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HOST RESPONSE AND INFLAMMATION

Pseudomonas aeruginosa Infection Impairs NKG2D-Dependent NK Cell Cytotoxicity through Regulatory T-Cell Activation

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ABSTRACT Natural killer (NK) cells play a key role in both antibacterial and antitumor immunity. *Pseudomonas aeruginosa* infection has already been reported to alter NK cell functions. We studied *in vitro* the effect of *P. aeruginosa* on NK cell cytotoxic response (CD107a membrane expression) to a lymphoma cell line. Through positive and negative cell sorting and adoptive transfer, we determined the influence of monocytes, lymphocytes, and regulatory T cells (Treg) on NK cell function during *P. aeruginosa* infection. We also studied the role of the activating receptor natural killer group 2D (NKG2D) in NK cell response to B221. We determined that *P. aeruginosa* significantly altered both cytotoxic response to B221 and NKG2D expression on NK cells in a Treg-dependent manner and that the NKG2D receptor was involved in NK cell cytotoxic response to B221. Our results also suggested that during *P. aeruginosa* infection, monocytes participated in Treg-mediated NK cell alteration. In conclusion, *P. aeruginosa* infection impairs NK cell cytotoxicity and alters antitumor immunity. These results highlight the strong interaction between bacterial infection and immunity against cancer.

KEYWORDS immunity, infection, cancer, *Pseudomonas aeruginosa*, regulatory T cells, NK cells

The physiologic immune process to fight severe infections consists of first an inflammatory response in order to clear the pathogen and heal the affected tissue and second an anti-inflammatory response that aims to restore immune homeostasis. The second phase leaves an immunological scar that can impair antitumor immunity (1, 2). There is increasing evidence that antitumor and anti-infectious immunity involve shared pathways—notably those involving immune evasion (3). In this setting, *Pseudomonas aeruginosa* infection has already been reported to alter antitumor immunity through natural killer (NK) cell function impairment (4).

NK cells are innate lymphoid cells that exhibit two main functions as follows: cytokine production and cytotoxic response via perforin/granzyme release through granule exocytosis in the extracellular milieu. NK cells patrol in the circulation and in tissues to sense and differentiate normal or abnormal cells (infected or tumor cells) through activating and inhibitory receptors. NK cell response usually depends on the balance between activating and inhibitory signals. The latter are mainly represented by major histocompatibility complex (MHC) class I molecules detected by killer cell immunoglobulin-like receptor (KIR) and allow self-tolerance. NK cells can also recognize activating ligands expressed on both tumor cells and pathogens (5–7).

P. aeruginosa is a Gram-negative opportunistic bacterium that is a major cause of

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severe pneumonia in immunocompromised patients. *P. aeruginosa* infection is an interesting model to study antitumor immunity alteration because it can interfere with signalization pathways in order to evade immunity (8). Moreover, its hypermutable genome explains its ability to persist in the host.

The mechanisms that explain how *P. aeruginosa* can alter antitumor immunity have to be addressed in order to orient future research and development to reduce the impact of infection on the occurrence of cancer. The aim of our study was to evaluate the effect of *P. aeruginosa* infection on NK cell cytotoxicity in response to a tumor cell line.

RESULTS

***P. aeruginosa* infection altered NK cell cytotoxic response to B221 cell line.** NK cells play a key role in immunity against bacteria (9) and tumors (4) through two main functions as follows: cytokine activity and the release of cytotoxic granules. We previously studied NK cell cytokine response after *P. aeruginosa* infection and found a direct modulation of the gamma interferon (IFN-γ) response induced by the bacteria (8). Focusing on cytotoxic response during antitumor immunity, we then evaluated the consequences of peripheral blood mononuclear cell (PBMC) infection with *P. aeruginosa* on NK cell cytotoxic activity toward an MHC class I-negative Epstein-Barr virus (EBV)-transformed human B lymphoma cell line (referred to as B221 cells). As a surrogate marker of cytotoxicity, we analyzed CD107a membrane expression, which corresponded to perforin and granzyme B granule release (10). In the absence of B221 cells, the cytotoxic activity of NK cells was low with or without *P. aeruginosa* infection (Fig. 1A and B; see also Fig. SA1 in the supplemental material). When exposed to B221 cells, CD107a activity significantly increased in noninfected NK cells. Interestingly, *P. aeruginosa* infection before B221 cell exposition significantly decreased CD107a$^+$ NK cells (Fig. 1A and B) compared with the noninfected cells. NK cell viability between infected and noninfected conditions was similar, ruling out a toxic effect of *P. aeruginosa* infection to explain these results (Fig. 1C). Overall, these results show that *P. aeruginosa* infection significantly impaired NK cell cytotoxic response to a tumor cell line, which encouraged us to further investigate the underlying mechanism. Unless otherwise stated, mobilization assays (i.e., CD107a staining) in the following experiments were all performed with B221 cells as target cells.

NKG2D is involved in NK cell cytotoxic response to B221 cell line. NK cells sense their environment through inhibitory or activating receptors. The cytotoxic response of NK cells is usually driven by an imbalance between inhibitory and activating ligands. In our model, since B221 cells do not express MHC class I molecule (inhibitory ligands), the cytotoxic response mainly depends on activating receptors. To explain the reduction of NK cell cytotoxicity, we hypothesized that *P. aeruginosa* infection altered the membrane expression of activating receptors. We first assessed whether B221 cells expressed specific ligands for NK cell activating receptor. The B221 cell line expressed one ligand for the 2B4 receptor (CD244, a transmembrane receptor belonging to the CD2 family) and one ligand for the NKp30 receptor (Fig. 2A). These 2 receptors have been reported to play a key role in cytotoxic response to antitumor immunity (11, 12). Interestingly, B221 cells also express 2 specific ligands (ULBP1 and ULBP4) of the natural killer group 2D (NKG2D) receptor, which has been reported to be involved in immune response against both tumors and *P. aeruginosa* infection (13, 14). Contrary to 2B4 and NKp30, the expression of NKG2D significantly decreased after *P. aeruginosa* infection (Fig. 2B to D), which could explain the reduction of cytotoxicity after *P. aeruginosa* infection. Moreover, blocking NKG2D in sorted NK cells before exposition to B221 cells significantly decreased cytotoxic response to B221 cells (Fig. 2E) without altering the viability of NK cells (see Fig. SA3A for viability). As a result, the reduction of NKG2D expression could account for the altered cytotoxicity of NK cells toward B221 cells after *P. aeruginosa* infection.

