IL-18 Is Involved in Eosinophil-Mediated Tumoricidal Activity against a Colon Carcinoma Cell Line by Upregulating LFA-1 and ICAM-1

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Eosinophils are multifunctional leukocytes that are involved in innate and adaptive immune responses through the expression of various receptors and mediators. Previously, we showed that human eosinophils and T cells shared cytotoxic activities against tumor cells that involved the γ-δ TCR and cell–cell contact. In this study, we investigated the molecules involved in eosinophil–tumor cell interactions. Given the role of IL-18 in cell adhesion and in protecting against colon cancer, we evaluated its role in eosinophil-mediated cytotoxicity against Colo-205, a human colon carcinoma cell line. We found that human eosinophils exerted dose- and time-dependent tumoricidal activity against Colo-205 cells. Neutralization of IL-18 significantly reduced eosinophil-mediated Colo-205 apoptosis and inhibited cell–cell adhesion. Moreover, addition of rIL-18 led to upregulation of CD11a and ICAM-1 adhesion molecules, which were involved in the contact between eosinophils and Colo-205 cells. Our results indicated that IL-18 was involved in the eosinophil-mediated death of Colo-205 by facilitating contact between effector and target cells. These data underscored the involvement of an additional mediator in eosinophil-mediated antitumor cytotoxicity. Our findings support existing evidence that eosinophils could play a beneficial role in the context of colon cancer. ***The Journal of Immunology*****, 2015, 195: 2483–2492.**

Eosinophils are multifunctional leukocytes that participate in innate and adaptive immune responses through the expression of various receptors and mediators (1). Although indirect, growing evidence supports the participation of eosinophils in antitumor immunity, notably in the context of colon cancer (2, 3). Several epidemiological studies suggested a correlation between the presence of tumor-associated tissue eosinophilia (TATE) and colorectal carcinoma, and the correlation showed good prognostic value (4–7). TATE was considered an independent prognostic indicator; thus, increased eosinophil counts indicated increased survival (5, 6). Recently, we (8, 9) and other investigators (10) showed that human eosinophils expressed receptors and mediators, such as 2B4, TCRγδ/CD3 complex, and granzyme A, which were shared with lymphocytes and involved in antitumor defense. Eosinophil stimulation through these receptors induced tumor cell death in vitro.

IL-18 is a member of the IL-1 family of cytokines. It is expressed as pro–IL-18, a biologically inactive form and is transformed to the active form primarily by caspase-1 (11). IL-18 was first identified as an IFN-γ–inducing factor based on its ability to enhance the Th1-type immune response by stimulating NK cells and T cells (12). Upon binding to the specific IL-18R complex (IL-18R), IL-18 stimulates a wide range of immune cells, including $CD4^+$ T cells (13), $CD8^+$ T cells (14), NK cells (15), γδ T cells (16), neutrophils (17), and eosinophils (18). This activity results from the induction of Fas ligand production and the generation of multiple secondary proinflammatory cytokines, chemokines, cell adhesion molecules, and NO species (19). Several reports showed that rIL-18 exerted potent antitumor effects in animal models of melanoma, lymphoma, sarcoma, and carcinoma (20–24). In those models, systemic IL-18 administration or IL-18 gene transfer inhibited tumor growth and prolonged the survival of tumor-bearing mice. In particular, IL-18 appeared to play a major role in intestinal homeostasis and defense against colon cancer (25). First, in vitro, IL-18 enhanced NK cell–mediated death of MC38 cells, a murine colon carcinoma cell line (15). Second, in vivo, *il18*$^{-/-}$ mice showed a higher frequency of tumor growth compared with wild-type mice (26, 27). Finally, administration of rIL-18 or an in vivo IL-18 gene transfer led to immune rejection of colon tumors in mice (20, 28–30).

The antitumor properties of IL-18 involve NK cells and cytotoxic $CD4^+$ and $CD8^+$ T cells (31). However, to our knowledge, the effects of IL-18 on eosinophil-mediated cytotoxicity have not been reported. Cell–cell contact is one of the main forms of communication between immune cells and cancer cells. In this context, IL-18's effects on tumor cells required direct contact between effector cells and target cells (15). Similarly, the effects of cytotoxic eosinophils on colon carcinoma cells depend on LFA-1 (CD11a/CD18)-mediated cell–cell contact (9). In the current study, we further investigated human eosinophil–mediated cytotoxicity against Colo-205 cells and showed that IL-18 was involved in the antitumor properties of eosinophils against Colo-205 cells, in particular, by promoting cell–cell contact between these two cell types.

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Received for publication November 19, 2014. Accepted for publication June 29, 2015.

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The online version of this article contains supplemental material.

Abbreviations used in this article: All, allergic donor; HES, hypereosinophilic syndrome; JAM-A, junctional adhesion molecule A; MFI, median fluorescence intensity; ND, normal donor; PI, propidium iodide; TATE, tumor-associated tissue eosinophilia.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1402914

Materials and Methods

Eosinophil purification

After participants provided informed consent, peripheral venous blood was collected from normal donors (NDs), allergic donors (Alls), or patients with hypereosinophilic syndrome (HES; clinical trials.gov identifier: NCT01713504). Human eosinophils were isolated as previously described (32) on a Percoll gradient (GE Healthcare, Uppsala, Sweden), followed by negative immunomagnetic selection with anti-CD16–coated MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Purification was optimized when necessary by adding anti-CD2–, anti-CD19–, and anti-CD14–coated MicroBeads (Miltenyi Biotec). Eosinophil purity was assessed by staining cytospin preparations with May–Grünwald–Giemsa; the purity was >98%. Eosinophils were cultured overnight at 37°C in 5% CO_2. The culture medium consisted of RPMI 1640 without phenol red, supplemented with 10% FCS, 25 mM HEPES buffer, 2 mM L-glutamine, 10 mM sodium pyruvate, and 10 μg/ml gentamicin (Life Technologies, Grand Island, NY; hereafter called "complete medium").

Cell lines

The Colo-205 and Caco-2 cell lines (human colon carcinomas) were purchased from the American Type Culture Collection (Manassas, VA). We obtained the T24 (human urinary bladder carcinoma) and L428 (Hodgkin's lymphoma) cell lines from Deutsche Sammlung von Mikroorganismen und Zellculturen (Braunschweig, Germany). HBL cells (LOCE-MM001; human melanoma cell line) were provided by Prof. G. Ghanem (Laboratoire d'Oncologie et de Chirurgie Expérimentale, Brussels, Belgium). Cells were grown at 37°C in 5% CO_2 in either RPMI 1640 or in DMEM supplemented with 10% FCS, 25 mM HEPES buffer, 2 mM L-glutamine, 10 mM sodium pyruvate, and 10 μg/ml gentamicin (Life Technologies).

