MIP-3α Expression in Macrophages Is NOD Dependent

M. Hausmann a C. Zeitler c A. Weber b M. Krebs a S. Kellermeier a P. Rosenstiel e C. de Vallière a K. Kosovac c M. Fried a E. Holler d G. Rogler a

a Division of Gastroenterology and Hepatology and b Institute of Pathology, University Hospital of Zurich, Zurich, Switzerland; c Department of Internal Medicine I, University of Regensburg, d Department of Haematology/Oncology, University Medical Centre, Regensburg, and e Institute of Clinical Molecular Biology, Christian-Albrechts-University, Kiel, Germany

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Abstract

Background: The first identified susceptibility gene for Crohn's disease, NOD2, acts as a sensor for the bacterial-wall peptidoglycan fragment muramyl dipeptide (MDP) and activates the transcription factor nuclear factor-κB (NF-κB). Upon NF-κB activation, intestinal macrophages (IMACs) induce expression of macrophage inflammatory protein (MIP)-3α to attract memory T lymphocytes. We therefore investigated the influence of NOD2 ligation of IMAC differentiation and functional MIP-3α induction. Methods: Human embryonal kidney HEK293 cells were transfected with NOD2 wild-type (NOD2 WT) and the NOD2 SNP13 variant (NOD2 L1007fsinsC) and stimulated with MDP. Recruitment of CD45R0+ and Th17 cells was determined by immunohistochemistry. Results: Endogenous NOD2 stimulation was followed by a dose-dependent increase in MIP-3α secretion in MONO-MAC-6 (MM6) cells. MIP-3α mRNA was also significantly (p < 0.05) induced in HEK293 transfected with NOD2 WT via MDP ligation. In vivo cell-cell contacts between IMACs and CD45R0+ memory T cells as well as recruitment of Th17 cells in patients of NOD2 variants were unchanged as compared to wild-type patients. Conclusion: Our data demonstrate a dose-dependent increase in MIP-3α secretion in the human myeloid cell line MM6 upon MDP. However, MIP-3α-driven recruitment of Th17 cells or CD45R0+ memory T lymphocytes is not affected in patients carrying heterozygous NOD2 variants.

Introduction

Antigens passing over the tightly regulated intestinal epithelial barrier are recognized and cleared by intestinal macrophages (IMACs). In healthy individuals monocytes differentiate into tolerant IMACs with a prototypic phenotype lacking activation markers such as toll-like receptors (TLRs) 2 and 4 [1]. This is important in maintaining effective tolerance to the natural microbial flora thus preventing chronic inflammation in response to commensal bacteria. Consequently no or only low activation of pro-inflammatory transcription factors such as nuclear factor-κB (NF-κB) is found in normal mucosa [2]. But normal IMACs may still express intracytoplasmatic pattern recognition receptors (PRRs) such as TLR9 or NOD2. This enables IMACs to react to invading bacteria. NOD2
detects the bacterial-wall component muramyl dipeptide (MDP). MDP sensing via the leucine-rich repeat domain of NOD2 induces activation of NF-κB in monocytes/macrophages as well as in intestinal epithelial cells [3–6]. By activation of the NF-κB signaling pathway numerous proinflammatory genes are induced, these include interleukin (IL)-1β, IL-6, IL-8, TNF, GM-CSF or chemokines and their receptors, such as macrophage inflammatory protein (MIP)-3α [7–9].

Variants of NOD2 were the first genetic susceptibility factors identified for Crohn’s disease (CD), an inflammatory bowel disease (IBD). The three most frequent single-nucleotide polymorphisms (SNP) are SNP 8, 12 and 13 [10, 11]. These SNPs are associated with ineffective MDP binding and a subsequent inability to activate defense mechanisms which results in reduced secretion of antimicrobial peptides and chemokines and increased bacterial translocation in animal models [5, 12, 13]. Using FISH technology, we detected mucus-associated bacteria in CD patients’ mucosa but not in control specimens [14]. Accumulation of endotoxin in the intestinal tissue of CD patients reflected the NOD2 genotype: patients with heterozygous SNP8 or SNP13 variants revealed notably stronger endotoxin staining than patients with SNP12 or the wild-type (WT) variant indicating more transepithelial bacterial translocation or ineffective clearance of bacterial products [14]. Increased activation of NF-κB was found in the mucosa of CD patients, and this was even greater in patients with NOD2 variants [14].

