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Cutting Edge: NKG2D Receptors Induced by IL-15 Costimulate CD28-Negative Effector CTL in the Tissue Microenvironment[1]

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Unlike primary T cells in lymph nodes, effector CD8$^+$ CTL in tissues do not express the costimulatory receptor CD28. We report that NKG2D, the receptor for stress-induced MICA and MICB molecules expressed in the intestine, serves as a potent costimulatory receptor for CTL freshly isolated from the human intestinal epithelium. Expression and function of NKG2D are selectively up-regulated by the cytokine IL-15, which is released by the inflamed intestinal epithelium. These findings identify a novel CTL costimulatory pathway regulated by IL-15 and suggest that tissues can fine-tune the activation of effector T cells based on the presence or absence of stress and inflammation. Uncontrolled secretion of IL-15 could lead to excessive induction of NKG2D and thus contribute to the development of autoimmune disease by facilitating the activation of autoreactive T cells. ***The Journal of Immunology,*** **2001, 167: 5527–5530.**

The clonal expansion of naive, Ag-stimulated T cells in lymph nodes is controlled by a set of costimulatory signals induced by the innate immune response to microbial and necrotic cell death products (1–3). However, little is known about possible mechanisms regulating the activation of Ag-stimulated effector T cells in the tissue environment. This is in part due to difficulties in recovering sufficient numbers of such tissue effector T cells to perform functional studies. We obtained large fragments of human intestine from healthy individuals undergoing gastric bypass for morbid obesity and isolated the population of TCR$\alpha\beta^+$CD8$\alpha\beta^+$ T-intraepithelial lymphocytes (IEL),[4] which is dominated by multiple expanded clones in an armed CTL effector state (reviewed in Ref. 4). Because several reports in mouse and human imply that NK receptors (5–16) and IL-15 (17–20) play an important role in the activation and/or expansion of memory/effector T cells, we studied the expression and function of NKG2D receptors in freshly isolated CTL and their regulation by IL-15.

Tissue effector CTL were negative for CD28, the costimulatory receptor for naive T cells, but expressed low levels of NKG2D. In humans, NKG2D is the receptor for the MHC class I-like ligands ULBPs (21), and for MICA and MICB induced by stress and viral infection in permissive cells (6, 22, 23). NKG2D expression and function were selectively up-regulated by IL-15, a cytokine that is prominently secreted in the gut microenvironment during stress or infection (24–26). NKG2D engagement markedly enhanced T cell activation under conditions of suboptimal TCR engagement for a range of proliferative and effector functions. Furthermore, NKG2D could mediate redirected cytolysis, an NK-like activity of CTL revealed by exposure to IL-15. Our results identify a novel CTL costimulatory pathway in tissues that is regulated by IL-15, and suggest that tissues can fine-tune the activation of effector T cells based on the presence or absence of stress and inflammation.

Materials and Methods

mAbs and recombinant cytokines

Biotin- or fluorochrome-conjugated anti-CD3, CD8, CD45RO, CD28, TCR$\alpha\beta$, NKR-P1A (CD161), CD56, and CD16, unconjugated anti-CD3 (clone UCTH1, IgG1), and nonspecific mouse isotype controls were from BD PharMingen (San Diego, CA). Anti-NKG2D mAb 1D11 (IgG1) was used unconjugated or biotinylated (22). Antiphosphotyrosine mAb 4G10 was from Upstate Biotechnology (Lake Placid, NY) and goat anti-mouse F(ab′)$_2$ mAb from Jackson ImmunoResearch Laboratories (West Grove, PA). rIL-15, IL-12, IFN-γ, TNF-α, IL-10, TGF-β, and IL-2 were from BD PharMingen and R&D Systems (Minneapolis, MN).

Flow cytometric analysis

Biotinylated Abs were revealed with streptavidin-PE. Fluorescence was analyzed on a four-color FACSCalibur (BD Biosciences, Mountain View, CA), with statistical quadrants set to score as negative >99% of control Ig isotype-stained cells.

Lymphocyte isolation

IEL were purified from jejunal mucosa obtained from healthy individuals undergoing gastric bypass for morbid obesity as described previously (27). PBL were isolated from whole blood of healthy volunteers after Ficoll density gradient centrifugation (Amersham Pharmacia Biotech, Piscataway, NJ).

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[4] Abbreviation used in this paper: IEL, intraepithelial lymphocyte.