Cytotoxicity assays

Tumor cell lines were stained with PKH26 (Sigma-Aldrich, St. Louis, MO), according to the manufacturer's recommendations. Eosinophil-mediated cytotoxicity against PHK26-labeled cell lines was measured in complete medium at different E:T ratios and at different time points. Apoptosis was assessed after staining cells with Annexin V-FITC (Miltenyi Biotec) for 15 min at room temperature. Apoptosis and necrosis were distinguished by staining with propidium iodide (PI; Miltenyi Biotec). Analyses were performed on a BD Accuri C6 flow cytometer (Becton Dickinson, San Jose, CA) using CFlow Sampler software. Specific cell death was determined as experimental cell death (%) − spontaneous cell death (%).

For inhibition experiments, eosinophils and/or Colo-205 cells were preincubated with human rIL-18 binding protein-a or with neutralizing mAbs, anti–IL-18 (clone 125-2H) (MBL, Nagoya, Japan), anti–ICAM-1 (clone BBIG-I1), and anti-junctional adhesion molecule A (JAM-A; clone 654806; R&D Systems, Minneapolis, MN). The isotype control was mouse IgG1 (clone 11711; R&D Systems).

Quantitative real-time PCR

IL-18 mRNA expression was evaluated in eosinophils, Colo-205 cells, and cocultured cells. For coculture experiments, Colo-205 cells were plated and grown overnight in six-well tissue culture plates. The medium was replaced with complete medium, and 1.5×10^6/ml human eosinophils were added. After 3 h at 37°C, eosinophils were collected by rinsing the plates with PBS. Then, Colo-205 cells were harvested with 0.05% trypsin EDTA. The purity of each fraction was determined by detecting FITC-conjugated CD11a mAbs using flow cytometry. Purities were estimated to be >95% (eosinophils) and >85% (Colo-205 cells).

Total RNA was isolated from eosinophils and Colo-205 cells with TRIzol reagent (Life Technologies), according to the manufacturer's instructions. RNA (1 μg) was reverse transcribed to cDNA with a High-Capacity cDNA Reverse Transcription Kit (Life Technologies). Real-time PCR was performed on an ABI StepOnePlus Real-Time PCR System with Fast SYBR Green Master Mix (both from Applied Biosystems). The sequences of the primers (Sigma-Aldrich) were as follows: IL-18, 5′-CCAAGGAAATCGGCCTCTAT-3′ and 5′-TTGTTCTCACAGGAGAGAGTTGA-3′; caspase-1, 5′-TTTCCGCAAGGTTCGATTTTCA-3′ and 5′-GGCATCTGCGCTCTACCATC-3′; and β-actin, 5′-GGGTCAGAAGGATTCCTATG-3′ and 5′-GGTCTCAAACATGATCTGGG-3′. Threshold cycle values of the target genes were normalized to those of β-actin. For the coculture experiments, the relative change in gene expression was analyzed with the $2^{-\Delta\Delta Ct}$ method.

Flow cytometry

For indirect intracellular staining, purified eosinophils or Colo-205 cells were fixed with 2% paraformaldehyde (Thermo Scientific, Rockford, IL) and permeabilized in intracellular buffer (PBS with 1% BSA and 0.01% saponin). Nonspecific binding was blocked with mouse serum. The cells were incubated with the mouse monoclonal IgM anti–IL-18 Ab (clone 12E7.1) or mouse IgM isotype control (clone CG323; both from Millipore, Temecula, CA). After washing and blocking with goat serum, cells were incubated with a goat FITC-conjugated anti-mouse IgM Ab. For the coculture experiments (E:T ratio 1:1, 1.5 h), IL-18 release was inhibited by pretreatment with brefeldin A (Sigma-Aldrich) at 5 μg/ml for 2 h.

For cell surface staining, experiments were performed on ice in PBS with 1% BSA. After blocking with mouse serum, cells were incubated with mouse mAbs: FITC-conjugated anti–ICAM-1 (clone BBIG-I1), Alexa Fluor 488–conjugated anti–JAM-A (clone 654806; R&D Systems), FITC-conjugated anti-CD18 (clone L130), FITC-conjugated anti-CD11a (clone HI111; BD Bioscience), PE-conjugated anti–ICAM-2 (clone CBR-IC2/2), PE-conjugated anti–ICAM-3 (clone CBR-IC3/1; BioLegend, San Diego, CA), or PE-conjugated anti–IL-18Rα (clone H44; eBioscience, San Diego, CA), as well as matched isotype controls. Analysis was performed on a BD Accuri C6 flow cytometer (Becton Dickinson) with CFlow Sampler software. The median fluorescence intensity (MFI) was calculated as [(median Ab signal − median isotype-control signal)/median isotype-control signal] × 100.

Western blot

Pro–IL-18 and IL-18 were evaluated in the same samples of eosinophils and cocultured eosinophils and Colo-205. For coculture, each fraction was recovered as previously described. Cells lysates were prepared as previously described (33). A total of 10 μg protein was run on a 10–20% polyacrylamide gel under reducing conditions and transferred to a polyvinylidene difluoride membrane (both from Bio-Rad, Hercules, CA). Membranes were blocked with 5% milk in TBS with 0.05% Tween 20 for 1 h at room temperature and probed with primary mouse anti-human IL-18 (clone 25-2G; MBL), mouse anti-human pro–IL-18 (clone 74801.11; R&D Systems), and rabbit anti-human HSC-70 (clone K-19; Santa Cruz Biotechnology, Dallas, TX) Abs at 4°C overnight. After washing, membranes were incubated with secondary HRP-conjugated anti-mouse and anti-rabbit IgG Ab (Sigma-Aldrich) for 1 h at room temperature, followed by ECL detection (Life Technologies).

Cell-adhesion assay

Adhesion between Colo-205 cells and eosinophils was assessed by flow cytometry, as previously described (9). Briefly, eosinophils were labeled with CFSE (Invitrogen, Carlsbad, CA), and Colo-205 cells were stained with the fluorescent cell membrane dye PKH26. After a 1.5-h coincubation at 37°C (5:1 ratio), the percentage of Colo-205 cells that bound to eosinophils was calculated as the number of Colo-205 cells bound to eosinophils ($PKH26^+CFSE^+$ cells)/total number of Colo-205 ($PKH26^+$ cells) × 100. Conversely, the percentage of eosinophils bound to Colo-205 cells was calculated as $KH26^+CFSE^+$ cells/$CFSE^+$ cells × 100.

Statistical analysis

All results are expressed as mean ± SEM. Statistical analyses were performed with SPSS software (SPSS, Chicago, IL). The nonparametric Wilcoxon or Mann–Whitney test was used for comparisons between groups. Differences were considered significant when the p value was < 0.05.

Results

Human eosinophil–induced death in some tumor cell lines is concentration and time dependent

Previously, we demonstrated that human eosinophils, like T cells, exhibited cytotoxic potential against tumor cells that involved the γ-δ TCR and cell–cell contact (8, 9). In this study, we investigated the mechanisms of interaction between eosinophils and tumor cells. Epidemiological studies reported distinct relationships between eosinophilia and different types of tumors (3). Accordingly, we used flow cytometry to compare the tumoricidal effects of human eosinophils on different human tumor cell lines, including L428 (Hodgkin lymphoma), T24 (bladder carcinoma), HBL (melanoma), and Colo-205 and Caco-2 (colon carcinoma). Significant tumor cell apoptosis was observed when eosinophils were added to T24, HBL, Colo-205, and Caco-2 cell lines but not when added to L428 cells (Fig. 1A). This result suggested that the tumoricidal function mediated by human eosinophils was target specific. In accordance with previous epidemiological

studies, which showed that TATE was associated with a good prognosis in colorectal cancer, we found that Colo-205 and Caco-2 cells are both sensitive to eosinophil-mediated cytotoxicity. The highest levels of specific cell death were obtained in Colo-205 cells (Fig. 1B); therefore, we selected them for further investigations.