Alteration of NK cell cytotoxicity after *P. aeruginosa* infection is mediated by CD3$^+$ cells. Accessory cells have been reported to participate in NK cell functions (15).

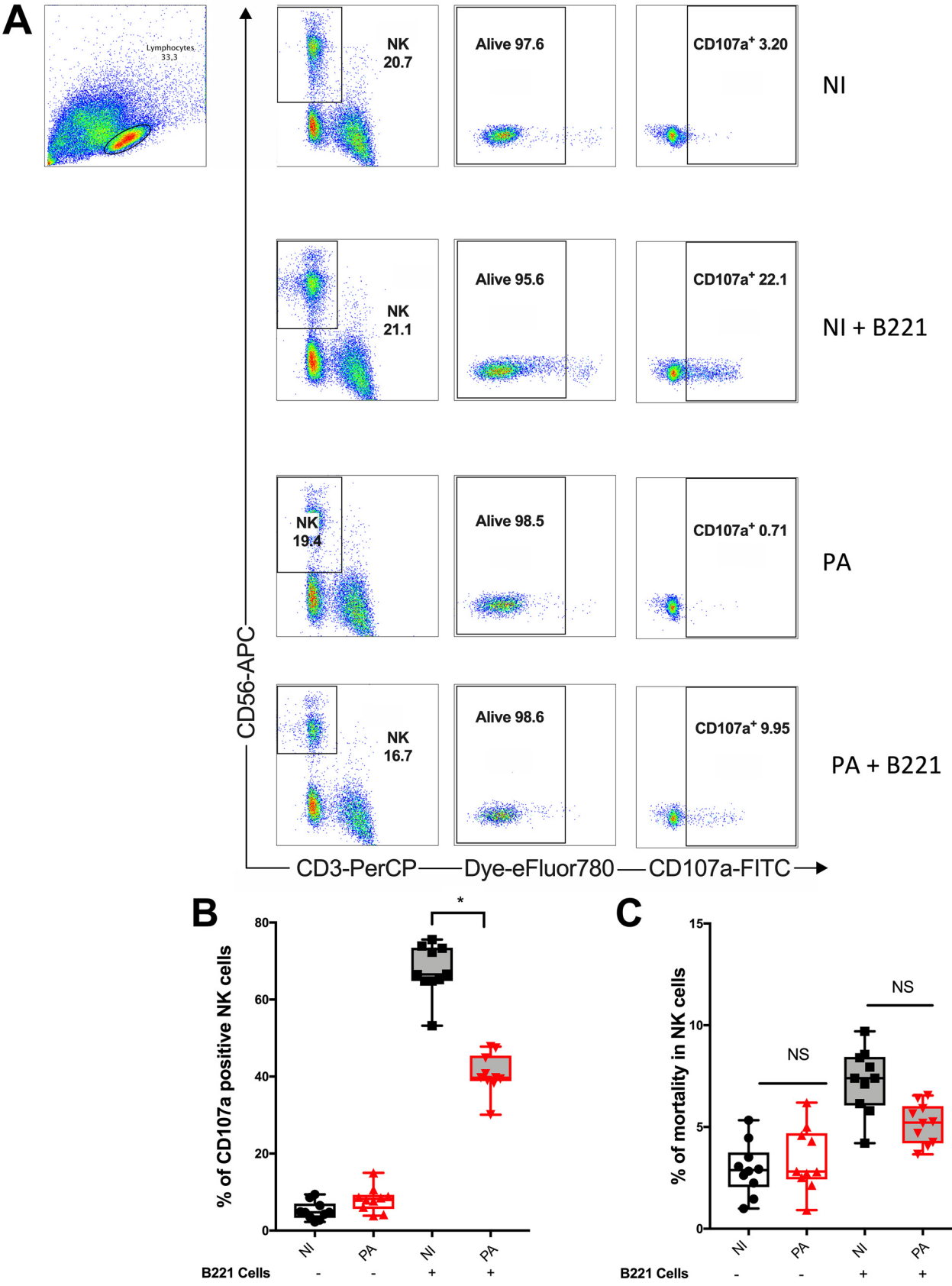

FIG 1 *Pseudomonas aeruginosa* infection-impaired NK cell cytotoxicity in response to B221 cell line. NK cell cytotoxic activity (CD107a) among PBMCs was assessed with or without infection and with (+) or without (−) B221 cell exposition (see Fig. SA1 in the supplemental material). (A) Representative

