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## Membrane-associated proteinase 3 on granulocytes and acute myeloid leukemia inhibits T cell proliferation

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### Abstract

Proteinase 3 (P3), a serine protease expressed by myeloid cells, localized within azurophil granules and also expressed on the cellular membrane of polymorphonuclear neutrophils (PMN), is the target of autoimmunity in granulomatosis with polyangiitis (GPA). PR1, an HLA-A2 restricted nonameric peptide derived from P3, has been targeted effectively in myeloid leukemia. We previously showed that overexpression of P3 in chronic myeloid leukemia (CML) induces apoptosis of high-affinity PR1-specific T cells, leading to deletional tolerance and leukemia outgrowth. In this study, we investigated the effect of membrane P3 (mP3)-expressing PMN and acute myeloid leukemia (AML) blasts on the proliferation of CD4 and CD8 T cells in vitro. We demonstrate that mP3-expressing PMN significantly inhibit autologous healthy donor T cell proliferation but does not affect cytokine production in activated T cells, and that this effect requires cell proximity and was abrogated by P3 blockade. This inhibition required P3 enzyme activity. However, suppression was not reversed by either the addition of catalase or the inhibition of arginase I. In addition to P3 blockade, anti-low density lipoprotein receptor-related protein 1 (LRP1) antibody also restored T cells' capacity to proliferate. Lastly, we show dose-dependent inhibition of T cell proliferation by mP3-expressing AML blasts. Together, our findings demonstrate a novel mechanism whereby PMN- and AML-associated mP3 inhibits T cell proliferation via direct LRP1 and mP3 interaction and we identify P3 as a novel target to modulate immunity in myeloid leukemia and autoimmune disease.

### Keywords

Neutrophils; Proteinase 3; T cell proliferation; AML

### Introduction

Polymorphonuclear neutrophils (PMN) are the most abundant leukocytes, accounting for 50–70% of leukocytes in circulating blood. PMN express numerous serine proteases, including proteinase 3 (P3), neutrophil elastase, and cathepsin G (1). These enzymes

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mediate intracellular killing of phagocytosed pathogens at sites of inflammation (1, 2). There is also evidence that PMN-derived serine proteases and their products impact the tumor microenvironment, and play an important role in regulating tumor cell proliferation, angiogenesis, and metastasis (3, 4). A number of studies have found increased numbers of PMN in the peripheral blood and tumors of patients with various cancers (5, 6). Intratumoral PMN are associated with poor prognosis in multiple tumor types, including renal cell carcinoma and lung cancer (3).

P3 has been shown to regulate cell proliferation. P3 can stimulate the proliferation of basophils and mast cells (7), and can activate apoptosis in human umbilical vein endothelial cells (8). P3 also plays a role in the autoimmune disease granulomatosis with polyangiitis (GPA), where it serves as an autoantigen (9). Moreover, P3 is overexpressed in AML and CML (10) and has been shown to play a role in leukemogenesis (11).

While P3 has a defined role in innate immunity, GPA, and leukemogenesis, little is known about the effect of P3 on T cells or how this protease might modulate host immune responses to cancer. In the present study, we investigate the effect of mP3-expressing PMN and AML on T lymphocytes in vitro and elucidate the mechanism by which mP3 exerts inhibitory effects on T cells. We show that mP3-expressing PMN inhibit proliferation of T cells via an enzyme-dependent mechanism. Inhibition is dose-dependent and correlates with mP3 expression. AML blasts expressing high level of mP3 inhibit T cell proliferation and such inhibition is abrogated by P3 blockade. Together these data support a new role for membrane-bound P3 expressed on PMN and AML in regulating adaptive T cell immunity.

## Materials and Methods

### Cells and patients

Blood of healthy adult donors was provided as leukocyte concentrates by Gulf Coast Regional Blood Bank and the University of Texas MD Anderson Cancer Center (MDACC) Blood Bank. Patient AML samples were collected after informed written consent was obtained in accordance with the Declaration of Helsinki under protocols approved by the MDACC Institutional Review Board (IRB). Before use, the AML cells were thawed, washed, and recovered by overnight incubation at 37°C in RPMI 1640 medium (Gibco, Grand Island, New York) supplemented with 10% FCS.

### Reagents

Anti-CD3 monoclonal antibody (mAb), anti-CD28 mAb, anti-CD4-APC, anti-CD8-APC-H7, anti-CD16-PE/Cy7, and anti-CD45-PB mAbs were purchased from BD Pharmingen (San Jose, California). Anti-CD4-PB and anti-CD8 APC-Cy-7 mAbs were from Tonbo Biosciences (San Diego, California). Anti-CD177 APC was purchased from AbD serotec (Munich, Germany). Anti-P3 antibodies included MCPR3-2 (Thermo Fisher Scientific, Fremont, California), WGM2 (Abcam Inc., Cambridge, Massachusetts) and human polyclonal cANCA (LSBio, Seattle, Washington). Mouse isotype control was purchased from R and D Systems (Minneapolis, Minnesota). Catalase was purchased from Athens Research and Technology (Athens, Georgia). N-Hydroxy-nor-L-arginine (Nor-NOHA) was

purchased from Cayman Chemical (Ann Arbor, Michigan). CFSE was obtained from Molecular Probes (Eugene, Oregon). Anti-LRP1 mAb was purchased from Thermo Fisher Scientific (Waltham, Massachusetts).

### Isolation and sort-purification of PBMC and PMN

PBMC and PMN were isolated from EDTA-anti-coagulated buffy coats of healthy donors by centrifugation on double-layer histopaque-1077/1119 (Sigma). The erythrocytes in the granulocyte layer were lysed with ammonium chloride buffer. All subsequent steps were performed at 4°C. The purity of the isolated PMN typically was >90% as determined by CD16 staining. Cell sorting of mP3-positive and mP3-negative PMN was done on a BD influx high-speed sorter with use of BD Software (BD Biosciences, Franklin Lakes, New Jersey). Briefly, PMN were stained with anti-CD16-PECy7 (Clone 3G8) for 15 minutes prior to addition of mouse anti-CD177-APC (Clone MEM-166) for 30 minutes, followed by subsequent washing steps. Cells were sorted for CD177<sup>+</sup>CD16<sup>+</sup> and CD177<sup>-</sup>CD16<sup>+</sup> populations to obtain mP3-expressing and mP3-lacking PMN, respectively (12), the purity of the sorted PMN typically was >90%. PBMC were stained with anti-CD4-PE (Clone RPA-T4, Biolegend, San Diego, California) and anti-CD8-APC (Clone RPA-T8, Biolegend) mAbs, then CD4 and CD8 T cells were sort-purified using BD influx high-speed sorter, the purity of CD4 and CD8 cells were >95%.

### Flow cytometric analysis of mP3

All steps were performed at 4°C. A total of 10<sup>6</sup> PMN were fixed with 0.5% paraformaldehyde (PFA) for 10 minutes, washed with PBS containing 1% BSA, and stained with anti-CD16 PEcy7 for 15 minutes prior to addition of mouse anti-P3 mAb (Clone MCPR3-2) conjugated with Alexa 488 or isotype control and anti-CD177 APC for 30 minutes, followed by subsequent washing steps. To exclude dead cells from the analysis, a Live/Dead Fixable Aqua kit (Invitrogen, Carlsbad, California) was used. PMN were gated on live CD16<sup>+</sup> cells. For mP3 expression on AML cells, anti-CD45 (Clone HI30) mAb was used instead of anti-CD16. Blast cells were identified by low to intermediate expression of CD45 (13). Cells were acquired on an LSRFortessa flow cytometer (BD Biosciences, Franklin Lakes, New Jersey). Data were analyzed using FlowJo analysis software (FlowJo, LLC, Ashland, Oregon).