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Cell cultures and NKG2D$^+$ cell lines

Cell culture.
Freshly isolated IEL were cultured in RPMI 1640 supplemented with 10% FCS and antibiotics, alone or in the presence of IL-15, at 15 ng/ml overnight and washed three times before the functional assays.

NKG2D$^+$ T-IEL cell lines.
To obtain NKG2D$^+$ T-IEL cell lines, IEL were isolated from surgical intestinal samples and labeled with anti-CD8, anti-TCR$\alpha\beta$ Abs to sort TCR$\alpha\beta^+$CD8$^+$ IEL, using a FACSVantage (BD Biosciences). Purified lymphocyte populations were stimulated with PHA at 1 μg/ml and a mixture of irradiated heterologous PBL and EBV-transformed human cell lines in RPMI 1640 supplemented with IL-2 (100 U/ml) and 10% human AB serum (Sigma-Aldrich, St. Louis, MO), as described by Vie et al. (28). Expression of NKG2D by the T-IEL cell lines was confirmed by flow cytometric analysis.

Ab-mediated stimulation assays

Cells (0.2×10^6) were cultured in 0.2 ml of culture medium in flat-bottom 96-well plates (Costar, Cambridge, MA) precoated overnight with 10 μg/ml (unless otherwise specified) anti-CD3 and/or anti-NKG2D. Control stimulations used an irrelevant IgG1 isotype mAb (BD PharMingen).

Proliferation of fresh IEL was measured by the uptake of [^{3}H]thymidine (ICN Biomedicals, Irvine, CA) added at 0.5 μCi/well after 48 h of culture.

IFN-γ production was measured in the 48-h culture supernatant using a specific ELISA (Beckman Coulter, Miami, FL) and values expressed in pg/ml by comparison to recombinant IFN-γ. For Fc-dependent redirected cytotoxicity assays, freshly isolated T-IEL or cell lines were incubated for 4 h with ^{51}Cr-labeled P815 cells at various E:T ratios in the presence of soluble anti-NKG2D (1 μg/ml) or anti-CD3 (1 μg/ml). Control mouse mAbs were MOPC-21 (IgG1; BD PharMingen) or the B9.12.1 IgG2a mAb against human HLA-class I ABC allotypes (Beckman Coulter). Maximum ^{51}Cr release was determined by addition of detergent (2% cetrimide) and spontaneous release ranged from 5 to 20% of the max. The percentage of specific cytotoxicity was $100 \times$ (cpm experimental $-$ cpm spontaneous)/(cpm maximum $-$ cpm spontaneous).

Tyrosine phosphorylation assay

Freshly isolated IEL were incubated at 37°C overnight alone or with 15 ng/ml IL-15. After washes, 5×10^6 cells were resuspended in 0.3 ml of RPMI 1640, incubated with anti-NKG2D or isotype control Ab for 1 h at 4°C, washed, and stimulated for 1 min at 37°C with 50 μg/ml goat anti-mouse Fab$'_2$ Ab (Jackson ImmunoResearch Laboratories). After quenching in ice-cold PBS containing NaF and Na_3VO_4 (each at 1 mM), cells were centrifuged and lysed for 20 min in cold lysis buffer containing fresh protease inhibitors (0.5% Triton X-100, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF, and 1 μg/ml each aprotinin, leupeptin, and pepstatin). Lysates were centrifuged for 20 min at 4°C in a microfuge to remove cell nuclei, and the supernatants were precleared on protein A-Sepharose (Amersham Pharmacia Biotech) for 1 h. Phosphotyrosine-containing proteins were then immunoprecipitated for 1 h at 4°C with 4G10 antiphosphotyrosine mAb coated onto protein A-Sepharose beads (Upstate Biotechnology). SDS-PAGE on 12% acrylamide gels and Western transfer were performed using standard methods and anti-phosphotyrosine Ab were detected with the ECL kit (Amersham Pharmacia Biotech).

Results

Memory/effector CD28$^-$CD8$^+\alpha\beta$ T-IEL express low levels of NKG2D

Freshly isolated jejunal IEL were mostly CD8$^+\alpha\beta$ T cells (>75%, not shown) and, like their PBL counterparts, expressed NKG2D, albeit consistently ($n = 8$) at levels substantially lower than those found on peripheral blood T cells, suggesting that NKG2D expression is down-regulated in the intestinal tissue (Fig. 1*a*). T-IEL uniformly expressed a memory/effector CD45RO$^+$CD28$^{-/low}$ phenotype, which was only found in a subset of circulating PBL (Fig. 1*b*).