MIP-3α – also called liver and activation-induced chemokine (LARC) [15], Exodus [16] or SCYA20 – is a CC chemokine which has been reported to attract memory T cells and immature dendritic cells (DCs) [17–23]. In contrast to many other chemokines MIP-3α has a specific receptor: it binds almost exclusively to the chemokine receptor CCR6, which is expressed on immature DCs and memory T cells, but also on B lymphocytes [22, 24–28]. Annunziato et al. [29] observed the presence of CCR6+ IL-17-producing T cells in the gut. Epithelial cells are thought to be an important source of MIP-3α production [16]. Other cell types, such as endothelial cells, fibroblasts, and monocytes are also known to express MIP-3α [28, 30]. High MIP-3α mRNA and protein expression are induced during the specific differentiation of IMACs as shown in previous studies from our laboratory [31]. Similarly, MIP-3α mRNA was found to be highly expressed in differentiated macrophages in the lung [28]. Izadpanah et al. [32] demonstrated constitutive MIP-3α mRNA expression in intestinal epithelium and an upregulation by pro-inflammatory cytokines or in response to infection with bacterial pathogens. Kwon et al. [33] found significantly elevated MIP-3α mRNA and protein levels in CD patients compared with controls or ulcerative colitis (UC) patients. CCR6 expression in T cells and chemotactic response to MIP-3α is thought to be restricted to the CD45R0+ memory T cell population [27]. These data suggest that CCR6 functions on memory T cells that home to mucosal sites as part of an immune response [26]. Professional antigen-presenting and MIP-3α-secreting IMACs may serve as a contact point for CD45R0+ memory T and B cells and subpopulations of DCs [31].

As translocated bacterial components can activate NF-κB in a NOD2 genotype-specific manner, we hypothesized that a lack of detection of invading bacteria or bacterial material could be followed by impaired recruitment of MIP-3α-dependent lymphocytes in individuals with NOD2 variants. The aim of the study was therefore to establish a link between NOD2-mediated MIP-3α expression and lymphocyte recruitment in CD patients.

Materials and Methods

Patients

Primary human cells were obtained from surgical specimens taken from healthy areas of the mucosa of patients undergoing surgery for colorectal carcinoma or from the mucosa of patients with CD. Histology was performed on surgical specimens by a pathologist. The medical treatment of the patients is listed in online supplementary table 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000335423). Of a total of 43 subjects, 18 were male and 25 were female. The patients were between 16 and 74 (mean 43.2 ± 13.8) years of age and treated with budesonide, 5-ASA, azathioprine and systemic steroids. The different pharmacotherapies had no apparent influence on the results of our assays as analyzed by clustering disease, treatment and gene variations. One-way ANOVA and post-hoc Bonferroni test were applied for analysis. This study was approved by the Ethics Committees of the University of Regensburg and the University of Zurich and performed in accordance with the Declaration of Helsinki.

Cell Lines and Primary Cells

The human embryonal kidney (HEK)293 cell line and the human acute monocytic leukemia cell line MONO-MAC-6 (MM6) were cultured under standard tissue culture conditions as described previously [34]. Surgical specimens from inflamed and normal mucosa were obtained by surgery. The study was approved by the University of Regensburg Ethics Committee. Specimens were obtained from the colons of patients after their agreement. Primary cells were isolated from surgical specimens as described [35, 36]. Briefly, intestinal epithelial cells were isolat-
ed from surgical specimens. Mucosal strips were incubated in 1 mmol/l EDTA (Sigma) for 10 min at 37°C and transferred to tubes. Tubes were shaken vigorously 5–10 times. Mucosal strips were removed by passing the slurry over a coarse mesh (400 µm; Carl Roth GmbH, Karlsruhe, Germany). The suspension containing the detached intestinal epithelial cells was passed over the mesh filter (80-µm pore size; Sefar, Kansas City, Mo., USA), and intact CEC crypts were eluted by inverting the filter in serum-free culture medium (keratinocyte serum-free medium; Gibco-BRL, Eggenstein, Germany).