### T cell proliferation

Proliferation of T lymphocytes was measured using CFSE (catalog number V12883, ThermoFisher) dye dilution assay. Human PBMC (10<sup>7</sup> cells per ml) were labeled with CFSE at a final concentration of 2 μM for 5 minutes at 25°C. CFSE-labeling was quenched by incubating cells in RPMI 1640 medium supplemented with 10% FCS for 5 minutes at 37°C. Cells were then washed three times in FCS-containing RPMI-1640 medium. To prevent leakage of intracellular proteinases (14), PMN used in the CFSE assay were fixed for 10 minutes at 4°C with 0.5% PFA unless indicated otherwise. A total of 4 × 10<sup>5</sup> CFSE-labeled PBMC or sort-purified CD8 or CD4 T cells were co-cultured with PMN, sorted mP3-expressing or mP3-lacking PMN at indicated PMN: PBMC ratios, for up to 5 days in the presence of anti-CD3/CD28 (Clone HIT3a and Clone 28.2, respectively) mAbs (5 μg/ml) in a 96-well U-bottom plate. In all proliferation assays, cells were gated on live CD4 and CD8

T cells and CFSE-low cells as a % CD4 or CD8 T cells were reported as % proliferating cells. CFSE-low gate placement was set just below CFSE-labeling level in unstimulated, undivided T cells. T cell apoptosis induced by PMN was determined using Annexin V (eBiosciences, San Diego) staining by flow cytometry on day 4 and day 5 of PBMC/PMN co-cultures at various ratios.

In some experiments, PMN were fixed before incubated with anti-P3 mAb (Clone WGM2) ( $10 \mu\text{g}/10^7$  PMN) for 30 minutes on ice and then added to the cell culture. In other experiments, the proliferation of T cells was assessed by flow cytometry using a BrdU Flow kit (BD Pharmingen). Seventy-two hours after stimulation, bromo-deoxyuridine (BrdU) was added to the co-culture for an additional 16 hours. CFSE-labeled PBMC activated by anti-CD3/CD28 mAbs were co-cultured with pre-fixed allogeneic AML cells at the indicated ratios with or without anti-P3 Ab human polyclonal anti-neutrophil cytoplasmic antibodies (cANCA) (LS-C38480, LSBio) for 5 days. Catalase (1000 U/ml) or nor-NOHA (100  $\mu\text{M}$ ) was added to the cell cultures along with unfixed PMN. Transwell experiments were performed in 96-well plates with 0.4  $\mu\text{m}$  pore size membranes (Corning Incorporated, Corning). Phenylmethane sulfonyl fluoride (PMSF, 329-98-6) and  $\alpha$ 1-Antitrypsin (A-6150) were purchased from Sigma. Elafin (ALX-201-240-C100) was purchased from Enzo (Farmingdale, New York).

Stimulated T cells were collected and stained with fluorochrome-conjugated antibodies against CD4 and CD8 in PBS containing 1% FBS for 30 minutes, then fixed in PBS with 1% PFA. Live/Dead Fixable Aqua kit (ThermoFisher Scientific) was used to exclude dead cells from the analysis. Cells were acquired on a BD LSRFortessa or FACS Canto II flow cytometer. PBMC stimulated with anti-CD3/CD28 mAbs were collected at various time points and stained for LRP1 (Clone 8G1, Calbiochem) surface expression.

### **Intracellular Flow Cytometry assessment of cytokine production**

To assess cytokine production by T cells, stimulated PBMC were incubated with PMN at the indicated ratio. Cells were co-cultured for a total of 16 hours with the addition of brefeldin A at a final concentration of 10  $\mu\text{g}/\text{ml}$  after the initial hour. Cells were washed and surface stained with fluorochrome-conjugated antibodies against CD4 (Clone SK4), CD8 (Clone RPA-T8) (BD bioscience) and Live/Dead Fixable Violet dead cell stain (Invitrogen, UK). Subsequently, the cells were washed, treated with Cytfix/Cytoperm and Perm/Wash (BD Biosciences) according to manufacturer instructions, and then stained intracellularly with IFN- $\gamma$ -FITC (Clone B27, BD biosciences), IL-2-PE (Clone MQ1-17H12) and TNF- $\alpha$ -PerCP-Cy5.5 (Clone Mab11) (both eBioscience, San Diego). Data were acquired on the LSRFortessa flow cytometer (BD Biosciences) and analyzed with FlowJo software.

### **Statistical analysis**

Unpaired *t*-test was used to compare two discrete groups. *P*-values < 0.05 were considered statistically significant. Data analysis was performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, California). Results were expressed as mean  $\pm$  SEM.

## Results

### PMN inhibit T cell proliferation in a dose-dependent manner

Tumor-associated neutrophils (TAN) can mediate broad immunosuppressive effects (3, 15), and the presence of TAN has been associated with poor clinical outcome (3). However, the precise mechanism of TAN-mediated immunosuppression is unknown (16–18). To investigate whether PMN from healthy donors affect T cell proliferation, we co-cultured PMN with autologous anti-CD3/CD28 stimulated peripheral blood mononuclear cells (PBMC) (containing >90% lymphocytes). At a ratio of 3:1 (PMN: PBMC) a significant decrease in proliferating cells was observed (19.5% CD8 and 17.7% CD4, respectively) compared to that seen at the 2:1 ratio (84.6% CD8 and 76.4% CD4, respectively) (Fig. 1A). This inhibition of proliferation was even more pronounced in the 4:1 and 5:1 ratios, establishing a dose-dependent relationship between PMN and PBMC in co-culture. Similar results were obtained from assays performed with nine different donors following co-culture of PBMC with PMN (Fig. 1B). Compared with activated PBMC alone (proliferating CD8 cells,  $90.2 \pm 1.5\%$  and CD4 cells,  $87.1 \pm 2.4\%$ , mean  $\pm$  SEM, respectively), co-incubation of PMN with PBMC at a 3:1 ratio resulted in significant inhibition of proliferation in both CD8 ( $29.2 \pm 9.6\%$  proliferating, mean  $\pm$  SEM) and CD4 ( $23.7 \pm 9.2\%$  proliferating, mean  $\pm$  SEM) T cells. Accordingly, co-incubation of PBMC with PMN decreased the percentage of T cells in S phase, while increasing the fraction of T cells in G<sub>2</sub>/M phase in a BrdU incorporation assay (Supplemental Fig. 1). We used fixed PMN in most assays to prevent leakage of intracellular proteinases, experiments were also carried out using unfixed PMN to reflect the physiological and pathological conditions (Fig. 1C). Unfixed PMN mediated dose-dependent inhibition of T cell proliferation similar to the results obtained with fixed PMN (Fig. 1B).

### Co-incubation of PBMC with PMN does not affect their cytokine production

Next, we used intracellular cytokine flow cytometry to investigate cytokine production of IFN- $\gamma$ , TNF- $\alpha$ , and IL-2 by T cells in response to co-culture of PMN. Cytokine profiles illustrated in Figure. 2A and 2B revealed upon stimulation with anti-CD3/CD28 mAbs the percentage of IFN- $\gamma$ -, TNF- $\alpha$ -, or IL-2-producing cells were as  $20.1 \pm 2.4\%$ ,  $9.4 \pm 2.6\%$  and  $13.7 \pm 5\%$ , mean  $\pm$  SEM, for CD8 and  $26.3 \pm 3.4\%$ ,  $17.3 \pm 3.3\%$  and  $5.9 \pm 0.7\%$ , mean  $\pm$  SEM, for CD4, respectively. As compared with T cells stimulated with anti-CD3/CD28 mAbs alone, co-culture of PMBC with PMN did not significantly change the percentage of IFN- $\gamma$ -, TNF- $\alpha$ -, or IL-2-producing CD8 and CD4 cells. A representative dot plots of IFN- $\gamma$ -, TNF- $\alpha$ -, or IL-2-producing CD8 (Fig. 2C) and CD4 cells (Fig. 2D) stimulated with or without anti-CD3/CD28 mAbs or co-cultured with PMN at 5:1 (PMN: PBMC) ratio are shown. These data suggest that PMN co-culture inhibits T cell proliferation but does not affect cytokine production in activated T cells.