IL-15 enhances expression of NKG2D by fresh T-IEL

Exposure to IL-15 resulted in a rapid 5- to 10-fold increase of NKG2D surface expression by T-IEL in all donors examined (Fig. 2*a*). This effect was superior to that observed after CD3 triggering.

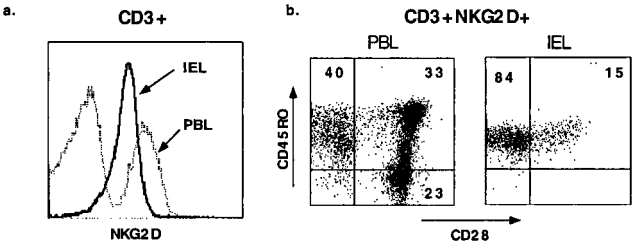

FIGURE 1. NKG2D expression by freshly isolated T-PBL and T-IEL. Dot plots are gated as indicated. *a*, NKG2D expression in freshly isolated CD3$^+$ IEL is lower than in CD3$^+$ PBL. *b*, The NKG2D$^+$CD45RO$^+$CD28$^{-/low}$ T-IEL phenotype is also found in a subset of PBL. Statistical quadrants are set with isotype control stained cells (not shown).

Consistent with this up-regulation, NKG2D signaling was also enhanced in three different experiments, as shown by the induction of a p110 phosphorylated protein upon mAb engagement of NKG2D on IL-15-treated T-IEL (Fig. 2*b*). We have reported that IL-15 also induced heterodimeric CD94/NKG2 receptors (7). However, other NK cell receptors such as KIR2DL, CD56, CD16, or NKR-PA1 were minimally or not affected (data not shown). Finally, these effects were observed only with IL-15 (Fig. 2*a*) or high doses of IL-2 (not shown), which also signals through the IL-15 receptor. Other cytokines such as TNFα, IL-7, IL-10, and IL-12 had no detectable effect, and IFN-γ had only modest effects (not shown).

Altogether, these results show that NKG2D is down-regulated in healthy T-IEL and is specifically up-regulated by IL-15.

IL-15 reveals NKG2D-mediated cytotoxicity by fresh T-IEL

Anti-NKG2D cross-linking did not induce redirected lysis of P815 cells by freshly isolated T-IEL, although, as previously reported

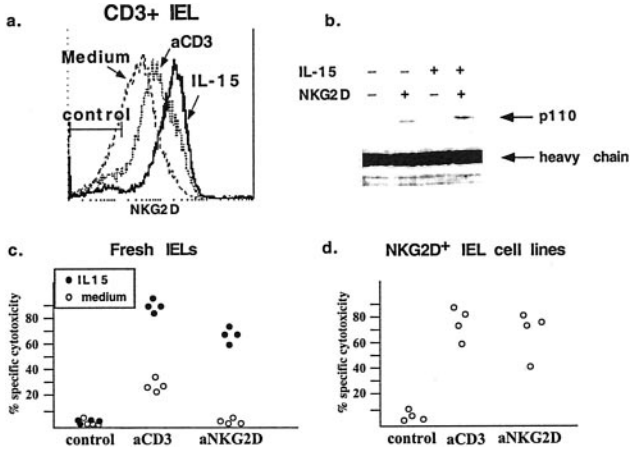

FIGURE 2. IL-15 up-regulates NKG2D expression and function in freshly isolated T-IEL. *a*, NKG2D expression by T-IEL cultured for 36 h in medium alone, or with plastic bound anti-CD3 (coated at 10 μg/ml) or IL-15 (15 ng/ml). *b*, Tyrosine phosphorylation by freshly isolated IEL stimulated with anti-NKG2D. IEL cultured overnight with or without IL-15 (15 ng/ml) were stimulated with anti-NKG2D or isotype control for 1 min before immunoprecipitation and immunoblotting with anti-phosphotyrosine Ab. IL-15 prestimulated cells showed markedly increased phosphorylation of a 110-kDa protein. *c*, NKG2D triggers cytolysis in fresh IL-15-stimulated T-IEL. Cytolysis of the FcγR$^+$P815 murine mastocytoma line was measured in a standard redirected ^{52}Cr release assay in the presence of anti-NKG2D (aNKG2D), anti-CD3 (aCD3), and isotype control (irrelevant mAb). IL-15 was used at a concentration of 15 ng/ml. (E:T ratio, 25:1). *d*, NKG2D mediates redirected cytolysis in T-IEL lines from four different donors in the absence of IL-15. (E:T ratio, 25:1).