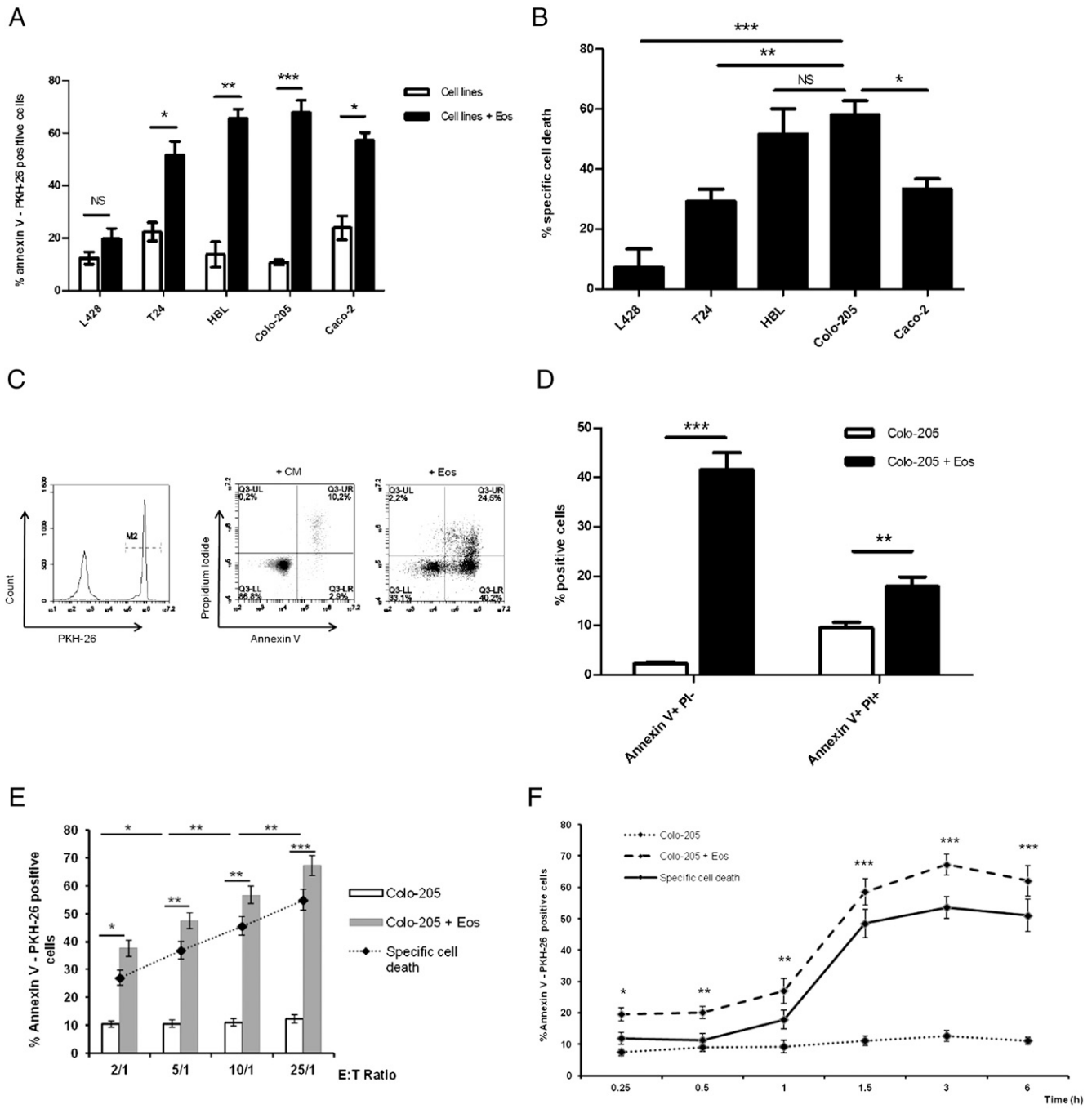

FIGURE 1. Purified human eosinophils exert tumoricidal activity. (**A** and **B**) Tumor cell lines L428 (All, $n = 1$; ND, $n = 2$, HES, $n = 2$), T24 (All, $n = 3$; ND, $n = 2$, HES, $n = 3$), HBL (All, $n = 1$; ND, $n = 3$, HES, $n = 1$), Colo-205 (All, $n = 6$; ND, $n = 6$; HES, $n = 4$), and Caco-2 (All, $n = 1$; ND, $n = 3$) were labeled with PKH-26 and cultivated in the absence or presence of human eosinophils (Eos) at an E:T ratio of 25:1 for 3 h. Cytotoxicity was assessed by annexin V staining. (A) White bars indicate spontaneous tumor cell death, and black bars indicate tumor cell death after coincubation with eosinophils. (B) Bar graph shows specific cell death among the different cell lines. (**C**) Representative dot plots of 13 independent flow cytometry experiments. Colo-205 cells were stained with PHK-26 to discriminate them from eosinophils. Colo-205 cell death was assessed by gating on M2 (*left panel*). Apoptotic cells (annexin V) were discriminated from necrotic cells (PI) in the absence (complete medium alone: +CM) (*middle panel*) or presence of eosinophils (+Eos) (*right panel*). (**D**) Apoptosis (annexin V^+ PI^-) and necrosis (annexin V^+ PI^+) were measured in Colo-205 cells double stained with annexin V–FITC and PI at a 25:1 ratio after 3 h of coculture. (**E**) Concentration dependence of cell death. Colo-205 cells were stained with annexin V and cultured for 3 h alone (white bars) or with eosinophils (gray bars) at different E:T ratios. Dotted line represents the specific cytotoxicity ($n = 6$–11). (**F**) Kinetics of eosinophil-induced Colo-205 cell death. Colo-205 ($PKH26^+$) cells were incubated with eosinophils at a 25:1 E:T ratio for 0.25–6 h. The percentage of annexin V^+ cells (dashed line) represents apoptotic cells. Spontaneous Colo-205 cell death (dotted line) and specific apoptosis (continuous line) are also shown ($n = 7$–21). Results are expressed as the mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, Mann–Whitney *U* test (A and B); Wilcoxon test (D–F).

