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The distinct response of γδ T cells to the Nod2 agonist muramyl dipeptide

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ABSTRACT

Purified γδ T cells are primed directly in response to pathogen associated molecular patterns (PAMPs) to better respond to secondary signals and increase expression of chemokine and activation-related genes. Transcripts encoding the innate receptor Nod2 were detected in bovine and human γδ T cells. Nod2 is the intracellular receptor for muramyl dipeptide (MDP), functions in regulating innate activities, and was thought to be expressed primarily in APCs. The response of γδ T cells to MDP was analyzed by microarray, Q-PCR, proteome array and functional priming assays. MDP had a consistent priming effect on γδ T cells, characterized by changes in transcripts and enhanced proliferation response to secondary signaling. Knockdown experiments implicated Nod2 as the receptor for MDP in γδ T cell-enriched bovine PBLs. The results indicate priming of γδ T cells by MDP, and offer definitive evidence of the expression of functional Nod2 in γδ T cells.

1. Introduction

Nucleotide-binding oligomerization domain (Nod) proteins are related to plant disease resistance genes and were recognized as cytoplasmic receptors for bacterial products [1]. Nod1 and Nod2 are receptors for peptidoglycan (PGN)-derived peptides g-D-glutamyl-meso-diaminopimelic acid from Gram-negative bacterial peptidoglycan [2,3], and muramyl dipeptide (MDP) [4,5], respectively. While Nod1 is more widely expressed in epithelial cells and APCs, Nod2 was primarily detected in APCs, such as monocytes and dentritic cells, in intestinal epithelial cells and only at very low levels in CD3+ T cells [6–8]. Nod2 mutations are associated with Crohn's disease, an inflammatory bowel disease in humans [9,10], suggesting that this gene has a critical function in immune regulation in the gut.

γδ T cells recognize unprocessed or non-peptide antigens, respond rapidly to infection, and localize to mucosal surfaces [11]. These characteristics suggest that although γδ T cells are lymphocytes, they express a myriad of innate phenotypic characteristics. We analyzed the transcriptional profiles of purified *in vitro*-expanded human γδ T cells after exposure to LPS [12] as well as differences in human γδ T cell subsets [13] using microarrays. Transcripts encoding the innate receptor Nod2 were detected in purified human and bovine γδ T cells in these analyses. Peripheral blood of adult humans contains very few γδ T cells that require expansion in culture before microarray analysis, whereas in neonatal calves up to 70% of the circulating lymphocytes are γδ T cells. mRNAs encoding numerous innate receptors, including Nod2, were more readily detected in γδ T cells isolated directly from peripheral blood of neonatal calves, suggesting that expansion in culture, or the adult source of human γδ T cells may diminish their expression. Furthermore, a similar, yet more robust, response to pathogen associated molecular patterns (PAMPs), was detected using freshly isolated neonatal bovine γδ T cells [12]. Lacking the reagents to detect Nod2 protein in bovine γδ T cells, we sought to characterize their response to the Nod2 agonist MDP.

We have recently defined γδ T cell priming as the subtle activation response of γδ T cells to these and other non-TCR agonists. Briefly, γδ T cell priming is characterized by increases in specific proteins, primarily GM-CSF, IL-8, and IL-2Rα, in a response that is not overt activation, but enables more rapid responses to secondary signals [14–17]. To gain an unbiased view of the effect of MDP on bovine γδ T cells, we analyzed changes in global transcription in response to MDP stimulation. Results suggested a consistent change in gene expression, and that γδ T cells were also primed by MDP to better respond to secondary signals, such as IL-2. Results using human cells also supported a direct response of human γδ T cells to MDP. *In vitro* priming assays demonstrated the functional significance of this priming response, and its specificity to γδ T cells. Using RNAi silencing in primary bovine PBLs, the expression of the Nod2 transcripts was reduced, which resulted in diminished responses to MDP by γδ T cell-enriched populations. These data indicated a distinct priming response of γδ T cells to MDP that was dependent on expression of Nod2.

Abbreviations: Nod, nucleotide-binding oligomerization domain; PAMPs, pathogen associated molecular patterns; MDP, muramyl dipeptide; PGN, peptidoglycan; IL-2Rα, IL-2 receptor α.

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2. Materials and methods

2.1. Cell isolation

All use of animals was in accordance with National Institute of Health guidelines and approved by the Institutional Animal Care and Use Committee (animal experiments) and the Institutional Review Board (human blood) of Montana State University. Bovine blood was collected from young calves and PBLs separated using Histopaque 1077 (Sigma) as previously described [18]. PBLs were monocyte depleted by adherence to plastic flasks for 1 h in cRPMI media (10% fetal bovine serum in RPMI supplemented with 1% each essential amino acids, penicillin/streptomycin, L-glutamine and 10 mM Hepes). γδ T cells from two calves (150 and 151) were sorted with MACS magnetic bead system (Miltenyi Biotec) as previously described [12] to a purity of >98%. Cells from several additional calves, including calf 129, were not monocyte depleted and were sorted using a VANTAGE SE cell sorter (BD Immunocytometry Systems) to >98% purity, as previously described [18] after labeling with a pan-γδ antibody (GD3.8) conjugated to FITC. Cells were rested overnight before stimulation. Human γδ T cells were expanded in culture and sorted as we have previously described [12].