Specimens for isolation of human lamina propria mononuclear cells (LPMNCs) were incubated for 30 min in 2 ml phosphate-buffered saline (PBS) with 1 mg/ml collagenase type I (≈ 336 U/ml), 0.3 mg/ml deoxyribonuclease (DNase I, Boehringer, Mannheim, Germany) and 0.2 mg/ml hyaluronidase without fetal calf serum (FCS) at 37°C. Cells were dispersed by passing through a 27-gauge needle by a 1- to 2-ml syringe, washed in 1.5 ml PBS with 500 µl FCS and finally submitted to Ficoll density gradient centrifugation for 20 min at 2,000 rpm (≈ 690 g, without brake) in a Heraeus centrifuge for the isolation of mononuclear cells. The infrasep was carefully removed and washed with PBS. Intestinal macrophages were labeled with immunomagnetic microbeads armed with CD33 antibody and purified twice with the help of type AS separation columns (Miltenyi Biotec) as described previously [37].

Peripheral blood lymphocytes and blood monocytes (CD33+), were isolated from healthy volunteers as described previously [36]. The purity of isolated primary cells was always >95%.

**RNA Extraction and Quantitative Real-Time PCR**

Total RNA was isolated using the RNeasy Mini Kit (#74106, Qiagen) following the manufacturer's recommendations. cDNA was synthesized with a high-capacity cDNA reverse-transcription kit according to the manufacturer's instructions (#4368814, Applied Biosystems). To determine expression, quantitative real-time PCR (qPCR) was performed under the following cycling conditions: 20 s at 95°C, then 45 cycles of 95°C for 3 s and 60°C for 30 s with the TaqMan Fast Universal Mastermix. Primers for real time PCR: MIP-3α for 5′ TGT CAG TGC TGC TAC TCC ACC T 3′, MIP-3α rev 5′ CTG TGT ATC CAA GAC AGT CAA 3′, MIP-3α probe, 6-FAM-5′ TGC GGC GAA TCA GAA GCA GCA A 3′-TAMRA. Human IL-8 (#HS01174103_m1, PE Applied Biosystems) and GAPDH (#4326317E, PE Applied Biosystems). Each sample was analyzed in triplicate. The comparative ΔΔCt method was applied to determine the quantity of the target sequences relative to the endogenous control GAPDH and a reference sample.

**ELISA**

Culture supernatants from MM6 and HEK293 cells were collected after MDP (#A9519, Sigma-Aldrich, Steinheim, Germany) or lipopolysaccharide (LPS) (#62325, Fluka, Buchs, Switzerland) stimulation. The supernatants were centrifuged and used for determination of IL-8 and MIP-3α by commercially available ELISA kits (human IL-8 colorimetric ELISA kit, Pierce, Rockford, Ill., USA and human CCL20/MIP-3α quantitative ELISA kit, R&D Systems, Minneapolis, Minn., USA, respectively).

**Vectors, Transfection and Stimulation**

For transfection 7.5 × 10^5 HEK293 were seeded in each well of a 6-well plate (Corning Inc., Costar®) in 1.5-ml media. Cells were cultured under standard tissue culture conditions as described above until cells were 60–80% confluent. pcDNA3.1 plasmids with inserts were constructed as described elsewhere [38]. For transient transfection, we used 2-µg expression vectors for NOD2WT gene (pcDNA3.1_NOD2/Card15) and for NOD2 SNP13 variant (pcDNA3.1_SNPI3). The control vector (pcDNA3.1) was from Invitrogen, Carlsbad, USA. Expression vectors were diluted in 97 µl serum-free media in sterile 1.5-ml plastic tubes. 3 µl Fugene 6™ (Roche) were added, mixed and incubated for 10 min at room temperature. The Fugene™-DNA mixture was added to the wells in a drop-wise manner. For transfection control cells were transfected with 2 µg pEGFP vector (BD Biosciences) in a separate well. Transfected cells were incubated for 24 h. For stimulation with MDP (#A9519, Sigma) substance was diluted in 97 µl serum-free media in sterile 1.5-ml plastic tubes. 3 µl Fugene 6™ (Roche) were added, mixed and incubated for 10 min at room temperature. The Fugene™-MDP mixture was added to the wells in a drop-wise manner. For stimulation with LPS (#62325, Fluka) substance was diluted in media containing serum in sterile 1.5-ml plastic tubes. Experiments with transfected HEK293 were terminated after 72 h.