(29), CD3 engagement was effective (Fig. 2*c*). In contrast, IL-15-prestimulated T-IEL exhibited potent NKG2D-mediated redirected lysis. However, as previously reported for T cell clones (23), NKG2D-mediated cytolysis by T-IEL lines was independent of IL-15 stimulation (Fig. 2*d*), suggesting a functional difference between freshly isolated T-IEL and T cell lines, and highlighting the limitations of studies with cultured lines.

Costimulatory function of NKG2D receptors expressed by fresh T-IEL

NKG2D triggering alone did not induce IFN-γ release or cell proliferation (Fig. 3, *a* and *b*). However, upon CD3/TCR stimulation of freshly isolated T-IEL, simultaneous engagement of NKG2D dramatically increased IFN-γ secretion 8-fold and proliferation 14-fold above CD3 stimulation alone (Fig. 3, *a* and *b*). Similar increases, ranging from 5- to 18-fold, were observed in three additional experiments (Fig. 3*c*). IL-15 stimulation increased TCR-mediated cytokine secretion and proliferation, as previously shown (30), but did not induce NKG2D-mediated IFN-γ secretion or cell proliferation. However, IL-15 enhanced T-IEL stimulation by the combination of CD3 and NKG2D synergistically, beyond the sum of its effects on CD3 and NKG2D alone, in three different experiments (Fig. 3, *a* and *b*).

Altogether, these data demonstrate that NKG2D can function as a potent costimulator of TCR-mediated activation of T-IEL.

Discussion

The clonal expansion of naive, Ag-specific T cells is critically regulated by costimulatory signals. T cell priming usually occurs in the lymph node environment where incoming, Ag-pulsed, activated dendritic cells have up-regulated their CD40 and B7 costimulatory molecules to provide the complement of accessory signals required for clonal expansion following TCR engagement (31). Because dendritic cell activation and up-regulation of costimulatory molecules is regulated by innate signals induced by microbial products and necrotic cell death, innate immunity controls the early phase of adaptive immunity (1–3).

In contrast, little is known about the signals that regulate differentiated effector lymphocytes once they reach the tissues. Indeed, tissue cells do not express B7 molecules, and most effector CTL do not express CD28. Although it could be argued that armed CTL can be triggered by Ag recognition alone, emerging evidence suggests that their activation may also be fine-tuned by novel costimulatory systems. Candidate receptor/ligand systems include the B7-like PD-L1 and PD-L2 molecules expressed by tissue cells and their PD-1 receptor on CTL (32, 33), as well as NK receptors and their tissue ligands (6, 8, 10).

In this study, we have investigated the function of NKG2D expressed by human CTL freshly isolated from the normal intestine. Our results extend to fresh tissue CTL the conclusions of a recent study using CMV-specific T cell clones and peripheral blood pp65/A2 tetramer, $CD28^{-}CD8^{+}\alpha\beta$ T cells to show that NKG2D

