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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 NOD1 × NOD2 skin was transplanted onto allogeneic hosts or when WT skin was transplanted onto NOD1 × NOD2 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.

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 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 (Akira, 2000; Beutler and Hoffmann, 2004). 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 (McKay et al., 2006; John and Nelson, 2007). 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 cells (Moreira and Zamboni, 2012; Holler et al., 2004; Holler et al., 2006). They respond to distinct peptidoglycan motifs and are thought to influence T cell differentiation by inducing DC maturation and cytokine production (Fritz et al., 2007; Fritz et al., 2006). Crosstalk between these NLRs and TLRs has been well described (Tada et al., 2005; Liu et al., 2013; Krishnaswamy et al., 2013). NOD1 ligand stimulation of DCs has been shown to prime T cell activation (Fritz et al., 2007) and NOD2-deficient DCs are not able to efficiently prime CD8+ T cells (Lupfer et al., 2014). NOD2 variants have been associated with defective antigen presentation in patients with Crohn's disease (Kramer et al., 2006) and been shown to regulate both peptidoglycan-induced arthritis (Rosenzweig et al., 2009) and autoimmune liver disease (Body-Malapel et al., 2008). 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.

Abbreviations: PRRs, pattern recognition receptors; NOD, nucleotide-binding oligomerization domain; APC, antigen presenting cell; DC, dendritic cell; TLR, toll-like receptor; NLR, NOD – leucine-rich repeat receptor.

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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 NOD1- and NOD2-deficient, and NOD1 × 2-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 published (Shigeoka et al., 2010).

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 5 μg/mL lipopolysaccharide (LPS), 10 μg/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 1 h 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 vs. 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, Minneapolis, MN). Stimulated dendritic cells were added to the upper chamber of transwells at a density of 2×10^5 cells/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 vs. NOD1 × 2-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 methods (McKay et al., 2006). 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 and 70% of the isolated LN cells and there were no differences noted in CD3, CD4 or CD8 T cells between the WT and NOD-deficient 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 NOD1 × 2-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 × 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 receptors (Netea et al., 2005). We first showed that DCs of WT mice contain both NOD1 and NOD2 receptors, and that both were significantly upregulated by LPS stimulation (Fig. 1).

We next asked whether the absence of NOD1 and NOD2 signaling impacted the activation of DCs, by using an in vitro model

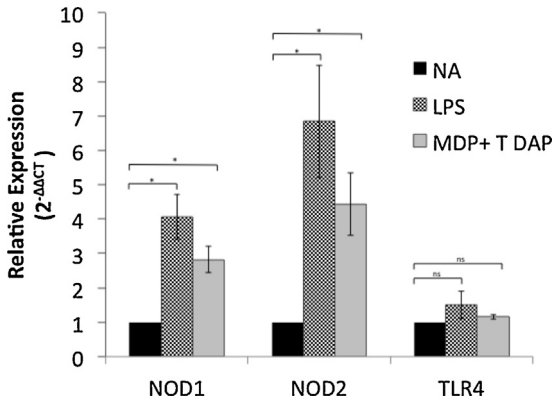

Fig. 1. Relative expression of NOD1, NOD1 and TLR4 in stimulated vs. unstimulated murine DCs. DCs were isolated and then either not stimulated (NA, black square) or stimulated for 1 h with LPS (10 μg/ml, checked square) or the NOD1 and NOD2 ligands MDP + TriDAP (MDP + T DAP, gray square). The relative 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.