

Letter to the Editor

The polyamine spermine promotes survival and activation of human eosinophils

To the Editor:

Allergic rhinitis and most phenotypes of asthma involve accumulation of eosinophils in the airways. The cytokines GM-CSF, IL-5, and IL-3 enhance eosinophil survival and contribute to initiation and maintenance of eosinophilic airway inflammation in asthmatic patients. However, their neutralization in clinical trials only partially depleted airway eosinophilia, suggesting the existence of other relevant life-supporting factors.¹ Spermine, spermidine, and putrescine are ubiquitous cationic polyamines with basic functions related to nucleic acid packaging, DNA replication, apoptosis, transcription, and

translation. Intracellular levels of polyamines are regulated by biosynthesis, catabolism, cellular uptake, and efflux. Polyamine synthesis is initiated by arginase, which converts arginine to ornithine. Putrescine is synthesized from ornithine and further converted to spermidine and spermine.²⁻⁴ Interestingly, arginase activity and polyamine levels are increased in both serum and sputum from asthmatic patients and in experimental models of asthma, suggesting a role in allergic airway diseases.⁴⁻⁶ Hence we aimed to investigate whether polyamines directly regulate human eosinophil survival and activation, focusing on spermine, the most active polyamine.

Eosinophils were isolated from venous blood of eosinophilic donors. For donors' characteristics, see Table E1 in this article's Online Repository at www.jacionline.org, and for the materials

