP, IL-6, and expression of programmed cell death ligands are all characteristics that have been associated with immunosuppression that occurs in pathological contexts such as tumor microenvironments. Thus, further studies will be required to explore whether the NKT cell-initiated pathway described here mainly provides beneficial immune regulation that helps to prevent autoimmunity, or whether this pathway also contributes to pathological immunosuppression in some circumstances.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Regulatory phenotype of NKT-instructed APCs

A) NKT-instructed APCs limit the proliferation of allogeneic T cells. Purified peripheral blood T cells were labeled with CFSE and cultured for 7 days with allogeneic NKT-instructed or cytokine-induced APCs. The plot shows percentages of live T cells that underwent cell division as estimated by dilution of CFSE signal; each pair of symbols represents an independent analysis of an allogeneic T cell-APC mixture. **B)** Cytokine-induced and NKT-instructed APCs produce similar levels of arginase. APCs were stimulated with LPS for 24 hours or left unstimulated, and the culture supernatants were assessed for arginase activity using a chromogenic assay that detects urea generated by the presence of enzyme (QuantiChrom, Bioassay Systems Inc.). **C and D)** APCs were matured with LPS for 8 hours, washed and cultured with allogeneic T cells in the presence of the

indicated concentrations of either the arginase inhibitor Nor-NOHA (B), or the iNOS inhibitor 1400W (C), for 7 days. Proliferation is shown as the percent of the live T cells (DAPI/CD3⁺) that showed reduced CFSE intensity. Results shown are from one representative experiment out of 2 independent analyses. **E)** NKT-instructed APCs do not induce increased numbers of regulatory T cells. Flow cytometric analysis of intracellular FoxP3 expression by allogeneic T cells after 7 days of culture with cytokine-induced or NKT-instructed APCs. Similar results were obtained in 3 independent experiments.

Figure 2. NKT-instructed APCs are refractory to conjugation with T cells

A) NKT-instructed APCs show an early defect in activation of allogeneic T cells. T cells were exposed to NKT-instructed or cytokine-induced APCs, and assayed for cell surface CD69 expression by flow cytometry at the indicated times. **B)** T cells and APCs were labeled with fluorescent dyes then centrifuged to initiate contact. After the indicated incubation times, the cells were vortexed vigorously to dissociate loosely adhered aggregates, and analyzed by flow cytometry to identify the percentage of the total T cells that were conjugated to APCs. **C)** Allogeneic T cells become incorporated into multi-cell clusters with cytokine-induced APCs, but not with NKT-instructed APCs. T cells were labeled with CFSE (shown in green) and APCs with DiD (shown in red). After 24 hours of co-culture at a 1:1 ratio, APC and T cell clustering was visualized using a fluorescent microscope at 20x magnification; scale bars in the lower left corners of the phase contrast plots represent approximately 100 microns. Results shown in parts B-D are each representative of at least 3 independently performed experiments.

Figure 3. Role of inhibitory B7 family proteins in the regulatory phenotype of NKT-instructed APCs

A) Expression of PD-L1 and PD-L2 by NKT-instructed APCs. APCs were stimulated with LPS for 8 hours and stained for surface PD-L1 and PD-L2 (solid histograms) or with isotype-matched negative control antibodies (open histograms). The result shown is one representative experiment out of 6 independent analyses. **B)** Blocking PD-L molecules promotes T cell proliferation in response to NKT-instructed APCs. The plot shows the fold increase in allogeneic T cell proliferation in response to NKT-instructed APCs in the presence of blocking antibodies against PD-L1 and/or PD-L2 compared to an isotype-matched control antibody. Each symbol represents the results from an independent analysis. **C)** Analysis of the effects of blocking PD-L molecules on the ability of allogeneic T cells to form multi-cellular clusters with NKT-instructed APCs. APCs (red) were cultured for 24 hours with T cells (green) in the presence of neutralizing antibodies against PD-L1 and PD-L2 (lower panels) or with matched isotype control antibodies (upper panels). Similar results were obtained in 3 independent experiments. **D)** Allogeneic T cells cultured with NKT-instructed APCs show upregulation of cell surface PD-1. The plot shows the percentage of T cells having up-regulated cell surface PD-1 after the indicated culture times in the presence of NKT-instructed APCs. Each point shows results from one independent analysis.