We performed flow cytometry to discriminate between apoptosis and necrosis. The levels of Colo-205 apoptosis and necrosis in the presence of eosinophils were compared with the spontaneous levels of Colo-205 cell death (Fig. 1C). Eosinophils induced significant levels of apoptosis and necrosis in Colo-205 cells after 3 h of coincubation (Fig. 1D). This effect was observed at all E:T ratios and at all time points tested (data not shown). Indeed, an E:T ratio of 2:1 induced 27.1 ± 2.7% specific lysis in Colo-205 cells, and a 25:1 E:T ratio induced 54.9 ± 3.8% specific lysis (Fig. 1E). Eosinophil-dependent cytotoxicity was observed within 15 min after coculturing, and it increased significantly to 62.1 ± 4.9% after 6 h (Fig. 1F). Taken together, these findings revealed that human eosinophils could induce concentration- and time-dependent cell death in several cell lines, but notably in the Colo-205 carcinoma cell line.

ICAM-1 and JAM-A are required for binding between Colo-205 cells and eosinophils

Our previous studies indicated that contact between Colo-205 cells and eosinophils was required for cytotoxicity (9). Although the adhesion molecules CD11a/CD18 were implicated in eosinophil binding to Colo-205 cells (9), the nature of the ligands expressed on Colo-205 cells remained to be determined. Flow cytometry analysis revealed that Colo-205 cells expressed ICAM-1 and JAM-A but not ICAM-2 or ICAM-3 (Fig. 2A, 2B).

To confirm that these cell-adhesion molecules participated in the binding of Colo-205 cells to eosinophils, we added neutralizing Abs to the cocultures. A significant reduction in Colo-205 cell binding to eosinophils was observed when tumor cells were preincubated with anti–ICAM-1– and anti–JAM-A–neutralizing Abs (Fig. 2C). However, only the anti–ICAM-1–neutralizing Abs induced a reduction in Colo-205 cell death (Fig. 2D). The addition of both neutralizing Abs did not reveal any synergism (data not shown), which suggested that, although both ligands were expressed, the role of ICAM-1 appeared to predominate.

The IL-18 cytokine is involved in eosinophil-mediated cytotoxicity

The protective role of IL-18 was described previously in murine models of colon cancer (31). Tumors were observed significantly more frequently in colons of *il-18*$^{-/-}$ mice than in colons of wild-type mice (26, 27). Also, IL-18 gene transfer enhanced immune rejection of tumors in mice (20, 29). Upon finding that human eosinophils induced cell death in Colo-205 cells, we investigated

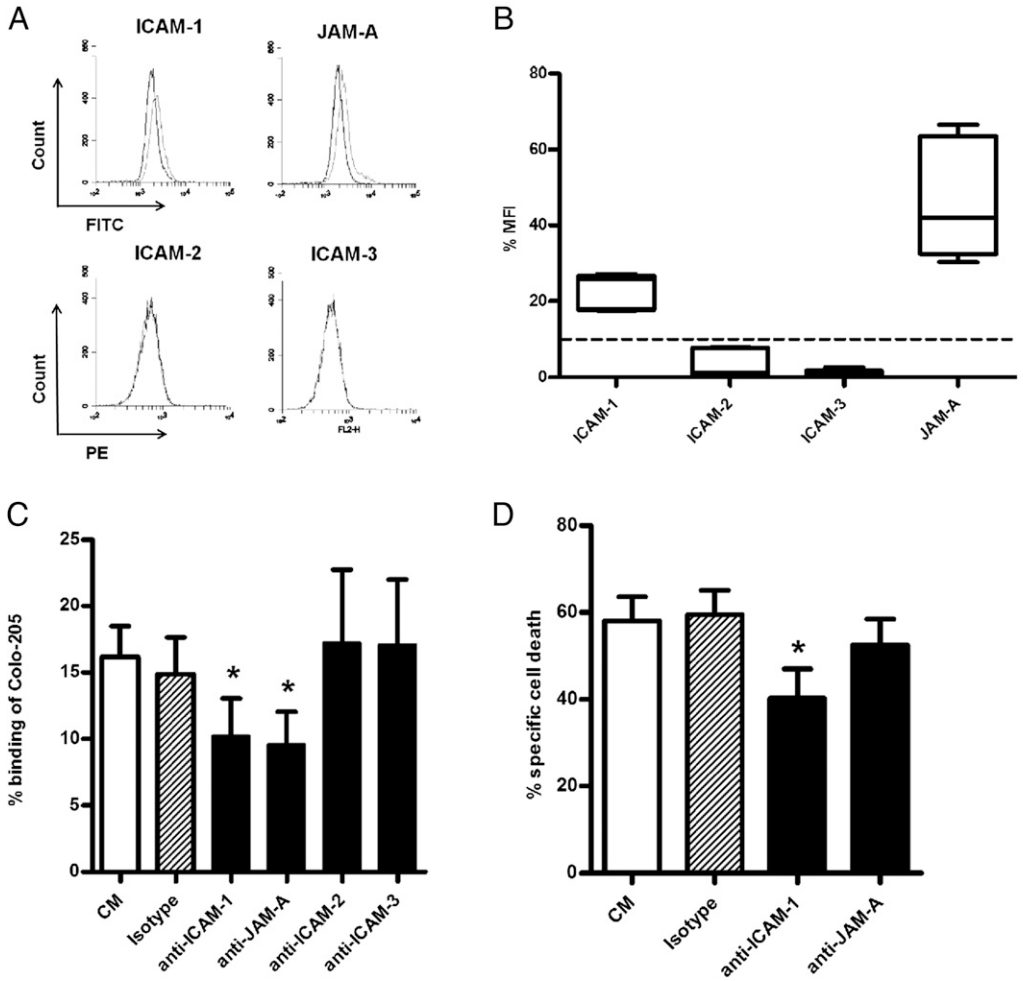

FIGURE 2. ICAM-1 and JAM-A expression and involvement in tumoricidal activity. (**A**) Representative line graphs for five independent flow cytometry experiments show ICAM-1, ICAM-2, ICAM-3, and JAM-A expression on Colo-205 cell surfaces. Specific Ab detection is indicated by gray lines, and detection of the matched Ab isotype controls is indicated by black lines. (**B**) Box plot represents the percentage change in MFI for the indicated adhesion molecules expressed on Colo-205 cell surfaces ($n = 5$). Dashed line represents significance threshold. (**C**) Effects of neutralizing mAbs anti–ICAM-1, anti–ICAM-2, anti–ICAM-3, and anti–JAM-A (10 μg/ml) on binding of Colo-205 cells to eosinophils after a 1.5-h coculture at and E:T ratio of 5:1 (All, $n = 3$; ND, $n = 3$). (**D**) Inhibition of eosinophil-mediated Colo-205 cell death by anti–ICAM-1 and anti–JAM-A mAbs (All, $n = 3$; ND, $n = 3$). Results are expressed as the mean ± SEM. *$p < 0.05$, Wilcoxon test. CM, complete medium; Isotype, control IgG Ab.

the implication of IL-18 in our in vitro model. First, we examined whether IL-18 was expressed in human eosinophils or Colo-205 cells with quantitative PCR and flow cytometry. Although both cell types expressed IL-18 mRNA (Fig. 3A), only eosinophils were positive for intracellular IL-18 staining in the flow cytometry assay (Fig. 3B). The IL-18 precursor (24 kDa) is processed by caspase-1, which cleaves the precursor into an active mature molecule (18 kDa) (11). Caspase-1 mRNA was only detected on human eosinophils (Fig. 3C).

