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Selective Mobilization of Cytotoxic Leukocytes by Epinephrine

Stoyan Dimitrov,*[,1] Tanja Lange,*[,†] and Jan Born*

It is well-known that acute stress, presumably as a first defense against pathogens, enhances PBMC counts by mobilizing these β2-adrenoceptor positive cells from the marginal pool. Yet, only select leukocyte subsets participate in this phenomenon of adrenergic leukocytosis and underlying mechanisms are obscure. In this study, we analyzed in human blood adhesion molecule and chemokine receptor profiles in 14 leukocyte subsets, and responsiveness of subsets to epinephrine in vivo and in vitro. Five subsets, namely, $CCR7^-CD45RA^+CD8^+$ effector T cells, $CD4^-CD8^-$ γ/δ T cells, $CD3^+CD56^+$ NKT-like cells, $CD16^+CD56^{dim}$ cytotoxic NK cells, and $CD14^{dim}CD16^+$ proinflammatory monocytes showed a rapid and transient increase after infusion of epinephrine at physiological concentrations. These cells were characterized by a $CD62L^-CD11a^{bright}CX3CR^{bright}$ phenotype, whereby expression of both CD11a and CX3CR1 was strongly correlated with adrenergic leukocytosis in vivo ($r = 0.86$ and 0.78, $p < 0.005$). The same subsets showed highest adherence to activated endothelium in vitro, which (except for proinflammatory monocytes) was reversed by epinephrine. We conclude that these five cytotoxic effector leukocyte subsets comprise the marginal pool by a CD11a/CX3CR1-mediated attachment to the endothelium. Epinephrine rapidly attenuates this attachment to allow demargination and release of the cells into the circulation that, because of their cytotoxic effector function, provide immediate protection from invading pathogens. ***The Journal of Immunology*****, 2010, 184: 503–511.**

Acute stress in humans is well-known to cause a rapid and transient increase in WBC counts, that is, leukocytosis, presumably reflecting the demargination of these cells from the marginal pool (1–4). Phenotypic analysis revealed that this mobilization of leukocytes is selective and primarily affects, among others, effector $CD8^+$ T cells and NK cells (3, 5). Immediate demargination of leukocytes from vascular endothelial cells is promoted by sympathetic nervous system (SNS) catecholaminergic activity stimulating PBMCs that express predominantly high-affinity β2-adrenoceptors (1, 3, 6–10). Interestingly, expression of β-adrenoceptors increases with immune cell activation and differentiation (10–14). Thus, β2-adrenoceptor-mediated mobilization of immune cells with cytotoxic effector potential has been proposed as a basic mechanism that allows cell redistribution to sites of injury during stress (3, 15, 16).

Aside from β2-adrenoceptor expression, the selective recruitment of leukocytes is determined by the specific profile of adhesion molecule expression on the cells. Stress as well as diurnal increases in SNS activity mobilize cells with low expression of L-selectin (CD62L) and high expression of integrin LFA-1 (CD11a) such as effector memory (EM) and effector $CD8^+$ T cells, $CD16^+CD56^{dim}$ cytotoxic NK cells, and $CD14^{dim}CD16^+$ proinflammatory monocytes, whereas their $CD62L^+CD11a^{dim}$ counterparts (e.g., naive T cells, $CD16^-CD56^{bright}$ immunomodulatory NK cells, and $CD14^+CD16^-$ conventional monocytes) are distinctly less sensitive to changes in catecholaminergic activity (10, 16–25). Along with the $CD62L^-CD11a^{bright}$ phenotype, effector immune cells are generally characterized by high expression of inflammatory chemokine receptors, such as CCR5, CXCR1, CXCR3, and CX3CR1 (26–28). Despite all these findings and that chemokines are main regulators of leukocyte traffic, the role of chemokine receptors in SNS-induced demargination of leukocytes has attracted surprisingly little attention (22, 24, 29–31).

We hypothesized that the mobilizing effect of catecholamines is linked to a specific profile of adhesion molecule and chemokine receptor expression. To identify such a profile in a comprehensive study, we examined in humans the effects of i.v. epinephrine infusion at concentrations within the physiological normal range on 14 different T cell, NK cell, and monocyte subsets. Epinephrine was chosen because it is well-known, via binding with high affinity to the β2-adrenoceptor of PBMCs, to play a key role in mediating acute stress-induced leukocytosis (1–3, 32–34). We show that epinephrine selectively mobilizes cells with cytotoxic effector functions, namely, $CCR7^-CD45RA^+CD8^+$ effector T cells (EFF), $CD4^-CD8^-$ γ/δ T cells, $CD3^+CD56^+$ NKT-like cells, $CD16^+CD56^{dim}$ cytotoxic NK cells, and $CD14^{dim}CD16^+$ proinflammatory monocytes. Analysis of adhesion molecule and chemokine receptor expression reveals a specific profile in these cells, characterized by high expression of CD11a and CX3CR1 (fractalkine receptor). The same subsets of cytotoxic effector cells show preferential adhesion to activated endothelium in vitro, which can be blocked by epinephrine. Our data suggest cytotoxic effector cells highly expressing CD11a and CX3CR1 to define a first line of immunological defense mobilized during acute stress.

Materials and Methods

Subjects and procedure of in vivo study

Subjects were eight healthy nonsmoking men (mean age 26 y; range, 22–35 y) presenting with a normal sleep/wake pattern and not taking any medication at the time of the experiments. None had a medical history of any relevant

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Abbreviations used in this article: CM, central memory; EFF, effector T cells; EM, effector memory; MFI, mean fluorescence intensity; SNS, sympathetic nervous system.

chronic disease or mental disorder. Acute illness was excluded by physical examination and routine laboratory investigation, including a chemistry panel, C-reactive protein concentration <6 mg/l, and a WBC count <9000/μl. The study was approved by the Ethics Committee of the University of Lübeck. All the men gave written informed consent prior to participating in accordance with the Declaration of Helsinki.

Subjects were i.v. infused for 30 min with sodium chloride (placebo) or the endogenous β-adrenoceptor ligand epinephrine (0.005 μg/kg/min, Suprarenin; Sanofi-Aventis, Bridgewater, NJ) on two different occasions, according to a double-blind within-subject design. The order of substance administration was balanced across subjects. Subjects were prepared for blood sampling 1 h before infusions started and remained in a supine position throughout the session. Infusions started in the evening at 9 PM when endogenous epinephrine concentration is low. Blood for determining hormones and immune parameters was sampled via a second catheter (inserted into the vein of the other arm) before (baseline at 9 PM), during (15 and 30 min), and after (60 and 90 min) the start of the infusion. Heart rate and electrocardiogram were continuously monitored to exclude any adverse effects.