### PMN-mediated inhibition of T cell proliferation is not associated with production of reactive oxygen species (ROS) or depletion of L-arginine

PMN are reported to inhibit T cell proliferation through the production of ROS and the depletion of L-arginine by the PMN-associated enzyme arginase I (4, 19, 20). To determine the role, if any, played by ROS and arginase I in PMN-mediated suppression of T cell

proliferation, we treated unfixed PMN with inhibitors in our co-culture system. Briefly, CFSE-labeled, anti-CD3/CD28 activated PBMC were co-cultured for 5 days with PMN alone, or with PMN treated with either catalase, a ROS inhibitor, or with nor-NOHA, an inhibitor of arginase I. Neither the addition of catalase nor nor-NOHA prevented the PMN-mediated suppression of T cell proliferation (Supplemental Fig. 2). The addition of catalase or nor-NOHA to PBMC alone had no effect on T cell proliferation induced by anti-CD3/CD28. Our data suggest that the inhibition of T cell proliferation after co-culture with freshly isolated PMN is not mediated by ROS or depletion of L-arginine.

### **PMN inhibit T cell proliferation in a cell contact-dependent manner**

Next we asked whether cell-cell contact was required for PMN-mediated T cell suppression. We utilized a transwell system with anti-CD3/CD28 stimulated PBMC in the lower compartment and PMN in the upper compartment, physically separated from the lower compartment by a 0.4  $\mu$ m membrane. As shown in Figure 3A, co-culture of PMN with PBMC at a 4:1 ratio significantly decreased proliferation of CD8 and CD4 T cells by  $82.3 \pm 7.2\%$  and  $85 \pm 4.3\%$ , mean  $\pm$  SEM, respectively, whereas PMN-mediated inhibition of T cell proliferation was merely  $8.5 \pm 1.9\%$  for CD8 and  $15.8 \pm 2.9\%$  for CD4 (mean  $\pm$  SEM) in the transwell culture. There was no significant soluble P3 detected in the culture media (data not shown). These data demonstrate that cell contact is required for maximal T cell inhibition by PMN. Representative histogram plots of CFSE profile are shown in Figure. 3B. Similar results were obtained when unfixed PMN were used (data not shown).

### **PMN-mediated T cell inhibition is prevented by anti-P3 blocking antibody and requires P3 enzyme activity**

We previously showed that high expression of P3 by CML selectively deletes high avidity PR1-cytotoxic T lymphocytes (CTLs) (21) and AML and CML patients had higher levels of soluble P3 in their serum compared to healthy donors (22). We examined if mP3 negatively regulates T cell proliferation, this inhibition could be blocked with an anti-P3 antibody. PBMC stimulated with anti-CD3/CD28 mAbs were co-cultured with PMN in the presence of anti-P3 mAb or isotype control. Compared to significant inhibition of T cell proliferation observed ( $85 \pm 2.7\%$  for CD8 and  $81 \pm 4.1\%$  for CD4, mean  $\pm$  SEM, respectively) at 3:1 ratio of PMN: PBMC, addition of anti-P3 antibody resulted in significant reduction in inhibition for CD8 ( $53 \pm 12.9\%$ , mean  $\pm$  SEM) and CD4 ( $54 \pm 12.3\%$ , mean  $\pm$  SEM) T cells, respectively (Fig. 4A). The anti-P3 antibody significantly reduced PMN-mediated suppression of T cell proliferation while isotype control showed no effect. These data indicate that membrane-bound P3 contributes to the inhibition of T cell proliferation by PMN. Representative histogram plots are shown in supplemental Figure 3.

It has been shown that fixation process does not affect catalytic activity of membrane-bound serine proteinases (14, 23). To determine whether the protease activity of mP3 was required for the inhibition of T cell proliferation, the serine proteinase inhibitors PMSF, Elafin, and  $\alpha$ 1-Antitrypsin (AAT) were used. In the presence of PMSF, at a ratio of 4:1 (PMN: PBMC), PMN-mediated inhibition of T cell proliferation was largely abrogated ( $69.3 \pm 3.1$  and  $62 \pm 3.5\%$  reduction in inhibition for CD8 and CD4, mean  $\pm$  SEM, respectively) (Fig. 4B). Added at large molar excess (14), Elafin, a reversible inhibitor for proteinase 3, resulted in a



14 ± 7.1% and 11.3 ± 4.5% reduction in inhibition in CD8 and CD4 T cells, respectively while AAT had no effect on the inhibitory activity of PMN (Fig. 4B). The result with Elafin and AAT is consistent with a previous report demonstrating that mP3 is not readily inhibited by circulating physiological inhibitors such as AAT (14). The addition of the inhibitors alone to PBMC did not have any effect on CD4 or CD8 T cell proliferation (data not shown). Taken together, these data demonstrate P3 enzyme dependence of the PMN-mediated anti-proliferative effect.

### mP3-expressing PMN mediate inhibition of T cell proliferation

Because the GPI-anchored CD177 (NB1) membrane protein and mP3 were co-expressed on the same subset of PMN (12) (Supplemental Fig. 4), we used an anti-CD177 mAb to sort PMN (98% purity) into mP3-expressing and mP3-lacking populations to confirm that T cell inhibition by PMN was mediated by mP3. PMN were fixed before sorting to prevent activation-induced changes in mP3 expression during cell sorting. Compared with activated PBMC alone, proliferation of T cells was significantly reduced by 57.6 ± 4.2% and 56.1 ± 2.8% (mean ± SEM) for CD8 and CD4, respectively in the presence of CD177-expressing PMN at a 3:1 PMN: PBMC ratio (Fig. 5A). A significant reduction in the inhibition of T cell proliferation was observed when the same ratio of CD177-negative PMN was added, with only a 29.7 ± 7.2% and 26.2 ± 7.8% (mean ± SEM) reduction in CD8 and CD4 T cell proliferation, respectively. The less suppression might be due to low-level mP3 expression on CD177-negative PMN (24), (Supplemental Fig. 4). Maximum inhibition of T cell proliferation observed in the CD177-expressing PMN co-cultures further supports a role for mP3 in mediating T cell inhibition. Representative CFSE profiles of CD8 and CD4 T cells are shown (Fig. 5B). To determine whether mP3-expressing PMN-mediated inhibition of T cell proliferation is sustained after removal of PMN, sorted CD177-expressing PMN were co-cultured with anti-CD3/CD28 activated, CFSE-labeled PBMC from healthy donors at a ratio of 3:1 for 24 hours. PMN were then depleted using anti-CD16 beads and PBMC cultures were continued for a total of 5 days in the presence of anti-CD3/CD28 mAbs. The proliferation of CD8 and CD4 T cells was restored following PMN depletion, demonstrating that PMN-mediated suppression of T cell proliferation was not permanent (data not shown).

We then performed a nonlinear regression analysis to determine the correlation between the number of mP3-expressing PMN as measured by flow cytometry and the percent inhibition of T cell proliferation in CFSE assays in 7 healthy donors (Fig. 5C). A sigmoidal function fit of the data resulted in a lower sum of residual errors ( $R^2 = 0.72$  or  $R^2 = 0.62$ , for CD8 and CD4 T cells, respectively) compared with a linear fit ( $R^2 = 0.65$ , or  $R^2 = 0.55$ ,  $p < 0.0001$ ). This suggests that there is a threshold effect of mP3-expressing PMN-mediated inhibition of T cell proliferation at a ratio of approximately 2.2. This interpretation also suggests that the inhibitory effects of mP3-expressing PMN would be operative at sites of acute inflammation, where the ratio of PMN to PBMC is above this threshold, but not in peripheral blood during normal homeostatic conditions where the ratio is typically 2.2 (25).