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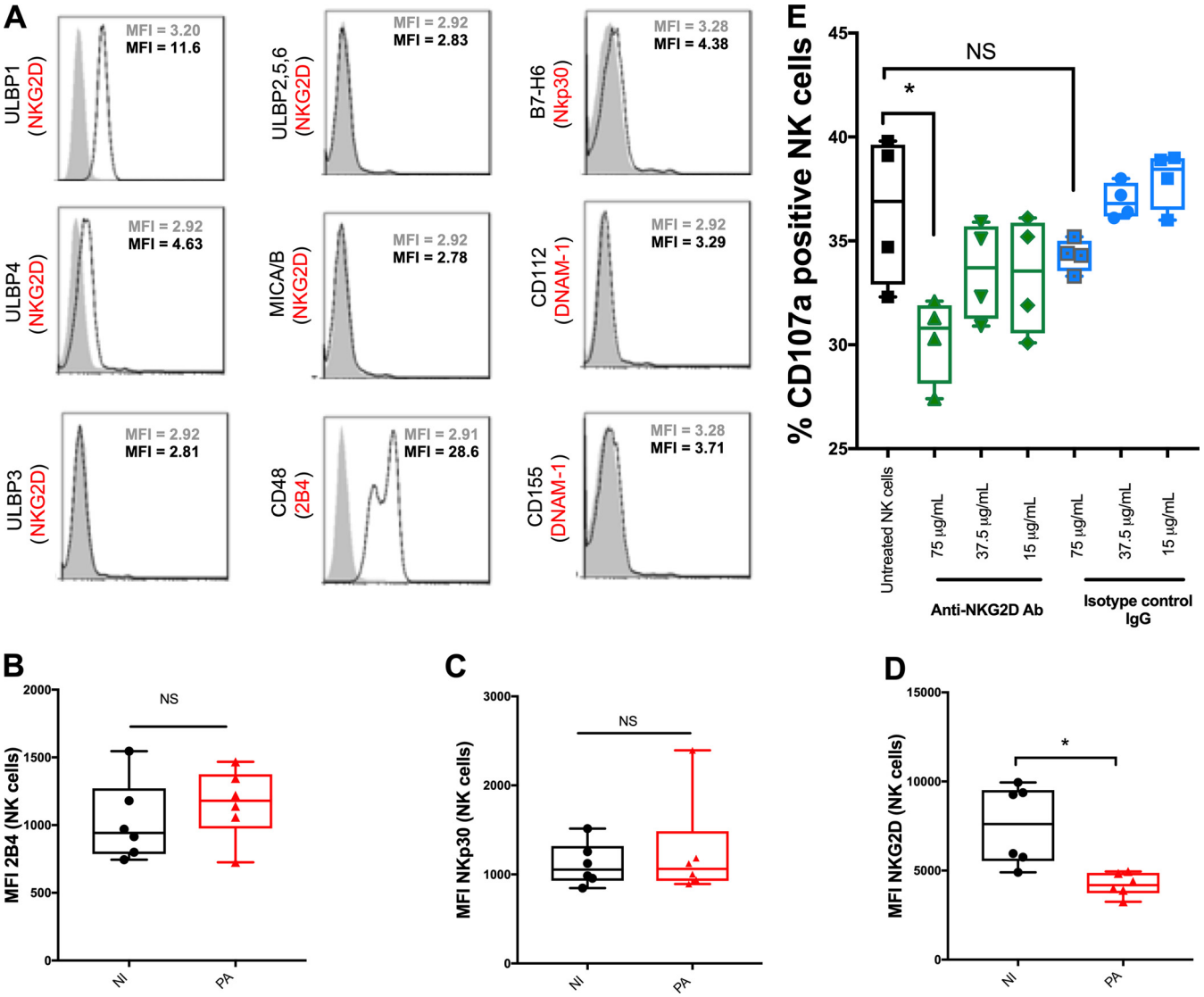

FIG 2 NKG2D-activating receptor is involved in NK cell cytotoxic response against B221 cells. (A) Histogram profiles for different ligands of activating NK cell receptors expressed on B221 cells as follows: ULPB1 to ULPB6 and MICA/B (ligands of NKG2D), B7-H6 (ligand of NKp30), CD48 (ligand of 2B4), and CD112 and CD155 (ligands of DNAM-1). Isotype-matched negative controls are shown as gray-filled curves and ligands as white-filled curves. (B, C, and D) 2B4, NKp30, and NKG2D mean fluorescence intensity (MFI) on NK cells in noninfected or 24-h *Pseudomonas aeruginosa*-infected (PA) PBMCs (without B221 cell exposition). (E) Representative histograms of CD107a activity in sorted NK cells in response to B221 cells with or without anti-NKG2D blocking antibody or its isotype. Data are shown as the median and interquartile range of 6 (B, C, and D) and 4 (E) distinct healthy donors. NK cells were analyzed in lymphocyte gate by flow cytometry after PerCp-CD3$^-$ APC-CD56$^+$ eFluor780$^-$ staining. *, $P < 0.05$; NS, nonsignificant difference; B221, 5-h exposition to B221 cells with a B221/NK ratio of 10:1 in order to keep the same ratio as in PBMCs (assuming 10% of NK cells in PBMC).

In order to determine their role in the impairment of NK cell cytotoxicity toward B221 cells after *P. aeruginosa* infection, we developed an adoptive transfer strategy (Fig. 3A). Transfer of sorted CD3$^+$ cells from infected PBMCs onto noninfected PBMCs altered NK cell cytotoxicity and NKG2D expression compared with that of their noninfected counterparts (Fig. 3B and C). These data suggested that NK cell cytotoxicity impairment after *P. aeruginosa* infection is a T cell-dependent mechanism.

FIG 1 Legend (Continued)
density plots illustrating CD107a expression in NK cells (PerCp-CD3$^-$ APC-CD56$^+$ eFluor780$^-$ in lymphocyte gate) by flow cytometry. Histograms illustrating CD107a$^+$ (B) and the mortality rate of NK cells (C) in noninfected (NI) or 24-h *Pseudomonas aeruginosa*-infected (PA) conditions. Data are shown as the median and interquartile range of 10 distinct healthy donors. *, $P < 0.05$; NS, nonsignificant difference; B221, 5-h exposition of PBMC to B221 cells with a B221/PBMC ratio of 1:1; APC-H7 eFluor 780, viability assessment.

