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Simultaneous deletion of NOD1 and NOD2 inhibits in vitro alloresponses but does not prevent allograft rejection

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Abstract

Pattern recognition receptors (PRRs) play an important role in host anti-donor responses to transplanted tissue. A key trigger of the host alloresponse involves recognition of foreign antigen presented on activated antigen presenting cells by the host T cells. Emerging data suggest that PRR blockade can abrogate host anti-donor responses by interfering with activation of antigen presenting cells, particularly activation of dendritic cells. Our study asked whether blockade of a well-characterized family of intracellular PRRs, the NOD family, interfered with alloantigen recognition and allograft rejection. We found that deletion of either NOD1 or NOD2 in antigen presenting cells (APCs) had no effect on induction of T cell proliferation to alloantigen, but that simultaneous deletion of NOD1 and NOD2 significantly inhibited T cell responses. There was however no effect of the NOD deletion on skin graft rejection when NOD1xNOD2 skin was transplanted onto allogeneic hosts or when WT skin was transplanted onto NOD1xNOD2 deficient recipients. The conclusion of this study is that in vitro alloresponses are negatively impacted by the simultaneous deletion of NOD1 and NOD2, but that allograft rejection across a stringent allo barrier is not affected. Our results suggest that the NOD family members, NOD1 and NOD2, play a collaborative role in T cell activation by alloantigen and that their blockade in vitro can inhibit T cell responses.

Keywords

Pattern recognition receptors; innate immunity; NOD1; NOD2; alloresponses; allograft rejection

Introduction

Host anti-donor immune responses are mediated by a complex cascade of cellular and molecular events triggered by host T cell recognition of donor antigens. T cells primed by donor antigen undergo proliferation, expansion and secretion of mediators that contribute to a robust inflammatory cascade, which left unchecked leads to acute rejection of the

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transplanted allograft. Although T cell activation contributes to acute rejection of transplanted allografts, it is innate pattern recognition receptors (PRRs) on donor APCs, such as dendritic cells (DCs) that are thought to be responsible for triggering initial T cell responses^{1,2}. PRRs, such as toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-leucine-rich repeat receptors (NLRs), have been identified as key triggers of DC activation and some have been found to play a role in the host response to a transplanted allograft^{3,4}. TLRs have been extensively studied for their ability to trigger adaptive immunity, but less is known about the role of NLR family members in DC function and induction of T cell-mediated alloresponses.

NOD1 and NOD2 are the best studied of the NLR protein family members that are expressed in a variety of immune and parenchymal cells⁵⁻⁷. They respond to distinct peptidoglycan motifs and are thought to influence T cell differentiation by inducing DC maturation and cytokine production^{8,9}. Crosstalk between these NLRs and TLRs has been well described¹⁰⁻¹². NOD1 ligand stimulation of DCs has been shown to prime T cell activation⁸ and NOD2-deficient DCs are not able to efficiently prime CD8+ T cells¹³. NOD2 variants have been associated with defective antigen presentation in patients with Crohn's disease¹⁴ and been shown to regulate both peptidoglycan-induced arthritis¹⁵ and autoimmune liver disease¹⁶. In the current study we investigated the role of NOD1 and NOD2 in APC-induced allogeneic T cell activation. Using a murine model, we asked whether defects in NOD1 and NOD2 signaling in APCs impacted either in vitro or in vivo alloresponses.

Materials and methods

Mice

All the mice used in these experiments were housed in the vivarium at UCSD and approved for use by the Institutional Animal Care and Use Committee of the UCSD Animal Research Center. All animals were handled according to the recommendations of the Humanities and Sciences and the standards of the Association for Assessment and Accreditation of Laboratory Animal Care. BALB/cBYJ and C57BL/6J (WT) were obtained from Jackson Laboratories, Bar Harbor MN. The NOD 1-deficient and NOD 2 - deficient, and NOD1x2-deficient mice were obtained from J. Matheson at the Scripps Research Institute. The NOD deficiency was confirmed by the absence of specific NODs by screening tail clips using primers specific for the NOD proteins, as previously published¹⁷.

Reverse Transcription-qPCR for detection of NOD1, NOD2, TLR4

Positively selected dendritic cells were isolated from WT (C57BL/6J) spleens (Jackson Laboratory, Bar Harbor, ME). Cells were stimulated with 5µg/mL lipopolysaccharide (LPS), 10µg/mL muramyl-dipeptide and/or L-Ala-gamma-D-Glu-mDAP (MDP + Tri DAP) from InvivoGen (San Diego, CA), or not activated (NA) and incubated for one hour at 37°C. Total RNA was isolated using the Quick-RNA MiniPrep kit from Zymo Research (Irvine, CA). Isolated RNA was purified using the TURBO DNase kit from Life Technologies (Rockville, MD). For the reverse transcriptase reaction, the Invitrogen SuperscriptIII cDNA Synthesis kit was used (Life Technologies, Rockville, MD). PCR amplification was

performed using Qiagen primers (Chatsworth, CA) and ssoAdvanced Universal SYBR Green Supermix (BioRad, Hercules, CA) for 40 cycles. RT-qPCR data were analyzed for relative increase in mRNA transcripts versus that found in unstimulated murine dendritic cells using an Eco Real Time PCR System (Illumina, San Diego, CA).