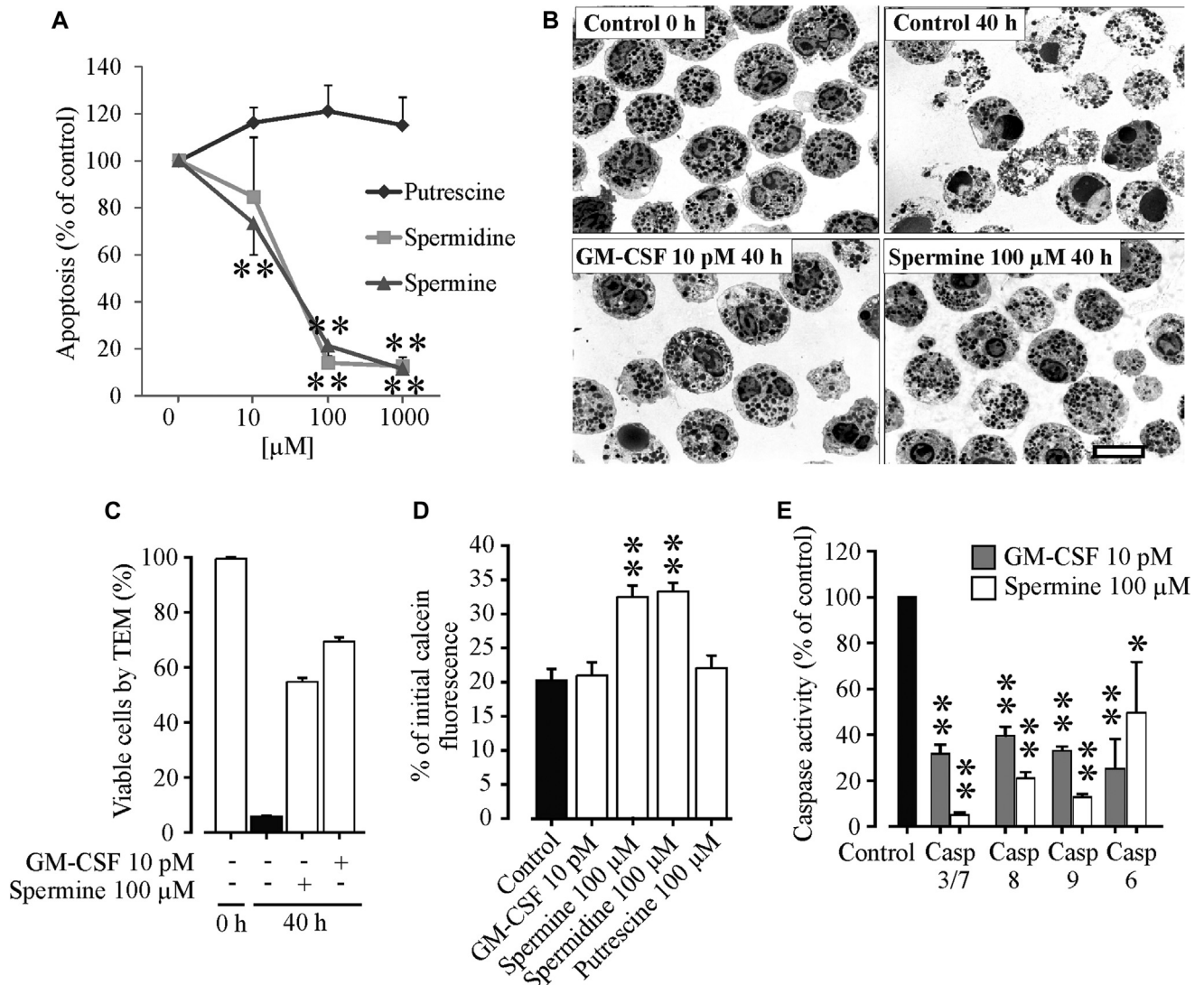


FIG 1. Spermine prolongs eosinophil survival by inhibiting mPT and caspase activity. Incubation times were 40 hours (A), 18 hours (D), and 16 hours (caspases 3/7, 8, and 9) or 40 hours (caspase 6; E). B, Representative figures from transmission electron microscopic analysis are shown. In Fig 1, D, mPT was determined based on calcein/CoCl₂ staining. Shown are means $\pm$ SEMs of 4 to 6 (Fig 1, A and D-E) and 2 (C) experiments, respectively. * $P < .05$ and ** $P < .01$ versus untreated controls.

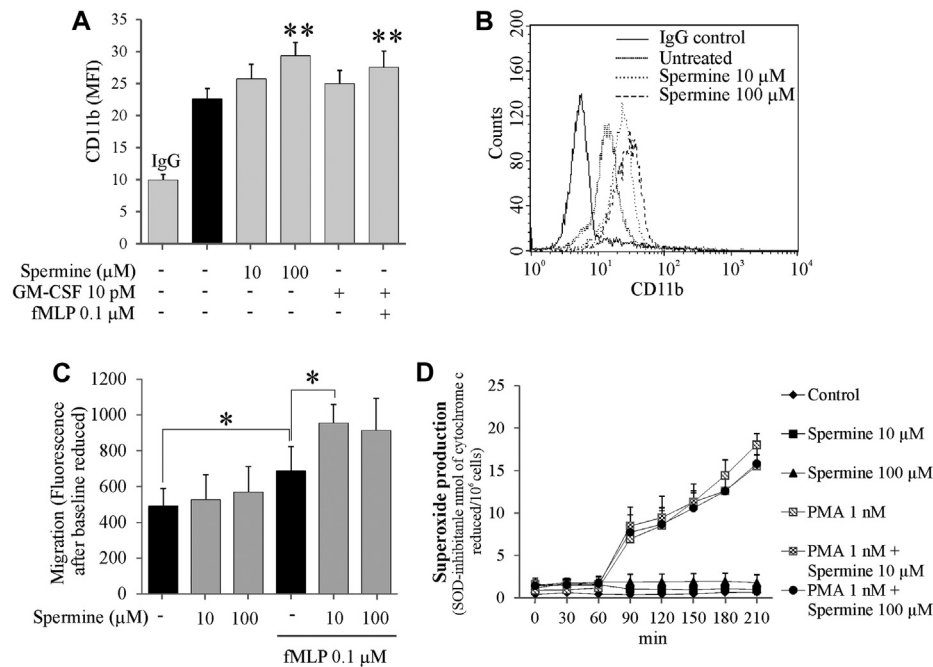


FIG 2. Spermine enhances eosinophil activation. Incubation times were 16 hours (**A** and **B**; fMLP added at the end for 15 minutes) and 2 hours (**C**). In Fig 2, **B**, representative overlaid plots of spermine effect in eosinophils from an asthmatic patient are shown. **D**, Phorbol 12-myristate 13-acetate (PMA) was added at the 1-hour time point. Shown are means $\pm$ SEMs of 6 to 8 experiments. * $P < .05$ and ** $P < .01$ versus control (ie, black bar; Fig 2, **A** and **C**).

and methods used in this study, see the [Methods](#) section in this article's Online Repository at www.jacionline.org.