Hormone assays

Whole blood was collected in plasma or serum tubes, kept on ice, and spun down within 10 min after collection. Plasma or serum were stored at −80°C until assay. Epinephrine and norepinephrine were measured in plasma by standard HPLC with subsequent electrochemical detection (Chromsystems, Munich, Germany) (35). Cortisol was measured in serum using a commercial assay (Immulite, DPC-Biermann GmbH, Bad Nauheim, Germany). The sensitivity and intra- and interassay coefficients of variation were as follows: cortisol, 0.2 μg/dl, <10%; epinephrine, 2.0 pg/ml, <5.6%; and norepinephrine, 5.0 pg/ml, <6.1%.

Abs

The following fluorochrome-conjugated Abs were used: CCR5 (Clone 2D7), CCR7 (3D12), CXCR1 (5A12), CXCR3 (1C6), CD3 (SK7), CD4 (SK3), CD8 (RPA-T8), CD11a (G43-25B), CD16 (3G8), CD45 (2D1), CD45RA (HI100), CD56 (B159), CD62L (SK11), HLA-DR (L243) all from BD Biosciences (San Jose, CA), CX3CR1 (2A9-1) from MBL International (Woburn, MA), CD11b (M1/70.15.11.5), CD49d (MZ18-24A9) from Miltenyi Biotec (Bergisch Gladbach, Germany), and CD14 (My4) from Beckman Coulter (Fullerton, CA). All isotype control Abs were purchased from BD Biosciences.

Flow cytometry

Based on previous reports, our analyses of leukocyte subsets in the in vivo administration study included naive, central memory (CM), EM and effector $CD4^+$ and $CD8^+$ T cells, $CD4^-CD8^-$ γ/δ T cells, NKT-like cells, immunomodulatory and cytotoxic NK cells, and conventional and proinflammatory monocytes. Because $CD4^-CD8^-$ T cells represent to the greatest extent γ/δ T cells, we refer to this population as $CD4^-CD8^-$ γ/δ T cells (36, 37).

Absolute counts of $CD3^+$ (total T cells), NKT-like cells ($CD3^+CD56^+$), immunomodulatory NK cells ($CD16^-CD56^{bright}$), cytotoxic NK cells ($CD16^+CD56^{dim}$), conventional monocytes ($CD14^+CD16^-$), and proinflammatory monocytes ($CD14^{dim}CD16^+$) were determined by a "lyse no-wash" flow cytometry procedure. To discriminate better $CD3^-CD16^+$ cells into either $CD16^+$ NK cells or $CD16^+$ monocytes, we used HLA-DR Ag [NK cells are negative for HLA-DR, whereas monocytes express this marker (23)]. An undiluted blood sample (50 μl) was immunostained with CD3, CD14, CD16, CD45, CD56, and HLA-DR Abs in Trucount tubes (BD Biosciences). After a 15-min incubation at room temperature, 0.45 ml FACS lysing solution (BD Biosciences) was added, followed by incubation for 15 min. No washing was performed to avoid cell loss. Finally, samples were mixed gently and at least 100,000 $CD45^+$ leukocytes were acquired on a FACSCanto II using FACSDiva Software (BD Biosciences). The absolute number of the cells per microliter of blood was calculated using the following formula: Cells per microliter = (acquired cell events in the respective gate) × (number of beads per tube)/(acquired bead events) × (sample volume [microliters]).

For detection of naive ($CCR7^+CD45RA^+$), CM ($CCR7^+CD45RA^-$), EM ($CCR7^-CD45RA^-$), effector ($CCR7^-CD45RA^+$), and γ/δ ($CD3^+CD4^-CD8^-$) T cells, whole blood was incubated with CCR7, CD3, CD4, CD8, and CD45RA Abs for 15 min. Cells were then lysed and washed twice before measuring on a FACSCanto II (BD Biosciences). The absolute counts were calculated based on percentage of the respective subpopulation and $CD3^+$ absolute counts obtained by the lyse no-wash procedure.

The adhesion molecule profile on PBMC subpopulations was measured in morning blood collected from six subjects. Cells were processed and analyzed in the same way as in the main experiments. Results were expressed as percentage from the respective cell population or as mean fluorescence intensity (MFI). Isotype controls were used to set the markers determining positive and negative populations.

Endothelial cell cultures

Vital HUVECs were purchased from Provitro (Berlin, Germany) and cultured in gelatin-coated 25 cm^2 tissue culture flasks (Sarstedt, Nümbrecht, Germany) in a humidified 37°C, 5% CO_2 environment using MCDB 131 culture medium (Invitrogen, Karlsruhe, Germany), supplemented with 8% FCS (Biochrom, Berlin, Germany), 2% heat-inactivated human serum, 100 μg/ml heparin, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2.5 μg/ml amphotericin B (all from Sigma-Aldrich, Seelze, Germany), and endothelial cell growth supplement (Promocell, Heidelberg, Germany). Tissue culture plates were coated with gelatin for 1 h at room temperature using autoclaved 1% gelatin solution made from gelatin powder (Serva, Heidelberg, Germany). HUVECs were used for experiments in the second to fifth passage.

Adhesion assay

PBMCs were isolated from heparinized whole blood obtained from seven healthy men. Blood was mixed with an equal volume of PBS and separated using 50-ml Leucosep tubes prefilled with Ficoll (Greiner Bio-One, Frickenhausen, Germany). PBMC fraction was rinsed twice with PBS containing 0.9 M Ca^{2+}, 0.5 M Mg^{2+}, and 0.5% BSA.

For the adhesion assay, HUVECs were detached using 0.05% trypsin and 0.02% EDTA, plated in 24-well culture plates precoated with gelatin using ~4×10^4 cells/well, and allowed to grow to confluence for 2 d. On the day of the binding assay, HUVECs were stimulated for 4 h with recombinant TNF-α and IFN-γ (at final concentrations of 25 ng/ml and 20 ng/ml, respectively) (Provitro) to enhance adhesion molecule and chemokine expression. At the end of the incubation, the HUVECs were rinsed twice with PBS containing 0.9 M Ca^{2+}, 0.5 M Mg^{2+}, and 0.5% BSA before adding 1 ml PBMCs (5×10^5 cells/well). Adhesion was manipulated by adding epinephrine at a final concentration of 10^{-8} M [1832 pg/ml, i.e., a dose that in foregoing studies proved maximal efficacy (2, 38)] and all tests were performed in triplicate. Cells were allowed to adhere for 30 min (37°C, 5% CO_2). Nonadherent cells were carefully removed, and the plate was washed three times, twice with PBS containing Ca^{2+} and Mg^{2+}, and one time with PBS without supplements. After the final wash, 250 μl trypsin/EDTA solution was added to obtain a single-cell suspension containing HUVECs and PBMCs. After 1 min of incubation (37°C, 5% CO_2), plates were shaken vigorously for an additional 1 min and 50 μl FCS was added, followed by 2 ml PBS containing 0.1% NaN_3. The cells were then separated equally into two tubes, centrifuged at 500*g* to throw away the supernatant, and stained with the same combinations of monoclonal Abs as used in the main study. Samples were acquired on a FACSCanto II using FACSDiva Software (BD Biosciences) until all events in the tubes were collected. As previously described (39), light scatter properties were used to distinguish PBMCs from HUVECs (HUVECs are larger and more granular than PBMCs). Events and percentages of the same T cell, NK cell, and monocyte subpopulations analyzed in the main study were counted and calculated.