Given the expression of IL -18 by eosinophils, we next examined whether IL-18 expression changed after coincubation with Colo-205 cells. We found that IL-18 mRNA expression in eosinophils increased by 4.3-fold when they were cultured with Colo-205 cells; however, no change in IL-18 expression was observed in Colo-205 cells (Fig. 3D). With regard to mRNA, the IL-18 protein content in eosinophils increased in response to contact with Colo-205 cells (Fig. 3E). After 3 h of coincubation, we observed an increase in mature IL-18 and a decrease in pro–IL-18 in eosinophils (Fig. 3F). No IL-18 expression was observed in the Colo-205 cells (data not shown). To investigate whether IL-18 was involved in the tumoricidal effect of eosinophils, we evaluated the inhibitory effects of an IL-18–neutralizing Ab and an IL-18–binding protein,

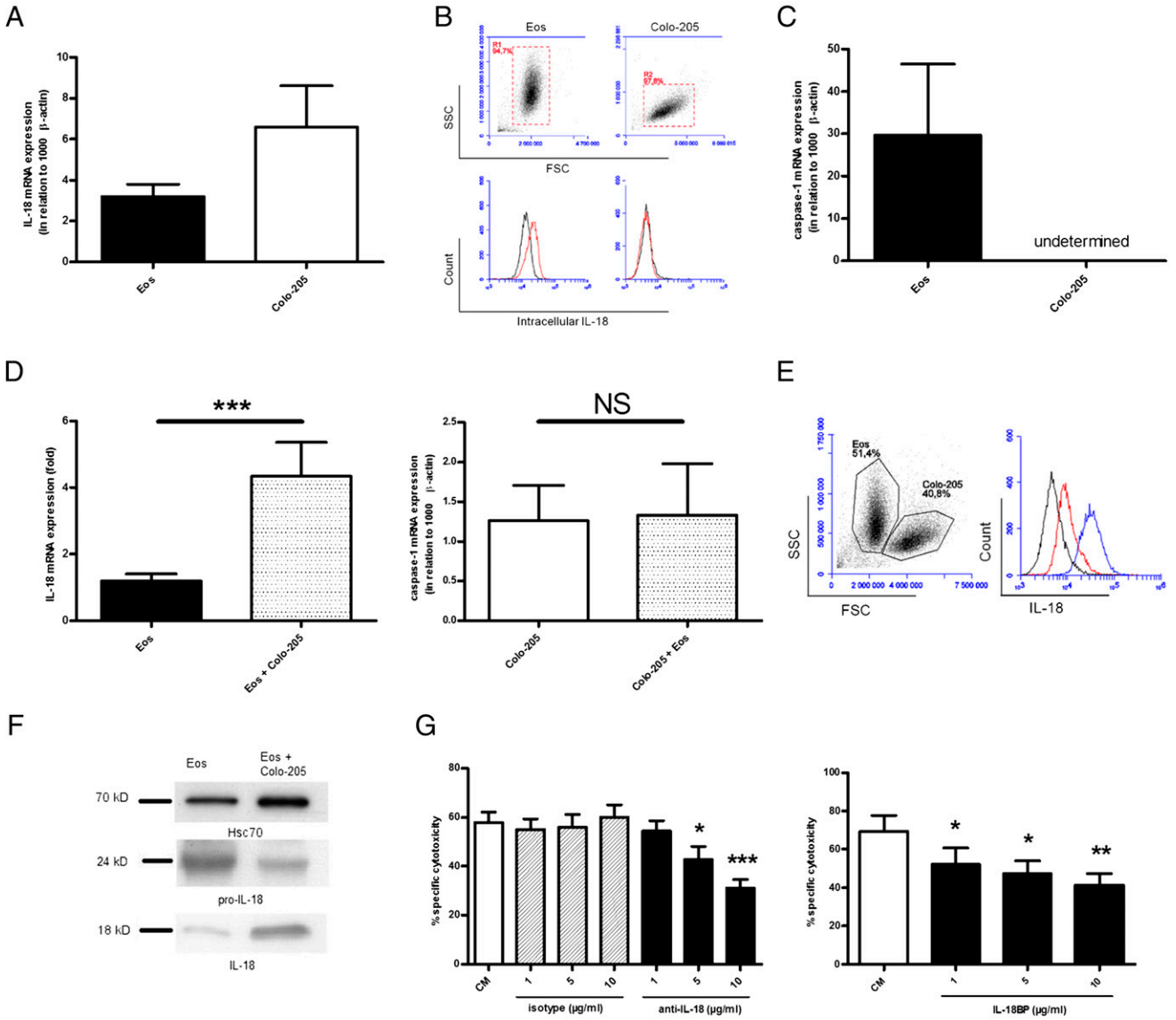

FIGURE 3. IL-18 expression in eosinophils and Colo-205 cells and its involvement in eosinophil tumoricidal activity. (**A**) IL-18 mRNA expression was analyzed in human eosinophils (Eos) (All, $n = 4$; ND, $n = 2$; HES, $n = 5$) and Colo-205 cells ($n = 6$) by quantitative real-time PCR. (**B**) Representative line graphs of six (Eos) or three (Colo-205 cells) independent flow cytometry experiments showing intracellular expression of IL-18. The cells stained with the control Ab are indicated by the black line, and the cells stained with anti–IL-18 mAb are indicated by the red line. (**C**) Caspase-1 mRNA expression was analyzed in human eosinophils (Eos) (All, $n = 3$; ND, $n = 1$; HES, $n = 3$) and Colo-205 cells ($n = 4$). (**D**) Changes in mean mRNA expression of IL-18 in eosinophils cocultured without (Eos) or with Colo-205 (*left panel*) or in Colo-205 cells cocultured without or with eosinophils (*right panel*) (All, $n = 4$; ND, $n = 2$). (**E**) Intracellular expression of IL-18 in eosinophils was assessed by gating on Eos (*left panel*). Representative line graph of three independent flow cytometry experiments showing intracellular expression of IL-18 in human eosinophils without (red line) or with Colo-205 cells (blue line) (*right panel*). The cells stained with the control Ab are indicated by the black line. (**F**) Representative image of three independent Western blot experiments showing expression of pro–IL-18 (24 kDa), mature IL-18 (18 kDa), and HSP-70 from the same samples of eosinophils after incubation for 3 h without (Eos) or with Colo-205 cells. (**G**) Eosinophil-induced cell death was inhibited with a neutralizing anti–IL-18 Ab (All, $n = 7$; ND, $n = 5$; HES, $n = 4$) (*left panel*) or with IL-18–binding protein (IL-18BP), a natural inhibitor (All, $n = 3$; ND, $n = 2$) (*right panel*). Data were obtained after 1.5 h of coculture at an E:T ratio of 25:1. Results are expressed as the mean ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, Wilcoxon test. CM, complete medium; isotype, control IgG Ab.

a natural inhibitor of IL-18. Both inhibitors caused dose-dependent reductions in eosinophil-mediated Colo-205 cell death (Fig. 3G), which indicated that IL-18 was involved in eosinophil-mediated Colo-205 cell death.