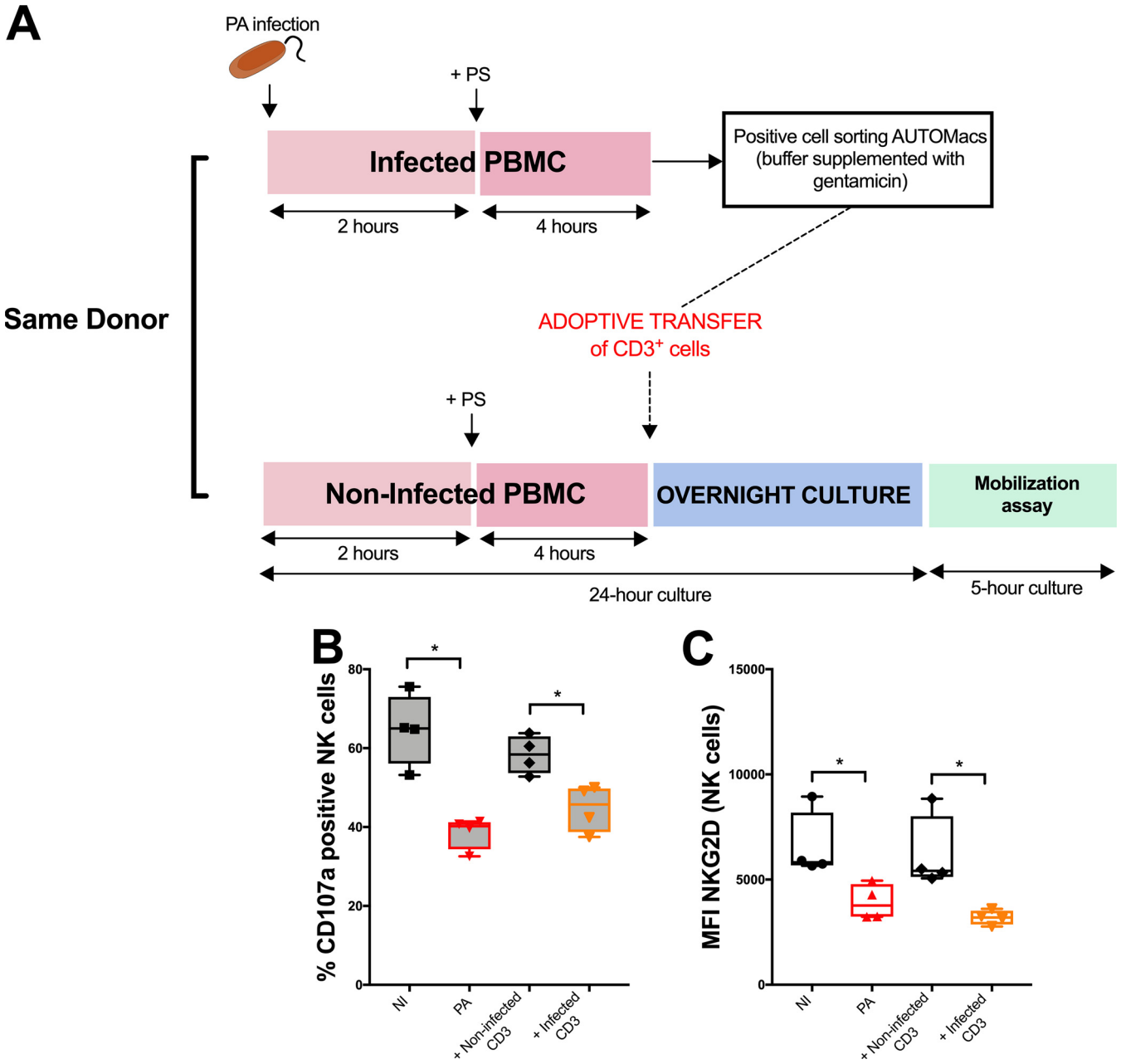

FIG 3 Involvement of T cells in NK cell cytotoxic impairment after *Pseudomonas aeruginosa* infection. (A) Comprehensive diagram explaining adoptive transfer strategy of infected (or noninfected) $CD3^+$ cells. (B) CD107a activity of NK cells after $CD3^+$ adoptive transfer when exposed to B221 cell line. (C) NKG2D mean fluorescence intensity (MFI) of NK cells after $CD3^+$ adoptive transfer (no exposition to B221 cell line for this analysis). Data are shown as the median and interquartile range of 4 distinct healthy donors. NK cells were analyzed in lymphocyte gate by flow cytometry after PerCp-$CD3^-$ APC-$CD56^+$ eFluor780$^-$ staining. *, $P < 0.05$; NI, noninfected PBMC; PA, PBMC after 24-h infection with *Pseudomonas aeruginosa*; B221, 5-h exposition to B221 cells with a B221/PBMC ratio of 1:1.

Treg participate in NK cell cytotoxicity impairment after *P. aeruginosa* infection. Among T lymphocytes, regulatory T cells (Treg) have been reported to regulate the cytotoxic activity of NK cells toward abnormal cells (16, 17). Treg were initially described as $CD4^+$ $CD25^+$ T cells (18) and more recently as $CD4^+$ $CD25^+$ $CD127^-$ cells (19). *P. aeruginosa* infection did not significantly modify the percentage of $CD4^+$ and $CD8^+$ T cells among lymphocytes (Fig. 4A) but increased the percentages of both $CD25^+$ and $CD25^+$ $CD127^-$ cells among $CD4^+$ T cells (Fig. 4B and C; see also Fig. SA3B to D for numbers). Thus, we hypothesized that $CD4^+$ $CD25^+$ depletion (see Fig. SA2 in the supplemental material) before infection would prevent the induction of $CD4^+$