Statistical analysis

Analysis of effects of i.v. epinephrine infusion on leukocyte subsets was based on ANOVA, including a substance and time factor, followed by paired *t* tests. Paired *t* test was also used to analyze differences in leukocyte adhesion to activated endothelium after epinephrine or placebo treatment. For correlation analyses, Pearson's coefficients were calculated. Means across subjects were calculated for the 14 leukocyte subsets of interest for the "increase in vivo" (cell count after epinephrine/cell count after placebo at 9:30 PM) and correlated with the averaged CD11a, CXCR1, CX3CR1, and CD62L expression, respectively. CX3CR1 MFI was normalized by log transformation. The p values < 0.05 were considered significant. Data are presented as means ± SEM.

Results

Influence of epinephrine on the redistribution of leukocyte subpopulations in vivo

We i.v. infused eight healthy men in a balanced order with epinephrine (i.e., the major endogenous ligand of β2-adrenoceptors on PBMCs) and placebo (sodium chloride solution). Infusion started

at 9 PM and was discontinued after 30 min. Low doses of epinephrine (0.005 μg/kg/min) were chosen to induce increases in plasma concentrations of the hormone comparable with levels observed during mild stress (16) and near to the effective dose generating 50% of maximal NK cell detachment in vitro [(38); Fig. 1]. Plasma epinephrine concentration reached peak values of 50.71 pg/ml (2.8×10^{-10} M) at the end of infusion and, thereafter, immediately decreased to recover baseline values 30 min later, reflecting the short half life of this hormone in blood (~2 min). Epinephrine infusion did not affect plasma norepinephrine or cortisol concentration ($p > 0.5$; data not shown).

Within the $CD4^+$ and $CD8^+$ T cell subsets, infusion of epinephrine, compared with placebo, induced a selective and distinct increase in numbers of effector $CD8^+$ T cells ($p < 0.01$). The increase was short lived and focused on the end of the infusion when epinephrine levels were maximal (Fig. 2*H*). All other $CD4^+$ and $CD8^+$ T cell subsets remained unchanged ($p > 0.5$). The increase in effector $CD8^+$ T cell numbers was paralleled by significant increases in $CD4^-CD8^-$ γ/δ T cell and NKT-like cell counts during infusion of epinephrine ($p < 0.05$, Fig. 2*I*, 2*J*). Again, cell counts peaked at the end of the infusion and recovered normal values shortly afterward. As expected, most pronounced increases were revealed for cytotoxic NK cell numbers that were increased by 200% at the end of the epinephrine infusion period, and then rapidly returned to baseline values within 30 min ($p < 0.01$, Fig. 2*L*). Although to a smaller extent, epinephrine infusion also enhanced counts of proinflammatory monocytes ($p < 0.05$, Fig. 2*N*). In contrast, immunomodulatory NK cells and conventional monocytes remained unchanged by epinephrine ($p > 0.3$). The pattern of epinephrine-induced increases in the different subsets of T cells, NK cells, and monocytes was confirmed by analyses based on percentages of cells expressed with reference to the respective parent population (data not shown).

Expression of adhesion molecules and chemokine receptors in leukocyte subpopulations

Adhesion molecules and chemokine receptors control leukocyte migration and thus represent candidate mechanisms for the differential demargination of leukocyte subsets by epinephrine. We defined adhesion molecule and chemokine receptor profiles in all leukocyte subpopulations of interest in samples from healthy donors collected in the morning (results are summarized in Fig. 3). Cell surface expression of L-selectin (CD62L), integrins (CD11a, CD11b, and CD49d), and so-called inflammatory chemokine receptors (CCR5, CXCR1, CXCR3, and CX3CR1) were examined. As to the adhesion molecules, all cytotoxic effector cell subsets were well-characterized by low expression of CD62L and high levels of CD11a. Integrins CD11b and CD49d did not show any preferential expression on the cells of interest (Fig. 3*B*).

As to the chemokine receptors, CCR5 was absent in naive T cells, but was expressed preferentially on EM $CD4^+$ and $CD8^+$ T cells, $CD4^-CD8^-$ γ/δ T cells, NKT-like cells, and immunomodulatory NK cells. CXCR3 expression was not selective for any T cell subset, but it was expressed preferentially on immunomodulatory NK cells. In contrast, CX3CR1 (fractalkine receptor) expression

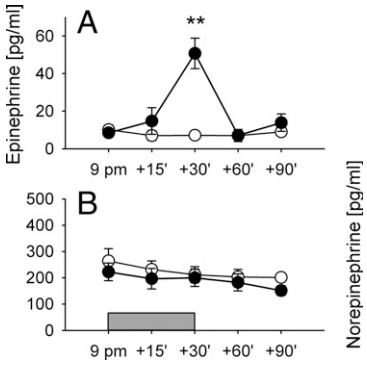

FIGURE 1. Low-dose epinephrine infusion to mimic levels observed during mild stress. Mean (± SEM) (*A*) epinephrine and (*B*) norepinephrine concentrations before (baseline, 9 PM), during (15 and 30 min, horizontal gray bar) and after (60 and 90 min) injection of placebo (sodium chloride, open circles) and epinephrine (0.005 μg/kg/min, filled circles); $n = 8$. $^{**}p < 0.01$ for pairwise comparisons between epinephrine and placebo conditions.