Spermine and spermidine, but not putrescine, prevented apoptosis-related DNA fragmentation in human eosinophils (Fig 1, **A**). For comparison, the well-known eosinophil survival-prolonging cytokine GM-CSF (10 pmol/L) decreased the number of apoptotic cells to $36.5\% \pm 7.4\%$ of control values ($n = 5$, $P < .05$). Spermine, with the highest number of positive charges, was the most potent polyamine, with a median effective concentration of $15.2 \pm 3.0 \mu\text{mol/L}$ (see Fig E1, **A**, in this article's Online Repository at www.jacionline.org), and was used in further studies.

Analysis of eosinophil morphology by means of bright-field microscopy further suggested that spermine promotes eosinophil survival (see Fig E1, **B**). This observation was confirmed by means of transmission electron microscopic analysis, which clearly showed that both spermine and GM-CSF inhibit apoptosis and increase the number of viable cells (Fig 1, **B** and **C**). Spermine did not cause primary necrosis, as shown in Table E2 in this article's Online Repository at www.jacionline.org. Our preliminary experiments also suggested that spermine inhibits apoptosis in eosinophils both from healthy donors and asthmatic patients (see Fig E2 in this article's Online Repository at www.jacionline.org).

The survival-promoting effects of spermine (median effective concentration = $15 \mu\text{mol/L}$ in the present study) are likely to be pathophysiologically relevant because levels of up to $30 \mu\text{mol/L}$ spermine were found in the blood of asthmatic patients.⁵ Interestingly, studies in mice suggest that the concentration of spermine is higher in the lungs compared with the systemic circulation (lung homogenate, 316 nmol/g [nanomoles per gram $\approx$ micromoles per liter]; blood, $4 \mu\text{mol/L}$).⁷ The present study is the first to show that

polyamines inhibit eosinophil apoptosis. However, their effect on cell death might depend on the cell type² because spermidine was reported to enhance survival of human PBMCs, and that effect was due to reduced necrosis rather than inhibited apoptosis.⁸

Next, we continued by elucidating the antiapoptotic mechanism of spermine. Inhibitors of the kinases Janus kinase 2 (JAK2), phosphoinositide 3-kinase (PI3K), extracellular signal-regulated kinase (ERK), and p38 and the transcription factor nuclear factor κB (NF- κB) did not alter the survival-promoting effect of spermine (see Tables E3-E5 in this article's Online Repository at www.jacionline.org), suggesting that spermine acts through a different mechanism when compared with the survival-prolonging cytokines GM-CSF and IL-5.¹ Spermidine has previously been shown to mediate survival of human blood mononuclear cells by inducing autophagy,⁸ but spermine had no such effect in human eosinophils in the present study (see Fig E3 in this article's Online Repository at www.jacionline.org).

Mitochondrial permeability transition (mPT) is a mechanism mediating the intrinsic form of apoptosis and found to be involved in spontaneous eosinophil apoptosis.⁹ The mitochondrial membrane has been reported to contain 2 binding sites for spermine.² Furthermore, spermine and spermidine, but not putrescine, prevented mPT in isolated mitochondria.² Therefore we tested whether polyamines inhibit mPT in human eosinophils using a method based on calcein acetoxymethyl ester (AM) and CoCl_2 .⁹ Calcein AM fluorescence is quenched by CoCl_2 , which only enters mitochondria with ongoing mPT. Treatment with spermine or spermidine ($100 \mu\text{mol/L}$) clearly increased calcein AM fluorescence in the presence of CoCl_2 when compared with that seen in untreated eosinophils or cells treated with putrescine or GM-CSF (Fig 1, **D**, and see Fig E4 in this article's Online

Repository at www.jacionline.org), indicating that spermine and spermidine, but not putrescine or GM-CSF, inhibit mPT, which is consistent with previous findings in isolated mitochondria.² Additionally, we found that spermine repressed activities of the effector caspases 3/7 and 6 and the initiator caspases 8 and 9 at similar or even greater efficiency when compared with GM-CSF (Fig 1, E). Thus the upstream mechanisms of eosinophil survival induced by spermine and GM-CSF seem to be distinct, but both pathways result in inhibition of caspases.