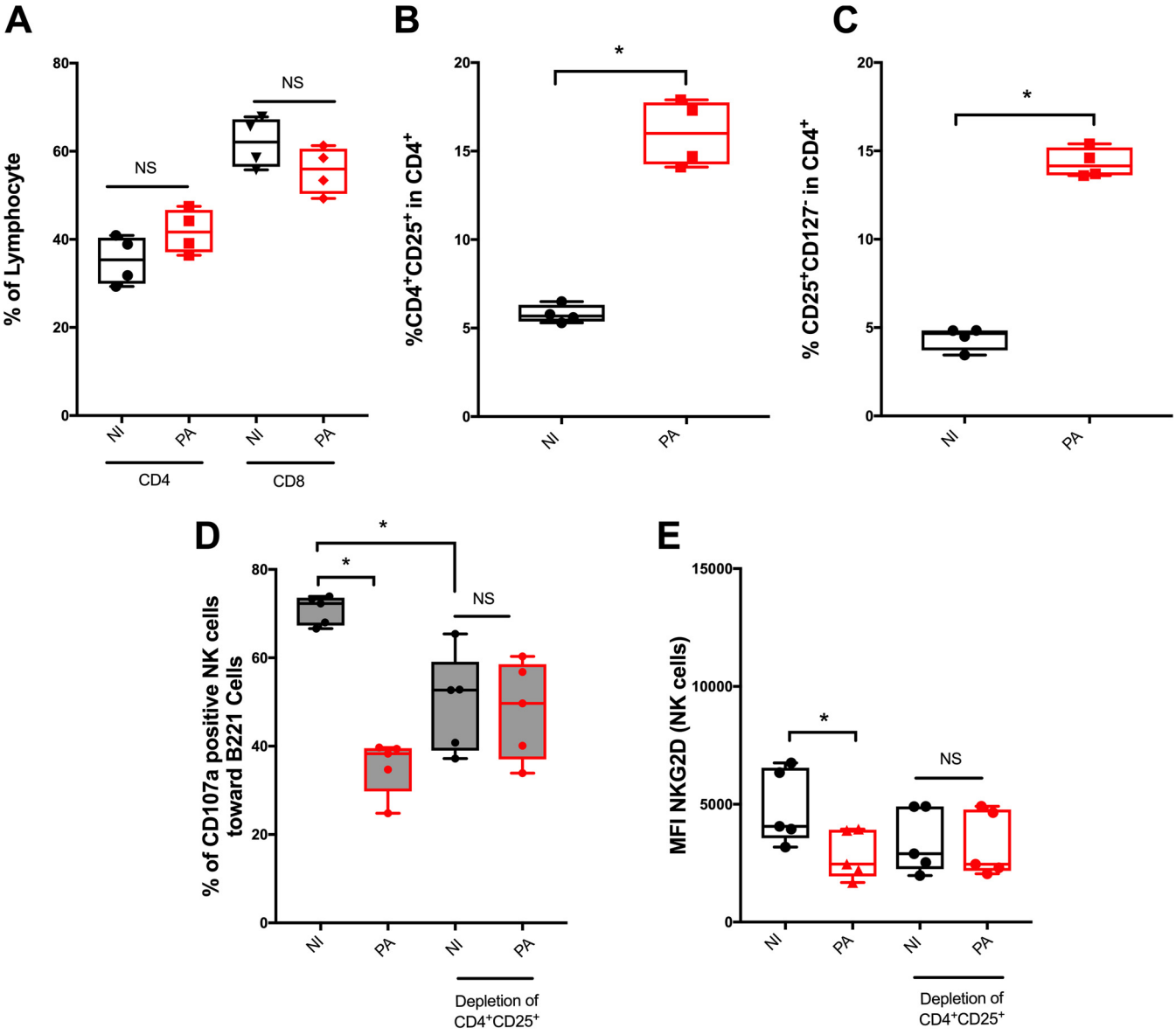

FIG 4 Regulatory T cell involvement in the reduction of NK cell cytotoxicity after *Pseudomonas aeruginosa* infection. Histograms illustrating the percentage of $CD4^+$ and $CD8^+$ cells among lymphocytes (A), $CD4^+$ $CD25^+$ cells (B), and $CD4^+$ $CD25^+$ $CD127^-$ cells among $CD4^+$ cells (C) in noninfected (NI) or 24-h *Pseudomonas aeruginosa*-infected (PA) conditions. Histograms illustrating CD107a activity (D) and NKG2D mean fluorescence intensity (MFI) (E) in noninfected (NI) or 24-h *Pseudomonas aeruginosa*-infected (PA) PBMCs depleted in $CD4^+$ $CD25^+$ cells before infection (see Fig. SA1 and SA2 in the supplemental material). Data is shown as the median and interquartile range of 4 (A, B, and C) and 5 (D and E) distinct healthy donors. NK cells were analyzed in lymphocyte gate by flow cytometry after PerCp-$CD3^-$ APC-$CD56^+$ eFluor780$^-$ staining. *, $P < 0.05$; NS, nonsignificant difference; B221, 5-h exposition to B221 cells with a B221/PBMC ratio of 1:1.

$CD25^+$ $CD127^-$ cells (Treg) and, therefore, the alteration of NK cell cytotoxicity. After $CD4^+$ $CD25^+$ depletion, *P. aeruginosa* infection did not alter NK cell cytotoxicity (Fig. 4D) or NKG2D expression (Fig. 4E) compared with that of the nondepleted condition. These results suggested that $CD4^+$ $CD25^+$ cells are key players in *P. aeruginosa*-mediated NK cell cytotoxicity alteration.

Infected $CD14^+$ cells are involved in regulatory T cell-mediated NK cell cytotoxicity impairment. In PBMCs, $CD14^+$ cells are mainly monocytes (with <2% circulating dendritic cells) (20). Thus, in the following experiments, the sorted $CD14^+$ cells are referred to as monocytes. The expression of costimulation molecules (CD80/86) on antigen presenting cells (APC), such as monocytes, is essential for the induction and the survival of Treg in the periphery (21). Transfer of $CD14^+$ cells (Fig. 5A) from infected