**Antibodies**

For the identification of human IMACs in immunohistochemistry, mouse anti-human macrophage CD68 (M0814, clone KP1, IgGx, monoclonal, DAKO, Hamburg, Germany, final concentration 0.5 µg/ml) was applied. Mouse anti-human CD45R0 (M 0742, clone UCHL1, IgG2a, monoclonal, DAKO, final concentration 0.2 µg/ml) was used to identify human memory cells. Goat anti-human IL-17 (AF-317-NA, IgG, R&D Systems, dilution 1:100) was applied for the identification of human IL-17-positive cells.

Monoclonal mouse anti-human NOD2 (#114445, IgG, Cayman Chemical, Ann Arbor, Mich., USA, dilution 1:1000) and monoclonal mouse anti-human actin (#sc-2005, Santa Cruz, Heidelberg, dilution 1:5,000) were used for Western blots.

Peroxidase-conjugated goat anti-mouse IgG antibody (A-4416, Sigma, final concentration 0.2 µg/ml) or rabbit anti-goat (P0449, DAKO, dilution 1:300) was used as secondary antibody in immunohistochemistry and goat anti-mouse IgG antibody (#sc-2005, Santa Cruz, Heidelberg, dilution 1:5,000) as secondary antibody in Western blotting.

**Immunohistochemistry**

Surgical specimens in paraffin blocks were cut (5 µm) and mounted on superfrost slides (Menzel-Gläser, Braunschweig, Germany). Sections were deparaffinized in xylene and gradually hydrated. The slides were exposed to 0.3% hydrogen peroxide in PBS for 5 min to inactivate endogenous peroxidase. Sections were treated using the Bond–maX automated staining system (Leica Microsystems). For brown staining, the primary antibody was detected using the RefineDAB method (Vision Biosystems) with an H2 Buffer (Vision Biosystems) heat-induced epitope retrieval (98°C). For red staining, the primary antibody was detected using alkaline phosphatase polymer and new Fuchsin as substrate. Slides were counterstained with hematoxylin, dehydrated and mounted.
MIP-3α secretion was induced after 8 h stimulation in a dose-dependent manner (fig. 1). This confirmed our hypothesis that MDP stimulates MIP-3α secretion in myeloid cells. To cross-check our hypothesis we examined MIP-3α induction upon MDP stimulation in the NOD2 negative cell line HEK293T. HEK293T cells were stimulated in a single experiment for 24 h with 1 μg/ml MDP. No induction of MIP-3α and IL-8 secretion was obtained compared to nonstimulated cells (not shown).

**NOD2 Variants Impair MDP-Mediated MIP-3α Induction**

To investigate the influence of NOD2 on the expression of MIP-3α, we transfected HEK293T with SNP13 variants. One variant expressed the NOD2WT gene (pcDNA3.1_NOD2/CARD15) and the second expressed the NOD2 SNP13 variant (pcDNA3.1_SNP13). The ‘empty’ vector backbone served as control (pcDNA3.1). NOD2 expression in transiently transfected HEK293T was confirmed by Western blot (online suppl. fig. 2). Cells were stimulated with MDP. To confirm NF-κB activation IL-8 secretion was measured (not shown). MIP-3α expression was significantly (p < 0.05) induced by 1,000 ng/ml MDP stimulation (fig. 2). We also determined increased expression of MIP-3α mRNA upon expression of NOD2 SNP13 variant compared to vector control.

NOD2-dependent secretion of MIP-3α into the supernatant was also confirmed by ELISA. Cells expressing the control plasmid did not show any changes in basal MIP-3α secretion. Expression of both NOD2WT gene and NOD2 SNP13 variant resulted in a significantly (p < 0.05) elevated MIP-3α secretion (6.2- and 3.2-fold compared to control, respectively; fig. 3). MIP-3α mRNA expression was significantly (p < 0.05) induced by 100 ng/ml MDP stimulation for NOD2WT gene (19.9-fold compared to unstimulated cells also transfected with NOD2WT gene; fig. 3). Higher MDP concentrations led to an increase in the secretion of MIP-3α in NOD2 SNP13 variant transfected HEK293T, achieving the values for NOD2WT gene following MDP stimulation with 1,000 and 2,000 ng/ml MDP. These results confirmed our hypothesis that MDP stimulates MIP-3α secretion in a NOD2 variant-dependent manner.