Spermine has also been shown to regulate immune cell functions in previous studies.¹⁰ Therefore our next aim was to investigate whether spermine, in addition to its survival-promoting effects, is able to activate eosinophils. Indeed, treatment with spermine enhanced cell-surface levels of the adhesion molecule integrin CD11b (Fig 2, A and B). Spermine treatment alone showed no effect on eosinophil migration or superoxide production (Fig 2, C and D), but it increased the chemotactic effect of the bacteria-derived peptide formyl-methionyl-leucyl-phenylalanine (fMLP; Fig 2, C). However, assessment of eosinophil activation based on superoxide production might be limited by the strong stimuli required to trigger this production. Altogether, for the first time, our data show that spermine upregulates integrin expression and stimulates chemotaxis in human eosinophils in addition to the significant survival-promoting effect.

In summary, polyamines are associated with several inflammatory conditions, such as asthma.⁴ The role of polyamines in asthmatic patients is unclear, but they might contribute to airway remodeling and hyperresponsiveness,^{4,6} both of which are conditions in which eosinophils might play a role. The present results introduce spermine as a novel eosinophil survival-prolonging factor that acts by inhibiting cells from apoptosis-related mPT. Furthermore, spermine was found to promote eosinophil activation. These effects are likely to have bearing on all eosinophilic diseases and might also open possibilities for novel therapeutic interventions.

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METHODS

Human eosinophil isolation and culture

Eosinophils were isolated from venous blood (100 mL) of eosinophilic donors (for patients' characteristics, see Table E1). All donors provided written informed consent to the study protocol approved by the Ethics Committee of Tampere University Hospital. Eosinophils were isolated by using immunomagnetic CD16 negative selection under sterile conditions, as previously described.^{E1,E2} The purity of eosinophils after the isolation process was at least 99%. If not otherwise stated, cells were suspended at 10^6 /mL and cultured in Dutch modification of RPMI 1640 containing 10% FBS, antibiotics, and L-glutamine at +37°C in a 5% CO₂ atmosphere for the indicated times.

Apoptosis assays

For transmission electron microscopic analysis, 4×10^6 eosinophils/mL were cultured in the presence or absence of spermine or GM-CSF for the indicated times and fixed by adding formaldehyde. After 2 hours of incubation at room temperature, the cells were centrifuged at 470g for 10 minutes at room temperature, and the supernatant was discarded. The cells were suspended in 1.5 mL of electron microscopy fixative and stored at +4°C until transmission electron microscopic analysis was performed, as described previously.^{E3}

Flow cytometric analysis of permeabilized propidium iodide-stained cells was used to determine DNA fragmentation, a specific sign of apoptosis, as previously described.^{E4} Cells with reduced DNA content were considered apoptotic.

For morphologic analysis, eosinophils were spun onto cytospin slides (25g for 5 minutes), fixed in methanol for 15 minutes, and stained with May-Grünwald-Giemsa. Shrunken cells with both nuclear coalescence and chromatin condensation were considered apoptotic.

mPT

mPT was determined by using the MitoProbe Transition Pore Assay Kit (Molecular Probes, Eugene, Ore), a technique based on calcein AM and CoCl₂,^{E5} as previously described.^{E4} Calcein fluorescence is expressed as a percentage of its initial value as follows: (MFI in the presence of calcein AM and CoCl₂/MFI in the presence of calcein AM) $\times$ 100, where MFI is defined as mean fluorescence intensity. Reduced calcein fluorescence indicates mPT pore opening, and increased calcein fluorescence indicates inhibited mPT. Ionomycin was used as a positive control, and calcein fluorescence in the presence of ionomycin (100 μ mol/L) was always less than an MFI of 7, indicating total mPT.

Caspase activity assays

The activities of caspases 3/7, 8, and 9 were determined by using Caspase-Glo 3/7, 8, and 9 Assays (Promega, Madison, Wis), as previously described.^{E2} Caspase 6 activity was determined after 40 hours of incubation by using the Caspase-6 Fluorometric Assay Kit, as instructed by the manufacturer (Assay Designs, Ann Arbor, Mich).