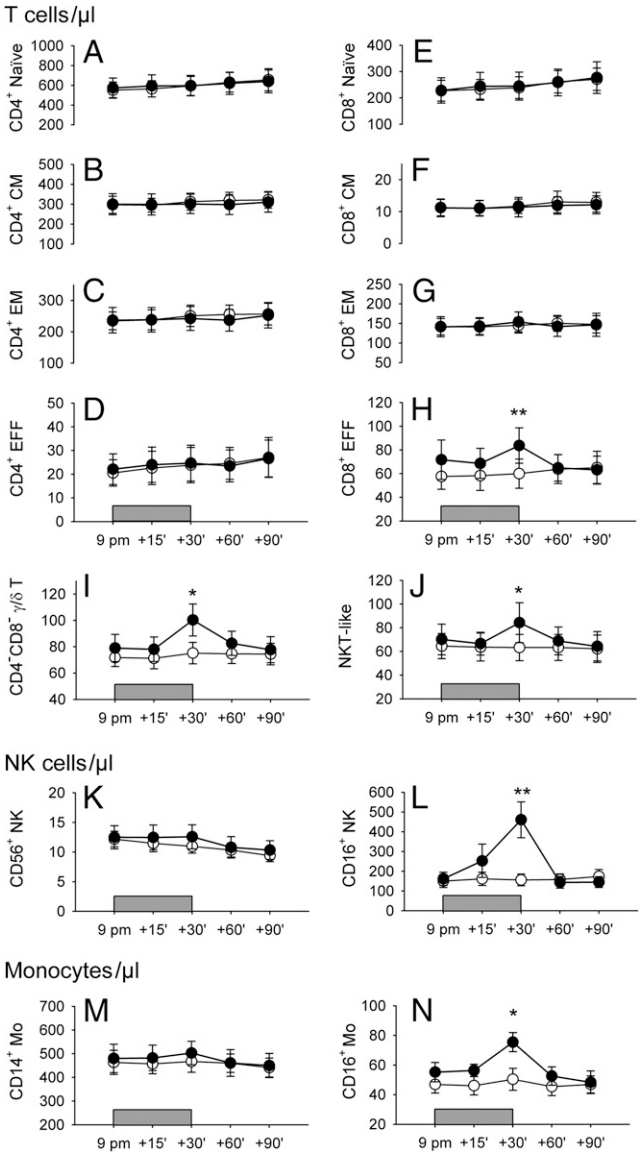

FIGURE 2. Epinephrine selectively mobilizes cytotoxic effector cells. Mean (± SEM) numbers of (*A*, *E*, naive [$CCR7^+CD45RA^+$]), (*B*, *F*, CM [$CCR7^+CD45RA^-$]), (*C*, *G*, EM [$CCR7^-CD45RA^-$]), and (*D*, *H*, EFF [$CCR7^-CD45RA^+$]) $CD4^+$ (*left*) and $CD8^+$ (*right*) T cells, of (*I*) γ/δ T cells ($CD3^+CD4^-CD8^-$), (*J*) NKT-like cells ($CD3^+CD56^+$), (*K*) immunomodulatory NK cells ($CD56^+$ NK, $CD16^-CD56^{bright}$), (*L*) cytotoxic NK cells ($CD16^+$ NK, $CD16^+CD56^{dim}$), (*M*) conventional monocytes ($CD14^+$ Mo, $CD14^+CD16^-$), and (*N*) proinflammatory monocytes ($CD16^+$ Mo, $CD14^{dim}CD16^+$) after a 30-min i.v. infusion (horizontal gray bar) of placebo (sodium chloride, open circles) and epinephrine (filled circles); $n = 8$, $^*p < 0.05$, $^{**}p < 0.01$ for pairwise comparison between epinephrine and placebo conditions.

clearly characterized those cytotoxic effector immune cells that were mobilized by epinephrine infusion (black bars in Fig. 3*B*). Representative flow cytometry data demonstrating the selective expression of CX3CR1 in cytotoxic effector cells are shown in Fig. 4. Expression of CXCR1 (IL-8 receptor) showed a pattern comparable to that of CX3CR1, except that it was absent in proinflammatory monocytes (Fig. 3*B*).

To further explore the functional significance of these markers for epinephrine-induced mobilization, we correlated the expression of adhesion molecules and chemokine receptors in the 14 leukocyte subsets of interest with the increase in respective cell counts after epinephrine administration. Pronounced positive correlations were revealed for CD11a ($r = 0.86$, $p = 0.00015$) and CX3CR1 expression ($r = 0.78$, $p = 0.0015$, Fig. 3*C*, 3*D*), suggesting a dominant contribution of these two molecules in mediating the epinephrine-induced demargination of leukocytes. Correlations were in negative direction for CD62L ($r = -0.66$, $p < 0.05$) and only approached significance for CXCR1 ($r = 0.53$, $p = 0.064$), (which does not exclude that CXCR1 adds to the exaggerated epinephrine-induced mobilization in vivo of cytotoxic NK cells). CD11a and CX3CR1 showed a high degree of coexpression ($r = 0.84$, $p < 0.001$).

Influence of epinephrine on the redistribution of T cells expressing different chemokine receptors in vivo

As a further test of the importance of CX3CR1 for mobilizing leukocytes, we studied the effect of epinephrine infusion specifically on total T cells expressing this chemokine receptor. $CX3CR1^+$ T cells were substantially mobilized after epinephrine administration ($p < 0.01$, Fig. 5*D*). In contrast, $CX3CR1^-$ as well as $CCR5^+$ and $CXCR3^+$ T cell counts were not influenced ($p > 0.3$), and $CXCR1^+$ T cells were only marginally increased ($p < 0.1$). Virtually the same differential effect of epinephrine was revealed when NK cells and monocytes were categorized based on CX3CR1 expression, because the CX3CR1 receptor was present on practically all cytotoxic NK cells and proinflammatory monocytes (results not shown).

Characterization of leukocyte adhesion to activated endothelium

We characterized in vitro adhesion of the cell subsets of interest to activated HUVECs after incubating the endothelial cells with PBMCs for 30 min. The proportion of cells of a subset (with reference to total PBMCs) was compared in the fraction prior to incubation and in the adherent fraction after incubation. For the main total populations (T cells, NK cells, and monocytes), we found distinct enrichment of NK cells (from 12 ± 2% to 31.5 ± 3.1%) and monocytes (from 9.4 ± 1.1% to 24.2 ± 1.6%) in the adherent fraction ($p < 0.001$, Table I). This enrichment was primarily due to cytotoxic, but not immunomodulatory NK cells, and was more pronounced for proinflammatory than conventional monocytes. In contrast, percentage of T cells decreased from 63.3 ± 2.6% prior to adhesion to 31.1 ± 2.5% in the adherent fraction ($p < 0.001$). This decline was due to a large extent to a drop of