### LRP1 blockade prevents PMN-mediated inhibition of T cell proliferation

MP3 co-expresses with calreticulin (CRT) on apoptotic PMN. Their association on PMN disrupts binding of CRT to LRP1 on macrophages and impairs CRT-LRP1-mediated phagocytosis (26). Moreover, LRP1 was shown to involve regulating T cell motility (27). Therefore we sought to determine whether mP3-expressing PMN-mediated T cell inhibition involved LRP1 on T cells. Flow cytometry analysis revealed expression of LRP1 increased upon stimulation with anti-CD3/CD28 mAbs. Up-regulation of LRP1 surface expression was most significant on day 2 on the surface of activated CD8 (mean= 3.4 fold) and CD4 (mean= 4.8 fold) T cells compared with un-stimulated CD8 and CD4 T cells (Fig. 6A). Next, we examined the role of LRP1 in PMN-mediated inhibition of T cell proliferation. PMN were added in a 3:1 ratio to CFSE-labeled PBMC stimulated with anti-CD3/CD28 mAbs in the presence of anti-LRP1 mAb or an isotype control mIgG for 5 days. Blockade of LRP1 significantly reduced PMN-mediated inhibition of CD8 and CD4 T cells when compared with PBMC cultured with PMN (Fig. 6B) ( $33.1 \pm 9.7\%$  versus  $88.4 \pm 3.9\%$  and  $29.5 \pm 13.1\%$  versus  $84.5 \pm 5.4\%$  for CD8 and CD4, mean  $\pm$  SEM, respectively). Isotype control did not affect inhibitory effect of PMN. In order to exclude the possible indirect or direct role of non-T cells, especially monocytes comprised in PBMC, we performed co-culture experiments with sort-purified CD8 and CD4 T cells from PBMC (Fig. 6C). Compared with significant inhibition of T cell proliferation observed ( $80.2 \pm 0.1\%$  for CD8 and  $73.7 \pm 3.2\%$  for CD4, mean  $\pm$  SEM, respectively) at 3:1 ratio of PMN: T cells, addition of anti-LRP1 mAb resulted in significant reduction in inhibition for CD8 ( $30 \pm 0.4\%$ , mean  $\pm$  SEM) and CD4 ( $38.5 \pm 0.7\%$ , mean  $\pm$  SEM) T cells, respectively. These results are comparable to the results obtained using PBMC as the source of T cells. This demonstrates a role for LRP1 in PMN-mediated inhibition of T cell proliferation, possibly through the interaction between LRP1 on activated T cells and mP3 on PMN.

### MP3<sup>+</sup> AML mediates inhibition of T cell proliferation and proliferation is restored via P3 blockade

Next, we sought to examine whether primary AML blasts suppressed T cell proliferation in a manner analogous to mP3-expressing PMN. Because P3 is co-expressed with CD177 on the membrane of resting PMN, we first examined whether co-expression also occurs on primary AML blasts. The level of mP3 expression varied greatly between AML blasts from 19 patients we investigated and the fraction of mP3-expressing blasts correlates with the MFI of mP3 on AML blasts (Fig. 7A). In contrast to the co-expression on PMN from healthy donors mP3 expression was not associated with CD177 on AML. Figure 7B shows representative flow cytometry plots of blasts from 5 AML patients and PMN from 1 healthy donor. We further investigated the impact of AML blasts on T cell proliferation by comparing mP3-high (AML#4) with mP3-low (AML#3) both contained >90% blast cells as documented in patients' medical records. Before co-cultured with CFSE-labeled PBMC in the presence of anti-CD3/CD28 mAbs, allogeneic AML blasts were fixed with 0.5% PFA for 10 minutes at 4°C to prevent T cell-activation (less than 5% T cells present within the samples) and to keep in line with the treatment of PMN. Proliferation of CD8 and CD4 T cells co-cultured with mP3-high AML was significantly inhibited while the percentage of proliferating CD8 and CD4 T cells co-cultured with mP3-low AML was comparable to PBMC stimulated with anti-CD3/CD28 mAbs alone (Fig. 7C). As expected, anti-P3 Ab



significantly restored T cells' capacity to proliferate. In conclusion, mP3-high AML demonstrated suppression of T cell proliferation, which could be abrogated by P3 blockade.

## Discussion

In this report, we demonstrate that cell-associated P3 has inhibitory properties on T cells. Specifically, mP3-expressing PMN inhibited T cell proliferation, which was abrogated through mP3 blockade. Co-incubation of sort-purified mP3-expressing PMN with PBMC at a ratio of 3:1 significantly inhibited proliferation of both CD4 and CD8 T cells, whereas mP3-dim/low PMN had a less potent effect. The inhibitory effect of PMN-associated mP3 was associated with altered T cell cycle kinetics. Gabillet et al. (26) showed that LRP blockade decreased the phagocytosis of apoptotic neutrophils from healthy donors with PMN co-expressing P3 and CRT on the membrane, but not from patients with GPA, who possess anti-P3 antibodies that interfere with the CRT-LRP interaction. This suggests that a direct association between P3 and LRP (or LRP-containing complexes) might occur (26). In support of this hypothesis, we showed that LRP1 blockade effectively prevented suppression of T cells by mP3-expressing PMN. This indicates a potential mechanism for the inhibition of T cells that relies on the interaction between mP3 on PMN and LRP1 on activated T cells. We also showed that AML blasts inhibited T cell proliferation, and that this effect was dependent on the expression of mP3 by AML. These findings provide one mechanism by which myeloid leukemia can evade the anti-leukemia T cell immune response. Taken together, our data suggest that P3 plays an important role in the regulation of adaptive immune responses by innate immunity.

Our data further indicate that PMN-mediated inhibition of T cell proliferation is dependent on the enzymatic activity of mP3. Though ROS and arginase I have been implicated in PMN-mediated T cell suppression (28), our data suggest that PMN-mediated inhibition of T cell proliferation is independent of these molecules. It is possible, though, that the discrepancy between our results and others are due to differences in the condition of PMN since activated PMN were used in other studies (20, 29, 30). Furthermore, our data indicate that the impact of mP3 on T cells is distinct from P3-mediated effects on other cell types, including endothelial cells (31, 32). For example, the catalytically inactive form of P3, as well as the active enzyme, was shown to induce P3-mediated apoptosis of endothelial cells. Of note, PMN-associated mP3 inhibited T cell proliferation but did not induce T cell apoptosis in our assays, therefore the mP3-mediated inhibition of T cell proliferation described here is distinctive from Indoleamine 2,3-dioxygenase (IDO) pathway that inhibits proliferation of T cells and induces T cell apoptosis (33).

Clinical studies have demonstrated that the presence of P3 within the tumor microenvironment is associated with poor clinical outcomes, including reduced survival, further supporting the immunosuppressive role of P3 (34). Padrines et al. (35) showed that P3 enhances the biological activity of IL-8 by cleaving the N-terminal domain, suggesting that P3 may play a role in potentiating the pro-tumor angiogenic properties of IL-8, an effect that has been demonstrated in human lung cancer (36). In addition, increased PMN were shown to be a poor prognostic factor for progression free survival (PFS) and overall survival (OS) in advanced-stage colorectal adenocarcinoma and melanoma (16–18). Here, we

showed that mP3 on PMN inhibited T cell proliferation at a low concentration, estimated at 7–8 nM (14), which suggests that mP3 may play an important role in regulating T cell immunity in inflammatory physiological conditions, including inflammation associated with malignant and autoimmune disease. Our data further suggest that in homeostatic conditions, when mP3 is constitutively expressed on a subset of PMN, there is a threshold effect that dictates the number of mP3-expressing PMN that are required to cause T cell inhibition (Fig. 5C).