**MIP-3α mRNA Expression in Human Intestinal Mucosa**

Previously, in an ex vivo model, we demonstrated that induction of MIP-3α during differentiation of human IMACs is followed by increased migration of CD45R0+ T

Statistical Analyses

Statistical analyses were performed using PASW statistics 18.0 (SPSS Inc., USA). One-way ANOVA and post-hoc Bonferroni test were applied for analysis of human samples. One-way ANOVA and Kruskal-Wallis one-way ANOVA on ranks were used for primary human cell experiments and human cell lines. Bars represent mean values with Whiskers displaying standard deviation. Differences were considered significant at p < 0.05 (cytokine and chemokine levels, quantification of immunohistochemistry). Luminescence of Western blots was quantified densitometrically with OptiQuant (Packard Instrument, USA). For quantitative analysis of cell recruitment in the intestinal mucosa and expression of CD68-, CD45R0- and IL-17-positive cells were counted and calculated from four high-power fields (hpf) at a magnification of ×200.

**Results**

**MDP-Induced MIP-3α Expression in the Myeloid Cell Line MM6**

We hypothesized that NOD2 stimulation via MDP is followed by increased MIP-3α secretion in IMACs. To investigate the influence of NOD2-mediated NF-κB activation on MIP-3α expression, we examined its induction in the human myeloid cell line MM6. As positive control IL-8 secretion upon LPS stimulation was confirmed for MM6 cells (online suppl. fig. 1). We stimulated MM6 with 0, 0.001, 0.01, 0.1, 1, 10 and 100 μg/ml MDP. MIP-3α secretion was induced after 8 h stimulation in a dose-dependent manner (fig. 1).
cells. We hypothesized that MIP-3α expression is also induced in IMACs in vivo during their differentiation in the lamina propria followed by the recruitment of CCR6+ cells. To confirm this hypothesis, MIP-3α mRNA expression was determined by real-time PCR in primary intestinal epithelial cells, LPMNCs and IMACs isolated from human intestinal mucosa from surgical specimens. To keep procedures similar, monocytes and intestinal macrophages were isolated via CD33, a unique surface marker present on both. Real-time PCR for MIP-3α mRNA synthesis showed high expression levels for both intestinal epithelial cells (17.6 ± 1.1 relative to blood lymphocytes set as one; fig. 4) and IMACs (12.4 ± 0.9). Similar MIP-3α mRNA expression levels were obtained from LPMNCs, including IMACs (11.9 ± 1.1). LPMNCs depleted of IMACs showed low MIP-3α mRNA expression levels (6.1 ± 1.1). Levels of MIP-3α mRNA synthesis found in peripheral blood lymphocytes were set as one and used as the control (fig. 4). Similarly, the level of MIP-3α mRNA expression was obtained from human blood monocytes isolated from peripheral blood lymphocytes (0 ± 0.5).

Recruitment of CD45RO+ and Th17 Cells Is Not Affected by Heterozygous NOD2 Gene Variants

Breakdown of the epithelial barrier and successive influx of bacterial wall components seem to play an essential role in the pathogenesis of CD. In vitro MDP is able to activate the NF-κB pathway via NOD2 followed by the release of MIP-3α, resulting in memory T lymphocyte recruitment. The in vitro experiments described above showed a functional deficit in MIP-3α expression in cells transfected with NOD2 variant SNP13. Since MIP-3α may be involved in the aggregation of CCR6+ lymphocytes during an immune response, we consequently investigated the recruitment of CD45RO+ cells and Th17 cells with respect to NOD2 genotypes. Immunohistochemistry showed that cell–cell contacts between IMACs and CD45RO+ memory T cells showed no difference between patients with NOD2WT gene (34.7 ± 17.8 cells/hpf) and patients with heterozygous mutations in NOD2 (31.8 ± 16.4 for SNP8, 53.0 ± 26.4 for SNP12, and 41.5 ± 20.2 cells/hpf for SNP13; fig. 5). Recruitment of Th17 cells was unchanged in patients with NOD2WT (12.5 ± 12.8 cells/
Discussion

Disturbance of the mucosal barrier and mucosal transport processes is thought to be a major factor in the pathogenesis of intestinal inflammation and IBD. Increased permeability of the epithelial barrier in ulcerative colitis and CD has been reported [39–42]. Local protective mechanisms of the healthy mucosa prevent the invasion of bacteria or specific pathogens from the intestinal lumen into the body. Protective factors such as mucus production [43] and synthesis of antimicrobial peptides are impaired in CD. The intestinal barrier becomes ‘leaky’, resulting in the invasion of luminal antigen. A number of knockout models of IBD confirm that bacteria are essential for the development of intestinal inflammation [44–46]. NOD2 is an intracellular sensor of bacterial MDP that has been shown to be the most important susceptibility factor for the onset and pathogenesis of CD.