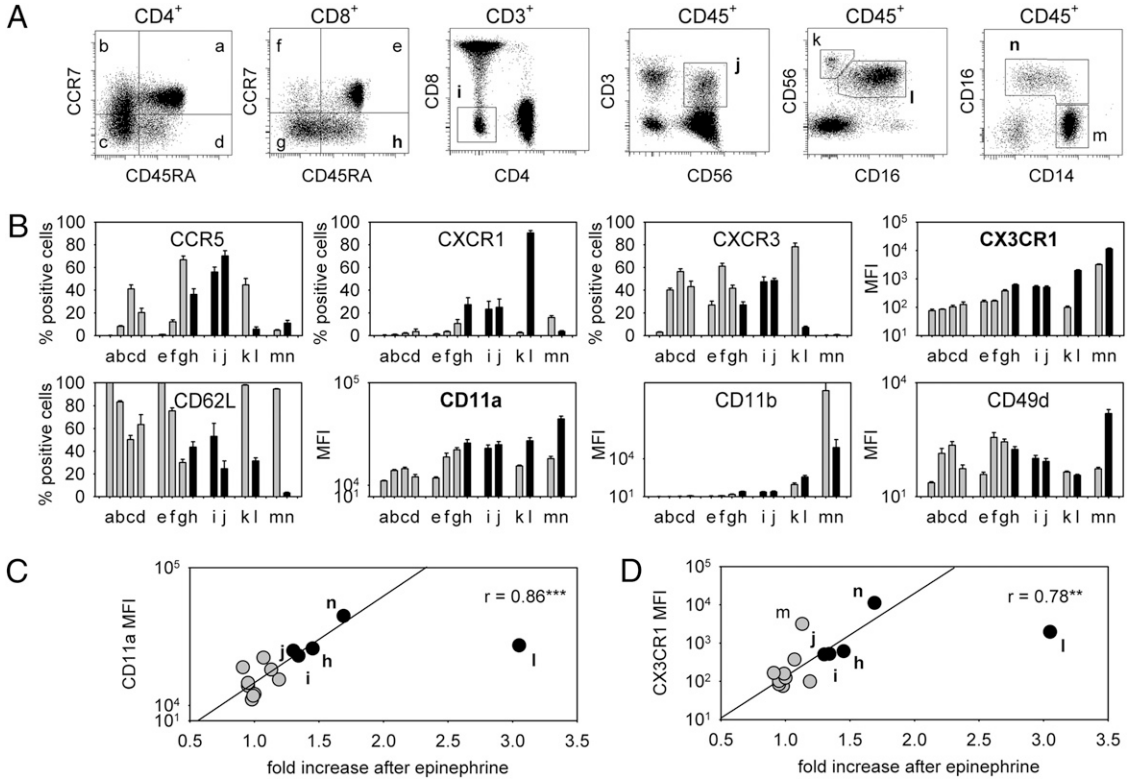

FIGURE 3. Adhesion molecule and chemokine receptor expression on leukocyte subsets. *A*, Representative dotplots of naive ($CCR7^+CD45RA^+CD4^+$ [a]), CM ($CCR7^+CD45RA^-CD4^+$ [b]), EM ($CCR7^-CD45RA^-CD4^+$ [c]), and effector ($CCR7^-CD45RA^+CD4^+$ [d]) Th cells; naive ($CCR7^+CD45RA^+CD8^+$ [e]), CM ($CCR7^+CD45RA^-CD8^+$ [f]), EM ($CCR7^-CD45RA^-CD8^+$ [g]), and effector ($CCR7^-CD45RA^+CD8^+$ [h]) cytotoxic T cells; γ/δ T cells ($CD3^+CD4^-CD8^-$ [i]), NKT-like cells ($CD3^+CD56^+$ [j]), immunomodulatory NK cells ($CD16^-CD56^{bright}$ [k]), cytotoxic NK cells ($CD16^+CD56^{dim}$ [l]), conventional monocytes ($CD14^+CD16^-$ [m]), and proinflammatory monocytes ($CD14^{dim}CD16^+$ [n]). Lowercase letters refer to respective cell subset; subpopulations mobilized by epinephrine in vivo are in bold. *B*, Surface expression of chemokine receptors (CCR5, CXCR1, CXCR3, and CX3CR1), L-selectin (CD62L), and three integrins (CD11a, CD11b, and CD49d). Mean (± SEM) percentages or MFI of cells for respective subset in six to eight healthy donors. Black bars indicate subpopulations mobilized by epinephrine in vivo; *y*-axis for CX3CR1 MFI is log-transformed. *C*, Correlations between CD11a and CX3CR1 expression and increases after epinephrine in vivo across different leukocyte subsets. Filled circles indicate subpopulations mobilized by epinephrine in vivo. For calculating correlation coefficients, cytotoxic NK cells (l) were excluded because of their clearly exaggerated response (3-fold increase) to epinephrine in vivo. $^{**}p < 0.01$, $^{***}p < 0.001$.

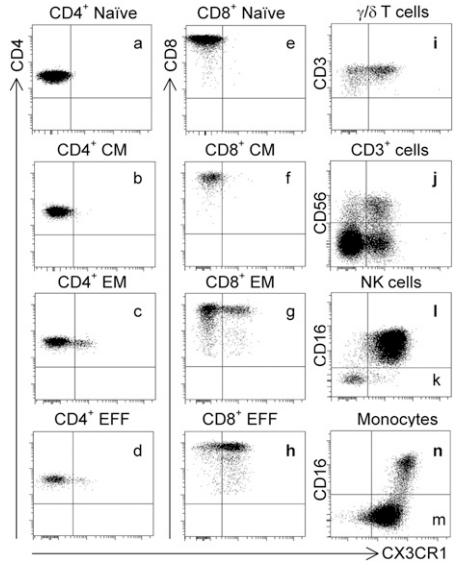

FIGURE 4. Representative examples of cell surface expression of CX3CR1 on leukocyte subpopulations. The lowercase letters indicating subpopulations are the same as in Fig. 3.

naive $CD4^+$ and naive $CD8^+$ T cells (Table I). Unlike naive T cells, proportions of more differentiated T cells with cytotoxic function, namely, EM and effector $CD8^+$ T cells, $CD4^-CD8^-$ γ/δ T cells and NKT-like cells, remained unchanged or were even increased in the adherent fraction (Table I).

Addition of epinephrine to the adhesion assay resulted in clearly reduced percentages of effector $CD8^+$ T cells, $CD4^-CD8^-$ γ/δ T cells, NKT-like cells, and cytotoxic NK cells and an increased percentage of conventional monocytes in the adherent fraction. This diminished adhesion of effector $CD8^+$ T cells, $CD4^-CD8^-$ γ/δ T cells, NKT-like cells, and cytotoxic NK cells in the presence of epinephrine was confirmed both for direct comparisons of absolute numbers of subpopulations in the adherent fraction with and without epinephrine (Fig. 6) and for the coefficients of adherence (indicating the ratio of cell numbers in the attached fraction to the cell numbers prior to adhesion, measured in percentage; see Table I, right two columns, $p < 0.05$ for all four subpopulations). Adhesion of the other T cell subpopulations, immunomodulatory NK cells, and both monocyte subpopulations was not significantly affected.