Our data also suggest a mechanism by which autoimmunity may be regulated in GPA. Since cANCA are known to bind P3 and correlate with disease activity in GPA (37, 38), our data suggest that blockade of P3 by cANCA abrogates the physiologic inhibitory effect of P3 on T cells, thereby contributing to the autoimmune manifestations of GPA. This is in line with the observation that increased mP3 expression in GPA impairs phagocytosis of apoptotic neutrophils thus leading to proinflammatory response (26). Taken together, these findings suggest a complex role for mP3 on PMN whereby P3 interacts with LRP1 on macrophages leading proinflammatory responses or via LRP1 on T cells resulting in inhibition thus averting inflammatory responses. It's a balancing act of P3 depending on the types of cells involved.

We previously reported that serum P3 is increased in AML and CML patients compared with healthy donors (22). This finding is consistent with prior report showing increased P3 expression by leukemia cells from patients with AML or CML (10). P3 was shown to play a role in leukemogenesis (39), and our findings suggest an additional leukemia-promoting effect of P3 via inhibition of the anti-leukemia T cell immune response. Importantly, we found that mP3-high AML inhibited the proliferation of T cells, an effect that could be prevented via P3 blockade. These results provide the first direct evidence that AML can inhibit T cell proliferation via membrane-bound P3, demonstrating one potential mechanism by which AML escapes T cell immunity. Such inhibition of T cell proliferation could shape patient outcomes after immune-based therapies such as allogeneic stem cell transplantation (aSCT) or vaccines.

Although our data indicate an inhibitory role for P3 on adaptive immune responses, previous reports suggested that P3 enhances adaptive immunity. For example, P3 was shown to induce dendritic cell maturation via protease-activated receptor-2 in patients with GPA, an effect hypothesized to promote autoimmunity (40). In addition, P3 can bind and activate IL-32, a recently discovered proinflammatory cytokine that has emerged as an important player in the innate and adaptive immune responses (41). Furthermore, we previously showed that P3 uptake by solid tumors enhanced their killing by PR1-CTL via cross-presentation (42). In the context of our current study, these reports emphasize the varying effects of P3 in different physiologic and pathologic states. The differences in the immune outcomes mediated by P3 could be determined by the cell type on which P3 exerts its effects and the other cytokines and proteases that are present within the tissue microenvironment. Moreover, P3 may be an alarmin (43) since it regulates the innate immune response (44–47), and our study expands on its role as an immune regulator by showing that it affects the adaptive immune response, specifically T cells.

In conclusion, our findings suggest that leukemia and neutrophil-associated P3 down-regulate T cell activity by inhibiting T cell proliferation. These findings are highly important in expanding our understanding of the biology of immune regulation in leukemia and other cancers, as well as in autoimmune disease. These results suggest that a novel therapeutic approach targeting P3 may be an effective immunotherapy for the treatment of leukemia and autoimmune disease.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## References

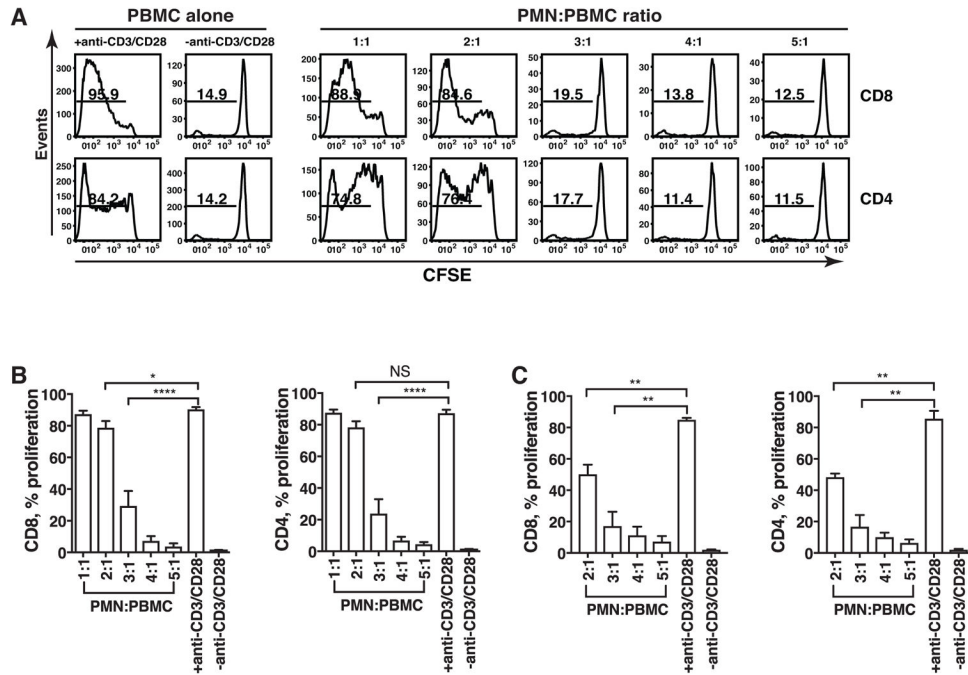
1. Pham CT. Neutrophil serine proteases fine-tune the inflammatory response. *Int J Biochem Cell Biol.* 2008; 40:1317–1333. [PubMed: 18180196]
2. van der Geld YM, Limburg PC, Kallenberg CG. Proteinase 3, Wegener's autoantigen: from gene to antigen. *J Leukoc Biol.* 2001; 69:177–190. [PubMed: 11272267]
3. Houghton AM. The paradox of tumor-associated neutrophils: fueling tumor growth with cytotoxic substances. *Cell Cycle.* 2010; 9:1732–1737. [PubMed: 20404546]
4. Muller I, Munder M, Kropf P, Hansch GM. Polymorphonuclear neutrophils and T lymphocytes: strange bedfellows or brothers in arms? *Trends in immunology.* 2009; 30:522–530. [PubMed: 19775938]
5. Dumitru CA, Moses K, Trellakis S, Lang S, Brandau S. Neutrophils and granulocytic myeloid-derived suppressor cells: immunophenotyping, cell biology and clinical relevance in human oncology. *Cancer immunology, immunotherapy : CII.* 2012; 61:1155–1167. [PubMed: 22692756]
6. Powell DR, Huttenlocher A. Neutrophils in the Tumor Microenvironment. *Trends in immunology.* 2016; 37:41–52. [PubMed: 26700397]
7. Witko-Sarsat V, Canteloup S, Durant S, Desdouets C, Chabernaud R, Lemarchand P, Descamps-Latscha B. Cleavage of p21waf1 by proteinase-3, a myeloid-specific serine protease, potentiates cell proliferation. *J Biol Chem.* 2002; 277:47338–47347. [PubMed: 12354776]
8. Pendergraft WF 3rd, Rudolph EH, Falk RJ, Jahn JE, Grimmmer M, Hengst L, Jennette JC, Preston GA. Proteinase 3 sidesteps caspases and cleaves p21(Waf1/Cip1/Sdi1) to induce endothelial cell apoptosis. *Kidney Int.* 2004; 65:75–84. [PubMed: 14675038]
9. Kallenberg CG. Pathogenesis of PR3-ANCA associated vasculitis. *J Autoimmun.* 2008; 30:29–36. [PubMed: 18162369]
10. Dengler R, Munstermann U, al-Batran S, Hausner I, Faderl S, Nerl C, Emmerich B. Immunocytochemical and flow cytometric detection of proteinase 3 (myeloblastin) in normal and leukaemic myeloid cells. *Br J Haematol.* 1995; 89:250–257. [PubMed: 7873374]
11. Greiner J, Bullinger L, Guinn BA, Dohner H, Schmitt M. Leukemia-associated antigens are critical for the proliferation of acute myeloid leukemia cells. *Clin Cancer Res.* 2008; 14:7161–7166. [PubMed: 19010831]