Figure 4. Extracellular ATP released during the interaction of NKT cells with monocytes stimulates monocyte calcium flux

A) Supernatant from a brief co-culture of monocytes with NKT cells induces strong intracellular calcium flux in fresh monocytes. The plot shows flow cytometric analysis of intracellular calcium flux, as assessed by a change in the fluorescence ratio of the indicator dyes Fluo-4 and Fura-red, in freshly isolated human monocytes exposed to culture supernatant from NKT cells and monocytes (red line), or culture medium containing GM-CSF and IL-4 (blue line), or medium alone (black line). **B)** Supernatants of NKT cells co-cultured with monocytes (black bar), or NKT cells cultured alone (white bar), or culture medium with no cells (grey bar) were analyzed for the presence of extracellular ATP using a luminescent kinase assay. The plot shows the means and standard deviations from 2 replicates. Similar results were obtained in 4 independent experiments. **C)** Supernatants from a co-culture of NKT cells and monocytes were treated for 10 minutes with the indicated concentrations of apyrase enzyme, then used to stimulate calcium flux in freshly

isolated monocytes. **D)** Monocytes were tested for viability after culture alone (grey bar), in the presence of factors produced by NKT cells (black bar), or in the presence of GM-CSF and IL-4 (white bar). The plot shows the percentage of the monocytes that stained positively for DAPI, an indicator of loss of viability. The results show the means and standard deviations from 3 independent analyses. **E)** Supernatants from NKT cells cultured with monocytes or on plates coated with anti-CD3 antibody were tested for the ability to induce calcium flux in freshly isolated monocytes. The plot shows the fold increase in fluxing monocytes for each type of supernatant, as calculated by dividing the percent of cells in flux at the maximum part of the peak by the percent in flux at the baseline, for four independent analyses. Paired samples are connected by dashed grey lines. **F)** Monocytes and NKT cells were co-cultured in medium alone (no mAb), or in the presence of blocking antibodies against CD1d, or CD40L, or with an isotype-matched negative control antibody (isotype). Supernatants collected after 8 hours were tested for the ability to induce calcium flux in freshly isolated monocytes compared to medium alone (neg ctrl). The plot shows the means and standard deviations from 4 independent experiments.

Figure 5. Blocking purinergic signaling during monocyte differentiation prevents the induction of the suppressive phenotype in NKT-instructed APCs

A) NKT-instructed APCs were generated in the presence or absence of suramin during the monocyte differentiation step, then washed and stimulated with LPS for 8 hours and stained for expression of PD-L1 and PD-L2 (filled histograms) or with isotype-matched negative control antibodies (open histograms). The data shown are from one representative experiment out of 6 independent analyses. **B)** NKT-instructed APCs that were generated as described in part (A) form multi-cellular clusters with allogeneic T cells. The results shown are from one representative experiment out of 3 independent analyses. **C)** NKT-instructed APCs generated as described in part (A) showed an increased ability to stimulate proliferation by allogeneic T cells compared to NKT-instructed APCs generated in the absence of suramin. **D)** Freshly isolated monocytes were cultured with 300 U/ml GM-CSF and 2 U/ml IL-13, in the presence or absence of 100 ng/ml IL-6 and 100 μ M ATP as indicated. APCs were stimulated with LPS for 8 hours and stained for surface PD-L1 and PD-L2 (solid histograms) or with isotype-matched negative control antibodies (open histograms). Results shown are from one representative experiment out of 4 independent analyses.

Figure 6. Human NKT cells induce the differentiation of monocytes *in vivo*

A) Flow cytometric analysis of APCs generated through exposure to soluble factors produced by NKT cells and monocytes (NKT trans-well, bottom row) compared to those generated by three days of direct co-culture with NKT cells (NKT contact, top row). **B)** Contact with NKT cells does not prevent the induction of the suppressive phenotype of NKT-instructed APCs. APCs were generated using a trans-well system that allowed exposure only to secreted factors from NKT cells and monocytes, or by direct co-culture with NKT cells followed by removal of the NKT cells by magnetic sorting, or by culturing with recombinant GM-CSF and IL-4 (cytokine induced). The plots show flow cytometric staining for CFSE of allogeneic T cells cultured with the indicated APCs for 7 days. Similar results were obtained in 2 further independent experiments. **C)** Detection of human monocytes and NKT cells after transfer into immunodeficient mice. The plots show flow cytometric analysis of spleen cells from a mouse that was not injected with human cells compared to a mouse that had been intraperitoneally injected with purified human monocytes and NKT cells 24 hours earlier. **D)** Immunohistochemical analysis showing localization of human cells in spleen and liver of immunodeficient mice. Mice were injected as in (C), and spleen and liver were harvested after 3, 24 or 60 hours post-injection, and tissue sections were stained with an antibody against human CD45 (dark brown colored cells). Cell nuclei are visualized by counterstaining with hematoxylin (blue coloration). All images represent 40X magnification. **E)** Induction of monocyte differentiation *in vivo*. Freshly isolated human peripheral blood monocytes were injected into immunodeficient mice in the presence of autologous human peripheral blood T cells alone, or autologous human peripheral blood T cells and human NKT cells. The contour plots show staining of the indicated markers on cells that were positive for human CD45 and negative for human CD3. Similar results were obtained in 10 independent analyses.