Dendritic cell function and T cell proliferation

Dendritic cells were isolated from spleens of either WT or Nod-deficient mice by positive selection using a CD11c+ MACS separation kit (R&D Systems, Minneapolis, MN). This purification method yields 90% purity of DCs. There were no differences in purity of CD11c DCs between the WT and NOD-deficient mice (data not shown). To test for proliferation, isolated DCs were plated on plastic tissue culture dishes and stimulated with LPS (5 µg/mL) (Enzo LifeSciences, Inc., Farmingdale, NY) for 5 days. Proliferation was detected on serial days by [3H] thymidine uptake. Stimulated cells were also stained CD86 (BD Biosciences, San Jose, CA) as a marker of activation. To test for migration, the DCs were seeded at density of 2×10^5 cells/well on a polycarbonate filter with 5-µm pore size in 24-well transwell chambers (Corning Costar, Cambridge, MA). The lower chambers contained 600 µL 10K RPMI media alone or with 250 ng/mL CCL21 (R&D Systems, Minn., MN). Stimulated dendritic cells were added to the upper chamber of transwells at a density of 2×10^5 cells per well in a total volume of 100 µL, incubated for 4 h at 37°C and migrated DCs detected by cell sorting, using CellQuest software (Becton Dickinson, Franklin Lakes, NJ). The ability of DCs from WT versus NOD1x2-deficient mice to stimulate allogeneic T cells (from H-2d BALB/cByJ mice) was detected using a standard mixed lymphocyte response (MLR), using previously published methods³. T cells were isolated from pooled peripheral LNs (axillary, brachial and inguinal nodes) of 3 mice per group. The T cell purity was between 50–70% of the isolated LN cells and there were no differences noted in CD3, CD4 or CD8 T cells between the WT and NODdeficient mice (data not shown). All mice were age and sex matched.

Allogeneic skin graft transplantation

Recipient mice (Balb/cByJ H-2d, or Nod1-def, Nod2-def or NOD1x2-def – all Nod-def mice were on H-2b background) were anesthetized and the flank hair shaved with electric clippers. A graft bed was prepared on the lateral thoracic region under aseptic conditions. The graft bed was prepared by careful removal of the epidermis and dermis to the level of the panniculus carnosus, keeping the vascular bed undisrupted. Donor tail skin was prepared by cutting the tail of a sacrificed donor mouse (WT, NOD1 x NOD2 deficient H-2b), incising circumferentially around the base of the tail and then down the dorsal surface and peeling off the donor skin. Equal-sized pieces of 5×3 mm were cut from the skin and kept in a wet sterile petri dish with PBS. The donor skin was then placed into the vascular bed, leaving a margin of 1–2 mm on all sides. Syngeneic and allogeneic donor skin was placed into the same graft bed. The grafted skin was then covered with sterile, antibiotic (bacitracin)-impregnated Vaseline gauze, covered with a bandage and then wrapped in cloth tape. The grafts were left undisturbed for 7 days. On day 7 the bandages were removed and the grafts were photographed on a daily basis. Rejection was scored as 90% necrosis of the grafted tissue. Survival fractions were determined using the Kaplan–Meyer method. Comparison of survival curves was performed using the log rank test provided by the Prism

4 software (GraphPad Software, La Jolla, CA). Median survival was also calculated using the Prism 4 software.

Results

NOD1 and NOD2 deficiency does not alter dendritic cell proliferation, maturation or migration, but does impact induction of in vitro T cell proliferation to alloantigen

NOD1 and NOD2 signals contribute to host innate and adaptive immunity, and crosstalk has been reported between these receptors¹⁸. We first showed that DCs of WT mice contain both NOD1 and NOD2 receptors, and that both were significantly upregulated by LPS stimulation (Figure 1).

We next asked whether the absence of NOD1 and NOD2 signaling impacted the activation of DCs, by using an in vitro model of TLR4-activated DCs. To eliminate potential crosstalk between NOD1 and NOD2 we used NOD1x2-deficient DCs. TLRs are known to prime for NOD-induced DC activation¹² and therefore LPS-induced proliferation and maturation of splenic DCs from NOD1x2-def mice was compared and contrasted to those from WT mice.