12. Bauer S, Abdgawad M, Gunnarsson L, Segelmark M, Tapper H, Hellmark T. Proteinase 3 and CD177 are expressed on the plasma membrane of the same subset of neutrophils. *J Leukoc Biol.* 2007; 81:458–464. [PubMed: 17077162]
13. Sutherland DR, Anderson L, Keeney M, Nayar R, Chin-Yee I. The ISHAGE guidelines for CD34+ cell determination by flow cytometry. *International Society of Hematotherapy and Graft Engineering. J Hematother.* 1996; 5:213–226. [PubMed: 8817388]
14. Campbell EJ, Campbell MA, Owen CA. Bioactive proteinase 3 on the cell surface of human neutrophils: quantification, catalytic activity, and susceptibility to inhibition. *J Immunol.* 2000; 165:3366–3374. [PubMed: 10975855]
15. Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L, Worthen GS, Albelda SM. Polarization of tumor-associated neutrophil phenotype by TGF-beta: “N1” versus “N2” TAN. *Cancer Cell.* 2009; 16:183–194. [PubMed: 19732719]
16. Schmidt H, Suci S, Punt CJ, Gore M, Kruit W, Patel P, Lienard D, von der Maase H, Eggermont AM, Keilholz U. Pretreatment levels of peripheral neutrophils and leukocytes as independent predictors of overall survival in patients with American Joint Committee on Cancer Stage IV Melanoma: results of the EORTC 18951 Biochemotherapy Trial. *J Clin Oncol.* 2007; 25:1562–1569. [PubMed: 17443000]
17. Paesmans M, Sculier JP, Lecomte J, Thiriaux J, Libert P, Sergysels R, Bureau G, Dabouis G, Van Cutsem O, Mommen P, Ninane V, Klastersky J. Prognostic factors for patients with small cell lung carcinoma: analysis of a series of 763 patients included in 4 consecutive prospective trials with a minimum follow-up of 5 years. *Cancer.* 2000; 89:523–533. [PubMed: 10931451]
18. Michael M, Goldstein D, Clarke SJ, Milner AD, Beale P, Friedlander M, Mitchell P. Prognostic factors predictive of response and survival to a modified FOLFOX regimen: importance of an increased neutrophil count. *Clin Colorectal Cancer.* 2006; 6:297–304. [PubMed: 17241514]
19. Kusmartsev S, Su Z, Heiser A, Dannull J, Eruslanov E, Kubler H, Yancey D, Dahm P, Vieweg J. Reversal of myeloid cell-mediated immunosuppression in patients with metastatic renal cell carcinoma. *Clin Cancer Res.* 2008; 14:8270–8278. [PubMed: 19088044]
20. Munder M, Schneider H, Luckner C, Giese T, Langhans CD, Fuentes JM, Kropf P, Mueller I, Kolb A, Modolell M, Ho AD. Suppression of T-cell functions by human granulocyte arginase. *Blood.* 2006; 108:1627–1634. [PubMed: 16709924]
21. Molldrem JJ, Lee PP, Kant S, Wieder E, Jiang W, Lu S, Wang C, Davis MM. Chronic myelogenous leukemia shapes host immunity by selective deletion of high-avidity leukemia-specific T cells. *J Clin Invest.* 2003; 111:639–647. [PubMed: 12618518]
22. Alatrash G, Ono Y, Sergeeva A, Sukhumalchandra P, Zhang M, St John LS, Yang TH, Ruisaard K, Armistead PM, Mittendorf EA, He H, Qiao N, Rodriguez-Cruz T, Liang S, Clise-Dwyer K, Wieder ED, Lizee G, Lu S, Molldrem JJ. The role of antigen cross-presentation from leukemia blasts on immunity to the leukemia-associated antigen PR1. *J Immunother.* 2012; 35:309–320. [PubMed: 22495388]
23. Owen CA, Campbell MA, Sannes PL, Boukedes SS, Campbell EJ. Cell surface-bound elastase and cathepsin G on human neutrophils: a novel, non-oxidative mechanism by which neutrophils focus and preserve catalytic activity of serine proteinases. *J Cell Biol.* 1995; 131:775–789. [PubMed: 7593196]
24. Hu N, Westra J, Kallenberg CG. Membrane-bound proteinase 3 and its receptors: relevance for the pathogenesis of Wegener’s Granulomatosis. *Autoimmun Rev.* 2009; 8:510–514. [PubMed: 19185066]
25. Satomi A, Murakami S, Ishida K, Mastuki M, Hashimoto T, Sonoda M. Significance of increased neutrophils in patients with advanced colorectal cancer. *Acta Oncol.* 1995; 34:69–73. [PubMed: 7532422]
26. Gabillet J, Millet A, Pederzoli-Ribeil M, Tacnet-Delorme P, Guillevin L, Mouthon L, Frchet P, Witko-Sarsat V. Proteinase 3, the autoantigen in granulomatosis with polyangiitis, associates with calreticulin on apoptotic neutrophils, impairs macrophage phagocytosis, and promotes inflammation. *J Immunol.* 2012; 189:2574–2583. [PubMed: 22844112]
27. Bergstrom SE, Bergdahl E, Sundqvist KG. A cytokine-controlled mechanism for integrated regulation of T-lymphocyte motility, adhesion and activation. *Immunology.* 2013; 140:441–455. [PubMed: 23866045]