Inhibition of IL-18 decreased binding between eosinophils and Colo-205 cells

Previous studies demonstrated that IL-18 played a role in mediating cell adhesion (34). Therefore, we investigated whether IL-18 was involved in cell–cell adhesion between eosinophils and Colo-205 cells. Inhibition of IL-18 with the IL-18–binding protein induced a significant reduction in the binding of Colo-205 cells to eosinophils after 1.5 h of coincubation at an E:T ratio of 5:1; Colo-205 cell binding was reduced by 45% in the presence of 10 μg/ml IL-18–binding protein (Fig. 4A). Similar results were obtained when the percentage of binding of eosinophils to Colo-205 cells was calculated. Addition of IL-18–binding protein decreased the binding of eosinophils to Colo-205 cells by 69.5% (Fig. 4B). Representative dot plots are shown in Supplemental Fig. 1. Taken together, our results revealed that IL-18 contributed to the interactions between human eosinophils and Colo-205 tumor cells in vitro.

IL-18 is a proinflammatory cytokine that acts on immune and epithelial cells through the IL-18R complex. This heterodimeric receptor is composed of a ligand-binding chain (IL-18Rα) and an accessory chain (IL-18Rβ). To determine whether eosinophils and tumor cells can communicate through IL-18 and IL-18R, we used flow cytometry to analyze the membrane expression of IL-18Rα. We found that both human eosinophils and Colo-205 cells expressed this receptor (Fig. 4C). The direct effect of IL-18 on Colo-205 cell death was investigated by incubating Colo-205 cells with human rIL-18 in the absence of eosinophils. No death of Colo-205 cells was detected in the presence of IL-18 without effector cells (data not shown), suggesting an indirect effect of IL-18 in eosinophil-mediated cytotoxicity.

IL-18 upregulated CD11a and ICAM-1

Given the involvement of IL-18 in human eosinophil–Colo-205 cell binding and the expression of IL-18R by both cell types, we next analyzed the effects of IL-18 on the expression of adhesion molecules involved in cell–cell contact. Specifically, we investigated the expression of CD11a, CD18, ICAM-1, and JAM-A. In Colo-205 cells, incubation with rIL-18 for 1.5 h led to an increase in ICAM-1 membrane expression (Fig. 5A), but no change was detected in JAM-A expression (Fig. 5B). In eosinophils, IL-18 induced a strong upregulation of CD11a, which increased by >50% (Fig. 5C); IL-18 also induced an increase in CD18 membrane expression but to a lesser extent (Fig. 5D). Representative line graphs are shown in Supplemental Fig. 2. Taken together, these findings revealed that IL-18 appeared to act on human eosinophils and Colo-205 cells by upregulating the membrane expression of adhesion molecules, both on the effectors and on the targets.

Cytotoxic and adhesion potentials of eosinophils differed based on the clinical status of donors

In this study, eosinophils were purified from Alls, NDs, or patients with HES. The membrane phenotype of human eosinophils varies with the maturation process, activation state, and clinical status of the donor (1, 35, 36). Therefore, we investigated the cytotoxic and adhesion potentials of human eosinophils among subgroups of donors.

With regard to eosinophil-mediated Colo-205 cell death, eosinophils from Alls were more efficient than eosinophils purified from NDs. On the contrary, eosinophils from patients with HES induced less Colo-205 cell apoptosis than did those from NDs (Fig. 6A). The same observation was observed with regard to adhesion between eosinophils and Colo-205 cells. In fact, adhesion was significantly higher with eosinophils from Alls compared with those from NDs, and it was significantly lower with eosinophils from patients with HES (Fig. 6B). In addition, membrane expression of LFA-1, which is involved in the Colo-205 cell/

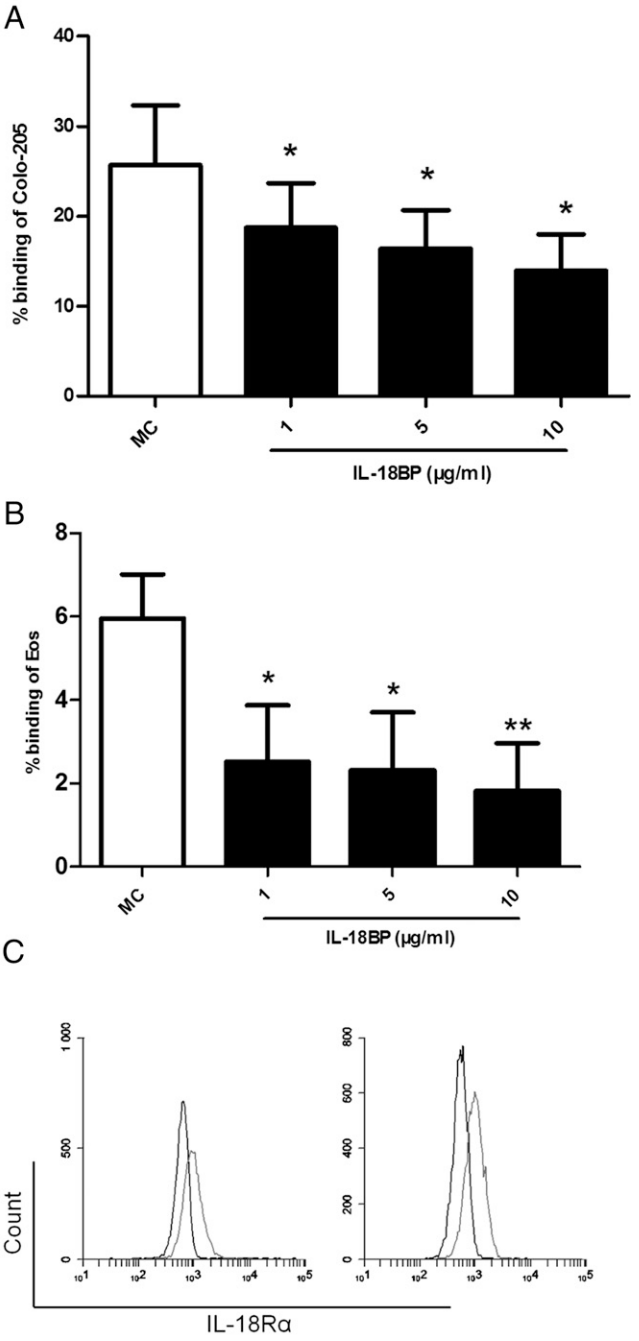

FIGURE 4. IL-18 mediates binding between eosinophils and Colo-205 cells. (**A** and **B**) Effect of IL-18 inhibition on binding between eosinophils and Colo-205 cells. $PKH26^+$ Colo-205 cells and $CFSE^+$ eosinophils were coincubated at 37°C for 1.5 h at an E:T ratio of 5:1 in the presence of IL-18–binding protein (IL-18BP). The population containing both eosinophils and Colo-205 was scored as double positive, and the percentages were determined for Colo-205 bound to eosinophils (A) and for eosinophils bound to Colo-205 cells (B) (All, $n = 4$; ND, $n = 1$). Results are mean ± SEM. (**C**) Representative line graphs of five (eosinophils; *left panel*) or three (Colo-205 cells; *right panel*) independent flow cytometry experiments showing IL-18Rα expression. The cells stained with isotype-control Ab are indicated in black, and the cells stained with anti–IL-18Rα mAb are indicated in gray. $*p < 0.05$, $**p < 0.01$, Wilcoxon test.

