Activation of the Receptor NKG2D Leads to Production of Th17 Cytokines in CD4+ T Cells of Patients With Crohn's Disease

BENJAMIN PARIENTE,* IULIA MOCAN,* MATTHIEU CAMUS,* CHARLES–ANTOINE DUTERTRE,‡ JULIEN ETTERSPERGER,* PIERRE CATTAN,§ JEAN–MARC GORNET,‖ NICOLAS DULPHY,* DOMINIQUE CHARRON,* MARC LÉMANN,‖ ANTOINE TOUBERT,* and MATTHIEU ALLEZ*,‖

*INSERM, Equipe AVENIR U940 Hôpital Saint-Louis, Paris; ‡INSERM, UMRS 872, Centre de Recherche des Cordeliers, Université Pierre et Marie Curie-Paris 6, Paris; and Departments of §Surgery and ‖Gastroenterology, Hôpital Saint-Louis, Université Denis-Diderot Paris 7, Paris, France

BACKGROUND & AIMS: The natural killer group 2 member D (NKG2D) is a stimulatory receptor expressed on a subset of mucosal and peripheral CD4+ T cells in patients with Crohn's disease (CD) and other inflammatory diseases. Ligand activation of NKG2D in patients induces CD4+ T cells to release T-helper (Th) 1 cytokines and become cytotoxic. We investigated the Th17 cytokines produced by T cells that express NKG2D in blood and intestinal mucosa samples from patients with CD. **METHODS:** We isolated CD4+ T cells from peripheral blood and lamina propria samples of patients with CD or ulcerative colitis (UC) and healthy individuals (controls). We analyzed the phenotype and functions of the CD4+NKG2D+ T cells and the cytokines they produce in response to NKG2D stimulation. **RESULTS:** In patients with CD, CD4+ T cells that express NKG2D produced high levels of interleukin (IL)-17 and IL-22 and expressed high levels of CCR6, the IL-23 receptor, CD161, and RORC (a transcription factor that regulates expression of Th17 cytokines). CD4+ T cells that produced IL-17 expressed high levels of NKG2D and CD161. Costimulation of NKG2D and the T-cell receptor (TCR) significantly increased production of IL-17 and tumor necrosis factor α by CD4+ T cells, compared with activation of only the TCR. CD4+NKG2D+ T cells also responded to Th17 polarization. **CONCLUSIONS: NKG2D is a functional marker of CD4+ T cells that produce IL-17 in patients with CD, via costimulation of the TCR and NKG2D. Reagents developed to block NKG2D might reduce gastrointestinal inflammation in patients with CD.**

Keywords: Intestine; T-cell Signaling; Immune Response; Inflammatory Bowel Diseases.

Inflammatory bowel diseases (IBDs) comprise Crohn's disease (CD) and ulcerative colitis (UC), which are both characterized by uncontrolled immune responses toward the intestinal flora.[1] The pathogenesis of IBD remains elusive but is clearly influenced by genetic and environmental factors.[1–3] Abnormal innate immune responses have been described in CD, including specific defects in the epithelium and in macrophages.[2,4] However, the inflammatory process is T-cell driven, and chronic intestinal inflammation may be due either to impaired regulatory T-cell activity or excessive effector T-cell function.[5–7]

Until recently, CD was described as a T-helper (Th) 1 disease, characterized by high interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and interleukin (IL)-12 levels.[8] The latest data highlighted the role of the Th17 subset in CD, as well as their cytokine signature IL-17 (IL-17A).[9,10] A number of murine models of experimental colitis showed the predominant role of Th17 response. Indeed, inhibition of the Th17 but not of the Th1 response was effective in prevention of colitis.[10] In addition, transfer of Th17 cells induced more severe disease than Th1 cells did in SCID mice.[11] Th17 cells have characteristic features, like elevated expression of IL-23 receptor (IL-23R), CCR6 integrin, and RORγt transcription factor in mice or RORC in humans.[12] The IL-23 cytokine is predominantly involved in Th17 development of previously activated cells. Moreover, the *IL-23R* gene has been identified as an IBD susceptibility gene, with an uncommon coding variant that confers strong protection against CD.[13] Also in CD, IL-22 has been shown to be elevated in the inflamed mucosa and exhibits proinflammatory properties.[14] Notably, Th17 cells have been reported to be the main producers of IL-22.[15]

Recent data have shown an abnormal expression of natural killer (NK) receptors on T cells in patients with CD.[16,17] Kleinschek et al have described a subset of CD4+ T cells involved in chronic intestinal inflammation in CD that carry the C-type lectin-like NK receptor CD161.[18] These CD4+CD161+ T cells display an activated Th17 phenotype, with increased expression of IL-17, CCR6, and IL-23R. Circulating CD161+ Th17 cells are imprinted for gut homing, as indicated by high levels of integrin β7 expression. We have recently identified a subset of effector CD4+ T cells mediating inflammatory response in CD and expressing the NK activating receptor natural killer group 2 member D (NKG2D).[16] CD4+NKG2D+ T cells are functionally active through interactions with NKG2D ligands. The nonclassic major histocompatibility complex class 1–like molecules

Abbreviations used in this paper: HC, healthy controls; IFN, interferon; IL, interleukin; LP, lamina propria; LPL, lamina propria lymphocytes; NK, natural killer; NKG2D, natural killer group 2 member D; PB, peripheral blood; PBL, peripheral blood lymphocytes; PMA, phorbol myristate acetate; Q-RT-PCR, quantitative real-time reverse-transcription polymerase chain reaction; TCR, T-cell receptor; Th, T-helper; TNF, tumor necrosis factor.