Discussion

We aimed at a comprehensive characterization of the cell subsets and mechanisms involved in the epinephrine induced mobilization of immune cells in humans, a phenomenon well-known to accompany the organism's response to acute stress. Cell surface markers were used to perform phenotypical analyses of 10 T cells, 2 NK cells, and 2 monocytes subsets that greatly differ in their function. We show that stresslike increases in epinephrine concentrations invoke a specific increase in immune subpopulations with cytotoxic effector potential, namely, effector $CD8^+$ T cells ($CD3^+CCR7^-CD45RA^+$), γ/δ T cells ($CD3^+CD4^-CD8^-$), NKT-like cells ($CD3^+CD56^+$), cytotoxic NK cells ($CD16^+CD56^{dim}$), and proinflammatory monocytes ($CD14^{dim}CD16^+$). We show that these leukocyte subpopulations are characterized by a common adhesion molecule and chemokine receptor profile with a $CD62L^-$ $CD11a^{bright}CX3CR1^{bright}$ phenotype. CD11a and CX3CR1 expression correlates with the degree of adrenergic mobilization.

It has been repeatedly demonstrated that the number of immune cells increases after physical exercise or mental stress, with the most pronounced increases occurring in NK cell and $CD8^+$ cytotoxic T cell counts (3, 5, 16, 34), whereas both populations are decreased when endogenous catecholamines are suppressed by stellate ganglion block (40). These effects are caused by adrenal medullary epinephrine binding to β2-adrenoceptors on PBMCs, with high and intermediate levels of β2-adrenoceptor expression on NK cells and cytotoxic T cells, respectively (1, 3, 7, 8, 19, 33, 41). Our findings substantially expand these previous findings in showing that the epinephrine-induced increase in circulating immune cells is highly specific to cytotoxic effector subtypes, namely, effector $CD8^+$ T cells, γ/δ T cells, NKT-like cells, cytotoxic NK cells, and proinflammatory monocytes, whereas no change is seen for cells lacking any cytotoxic potential, namely, $CD4^+$ T cells, $CD8^+$ T cells at their early stage of differentiation, immunomodulatory NK cells, and conventional monocytes. Consistent with previous observations (3, 16, 25, 42), cytotoxic NK cells show the most pronounced increase after epinephrine.

The epinephrine-induced mobilization of effector $CD8^+$ T cells is consistent with previous studies of increased effector $CCR7^-$/$CD62L^-CD45RA^+$ $CD8^+$ T cell numbers after stress or catecholamine administration (19, 22, 25). These effector $CD8^+$ T cells represent a stage of T cell differentiation occurring late in the immune response, and are hence called terminally differentiated cytotoxic T cells. They do not proliferate in culture and express markers such as the killer cell lectin-like receptor G1 and CD57, defining their replicative senescence. Recent studies indicated a mobilization of $CD8^+$ T cells expressing the killer cell lectin-like receptor G1 and CD57 after physical exercise (25, 43,

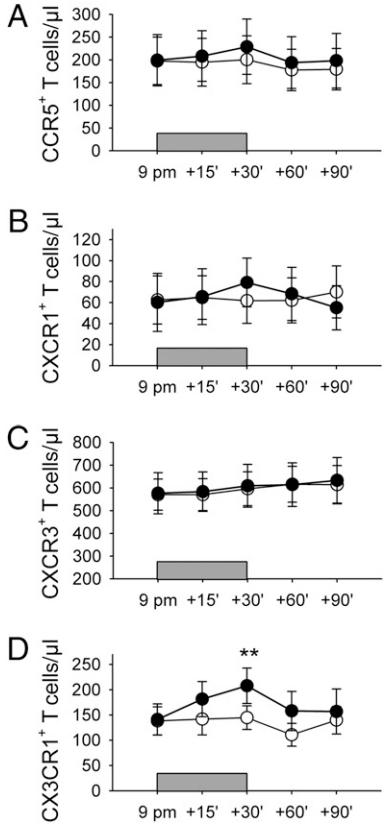

FIGURE 5. Epinephrine selectively mobilizes $CX3CR1^+$ T cells. Mean (± SEM) numbers of (*A*) $CCR5^+$, (*B*) $CXCR1^+$, (*C*) $CXCR3^+$, and (*D*) $CX3CR1^+$ T cells after a 30-min i.v. infusion (horizontal gray bar) of placebo (sodium chloride, open circles) and epinephrine (filled circles); $n = 8$. $^{**}p < 0.01$ for pairwise comparison between epinephrine and placebo conditions.

Table I. *Adhesion of leukocyte subpopulation to activated HUVECs in the absence or presence of epinephrine*

	Subpopulations	Cells Prior to Adhesion %[a]	Adherent Fraction %[b]		Coefficient of Adherence %[c]	
			Medium[d]	Epinephrine[d]	Medium	Epinephrine[d]
	PBMC	—	—	—	11.6 (1.6)	10.7 (1.4)
	T cells	63.3 (2.6)	31.1 (2.5)‡	31.2 (2.6)	5.6 (0.7)	5.1 (0.6)
	Th cells	42.3 (2.8)	15.0 (2.2)‡	16.0 (2.3)	3.6 (0.3)	3.5 (0.4)
a	Naive	17.8 (1.3)	2.1 (0.7)‡	2.5 (0.6)	0.8 (0.4)	0.7 (0.2)
b	CM	13.6 (1.1)	7.3 (1.1)‡	7.6 (1.2)	5.4 (0.4)	5.1 (0.6)
c	EM	5.5 (0.3)	4.4 (0.5)*	4.6 (0.6)	8.1 (0.9)	7.9 (1.3)
d	Effector	3.4 (0.4)	0.5 (0.1)‡	0.6 (0.1)	1.5 (0.4)	1.7 (0.3)
	Cytotoxic T cells	18.1 (2.1)	10.8 (1.6)†	10.7 (1.5)	6.1 (0.6)	5.6 (0.6)
e	Naive	11.1 (1.8)	2.8 (0.4)†	3.1 (0.4)	3.0 (0.5)	2.8 (0.4)
f	CM	3.2 (0.4)	3.2 (0.6)	3.1 (0.6)	9.8 (1.1)	9.1 (1.3)
g	EM	1.6 (0.2)	2.5 (0.4)*	2.4 (0.4)	15.6 (1.8)	14.0 (2.2)
h	Effector	**1.7 (0.4)**	**1.7 (0.4)**	**1.5 (0.4)**†	**12.2 (2.5)**	**9.4 (1.6)***
i	**γ/δ T cells**	**2.5 (0.3)**	**4.1 (0.5)**†	**3.6 (0.4)***	**17.6 (2.2)**	**13.3 (1.4)**†
j	**NKT-like cells**	**2.9 (0.4)**	**3.5 (0.6)**	**2.9 (0.5)***	**13.6 (2.3)**	**10.3 (1.3)***
	NK cells	12.0 (2.0)	31.5 (3.1)‡	28.4 (2.9)†	32.6 (4.4)	27.1 (3.5)†
k	Immunomodulatory	0.6 (0.1)	0.8 (0.1)	0.7 (0.1)	14.9 (1.8)	12.5 (1.4)
l	**Cytotoxic**	**11.3 (1.9)**	**30.8 (3.1)**‡	**27.7 (2.9)**†	**33.5 (4.4)**	**27.9 (3.5)**†
	Monocytes	9.4 (1.1)	24.2 (1.6)‡	27.2 (1.9)*	30.1 (3.5)	31.5 (3.8)
m	Conventional	8.2 (1.0)	19.3 (1.8)‡	21.9 (1.9)*	27.4 (3.4)	29.0 (3.5)
n	**Proinflammatory**	**1.3 (0.2)**	**5.0 (0.5)**‡	**5.3 (0.4)**	**47.6 (3.4)**	**47.5 (6.4)**