CD11b expression

For detection of CD11b surface expression, cells (10^6 /mL) were incubated with or without spermine or GM-CSF for 16 hours. At the end of incubation, fMLP was added for 15 minutes to the indicated samples (for positive control). Cell-surface expression of CD11b was determined, as previously described.^{E6}

Migration assay

Eosinophils were suspended at 10^6 /mL, and cell migration toward 10% FBS, spermine, and/or fMLP was determined by using the Cytoselect Cell Migration Assay (Cell Biolabs, San Diego, Calif) at 2 hours, as previously described.^{E6} The assay was carried out according to the manufacturer's instructions. Fluorescence was measured from the migrated lysed cells stained with CyQuant GR dye with a microplate reader at 485/535 nm. Baseline

fluorescence (average fluorescence intensity of the wells containing serum-deprived medium) was deducted from the average fluorescence intensities of the other wells.

Superoxide generation

Generation of superoxide anions was determined based on superoxide dismutase (SOD)-inhibitable reduction of cytochrome c levels, as previously described.^{E6} After purification, eosinophils were resuspended at 0.5×10^6 cells/mL in HBSS with 10 mmol/L HEPES. Cytochrome c (100 μ mol/L) and SOD (0.6 μ mol/L) or vehicle and spermine or vehicle was added at the beginning of incubation. Corresponding samples that differed only by the presence or absence of 0.6 μ mol/L SOD were prepared to assess the specific formation of superoxide. Absorbance at 550 nm was read on a microplate reader immediately (0 minutes) and at indicated time points after incubation at +37°C. Phorbol 12-myristate 13-acetate (PMA) was added to the indicated samples at 60 minutes, and measurements continued for 2.5 hours. The amount of superoxide generated was calculated as nanomoles of cytochrome c reduced per million cells by using an extinction coefficient of 2.1×10^{-4} M⁻¹ cm⁻¹. Finally, values from the SOD samples were deducted from the values of corresponding samples without SOD.

Western blotting

Eosinophils were washed with ice-cold PBS and lysed in ice-cold RIPA (radioimmunoprecipitation assay) buffer with a mix of protease inhibitors, as previously described.^{E1} After 15 minutes of incubation at +4°C, lysate was centrifuged at 12,000g for 5 minutes. Supernatant was mixed in SDS loading buffer and boiled for 10 minutes. The same amount of protein was loaded into each well in SDS-PAGE, and Western blotting was conducted, as previously described.^{E1}

Materials

Materials were purchased, as previously described,^{E1,E6} or as follows: spermine, spermidine, propidium iodide, SOD from bovine erythrocytes, cytochrome c from bovine heart, and fMLP (Sigma-Aldrich, Saint Louis, Mo); putrescine (Alexis, L  ufelfingen, Switzerland); anti-CD16 microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany); Cytoselect Cell Migration Assay (Cell Biolabs); fluorescence isothiocyanate-conjugated anti-human CD11b and fluorescein isothiocyanate-conjugated mouse IgG control (EuroBioSciences GmbH, Friesoythe, Germany); the MitoProbe Transition Pore Assay Kit (Molecular Probes, Eugene, Ore); LC3 rabbit polyclonal antibody (Abgent, San Diego, Calif); β -actin (Santa Cruz Biotechnology, Santa Cruz, Calif); rhGM-CSF (R&D Systems, Minneapolis, Minn); Caspase-Glo 3/7, 8, and 9 Assays (Promega); Caspase-6 Fluorometric Assay Kit (Assay Designs); wortmannin, PD98059, PD169316, AG-490, and WHI-P154 (Merck, Darmstadt, Germany); and SB203580 (Tocris Bioscience, Bristol, United Kingdom). Polyamines were dissolved in cell-culture medium or HBSS. All inhibitors and fMLP were dissolved in dimethyl sulfoxide, and similar concentrations of dimethyl sulfoxide (maximum of 1%) were added to the control cultures.

Statistics

Statistics were performed with GraphPad InStat software (GraphPad Software, La Jolla, Calif), and differences between groups were analyzed by using repeated-measures 1-way ANOVA with the Dunnett or Student-Newman-Keuls posttest or by using the paired *t* test. Results are presented as means $\pm$ SEMs, and *P* values of less than .05 were considered significant.