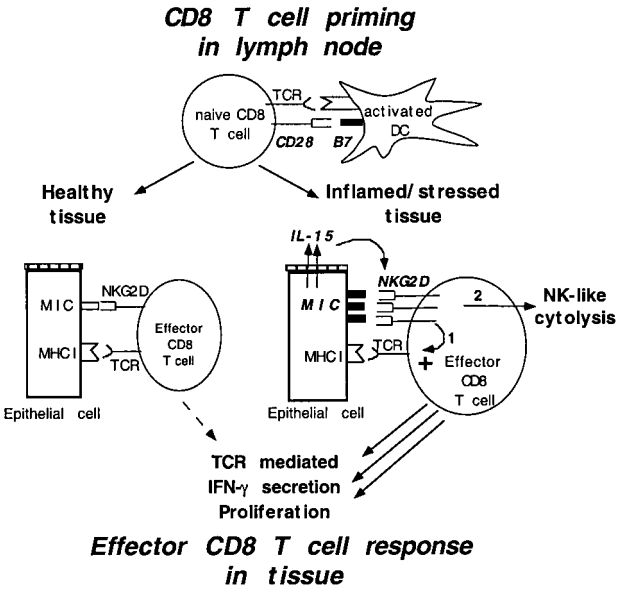

FIGURE 4. Tissue control of CTL effector functions and proliferation by NKG2D. Whereas naive CD8 T cells encountering Ag-loaded dendritic cells (DC) in lymph nodes use the CD28/B7 costimulatory pathway, effector CTL in the tissue microenvironment are controlled by a distinct regulatory pathway that reads out the induction of the nonclassical MHC-like molecules MIC and ULBP. NKG2D binds to ULPB and MIC molecules induced upon stress and viral infection. IL-15, a cytokine secreted by epithelial cells in inflammatory and infectious conditions, up-regulates the NK receptor component of this costimulatory system (1) and confers to NKG2D the ability to mediate NK-like cytolysis (2). This new level of control of adaptive effector functions and proliferation by innate immunity selectively promotes the activation and expansion of CTL in tissues undergoing stress and infection.

engagement, in the context of a MICA/MICB-inducing viral infection of target cells, exerted marked costimulatory properties on TCR stimulation (6).

Furthermore, a novel aspect of our study is the finding that IL-15, a cytokine secreted by intestinal epithelial cells and induced upon inflammation and viral infections (reviewed in Refs. 24 and 26), selectively up-regulates the expression and function of NKG2D. IL-15 functions as a key regulator of effector CTL activation and expansion by arming the NKG2D costimulatory pathway under inflammatory conditions. In addition, IL-15 confers NK-like activity to CTL, unveiling the cytotoxic properties of NKG2D. This might well explain the previous reports showing that IEL could kill tumoral and virally infected intestinal epithelial cells (27, 34), which potentially express MIC proteins (6, 22), in an Ag-independent manner.

Altogether, these experiments support a scenario whereby tissues themselves take control of the activation and expansion of adaptive immune CTL, favoring effector CTL functions whenever

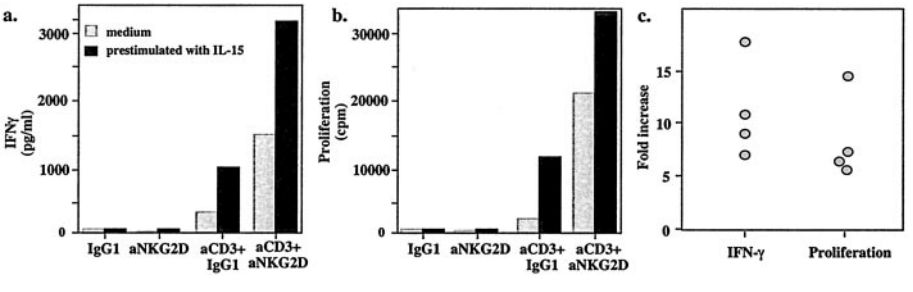

FIGURE 3. Costimulatory functions of NKG2D. Freshly isolated T-IEL were incubated with plate-bound Ab or isotype control in the presence or absence of IL-15, as indicated. *a*, IFN-γ secretion. *b*, Proliferation. *c*, Compiled results from four different donors in the absence of IL-15 stimulation.

agents causing damage and inflammation are present (Fig. 4). In healthy conditions, these costimulatory pathways are repressed at several levels, preventing uncontrolled T cell activation. Stress, inflammation, and IL-15 release promptly induce and/or arm the various components of these costimulatory systems. This new level of control of adaptive effector cells by local innate immune signals has two interesting properties: it can enhance the CTL response to Ags recognized with low affinity or present at low concentrations when damaging agents are present, and it diminishes the risk of protracted or bystander autoimmune responses after they have been cleared. In contrast, dysregulation of this pathway, for example through uncontrolled secretion of IL-15, might participate in the induction or persistence of autoimmune responses by reducing the threshold of TCR activation by low-affinity self-ligands or by increasing NK-like functions. Indeed, several studies have implicated IL-15 in rheumatoid and psoriasis (26, 35, 36). In addition, in celiac disease, a gluten-sensitive enteropathy (reviewed in Ref. 4), high levels of IL-15 are found in the damaged gut epithelium (7) in association with an increased expression of NKG2D by intestinal CTL and of MIC by intestinal epithelial cells (V. Groh, T. Spies, and B. Jabri, manuscript in preparation). These findings suggest that NKG2D and MIC may contribute to the destruction of intestinal epithelial cells in celiac disease and other immunopathological conditions.

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