Interestingly, we found that the simultaneous deletion of NOD1 and 2 signaling had no effect on DC proliferation (Figure 2, Panel A) or on LPS-induced CD86 expression in response to LPS (Figure 2, Panel B) or MDP and Tri-DAP (data not shown because there was no significant proliferation in response to these ligands). DC migration is a key step in the in vivo induction of an inflammatory response to transplanted tissue, so we also compared and contrasted chemokine-induced migration between DCs from NOD1x2-deficient versus WT mice. Figure 1C shows that there were no differences detected in chemokine-induced migration of the DCs between the two groups.

NOD1xNOD2 deficient APCs do not efficiently stimulate allogeneic T cells

Since NOD1 and NOD2 have been shown to be important for APC-induced T cell priming in other models¹², we next tested whether the absence of either of these receptors (or simultaneous absence of both receptors) in APCs affected their ability to stimulate allogeneic T cells. T cell proliferation to alloantigen was assayed in a standard mixed lymphocyte reaction (MLR) assay, in which BALB/cByJ (H-2d) T cells were exposed to irradiated APCs from C57BL/6J (H-2b), NOD1 (H-2b), NOD2 (H-2b), or NOD1xNOD2 (H-2b) deficient mice. The results show that the absence of either NOD1 or NOD2 in APCs did not impact their ability to stimulate the proliferation of allogeneic T cells (Figure 2A). In contrast, the simultaneous deficiency of both NOD1 and NOD2 proteins (NOD1x2-def) led to a significant decrease in ability to activate allogeneic T cells (Figure 2B). Analysis of the supernatants from the MLRs showed that there was significantly less IFN- γ produced in the cultures stimulated with NOD1x2-def APCs (Figure 2C), but there was no difference in TNF α , IL-2 and IL-6 secretion noted at the time of the assay (data not shown).

Allogeneic skin grafts lacking both NOD1 and NOD2 are not protected from rejection

Donor APCs play an important role in vivo by initiating host responses to transplanted solid organ allografts. Following skin transplantation sentinel DCs that reside in the transplanted

donor tissue are activated by PRR ligands, leading to their activation and subsequent migration to host draining lymph nodes¹⁹. Blockade of DC migration from the transplanted skin has been known for many years to prevent rejection of the allograft^{20,21}. Since the simultaneous absence of NOD1 and NOD2 affected the ability of APCs to effectively stimulate allogeneic T cells in vitro, we next asked whether the absence of these proteins in donor DCs (donor skin from NOD1xNOD2-deficient mice) would impact the rejection of skin grafts on allogeneic hosts. Figure 3, Panels A and B show that the absence of either NOD1 or NOD2, or the simultaneous deficiency of both NOD1 and NOD2 in the donor skin did not affect the tempo of skin graft rejection (Figure 3, Panel A). A representative picture of the skin grafts on day 12 after transplantation are shown in Figure 3, Panel B, with the syngeneic grafts shown on the left of the allogeneic grafts on the same recipient. In Figure 3, Panels C and D it is also shown that the absence of NOD 1, NOD2 or both NOD1 and NOD2 in the recipient had no effect on the tempo of allogeneic skin graft rejection, suggesting that indirect antigen presentation was also not impacted by the NOD deficiency.

Discussion

Since the intracellular innate immune receptors NOD1 and NOD2 have been shown to be important for T cell priming in both in vitro and in vivo models, we hypothesized that these well-characterized receptors would play an important role in adaptive immune responses to alloantigen. This study investigated the role of NOD1 and NOD2 proteins in DCs activation and alloantigen presentation and asked whether defects in either NOD1 or NOD2, or simultaneous deletion in both NOD1 and NOD2 signaling impacted in vitro and in vivo alloresponses.

Our first studies were directed to determine whether the absence of NOD1 and NOD2 altered DC maturation and activation. Synergistic stimulation of human DCs by TLR4 and NOD1/NOD2 agonists has been shown to activate WT DCs²². Our data demonstrated that the absence of either NOD1 or NOD2 had no effect on TLR4-induced DC proliferation. Likewise, there were no differences detected between the NOD-deficient DCs and WT DCs in expression of activation markers, or DC migration, suggesting that NOD1, NOD2 or the simultaneous absence of both NOD1 and NOD2 were not critical for DC activation in our model.

We next asked whether DCs deficient in NOD1 and NOD2 were able to efficiently stimulate alloantigen-dependent T cell proliferation. It is known that NOD2 in DCs can stimulate the development of an adaptive immune response, possibly by regulating the antigen-presenting capacity of DCs through autophagy²³. Our data found though that a deficiency in either NOD1 or NOD2 receptors had no impact on T cell proliferative responses. The simultaneous absence of both NOD1 and NOD2 receptors was however associated with a significant decrease in the ability to stimulate allogeneic T cells. The deficiency in T cell stimulation was also associated with a significant decrease in IFN γ secretion.