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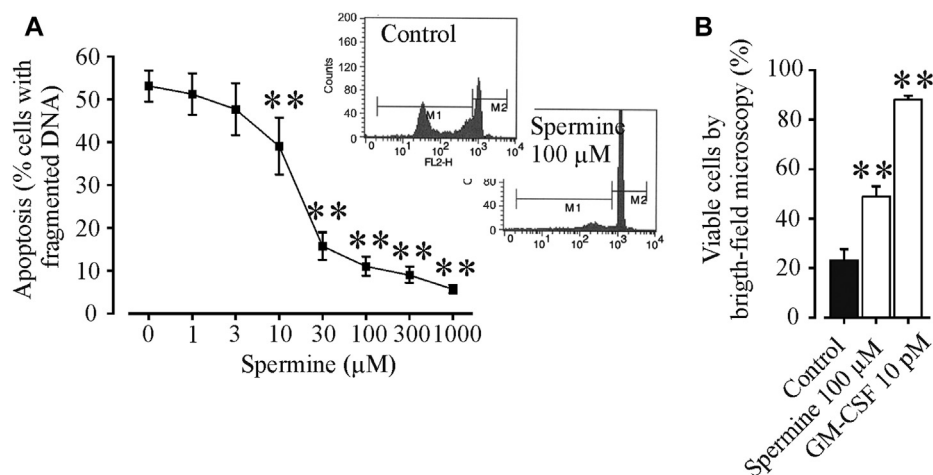


FIG E1. Spermine prolongs eosinophil survival. **A**, Apoptosis-related DNA fragmentation was determined by means of FACS analysis of permeabilized propidium iodide-stained eosinophils. Shown are means $\pm$ SEMs of 6 independent experiments and representative plots from a DNA fragmentation assay. Four of 6 patients had asthma, and 2 of 6 had allergy. **B**, Viability was determined by using bright-field microscopic analysis. Shown are means $\pm$ SEMs of 5 independent experiments. Four of 5 patients had asthma, and 1 of 5 had allergy. $**P < .01$ when compared with untreated control.

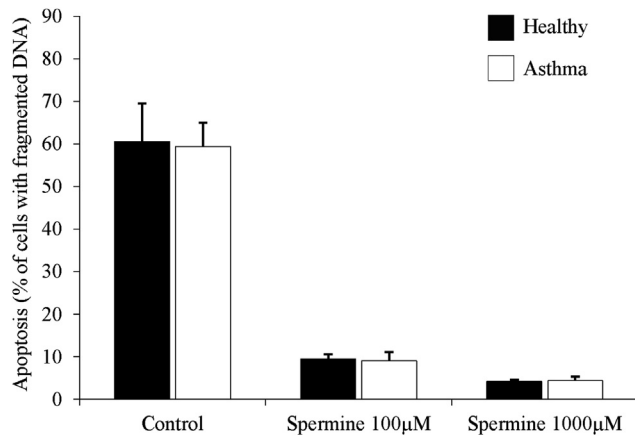


FIG E2. Spermine inhibits apoptosis-related DNA fragmentation in eosinophils from healthy subjects and asthmatic patients. Apoptosis was determined by using a DNA fragmentation assay after 40 hours of incubation. Shown are means $\pm$ SEMs. Experiments were carried out by using blood eosinophils from asthmatic patients ($n = 5$) or healthy subjects ($n = 3$).