28. Leliefeld PH, Koenderman L, Pillay J. How Neutrophils Shape Adaptive Immune Responses. *Front Immunol.* 2015; 6:471. [PubMed: 26441976]
29. Hock BD, Taylor KG, Cross NB, Kettle AJ, Hampton MB, McKenzie JL. Effect of activated human polymorphonuclear leucocytes on T lymphocyte proliferation and viability. *Immunology.* 2012; 137:249–258. [PubMed: 23025756]
30. Rotondo R, Bertolotto M, Barisione G, Astigiano S, Mandruzzato S, Ottonello L, Dallegri F, Bronte V, Ferrini S, Barbieri O. Exocytosis of azurophil and arginase 1-containing granules by activated polymorphonuclear neutrophils is required to inhibit T lymphocyte proliferation. *J Leukoc Biol.* 2011; 89:721–727. [PubMed: 21330347]
31. Pederzoli M, Kantari C, Gausson V, Moriceau S, Witko-Sarsat V. Proteinase-3 induces procaspase-3 activation in the absence of apoptosis: potential role of this compartmentalized activation of membrane-associated procaspase-3 in neutrophils. *J Immunol.* 2005; 174:6381–6390. [PubMed: 15879139]
32. Yang JJ, Preston GA, Pendergraft WF, Segelmark M, Heeringa P, Hogan SL, Jennette JC, Falk RJ. Internalization of proteinase 3 is concomitant with endothelial cell apoptosis and internalization of myeloperoxidase with generation of intracellular oxidants. *Am J Pathol.* 2001; 158:581–592. [PubMed: 11159195]
33. Timosenko E, Hadjinicolaou AV, Cerundolo V. Modulation of cancer-specific immune responses by amino acid degrading enzymes. *Immunotherapy.* 2017; 9:83–97. [PubMed: 28000524]
34. Desmedt C, Ouriaghli FE, Durbecq V, Soree A, Coloza MA, Azambuja E, Paesmans M, Larsimont D, Buyse M, Harris A, Piccart M, Martiat P, Sotiriou C. Impact of cyclins E, neutrophil elastase and proteinase 3 expression levels on clinical outcome in primary breast cancer patients. *International journal of cancer Journal international du cancer.* 2006; 119:2539–2545. [PubMed: 16929516]
35. Padrines M, Wolf M, Walz A, Baggiolini M. Interleukin-8 processing by neutrophil elastase, cathepsin G and proteinase-3. *FEBS Lett.* 1994; 352:231–235. [PubMed: 7925979]
36. Smith DR, Polverini PJ, Kunkel SL, Orringer MB, Whyte RI, Burdick MD, Wilke CA, Strieter RM. Inhibition of interleukin 8 attenuates angiogenesis in bronchogenic carcinoma. *The Journal of experimental medicine.* 1994; 179:1409–1415. [PubMed: 7513008]
37. van der Woude FJ, Rasmussen N, Lobatto S, Wiik A, Permin H, van Es LA, van der Giessen M, van der Hem GK, The TH. Autoantibodies against neutrophils and monocytes: tool for diagnosis and marker of disease activity in Wegener's granulomatosis. *Lancet.* 1985; 1:425–429. [PubMed: 2857806]
38. Dolman KM, Jager A, Sonnenberg A, von dem Borne AE, Goldschmeding R. Proteolysis of classic anti-neutrophil cytoplasmic autoantibodies (C-ANCA) by neutrophil proteinase 3. *Clin Exp Immunol.* 1995; 101:8–12. [PubMed: 7621597]
39. Skold S, Rosberg B, Gullberg U, Olofsson T. A secreted proform of neutrophil proteinase 3 regulates the proliferation of granulopoietic progenitor cells. *Blood.* 1999; 93:849–856. [PubMed: 9920833]
40. Csernok E, Ai M, Gross WL, Wicklein D, Petersen A, Lindner B, Lamprecht P, Holle JU, Hellmich B. Wegener autoantigen induces maturation of dendritic cells and licenses them for Th1 priming via the protease-activated receptor-2 pathway. *Blood.* 2006; 107:4440–4448. [PubMed: 16478888]
41. Csernok E, Holle JU, Gross WL. Proteinase 3, protease-activated receptor-2 and interleukin-32: linking innate and autoimmunity in Wegener's granulomatosis. *Clin Exp Rheumatol.* 2008; 26:S112–117. [PubMed: 18799068]
42. Alatrash G, Mittendorf EA, Sergeeva A, Sukhumalchandra P, Qiao N, Zhang M, St John LS, Ruisaard K, Haugen CE, Al-Atrache Z, Jakher H, Philips AV, Ding X, Chen JQ, Wu Y, Patenia RS, Bernatchez C, Vence LM, Radvanyi LG, Hwu P, Clise-Dwyer K, Ma Q, Lu S, Mollndrem JJ. Broad cross-presentation of the hematopoietically derived PR1 antigen on solid tumors leads to susceptibility to PR1-targeted immunotherapy. *J Immunol.* 2012; 189:5476–5484. [PubMed: 23105141]
43. Bianchi ME. DAMPs, PAMPs and alarmins: all we need to know about danger. *J Leukoc Biol.* 2007; 81:1–5.

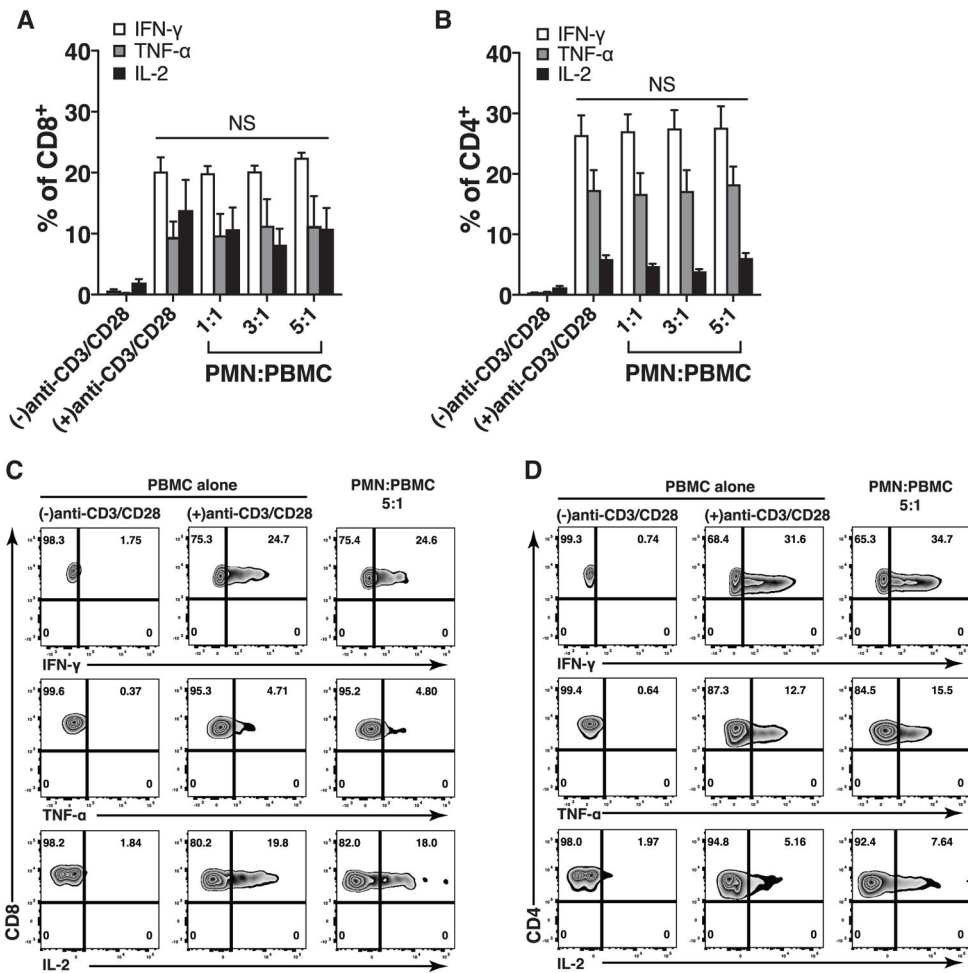
44. Bank U, Kupper B, Reinhold D, Hoffmann T, Ansorge S. Evidence for a crucial role of neutrophil-derived serine proteases in the inactivation of interleukin-6 at sites of inflammation. *FEBS Lett.* 1999; 461:235–240. [PubMed: 10567703]
45. Novick D, Rubinstein M, Azam T, Rabinkov A, Dinarello CA, Kim SH. Proteinase 3 is an IL-32 binding protein. *Proc Natl Acad Sci U S A.* 2006; 103:3316–3321. [PubMed: 16488976]
46. Uehara A, Sugawara S, Muramoto K, Takada H. Activation of human oral epithelial cells by neutrophil proteinase 3 through protease-activated receptor-2. *J Immunol.* 2002; 169:4594–4603. [PubMed: 12370398]
47. Meyer-Hoffert U. Neutrophil-derived serine proteases modulate innate immune responses. *Front Biosci.* 2009; 14:3409–3418.