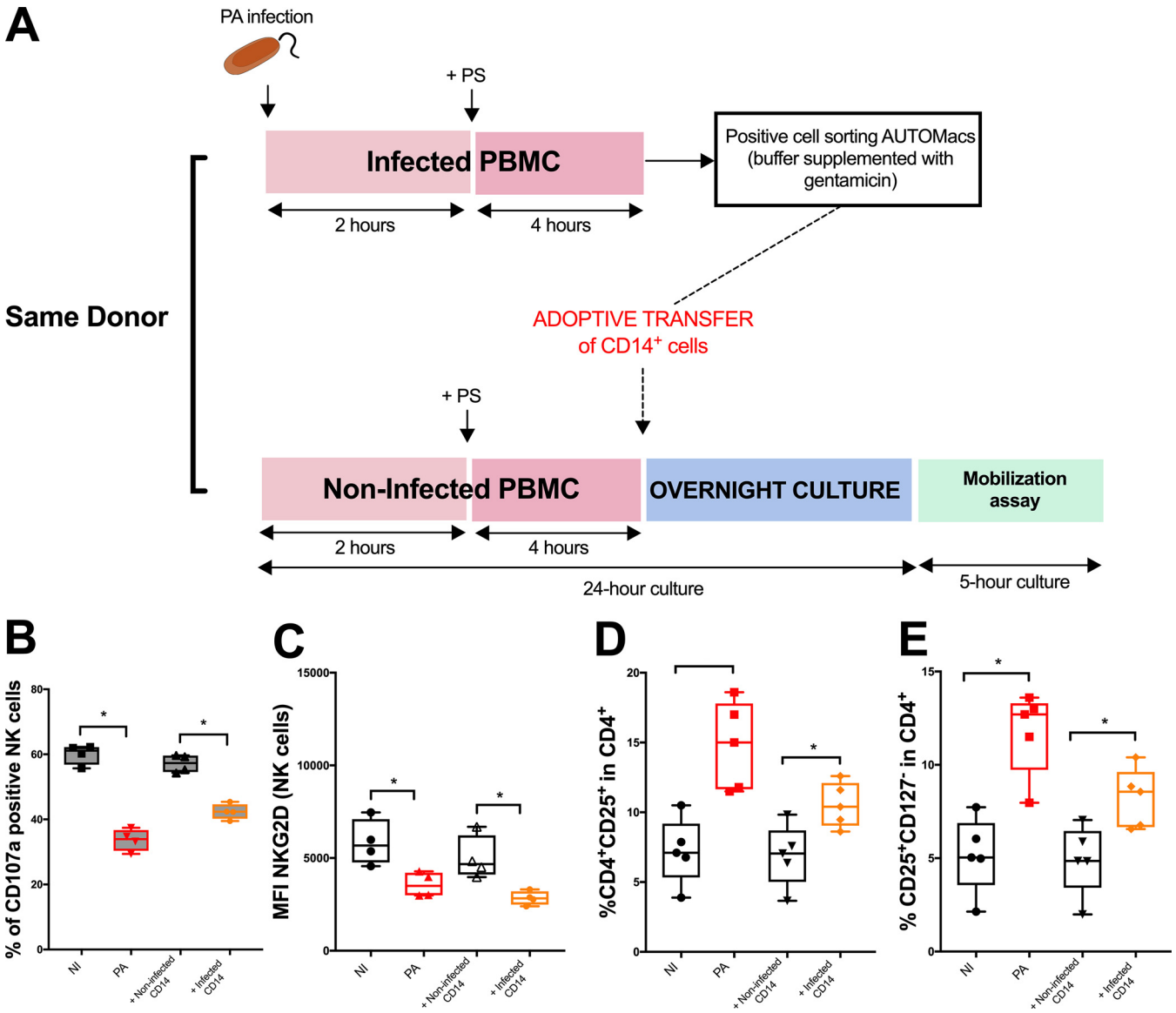

FIG 5 Effect of CD14$^+$ cell transfer on NK cell cytotoxicity and Treg frequency after *Pseudomonas aeruginosa* infection. (A) Comprehensive diagram explaining adoptive transfer strategy of infected (or noninfected) CD14$^+$ cells. (B) CD107a activity of NK cells after CD14$^+$ adoptive transfer when exposed to B221 cell line. (C) NKG2D mean fluorescence intensity (MFI) of NK cells after CD14$^+$ adoptive transfer (no exposition to B221 cell line for this analysis). Histograms illustrating the effect of CD14$^+$ adoptive transfer on the percentage of CD4$^+$ CD25$^+$ (D) and CD4$^+$ CD25$^+$ CD127$^-$ (E) cells among CD4$^+$ cells. *, $P < 0.05$; NS, nonsignificant difference; NI, noninfected PBMC; PA, PBMC after 24-h infection with *Pseudomonas aeruginosa*; B221, 5-h exposition of PBMC to B221 cells with a B221/PBMC ratio of 1:1. Data is shown as the median and interquartile range of 4 (B and C) and 5 (D and E) distinct healthy donors. NK cells were analyzed in lymphocyte gate by flow cytometry after PerCp-CD3$^-$ APC-CD56$^+$ eFluor780$^-$ staining.

PBMCs onto noninfected PBMCs altered NK cell cytotoxicity toward B221 cells and NKG2D expression (Fig. 5B and C) compared with that of their noninfected counterparts. This alteration was concomitant of an increase in the percentage of CD4$^+$ CD25$^+$ and CD25$^+$ CD127$^-$ cells among CD4$^+$ cells after CD14$^+$ infected cell transfer (Fig. 5D and E; see also Fig. SA3E, F, and G for numbers). These results support the involvement of monocytes in Treg-induced NK cell cytotoxicity impairment after *P. aeruginosa* infection.

Overall, the present results suggest that NK cell cytotoxicity in response to the B221 cell line is altered after *P. aeruginosa* infection by complex interactions involving CD4$^+$ CD25$^+$ and CD14$^+$ cells. The reduction of cytotoxic response was at least in part related to the alteration of NKG2D expression on NK cells.

DISCUSSION

Our study provides novel insight into our understanding of how bacterial infection could impair tumor immunity. Notably, we highlighted that *P. aeruginosa* infection led to Treg expansion involving monocytes, with subsequent reduction of NKG2D expression on NK cells, which altered NK cell cytotoxicity against a human B lymphoma cell line.