NOD2 is able to activate the NF-κB pathway followed by the release of MIP-3α, which attracts memory T lymphocytes [17–23].

In this study, we therefore asked (1) whether the expression of MIP-3α is associated with the NOD2 genotype and (2) what consequence stimulation with bacterial antigen after mucosal translocation has on recruitment of CCR6-positive immune cells.

We found that NOD2 stimulation via MDP was followed by a dose-dependent increase in MIP-3α expression in the myeloid cell line MM6. No induction of MIP-3α secretion was obtained in NOD2-negative HEK293 upon MDP stimulation but in vitro, MIP-3α mRNA was significantly (p<0.05) induced in HEK293 transfected with NOD2_pcDNA via MDP ligation. Increased MIP-3α protein secretion was confirmed by ELISA. In vivo, MIP-3α protein expression in human intestinal epithelial cells and IMACs was determined in a nonquantitative manner by immunohistochemistry and flow cytometry [31]. In this work, we could confirm MIP-3α mRNA expression ex vivo in freshly isolated intestinal epithelial cells and IMACs. MIP-3α mRNA expression was significantly (p<0.05) higher in primary human IMACs in comparison to monocytes. Previous in vitro experiments showed a functional deficit in Mip-3α expression in HEK293 cells transfected with NOD2 variant SNP13. We therefore investigated Th17 and memory cell recruitment with respect to NOD2 variants. In vivo cell-cell contacts between IMACs and CD45R0+ and recruitment of Th17 cells was unchanged in patients with heterozygous NOD2 variants as compared to NOD2WT individuals.

Altered immunological responses in the gut during inflammation and under normal conditions are reflected by different PRR subsets of IMACs and DCs. It seems unlikely that in CD only a single reactive endotoxin component accumulates in the mucosa. Multiple pathogen-associated molecular patterns or microbe-associated molecular patterns are detected by a number of PRRs shown to be expressed extracellularly. Intracellularly, a repertoire of PRRs accounts for NF-κB-driven immunological competence of IMACs and DCs during inflammation. Recognition of endotoxin induces a Th1 or Th17 pro-inflammatory immune response directed normally at pathogens [47–49]. Due to their expansion or homing Th17 cells are highly enriched in the intestine. Th17 cells express various trafficking receptors, but the MIP-3α receptor CCR6 is uniformly expressed by all subsets of Th17 cells. In mice, CCR6−/− Th17 cells are less efficient in migration to small intestinal lamina propria [50].

Short-term migration of CCR6−/− Th17 cells to the large...
intestine is not decreased. However, significantly fewer Th17 cells and an aberrant increase of Th1 cells are found in the intestine of Rag1−/− SCID mice injected with CCR6−/− Th17 cells compared to WT Th17 cells. Consistently, CCR6−/− Th17 cells induce more severe intestinal inflammation in Rag1−/− SCID mice [50]. CCR6 is not the only trafficking receptor that is important for migration and localization in the gut.

On the other hand, under normal conditions, ‘tolerant’ IMACs and DCs lack antigens connected with the recep-
NOD Ligation Stimulates Expression of MIP-3α

NOD2 polymorphisms have been identified as risk factors of graft-versus-host disease (GVHD) following allogeneic stem cell transplantation. Recent work shows that intestinal GVHD is associated with a stage-dependent decrease in CD4 T cell infiltrates in the lamina propria [58]. The presence of NOD2 variants in the recipient is associated with a significant loss of CD4 T cells. Therefore, in future work it will be necessary to consider recruitment of other subpopulations of CCR6+ cells. Next to CCR6+ DCs and B lymphocytes recruitment of tissue CCR6+CD4+ T cells might be of interest.

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NOD Ligation Stimulates Expression of MIP-3α


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