Rows show mean (± SEM) of different leukocyte subpopulations. The lowercase letters refer to respective subsets in figures.

[a]Proportion of leukocyte subpopulations from total PBMCs prior to adhesion.

[b]Proportion of leukocyte subpopulations from total cells in the adherent fraction after seeding of 5×10^5 PBMCs on activated HUVECs in the absence (medium) or presence of epinephrine (10^{-8} M, 1832 pg/ml).

[c]Coefficient of adherence indicates the ratio of cell numbers in the attached fraction to the cell numbers prior to adhesion. Note that the subpopulations mobilized by epinephrine in vivo are in boldface type ($n = 7$).

[d]For pairwise comparisons between adherent cells/cells prior to adhesion and between both experimental conditions (medium/epinephrine)

$*p < 0.05$, $^{\dagger}p < 0.01$, $^{\ddagger}p < 0.001$.

44). Effector $CD8^+$ T cells can also express the NK cell marker CD56 that seems to define those cells with high direct cytolytic capacity (42, 45). Hence, the epinephrine-induced increase in $CD3^+CD56^+$ NKT-like cells observed in the current as well as in previous studies (22, 42) might to some extent overlap with a mobilization of effector $CD8^+$ T cells.

Our findings of substantial mobilization of γ/δ T cells after epinephrine likewise agrees with the view of a first line of

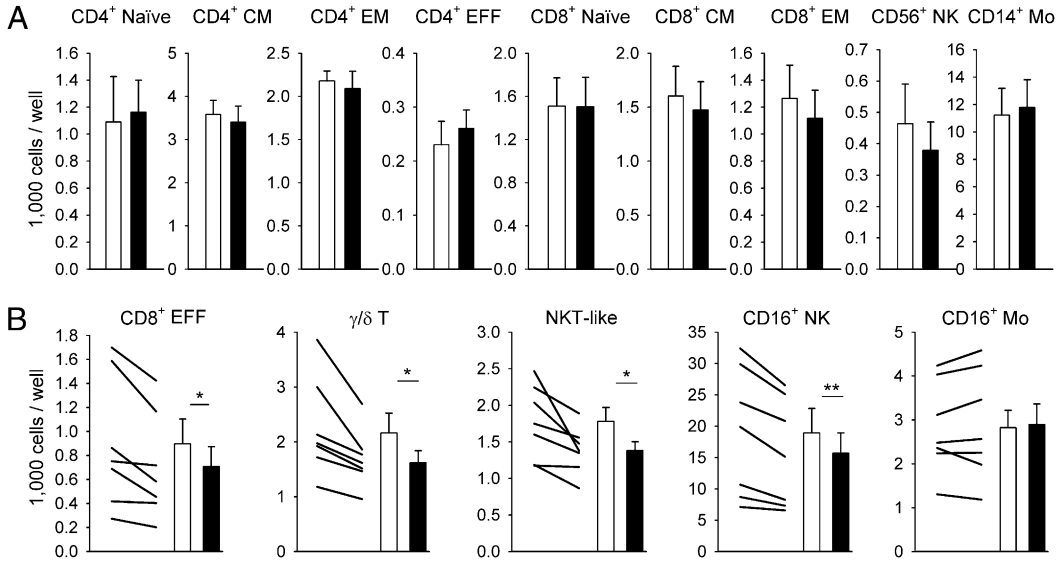

FIGURE 6. Epinephrine selectively decreases adhesion of cytotoxic effector leukocyte subpopulations to activated endothelium in vitro. Mean (± SEM) numbers of (*A*) subsets that were not mobilized by epinephrine in vivo: $CD4^+$ and $CD8^+$ naive ($CCR7^+CD45RA^+$), CM ($CCR7^+CD45RA^-$), EM ($CCR7^-CD45RA^-$), $CD4^+$ EFF ($CCR7^-CD45RA^+$) T cells, immunomodulatory NK cells ($CD56^+$ NK, $CD16^-CD56^{bright}$), conventional monocytes ($CD14^+$ Mo, $CD14^+CD16^-$). *B*, Cytotoxic effector leukocyte subsets that increased after epinephrine in vivo: $CD8^+$ EFF ($CCR7^-CD45RA^+$) T cells, γ/δ ($CD3^+CD4^-CD8^-$) T cells, NKT-like cells ($CD3^+CD56^+$), cytotoxic NK cells ($CD16^+$ NK, $CD16^+CD56^{dim}$), and proinflammatory monocytes ($CD16^+$ Mo, $CD14^{dim}CD16^+$) attached to HUVECs after a 30-min incubation with medium only (open bars) and epinephrine (filled bars). For (*B*) individual values for both experimental conditions are connected by thin lines on the left. $*p < 0.05$, $**p < 0.01$.

immunological defense promoted through the catecholamine (46). $CD3^+CD4^-CD8^-$ γ/δ T cells constitute a unique subset of T cells that exhibit spontaneous non-MHC restricted cytotoxicity. Although their function is not fully understood, they likely serve as an immediate defense against foreign pathogens (47). Moreover, the observed increase in cells after epinephrine administration was restricted to cytotoxic NK cells and proinflammatory monocytes that complements previous studies (16, 20, 21, 24, 25, 42), whereas no change was seen for immunomodulatory NK cells or conventional monocytes. Like effector $CD8^+$ T cells, γ/δ T cells, and NKT-like cells, both cytotoxic NK cells and proinflammatory monocytes are important cytotoxic effector cells in the early immune defense (28, 48–52). Of note, all five subsets with high effector activity produce IFN-γ and TNF-α, show high tissue-migrating potential, and, except for proinflammatoty monocytes, share phenotype signs of senescence ($CD27^-$, $CD28^-$) and high cytotoxicity (granzyme, perforin). In combination, these subsets thus build up an acute defense system whose mobilization on epinephrine release allows for efficient surveillance of tissues and rapid accumulation at sites of injury and infection (22, 25–28, 42, 45, 48, 52–54). In this context, it would be of interest to examine the effect of epinephrine also on other cytotoxic effector leukocytes, for example, $CD8^+$ type 1 NKT cells showing strong antitumor cytotoxicity (54, 55).