NK cells play a key role in both antibacterial and antitumor immunity. Our team has already reported the key role of NK cells to defend against *P. aeruginosa in vitro* (8) or *in vivo* (9). There is increasing evidence of complex interaction between antitumor and antibacterial immunity (22). For example, in a murine model of melanoma, *P. aeruginosa* infection has been reported to depress tumor control and enhance its metastatic diffusion through NK cell function impairment (4). The authors suggested that *P. aeruginosa* can provoke a direct phagocytosis-induced apoptosis of NK cells via caspase 9 activation, whereas in the present study, *P. aeruginosa* infection altered NK cell cytotoxicity without markedly impairing NK cell viability (Fig. 1C). These differences could be explained by 8-h infections in antibiotic-free medium of the NK92 cell line with high multiplicity of infection (MOI) (500 bacteria for 1 NK cell) and with a different *P. aeruginosa* strain in the Chung et al. model (4). However, taken together, these results strongly suggest the need to conduct further research on the interaction between bacteria and tumor immunity.

NK cell antitumor function is correlated with the level of expression of activating receptors and the amount of circulating tumor-infiltrating NK cells (23). NK cell response usually depends on the balance between inhibitory and activating receptors. Nevertheless, natural killer group 2D (NKG2D), a C-type lectin-like molecule, is considered to be a "dominant" activating receptor since it can trigger cytotoxic activity despite simultaneous inhibitory signals (13). NKG2D is involved in antitumor as well as antibacterial immunity. Regarding antibacterial defenses, NKG2D has been reported to participate in the bacterial clearance in a murine model of *P. aeruginosa* pneumonia (24). Regarding antitumor immunity, NKG2D recognizes various activating ligands (NKG2D-L) derived from nonclassical MHC class I molecules expressed on several histologic types of tumors including melanoma (25) and lacking on normal tissues. Interestingly, a tumor can release transforming growth factor β (TGF-β) (26) or soluble NKG2D-L (27) leading to NKG2D internalization to escape NK cell immunity. Thus, NKG2D is a candidate receptor to study the interaction between bacterial infection and tumor immunity.

Accessory cells are key regulators of NK cell function (17). Consistent with our results, the increased number and frequency of Treg was correlated to cancer progression and inversely correlated to the function of NK cells and NKG2D expression (28, 29). The underlying mechanisms remain uncertain. Interleukin-2 (IL-2) neutralization via CD25 receptor or membrane-bound TGF-β on Treg were reported to reduce NKG2D expression (30, 31) and alter NK cell immunity against cancer expressing NKG2D ligands (32). IL-2 supplementation at increasing doses did not prevent the drop of cytotoxicity after infection and did not support CD25 involvement in our model (data not shown). The role of TGF-β has not yet been addressed. In the same way, monocytes increase regulatory T cell activation through cytokine or reactive oxygen species (ROS) release during inflammatory response (33, 34). We confirmed the role of infected monocytes in regulatory T cell expansion.

Our *in vitro* study has limitations. The CD107a mobilization assay is a surrogate marker of degranulation, but its detection is correlated with target lysis assessed by ^{51}Cr release (35). Considering coculture as a tumor model may be questionable. However, the study of circulating NK cell function from PBMCs is an accurate approach given their critical roles in preventing metastasis diffusion (36). In our model, the involvement of NKG2D in the cytotoxic response must be tempered considering that blocking antibodies did not suppress but only partly decreased CD107a expression. Our results pled for a predominant accessory cell-dependent alteration of NK cell functions,

but specific pathways were not addressed and will require further studies. In the noninfected conditions, the proportion of CD107a-positive cells was significantly lower after $CD4^+$ $CD25^+$ depletion. The sorting process could explain this difference, but a specific effect of this subset on CD107a activity cannot be excluded. However, after $CD4^+$ $CD25^+$ depletion, the similar proportion of CD107a-positive cells in infected or noninfected conditions suggests that the reduction of CD107a activity after *P. aeruginosa* infection involves $CD4^+$ $CD25^+$ cells.

Regarding Treg characterization, we did not study FOXP3 intracellular staining. However, the $CD4^+$ $CD25^+$ $CD127^-$ T cells are known to express the highest level of FOXP3 (37). Finally, despite systematic control of the supernatant on agar plates, we cannot exclude undetectable residual bacterial pathogen-associated molecular patterns (PAMPs) that could have participated in the results during adoptive transfer experiments.

In conclusion, we found that *P. aeruginosa* infection can alter the cytotoxic function of NK cells against tumor cells by a $CD4^+$ $CD25^+$-dependent activation loop, which is initiated by monocytes. Hence, manipulating $CD4^+$ $CD25^+$ cells or monocytes stands as an interesting therapeutic target to enhance antitumor immunity and to correct postinfectious immune defects (38).

MATERIALS AND METHODS

PBMCs from healthy donors, 721.221 cell line, and cell sorting. All volunteer blood donors were recruited at the blood transfusion center (Nantes, France). Human peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by gradient centrifugation on Ficoll-Hypaque and frozen (Lymphoprep, Norway). Before sorting or infection, PBMCs were cultured at 37°C in 5% CO_2 overnight in RPMI 1640 medium (Gibco) containing glutamine (Gibco) with 10% fetal bovine serum (Gibco; <10 endotoxin units [EU]/ml endotoxin contamination), penicillin-streptomycin (PS) free, and supplemented with 100 U/ml IL-2 (Proleukin [aldesleukin]; Chiron). This medium is referred to as "IL-2 medium" throughout.

Human PBMCs were sorted with an autoMACS pro separator (Miltenyi Biotec, Germany) and the corresponding isolation kits as follows: untouched NK cells, $CD14^+$, $CD3^+$, and $CD4^+$ $CD25^+$ cells. Cell sorting assessment yielded to a mean (±standard deviation [SD]) purity of 86% (±6%) for NK cells and 90% (±4%) for $CD14^+$ or $CD3^+$ cell isolation. After $CD4^+$ $CD25^+$ cell selection, the negative cell fraction contained <0.5% of residual $CD4^+$ $CD25^+$ cells. The cells were kept in IL-2 medium during cell sorting.