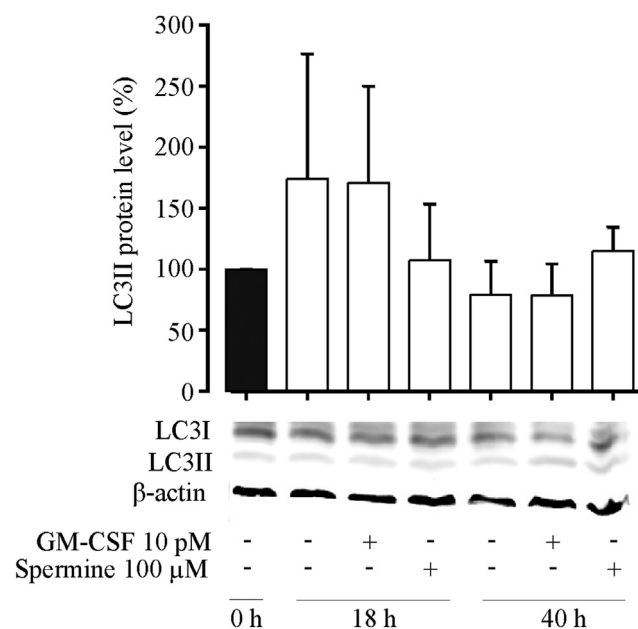


FIG E3. Spermine has no effect on eosinophil autophagy. After indicated periods of incubation, cells were lysed and autophagy was assessed by determining levels of LC3II protein by using Western blotting. LC3II levels were normalized against the untreated 0-hour sample. β-Actin was used as a loading control, and shown are bands of 16 (LC3-I), 14 (LC3-II), and 43 kDa (β-actin). The bars present means $\pm$ SEMs of 4 independent experiments.

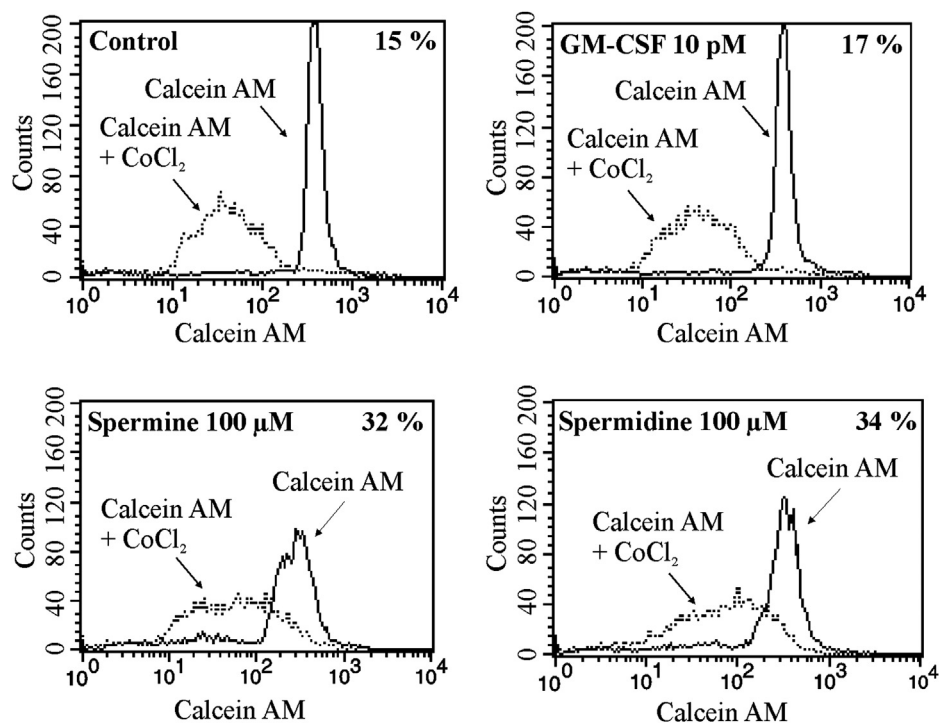


FIG E4. Spermine and spermidine inhibit mPT. Shown are representative overlaid plots of the calcein AM/CoCl₂ assay in which percentages indicate the percentage of initial calcein fluorescence. An increase in calcein AM fluorescence in the presence of CoCl₂ indicates inhibited mPT and *vice versa*.

TABLE E1. Blood donor characteristics

Characteristic	
No. of subjects	43
Mean age (y [range])	46 (22-74)
Sex (no.), male/female	14/29
Health status, no. (%)	
Healthy	8 (19)
Asthmatic*	23 (53)
Use of ICS, no, (%)	16 (70)
Allergic subjects without asthma†	12 (28)

ICS, Inhaled corticosteroids.

*Allergic or nonallergic.

†Patients with allergic rhinitis, allergic rhinoconjunctivitis, or both.