2.2. Bovine oligonucleotide microarray

Sorted bovine γδ T cells from three different calves (Calf numbers 150, 151, and 129) were stimulated with either PBS or MDP (BioChemika, ⩾99.0% pure by thin layer chromatography, 10 μg/ml) for 4 h. The cells were lysed and genomic DNA was homogenized using QIAshredder spin columns (Qiagen). RNA was extracted using the RNeasy mini columns (Qiagen) according to the manufacturer's protocol for use in either microarray or real time RT-PCR analysis. Prior to amplification for microarrays, RNA quality was confirmed using an Agilent 2100 bioanalyzer (Palo Alto, CA). RNA was extracted, amplified and used to probe (six different Affymetrix GeneChip® Bovine Genome Arrays (Affymetrix, Santa Clara, CA) that represents approximately 23,000 transcripts based on Unigene build 57 (April 2004) and Genbank sequences. cDNA amplification and synthesis of biotin-labeled cRNA was performed with the One-cycle target labeling protocol with 1.7 μg total RNA as described in the GeneChip® Expression Analysis Technical Manual (March 2004). Hybridization was performed with 15 μg cRNA. Washing and staining was performed in the GeneChip® Fluidics Station 450 using the Midi_euk2v3 protocol. Chip scans were performed on the Affymetrix GeneChip® Scanner 3000. GeneChip® Operating Software (GCOS v.1.1, Affymetrix) [19,20] was used for data collection. Further analysis was done using GeneSpring (Silicon Genetics) with RMA preprocessing and Microsoft Excel. All data was normalized to the median and filtered on expression levels (>100 raw) and fold change (>2-fold change).

Real time RT-PCR was performed as previously described [18]. Primers were designed with Primer Express, primer design software from Applied Biosystems. The reverse transcription reactions were performed with Superscript III (Invitrogen) and approximately 700 ng of RNA according to the manufacturer's protocol. Relative specific mRNA in the γδ T cells was quantified by measuring SYBR green incorporation during real time quantitative RT-PCR using the relative standard curve method. Primers specific for 18S RNA were used as the endogenous control. The PCR was set up in triplicate, cycled and data collected on the MyiQ Real Time PCR Detection System (Bio-Rad).

2.3. Supernatant protein measurement

In vitro-expanded as previously described [12], VANTAGE-purified γδ T cells from seven different human subjects were densely plated (5×10^6 cells per ml) and stimulated for 22–24 h. The supernatant fluids from these cultures were collected, frozen at −80 °C, and sent to Pierce for Searchlight Proteome array analysis to measure representative protein concentrations as suggested by the microarray data.

2.4. Priming assay

Cells used in the priming assay were either Histopaque purified bovine PBMCs that were monocyte depleted using either MACS human CD14 microbeads (Miltenyi Biotec) and LD depletion columns as described by the manufacturer, or by adherence to plastic flasks, or VANTAGE sorted bovine peripheral blood γδ T cells. Cells were washed with HANKS buffer and loaded with 0.25 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) in the dark for 5 min. Cells were assayed in duplicate or triplicate wells, depending on the sort yield and primed with PBS or MDP (10 μg/ml) in XVIVO (serum-free media, Cambrex, Walkersville, MD) media for 48 h at 37 °C. Priming with LPS was used as a positive control (data not shown, [14]). Priming media was removed and replaced with media containing IL-2 (1 ng/ml) then cells were cultured another 72 h at 37 °C. Cells were then stained with GD3.8 (anti-pan-γδ TCR), or GD3.8 and GD3.5 (specific for WC1, a bovine γδ T cell subset specific marker) for subset specific staining [18] using standard flow cytometry techniques and read on a FACS Calibur HTS using high-throughput settings. Gates were placed on lymphocyte populations in scatter plots, $GD3.8^+$ cells, and $GD3.5^+$ and $GD3.5^-$ cells as appropriate for analysis of CFSE staining.

2.5. RNAi knockdown of Nod2

Three different dicer substrate RNAi Duplexes specific for bovine Nod2 were designed and synthesized commercially (Integrated DNA Technologies). The duplexes were electroporated into monocyte depleted (by adherence to plastic) bovine PBLs using the Nucleofector Device and Human T Cell Kit (Amaxa, Program T-23) according to the manufacturer's protocols. Standard flow cytometric evaluation following Nucelofector electroporation with an Alexa 488-labeled control siRNA (Qiagen), suggested transfection was very efficient, but lethal to most cells. To achieve sufficient live transfected cells and to eliminate variation between cuvettes, four cuvettes each with 5×10^6 cells were used for each siRNA transfection. Electroporated cells were incubated for 18 h in XVIVO (serum-free) media, then underlaid with approximately 5 ml Histopaque 1077, and centrifuged at 1600 rpm for 20 min to separate live and dead cells. Live cells (as determined by forward and side scatter using flow cytometry) were aspirated from Histopaque, and either suspended in XVIVO at 1×10^6 cells per ml and stimulated with PBS, LPS or MDP for 4 h for Nod2 functional assessment, or immediately lysed in Buffer RLT (Qiagen) for knockdown assessment, both were determined in cells from at least three different calves. RNA extraction and real time RT-PCR were then performed as described above. Fold change in GM-CSF expression after stimulation with MDP relative to PBS in cells transfected with siRNAs specific for either TLR4 or Nod2 was used as the indicator of Nod2 function.

2.6. Statistical analyses

Statistical analyses were performed with a two-tailed paired *t* test. The proteome array was run in duplicate and the average protein concentrations for cells stimulated with PBS or MDP from the seven different subjects were compared using the paired *t* test. In the Priming assay, mean values of percent of gated cells were calculated with 1–3 experimental replicates (sample wells) from at least three individual calves (biological replicates). Extensive experience with this assay indicates that the percent of gated cells follows an approximately normal distribution. Percentages of gated