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MICA and MICB, together with ULBPs (UL16 binding proteins), constitute major ligands of NKG2D in humans. Their increased expression has been correlated with cellular stress[19,20] and with a subsequent NKG2D-driven $CD4^+$ T-cell cytotoxicity in autoimmune diseases.[16,21] Nevertheless, their functional implication in CD deserves further studies. $CD4^+NKG2D^+$ T cells expand in the lamina propria (LP) and the peripheral blood (PB) of patients with CD, but not in patients with UC or in healthy controls (HCs). $CD4^+NKG2D^+$ T cells exhibit specific cytotoxic activity and produce IFN-γ in the presence of MICA-bearing cells.[16] The implication of $CD4^+NKG2D^+$ T cells in gut inflammation has been further shown in a murine model of transfer-induced colitis.[22,23] In these studies, administration of a specific NKG2D blocking antibody decreased NKG2D expression on $CD4^+$ T cells and attenuated the development of colitis, highlighting NKG2D as a possible therapeutic target in IBD.

The purpose of our study was to further characterize the functional properties of $CD4^+NKG2D^+$ T cells and their relationship with Th17 cells as well as $CD4^+$ T cells expressing CD161. We show that $CD4^+NKG2D^+$ T cells represent a major source of IL-17 in CD and have typical features of Th17 cells, including high CD161 expression. In patients with CD, IL-17–producing $CD4^+$ T cells preferentially expressed NKG2D and CD161 at their surface, with NKG2D being more specific for this population. NKG2D stimulation by its ligands significantly enhances the IL-17 secretion triggered by the T-cell receptor (TCR). Altogether, these results highlight the functional plasticity of $CD4^+$ T-cell subsets in CD and identify $CD4^+NKG2D^+CD161^+$ cells as a major proinflammatory T-cell population. Targeting NKG2D might have a significant impact on Th17 pathways in CD and gut inflammation.

Patients and Methods

Patients With IBD and Controls

Forty-nine patients with moderate to severe active CD, 12 patients with active UC, and 15 HCs were included in this retrospective study. All of the patients were hospitalized in the Department of Gastroenterology at Hôpital Saint-Louis in Paris, France. Patient characteristics are given in Table 1. Among the 49 patients with CD, 20 had ileal (n = 17) or colonic resections (n = 3). This study was approved by the Ethical Committee of Hôpital Saint-Louis (IRB 00003835), and all subjects gave written informed consent.

Table 1. Clinical Characteristics of Patients With CD and UC

	Patients With CD (n = 49)	Patients With UC (n = 12)
Age (*y*)[a]	34.9 ± 12.5	37 ± 20.5
Sex (M/F)	15/34	7/10
Location of lesions		
Ileum only	18	
Ileum and colon	18	
Colon	12	12
Anus only	16	
Indication of surgical resection (n = 20)		
Small bowel obstruction	15	
Abscess	5	
Active disease		
Harvey–Bradshow[a]	8.7 ± 5.5	—
Truelove–Witts[b]	—	3 (1–4)
C-reactive protein (*mg/L*)[b]	49.2 (3–170)	11 (3–39)
Ongoing treatments		
Corticosteroids	8	1
Azathioprine, 6-mercaptopurine, methotrexate	15	6
Infliximab, Adalimumab	5	1

[a]Mean ± SD.
[b]Median (extremes).

Isolation of Intestinal and Blood Lymphocytes

LP lymphocytes (LPLs) and PB lymphocytes (PBLs) were isolated with the same method as we previously described.[16] Techniques are detailed in Supplementary Materials and Methods.

Multiparametric Flow Cytometry

For surface staining, lymphocytes (PBLs, LPLs) were resuspended in fluorescence-activated cell sorter buffer (phosphate-buffered saline, 5% fetal calf serum) and incubated for 30 minutes at 4°C with different mixtures of antibodies. The surface antibody mixtures included antibodies conjugated with fluorescein isothiocyanate, phycoerythrin, phycoerythrin-Cy5, phycoerythrin-Cy7, allophycocyanin, allophycocyanin-H7, Pacific Blue, or AmCyan and directed against CD3, CD8, CD4, CD161 (BD Biosciences, Le-Pont-De-Claix, France), NKG2D (Beckman Coulter, Miami, FL), IL-23R and CCR6 (R&D Systems, Abingdon, England), and relevant isotype controls (R&D Systems, Abingdon, England).

For functional studies, lymphocytes (PBLs, LPLs) were stimulated for 4 hours with 25 ng/mL phorbol myristate acetate (PMA) and 1 μg/mL ionomycin and incubated with 5 μg/mL brefeldin A before intracellular staining. Cells were stained with CD3, CD4, CD8, NKG2D, and CD161 as shown previously. Cells were subsequently permeabilized in a saline buffer containing 0.1% saponin for 5 minutes. Cells were then incubated with fluorescein isothiocyanate–conjugated antibodies directed against IL-17A (Clinisciences, Montrouge, France), Alexa 647 anti–IFN-γ (BD Biosciences) and allophycocyanin anti–IL-22 (R&D Systems), and relevant isotype controls. Eight-color analyses were performed using BD FACS Canto-II and FACS Diva software (BD Biosciences).

Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction Analysis

LPLs from 4 patients with active CD were stained for surface CD3, CD4, and NKG2D, and different T-cell populations ($CD4^-$, $CD4^+NKG2D^+$, and $CD4^+NKG2D^-$) were sorted using a BD FACSAria II cell sorter (Becton Dickinson, San Jose, CA).

Total RNA from purified populations was extracted using a QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany). Quantitative real-time reverse-transcription polymerase chain