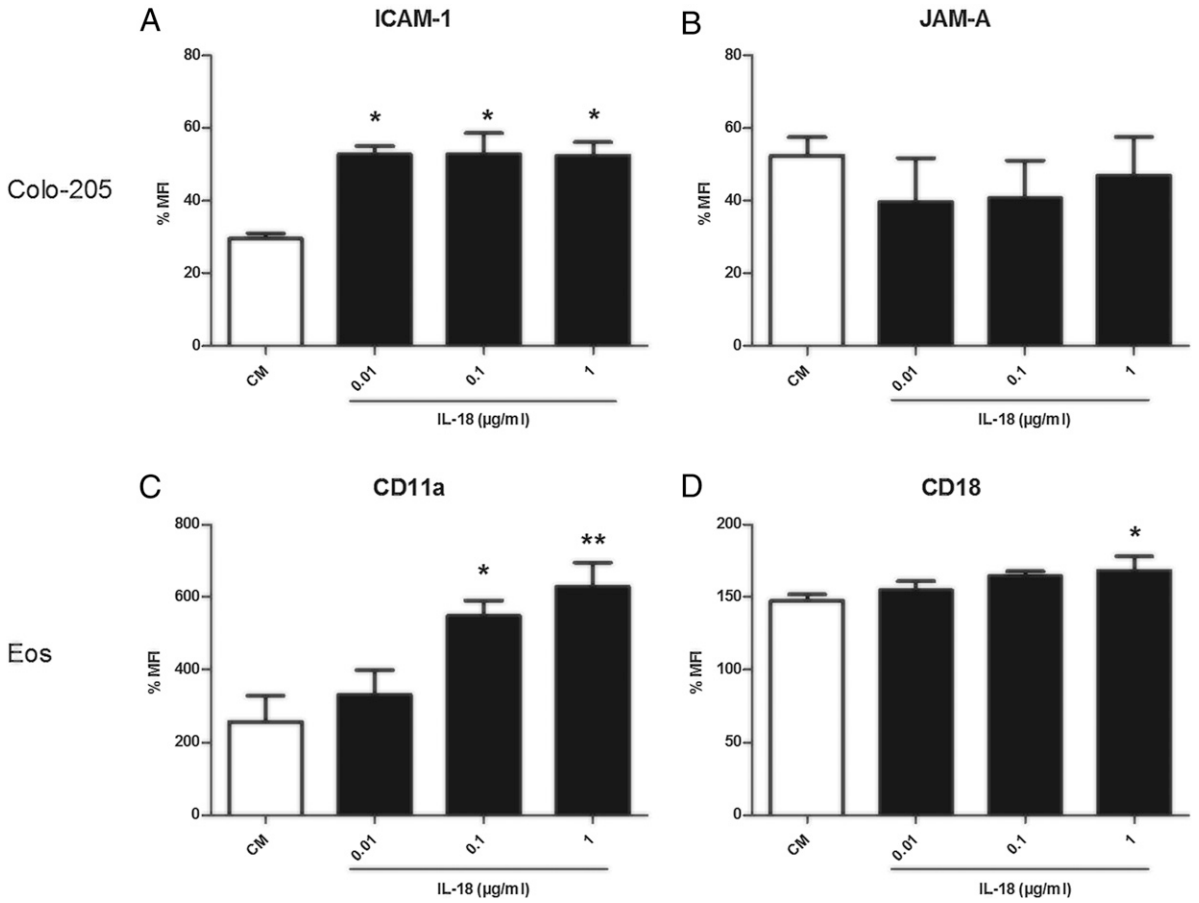

FIGURE 5. IL-18 upregulates the expression of ICAM-1 and CD11a. Purified eosinophils and Colo-205 cells were incubated for 1.5 h at 37°C with rIL-18. Flow cytometry data show the Colo-205 cell surface expression ($n = 4$) of ICAM-1 (**A**) and JAM-A (**B**), as well as the eosinophil (Eos) expression (All, $n = 4$; ND, $n = 1$) of CD11a (**C**) and CD18 (**D**). The percentage change in MFI is shown. Results are expressed as the mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, Wilcoxon test.

eosinophil adhesion process, was evaluated by flow cytometry. Eosinophils purified from Alls expressed more CD11a and CD18 than did those from NDs, and eosinophils from patients with HES had a lower membrane expression of LFA-1 than did NDs (Fig. 6C). Therefore, these results suggest heterogeneity for eosinophil-mediated Colo-205 cell death and adhesion between these cells based on the clinical status of the donor.

Discussion

Antitumor immunity, and particularly immune surveillance, implies both innate and adaptive immune responses from the tissue peritumoral microenvironment, which involves different cell types. Although the essential roles of lymphocytes, NK cells, and dendritic cells have been largely documented (37), new cell types that express tumoricidal activity have emerged, including eosinophils (3). In a previous study, we showed that human eosinophils could induce Colo-205 cell death through a process that required LFA-1–mediated cell–cell contact (8, 9). In the current study, we found that IL-18 was involved in eosinophil-mediated cytotoxicity, and we showed that IL-18 could upregulate binding between eosinophils and Colo-205 cells.

Limited studies in mouse models suggested that eosinophils were involved in antitumor immunity (3). However, because of the strong disparity between human and mouse eosinophils (8, 38), those results were not totally relevant to clinical situations. Several studies attempted to assess the prognostic value of TATE to support, even indirectly, the tumoricidal properties of eosinophils and the benefit that they might provide to patients. However, depending on the tumor type and stage of progression, studies showed that increased numbers of eosinophils could be associated with either a good or a poor prognosis. Similarly, our data indicated that the antitumor activity of human eosinophils depended upon the target cells. Our results were consistent with previous epidemiological studies that showed TATE was associated with a good prognosis in colon cancer (4–7), because we showed that human eosinophils induced death in Colo-205 cells. In contrast, TATE was associated with a poor prognosis in Hodgkin's lymphoma (39, 40). This observation was consistent with our finding that eosinophils did not appear to be cytotoxic against L428 cells, a Hodgkin's lymphoma cell line. Thus, taken together, our results pointed to the specificity of eosinophil-mediated cytotoxicity, which may be relevant to clinical observations.

Like T cells and NK cells, eosinophil binding to tumor cells is required for cytotoxicity. This binding provides stable contact with target cells (9, 41). We showed previously that the β_2 integrin LFA-1 played a crucial role in the cytolytic effect of eosinophils on Colo-205 cells (9). The interaction between LFA-1 and its ligands, ICAMs 1–3 and JAM-A, was shown to enhance leukocyte cell–cell binding (42, 43). In the basal state, Colo-205 cells express ICAM-1 and JAM-A. Our results indicated that both ligands contributed to cell–cell binding, but only ICAM-1 was involved in the cytolytic effect of eosinophils on Colo-205 cells. JAM-A is a transmembrane glycoprotein that belongs to the Ig superfamily.