TABLE E2. Effect of spermine on primary necrosis

Cell death from primary necrosis (%)	
Control	1.6 ± 0.3
Spermine, 1 mmol/L	1.7 ± 0.2
GM-CSF, 10 pmol/L	2.1 ± 0.1

After 1 hour of incubation, eosinophils were suspended in HBSS and supplied with propidium iodide (final, 20 µg/mL). After 5 minutes, FACScan analysis was carried out. Eosinophils with heat-induced necrosis (1 hour of incubation at +62°C) were used as positive controls. Cells positive for propidium iodide were considered necrotic. Shown are means ± SEMs of 6 experiments with cells obtained from different donors.

TABLE E3. Effects of inhibitors of Janus kinase (AG-490 and WHI-P154) and phosphoinositide 3-kinase (wortmannin) on spermine-induced eosinophil survival

	Apoptosis (% of cells with fragmented DNA)
Control	62.0 ± 5.1
AG-490, 100 μmol/L	34.1 ± 3.9*
WHI-P154, 100 μmol/L	84.7 ± 3.2*
Wortmannin, 100 nmol/L	59.4 ± 4.0
Spermine, 100 μmol/L	12.2 ± 1.6†
Spermine, 100 μmol/L, plus AG-490, 10 μmol/L	7.9 ± 1.3
Spermine, 100 μmol/L, + AG-490, 100 μmol/L	4.7 ± 0.6
Spermine, 100 μmol/L, plus WHI-P154 10 μmol/L	12.7 ± 3.1
Spermine, 100 μmol/L, plus WHI-P154, 100 μmol/L	18.3 ± 2.9
Spermine, 100 μmol/L, plus wortmannin, 10 nmol/L	12.6 ± 1.6
Spermine, 100 μmol/L, plus wortmannin, 100 nmol/L	14.0 ± 1.9

Eosinophil apoptosis was assessed by means of propidium iodide staining and a DNA fragmentation assay after 40 hours of incubation. Shown are means ± SEMs of 6 experiments conducted with cells from different donors.

* $P < .01$ and

† $P < .001$ versus control cells in the absence of spermine.

TABLE E4. Effect of inhibitor of the nuclear factor κ B pathway (BMS-345541) on spermine-induced eosinophil survival

	Apoptosis (% cells with fragmented DNA)
Control	66.4 $\pm$ 6.2
BMS-345541, 10 μ mol/L	89.9 $\pm$ 1.9*
Spermine, 100 μ mol/L	2.7 $\pm$ 0.4*
Spermine, 100 μ mol/L, plus BMS-345541, 10 μ mol/L	4.8 $\pm$ 0.8
GM-CSF, 10 pmol/L	20.0 $\pm$ 5.0*
GM-CSF, 10 pmol/L, plus BMS-345541, 10 μ mol/L	80.5 $\pm$ 3.0†

Eosinophil apoptosis was determined by using a DNA fragmentation assay. After 40 hours of incubation, cells were permeabilized and stained with propidium iodide. Flow cytometric analysis was carried out. Shown are means $\pm$ SEMs of 6 experiments conducted with cells from different donors.

* $P < .01$ compared with control cells.

† $P < .001$ compared with cells treated with GM-CSF.

TABLE E5. Effects of inhibitors of p38 (SB203580 and PD169316) and the extracellular signal-regulated kinase pathway (PD98059) on spermine-induced eosinophil survival

	Apoptosis (% of cells with fragmented DNA)
Control	49.9 ± 0.7
SB203580, 10 μmol/L	52.0 ± 4.5
PD169316, 1 μmol/L	55.3 ± 1.6
PD98059, 10 μmol/L	48.2 ± 0.7
Spermine, 100 μmol/L	6.2 ± 2.1
Spermine, 100 μmol/L, plus SB203580, 10 μmol/L	5.7 ± 2.2
Spermine, 100 μmol/L, plus PD169316, 1 μmol/L	5.9 ± 2.2
Spermine, 100 μmol/L, plus PD98059, 10 μmol/L	6.5 ± 2.3

Apoptosis was assessed by using a DNA fragmentation assay. After 40 hours of incubation, cells were permeabilized and stained with propidium iodide, and flow cytometric analysis was carried out. Shown are means ± SEMs of 3 experiments conducted with cells from different donors.