The 721.221 cell line (referred to as B221 cells; from EFS-PL laboratory, Nantes, France) is an MHC class I-negative EBV-transformed human B lymphoma cell line. This cell line was cultured at 37°C in 5% CO_2 in the same medium as PBMCs without IL-2 supplementation and was used as a positive control to assess NK cell cytotoxic granule release (39).

Infection. Before infection, PBMCs or sorted cells were seeded in 24-well plates (1×10^6 per well in 1 ml) in IL-2 medium.

Infections were performed with the PAO1 strain, which is a clinical strain of *Pseudomonas aeruginosa* (number 15692) whose genome has been fully sequenced (40). The PAO1 strain was grown overnight in brain heart infusion medium at 37°C. Bacterial inoculum was calibrated by nephelometry. After 2 h of coculture in IL-2 medium with a multiplicity of infection (MOI) for bacteria/PBMC of 25:1, the wells were supplemented with 10 mM PS to prevent bacterial overgrowth until the 24th hour (8). After 24 h of coculture, the NK cell mobilization assay was performed. Noninfected conditions followed the same protocol (see Figure SA1 in the supplemental material). Bacterial loads were checked by inoculation of serial dilution on agar plates.

NK cell mobilization assay. CD107a mobilization assay was detected by flow cytometry. This assay reflects NK cell cytotoxicity given that CD107 is localized in a lipid layer surrounding cytotoxic granules and remains attached to the surface of cells after granule release (41). The cells from 24-h *P. aeruginosa*-infected and noninfected wells were numbered, resuspended in fresh IL-2 medium with PS, seeded in 96-well plates (0.5×10^6 per well in 100 μl), and exposed to B221 (IL-2 medium with PS, 0.5×10^6 per well in 100 μl) for 5 h at 37°C in 5% CO_2. The B221/PBMC ratio was 1:1 (indicating a B221/NK ratio of 10:1 assuming 10% NK cells among PBMC). Similarly, the control wells (without B221 cells) were supplemented with 100 μl of IL-2 medium with PS (see Figure SA1 in the supplemental material).

NKG2D blocking. The anti-hNKG2D antibody (IgG1, clone 149810; R&D) or its isotype (IgG1, clone NCG01; Invitrogen) were incubated with sorted NK cells (untouched NK cell isolation kit) for 1 h (4°C) in 100 μl of IL-2 medium with PS for final concentrations of 75, 37.5, and 15 μg/ml. The mobilization assay was subsequently performed as described above.

$CD3^+$ or $CD14^+$ adoptive transfer. After 6 h of infection with *P. aeruginosa* (including PS supplementation after 2 h) in IL-2 medium, PBMCs were treated with gentamicin for 1 h (final concentration of 2 mg/ml), which was adjusted to 1,000-fold the MIC of the antibiotic for the PAO1 strain (MIC = 2 mg/liter). The cells were then sorted ($CD3^+$ or $CD14^+$) using a positive selection kit, resuspended in fresh IL-2 medium with PS, and adoptively transferred onto noninfected PBMCs. The lack of residual viable bacteria

was controlled by pure inoculation of the supernatant on agar plates, and cultures were followed for 48 h. Adoptive transfer of noninfected $CD3^+$ or $CD14^+$ cells followed the same protocol.

Regulatory T cell depletion. PBMCs were depleted from $CD4^+$ $CD25^+$ cells using a positive selection kit. Then the negative fraction was infected for 24 h (see Fig. SA2 in the supplemental material).

Cell labeling. Antibodies were purchased from BD Biosciences unless otherwise stated. Data were collected with a 4-color FACSCalibur (BD Biosciences) and LSR II cytometer (Beckton Dickinson, Le Pont de Claix, France) and analyzed using FlowJo 6.2 software (FlowJo, LLC, Ashland, OR, USA). For PBMCs, NK cell gating was performed with anti-CD56-APC (NCAM16.2), anti-CD3-PerCP (SK7), and the corresponding isotype-matched control monoclonal antibody (MAb). NK cell-activating receptor phenotyping was performed with anti-NKp30 (Z21), anti-NKG2D (1D11), and anti-2B4 (2-69) MAbs. The expression of the ligands of NK cells activating receptors on B221 cells was determined using anti-ULBP1 (170818), anti-ULBP2,5,6 (165903), anti-ULBP3 (166510), anti-ULBP4 (709116), anti-B7-H6 MAbs (8750001; R&D Systems, Minneapolis, MN, USA), anti-MICA/B (6D4), anti-CD112 (R2.525, BD Biosciences, Le Pont de Claix, France), anti-CD48 (BJ40), and anti-CD155 (SKII.4; BioLegend, San Diego, CA, USA).

For mobilization assay, cells were stained during the 5-h exposition to B221 cells with an anti-CD107 MAb (clone H4A3). Viability was assessed by APC-fixable viability dye kit eFluor 780 staining (eBioscience).

Statistical analysis. Statistical analyses were performed with GraphPad prism software (La Jolla, CA, USA). Continuous nonparametric variables were expressed as the median (25th to 75th percentile). The Kruskal-Wallis test was used to compare multiple groups. Dunn's *post hoc* test was used to perform comparisons between the 2 groups. A P value of <0.05 was considered to be statistically significant.

Ethics statement. Written informed consent was obtained from all individuals (Biocollection Authorization Number DC-2012-1555). All experiments were approved by the Ethics Committee of Ouest IV, France (11/13) and performed in accordance with relevant guidelines and regulations.

Data availability. The data sets generated and/or analyzed during the current study are available at https://data.mendeley.com/datasets/2jrwyy4c28/draft?a=5b1c6245-9aea-4d91-b4eb-05b3e4a31d8e.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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M.V. and K.A. designed all of the experiments. M.V., A.B., G.D., A.R., C.J., T.C., B.G., J.C., C.R., and K.A. wrote the main manuscript text. All authors reviewed the manuscript. M.V., A.B., C.J., T.C., J.C., and G.D. performed the experiments.

We have no conflicts of interest to declare.

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