Rejection of donor skin graft involves migration of “passenger donor dendritic cells” from the transplanted graft to host lymph nodes, where activation of naïve host T lymphocytes initiates the cascade of graft rejection²⁴. To test whether a deficiency in NOD 1 and NOD2

impacted allograft rejection, we transplanted donor skin deficient in NOD1 and NOD2 proteins onto allogeneic hosts. Even though we had seen in vitro that blockade of both NOD1 and NOD2 signaling decreased T cell alloreactivity, there was no delay in the rejection of the NOD-deficient skin grafts. Furthermore, there were no differences in rejection of allogeneic WT skin grafts by NOD1x2 deficient hosts, suggesting no defects in cross-priming were conferred by the NOD-deficiency. These data suggest that, while a defect in T cell alloresponses required a simultaneous absence of NOD1 and NOD2 signaling, in vivo alloresponses were not affected by the deficiency in NOD1 and NOD2 receptors.

The conclusions of this study are that effective blockade of NOD signaling requires blockade of both NOD1 and NOD2 to inhibit T cell proliferation to alloantigen, but in vivo alloresponses require blockade of additional targets.

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Abbreviations

PRRs	pattern recognition receptors
NOD	APC
DC	dendritic Cell
TLR	toll-like receptor
NLR	nucleotide-binding oligomerization domain- leucine-rich repeat receptor

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Figure 1. Relative expression of NOD1, NOD1 and TLR4 in stimulated versus unstimulated murine DCs

DCs were isolated and then either not stimulated (NA, black square) or stimulated for 1hr with LPS (10ug/ml, checked square) or the NOD1 and NOD2 ligands MDP+TriDAP (MDP +T DAP, grey square). The relative expression of NOD1, NOD2 and TLR4 was assessed by RT-qPCR. This data represents one of three identical experiments. Error bars represent SDs. Significance was detected between unstimulated and stimulated groups as noted in the figure.

Figure 2. Dendritic cells from NOD1xNOD2-deficient mice demonstrate no defects in LPS-induced proliferation, expression of CD86 or CCL21-induced migration

(A). Dendritic cells isolated from spleens of WT (black bars) vs. NOD1xNOD2-deficient (striped bars), were stimulated with LPS (1ug/mL) for the indicated times. Proliferation was detected by ³H-thy uptake. Error bars represent SD of three samples. The graph represents one of three identical experiments. (B). CD86 expression on WT vs. NOD1xNOD2-deficient splenic DCs were stimulated with LPS (5 ug/mL) for 4 hrs, and stained with CD86 and analyzed by FACS. This represents one of 4 identical experiments. (C) Dendritic cell migration was detected in a transwell assay comparing WT vs. NOD1xNOD2-deficient DCs stimulated for 4 hrs with the chemokine CCL21 (250 ng/mL). Error bars represent SD of three samples. The graph represents one of three identical experiments.

Figure 3. NOD1 or NOD2 deficient APCs are able to induce proliferation of allogeneic T cells, but NOD1xNOD2-deficient APCs do not efficiently stimulate allogeneic T cells

(A). WT vs. NOD1-def or NOD2-def irradiated APCs (all H-2b) cultured with allogeneic T cells (H-2d) in MLR assay. Proliferation was detected by 3H-thy uptake on the 4th day of culture. Error bars represent SDs; graph represents 1 of 3 identical experiments. (B). WT (black bar) vs. NOD1xNOD2-def (checked bar) irradiated APCs (all H-2b) and cultured with increasing number of allogeneic T cells (H-2d). Proliferation was detected by 3H-thy uptake on the 4th day of culture. Error bars represent SDs; graph represents 1 of 3 identical experiments. (C). IFN-γ secretion into supernatants of MLR cultures of WT vs NOD1x2-def APCs and allogeneic T cells.

Figure 3.

Skin graft rejection in WT recipients of NOD-deficient recipients. (A). Percent survival of donor skin grafts (H-2b) in allogeneic recipients (BALB/cByJ, H-2d), n=6 mice/gp. (B). Representative photograph of allogeneic skin grafts (H-2b) on Balb/cBYJ recipient mice (left graft is syngeneic, right graft is allogeneic and noted by donor title over photograph). (C). Percent survival of donor skin grafts (Balb/c, BYJ H-2d) on allogeneic recipients (all H-2b), noted by donor in legend, n=6 mice/gp. (D). Representative photograph of allogeneic skin grafts on recipient mice noted by title over each photograph (left graft is syngeneic, right graft is allogeneic).