The fast mobilization [within 1 min (20)] and recovery of cytotoxic cell numbers in blood that has been observed in this study and in previous studies after stress, exercise, and infusion of catecholamines, reflects the mobilization of the cells from a quite dynamic compartment, namely, the marginal pool that is well-known to house a variety of T cells, NK cells, and monocyte subpopulations (3, 4). Consistent with previous examinations of stress (16), in the current study, peak epinephrine levels of 10^{-10} M induced a 3-fold increase in NK cell numbers. In vitro experiments revealed a clear dose dependency of this NK cell detachment after epinephrine (38), and it becomes likewise evident from a comparison across different in vivo experiments (1, 33, 42). Acute increases in PBMCs to stress and exercise have been consistently revealed to be mediated via epinephrine binding to β2-adrenoceptors, whereas noradrenergic influences and changes in blood flow seem to play minor roles (1–3, 33, 34, 38, 56).

What is the basis for the selectivity of the epinephrine induced increase specifically in cytotoxic cells? We hypothesized that a unique adhesion molecule and chemokine receptor profile make cytotoxic effector cells residents of the marginal pool, and that changes of these molecules via stimulation of β2-adrenergic receptors induce their demargination. In line with previous findings (4, 16, 17, 20, 21, 25, 53, 57, 58), in this study, epinephrine-sensitive cytotoxic cells showed a lack of CD62L and a high density of CD11a. Importantly, however, we revealed that cytotoxic effector cells also selectively express CXCR1 and especially CX3CR1 (26–28, 31, 48, 53, 58). The role of these inflammatory chemokine receptors in adrenergic leukocytosis is currently obscure although particularly CX3CR1 is a very likely candidate mediating demargination of cytotoxic effector cells by catecholamines: CX3CR1 replaces function of selectin as adhesion molecule on $CD62L^-$ cells (27, 30, 53), like all chemokine receptors its stimulation enhances the affinity of integrins such as CD11a (27, 59) and, like the β2-adrenoceptor, it is a G protein–coupled receptor that enables immediate influences of catecholamines on CX3CR1 signaling (60, 61). In fact, β2-adrenoceptors are generally expressed at high levels in cytotoxic effector cell populations like NK cells and effector $CD8^+$ T cells [characterized by a $CD62L^-CD11a^{bright}CX3CR1^{bright}$ phenotype (8, 10)]. Our data add to this view: Epinephrine in vivo mobilized only $CX3CR1^+$ T cells, whereas their $CX3CR1^-$ counterparts remained unaffected. Moreover, CX3CR1 as well as CD11a expression was strongly correlated with the degree of epinephrine-induced mobilization. In summary, cytotoxic effector cell populations show a high expression of β2-adrenoceptors, CD11a and CX3CR1 that correspond to a great capability of these cells to be mobilized by epinephrine. A number of experiments indicates that β2-adrenoceptor-mediated leukocytosis is associated with an increase in intracellular cAMP (3, 38, 41, 62, 63). This second messenger was shown to attenuate chemokine triggered integrin affinity within seconds (60, 61). We are thus tempted to speculate that PBMCs are attached to the endothelium via CX3CR1 triggered adhesive CD11a signaling and become immediately released into the circulation on epinephrine binding.

We applied an in vitro assay with HUVECs to further examine these mechanisms of adrenergic leukocytosis. Earlier studies using this assay revealed distinctly reduced cell adhesion of NK or T cell populations after administration of β2-adrenoceptor agonists (2, 41). Using the long-acting β2-adrenoceptor antagonist, GR81706, it was shown that this phenomenon is mediated via receptors on the leukocyte itself and not on the endothelium (62). Our in vitro experiments indicate that cytotoxic effector leukocytes display the highest adherence to activated endothelium, strongly suggesting that these cell populations form the predominant part of cells in the marginal pool also in the in vivo condition. We further showed that epinephrine selectively inhibits adhesion of effector $CD8^+$ T cells, γ/δ T cells, NKT-like cells, and cytotoxic NK cells to endothelium. Of the cytotoxic cells that are mobilized in vivo by epinephrine, proinflammatory monocytes were the only subset not affected by epinephrine in vitro. This discrepancy may reflect the specific conditions in vitro: 1) the activation of HUVECs and hence higher expression of, for example, intercellular cell adhesion molecule-1 and fractalkine; 2) the isolation of PBMCs in the in vitro assay that possibly leads to a further activation of monocytes and expression of α-adrenoceptors on these cells; 3) the epinephrine concentration that was >30-fold higher than in vivo, and thus possibly costimulated α-adrenoceptors with presumed opposite effects on leukocyte adhesiveness; and 4) the use of a static assay that does not accurately reflect the conditions of flow in vivo (7, 27, 28, 39, 64–66). These differences highlight that conclusions from the in vitro data have to be drawn with caution, as well as the need for additonal studies using different doses of epinephrine and selective α- and β-adrenergic agonists and antagonists.

In summary, we demonstrate that epinephrine in vivo selectively increases numbers of circulating cytotoxic leukocytes, including effector $CD8^+$ T cells, γ/δ T cells, NKT-like cells, cytotoxic NK cells, and proinflammatory monocytes. Except for proinflammatory monocytes, all these cytotoxic cell populations show reduced adhesion to endothelium after epinephrine administration in vitro. Importantly, our in vivo studies used small and short-lasting increases in epinephrine concentrations to establish conditions closely comparable with those observed during acute stress. They cannot be used to infer effects of chronic β-adrenoceptor stimulation as seen, for example, in patients with heart failure where catecholamines seem to exert effects in opposite direction with selectively reduced numbers of circulating NK cells and $CD8^+$ T cells (67, 68). On acute stress, increased adrenal medullary epinephrine release likely serves to selectively recruit $CD62L^-CD11a^{bright}CX3CR1^{bright}$ cytotoxic effector cells as the first line of defense against pathogens.

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Disclosures

The authors have no competing financial interests.

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