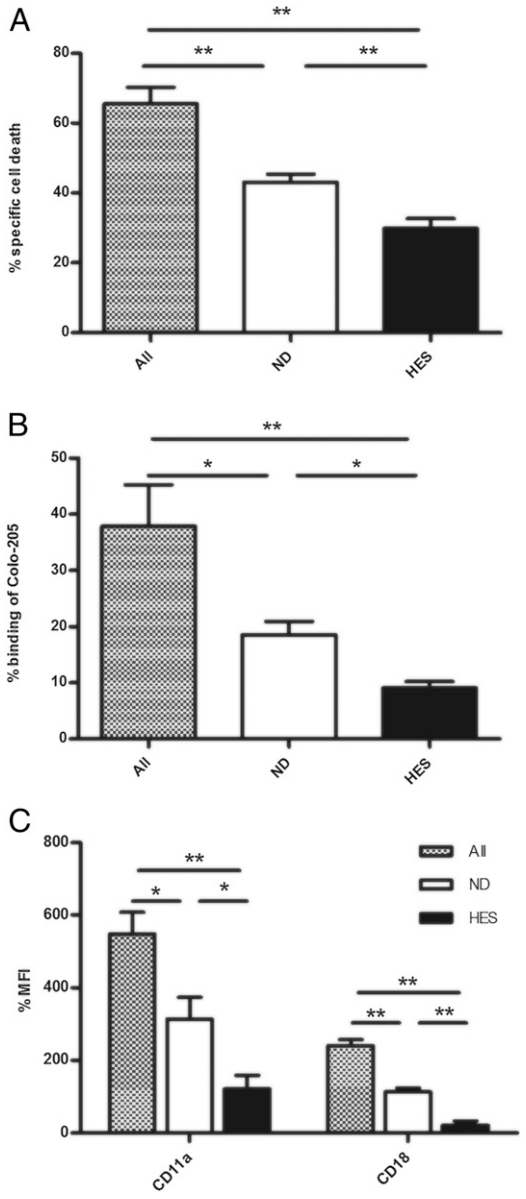

FIGURE 6. Heterogeneity of human eosinophils based on the status of donors. Human eosinophils were purified from Alls, NDs, or patients with HES. (**A** and **B**) Purified eosinophils and Colo-205 cells were incubated for 1.5 h at an E:T ratio of 25:1. Eosinophil-mediated Colo-205 cell death (All, $n = 6$; ND, $n = 6$; HES, $n = 4$) (A) and adhesion between eosinophils and Colo-205 (All, $n = 6$; ND, $n = 4$; HES, $n = 4$) (B) were assessed by flow cytometry. (**C**) The percentage change in MFI for the expression of CD11a and CD18 on eosinophil cell surfaces (All, $n = 5$; ND, $n = 5$; HES, $n = 4$). Results are mean ± SEM. $*p < 0.05$, $**p < 0.01$, Mann–Whitney U test.

It is widely distributed in tissues, including the gastrointestinal tract (44). JAM-A can form heterodimers with LFA-1, but it also forms homodimers. The homophilic interactions are implicated in diverse cellular processes, such as cell–cell adhesion (45). Thus, JAM-A–JAM-A binding may also have been involved in our cell-adhesion assay. ICAM-1 is both a cell surface glycoprotein that belongs to the Ig superfamily and a costimulatory molecule that provides signals to cytotoxic T lymphocytes and NK cells (46, 47). Increasing evidence suggests that ICAM-1 plays a functional role on the tumor cell surface. Consistent with our results, neutralization of ICAM-1 with an Ab or a small interfering RNA reduced both lymphocyte and NK cell adhesion to cancer cells, which then reduced their cytotoxicity against cancer cells (48, 49). Similarly, upregulation of ICAM-1 increased tumor susceptibility to lymphocyte adhesion and cell-mediated cytotoxicity (50, 51).

The involvement of IL-18, a systemic, multifunctional cytokine (34), in antitumoral immunity remains controversial (19). This cytokine appears to have both pro- and anti-tumor activities. On the one hand, IL-18 facilitated tumor cell immune escape by suppressing CD70, increasing metastatic potential, and promoting angiogenesis by stimulating production of CD44 and VEGF (52). On the other hand, IL-18 exerted antitumor effects by inducing an immune response to cancer cells, notably in colon cancer (20, 27–30). In addition to results obtained in murine models, a phase II trial showed that IL-18 was well tolerated and induced antitumor effects when used alone in patients with advanced cancer (53). Although recent studies focused on NK cells and cytotoxic T lymphocytes (31), the involvement of eosinophils was also envisaged, because eosinophils express IL-18Rα and respond to this cytokine (18, 54). The present study showed that inhibiting IL-18 with a neutralizing mAb or with the IL-18–binding protein led to a reduction in eosinophil-mediated cytotoxicity. The mechanism of inhibition was to decrease adhesion between eosinophils and Colo-205 cells. Because cell adhesion was required for IL-18–driven antitumor immunity (15), we investigated the effect of this cytokine on adhesion molecules expressed in effectors and targets. Our data showed that IL-18 upregulated ICAM-1 expression in Colo-205 cells and CD11a expression in human eosinophils. In tumor cells, ICAM-1 expression was inducible with cytokines. Thus, cytokines produced by infiltrating cells might influence the expression of ICAM-1 on tumor cells (55). This possibility was supported by the finding that the degree of mononuclear cell infiltration correlated significantly with the expression of ICAM-1 by stromal cells in breast cancer and renal cancer, as well as by malignant melanoma cells. Our results suggested that IL-18 upregulated ICAM-1 and CD11a on immune and cancer cells, which could lead to an increase in target cell lysis by immune cells (51, 56, 57). IL-18 is an inducer of IFN-γ (12). This cytokine is involved in antitumor immunity (58) and can induce ICAM-1 production on cancer cells (59). However, the effects of IL-18 on eosinophil-mediated cytotoxicity toward Colo-205 cells seem to be independent of IFN-γ (data not shown).

Eosinophils purified from Alls are more cytotoxic against Colo-205 cells than are eosinophils from NDs or HES patients. This functional heterogeneity may be related to an increase in binding and LFA-1 expression with eosinophils from Alls. However, the involvement of IL-18 in this heterogeneity remains to be evaluated.

In conclusion, this was the first study, to our knowledge, to demonstrate that IL-18 plays a major role in eosinophil-mediated lysis of colon carcinoma cells (Colo-205). Our results indicated that the mechanism of cell death required contact between tumor cells and human eosinophils. Therefore, the tumoricidal potential of eosinophils against various tumors, and notably in the evolution of colon cancer, should be investigated further in follow-up studies. Moreover, eosinophil activity should be linked to current immune therapeutics that involve IL-18.

Acknowledgments

We thank Sylvie Loiseau and Caroline Stremnitzer for technical support, Prof. G. Ghanem for providing the HBL cell line, and DigestScience.

Disclosures

The authors have no financial conflicts of interest.

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