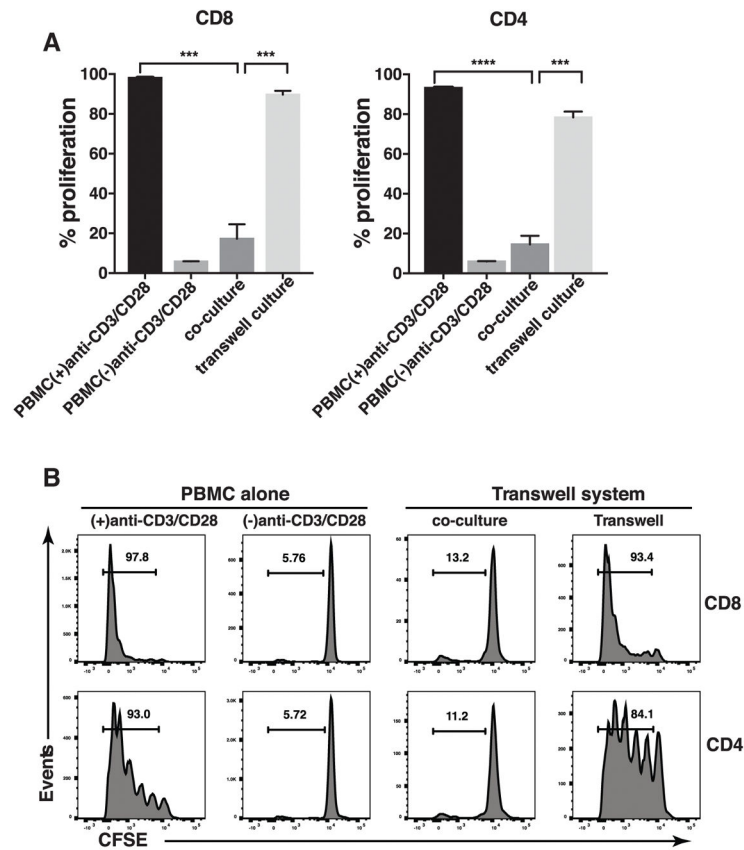


**Figure 1. PMN inhibit T cell proliferation in a dose-dependent manner**

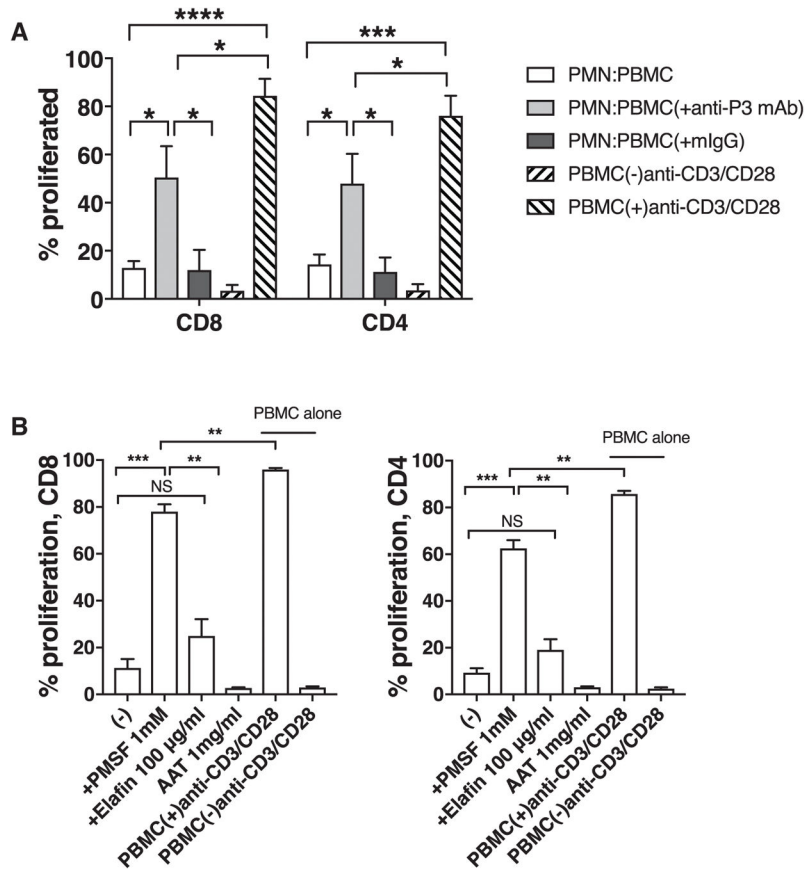
CFSE-labeled PBMC stimulated with or without anti-CD3/CD28 mAbs were co-cultured with increasing numbers of fixed PMN (Fig 1A, 1B) or unfixed PMN (Fig. 1C) for 5 days. Flow cytometry was used to determine CFSE dilution in live CD8 and CD4 T cells. (A) The result is a representative of nine different donors. Numbers on histograms represent the percentage of proliferating T cells. (B) Results from nine different donors ( $n=9$ ) are expressed as mean  $\pm$  SEM. \*\*\*\* $P < 0.0001$ , \* $P < 0.05$ ,  $P=NS$  (not significant). (C) CFSE-labeled PBMC were co-cultured with unfixed PMN in the presence of anti-CD3/CD28 mAbs at indicated ratios. Results are shown as mean  $\pm$  SEM ( $n=3$ ) from three different donors. \*\* $P < 0.01$ .



**Figure 2. PMN co-culture does not affect cytokine production in stimulated T cells**  
 (A and B) PBMC stimulated with or without anti-CD3/CD28 mAbs were co-cultured with increasing numbers of PMN for 16 hours, and the percentage of T cells producing cytokines was determined using intracellular staining of flow cytometry. Live CD8 and CD4 T cells were gated. Results are shown as mean  $\pm$  SEM ( $n=3$ ) from three different donors.  $P=NS$  (not significant). (C and D) Dotplots show a representative data of three different donors as shown in the cumulative data graph (top panels). Fixed PMN were used.

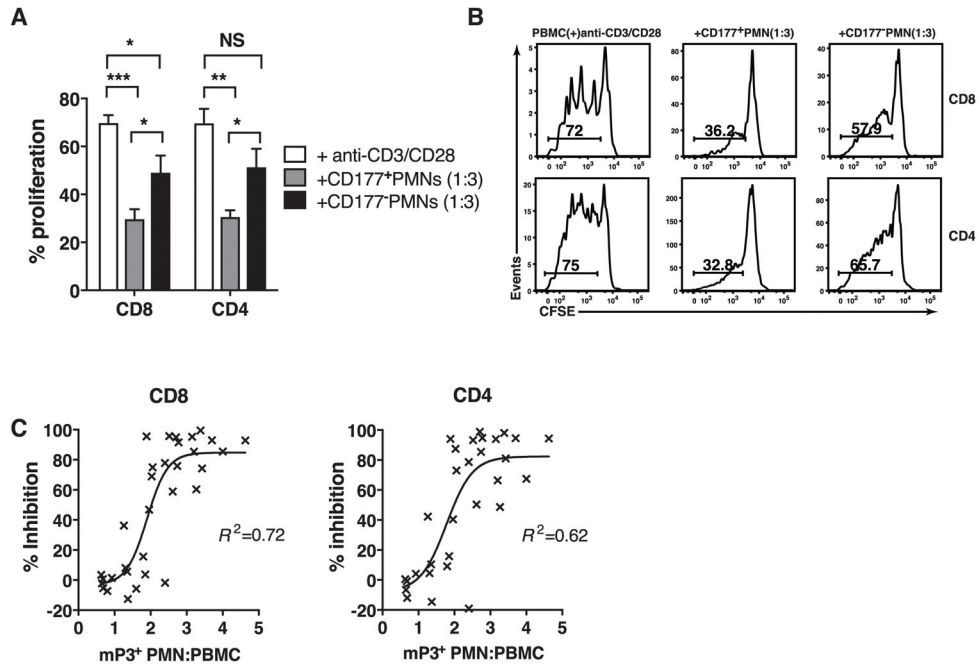


**Figure 3. PMN mediated-inhibition of T cell proliferation requires cell-cell contact**  
 Flow cytometric analysis of T cell proliferation in the presence of PMN using a transwell system. (A) PMN were mixed with CFSE-labeled PBMC at a 4:1 ratio in co-culture with anti-CD3/CD28 (co-culture). To separate PBMC and PMN, PBMC were cultured in the bottom chamber and PMN were placed in the top chamber of a 96-well transwell culture plate (Transwell). Representative results from one of three different donors performed in triplicates are expressed as mean ± SEM. \*\*\*\* $P < 0.0001$ , \*\*\* $P < 0.01$ . (B) Representative results of three different donors are shown. Numbers on histograms represent the percentage of proliferating T cells. Fixed PMN were used.



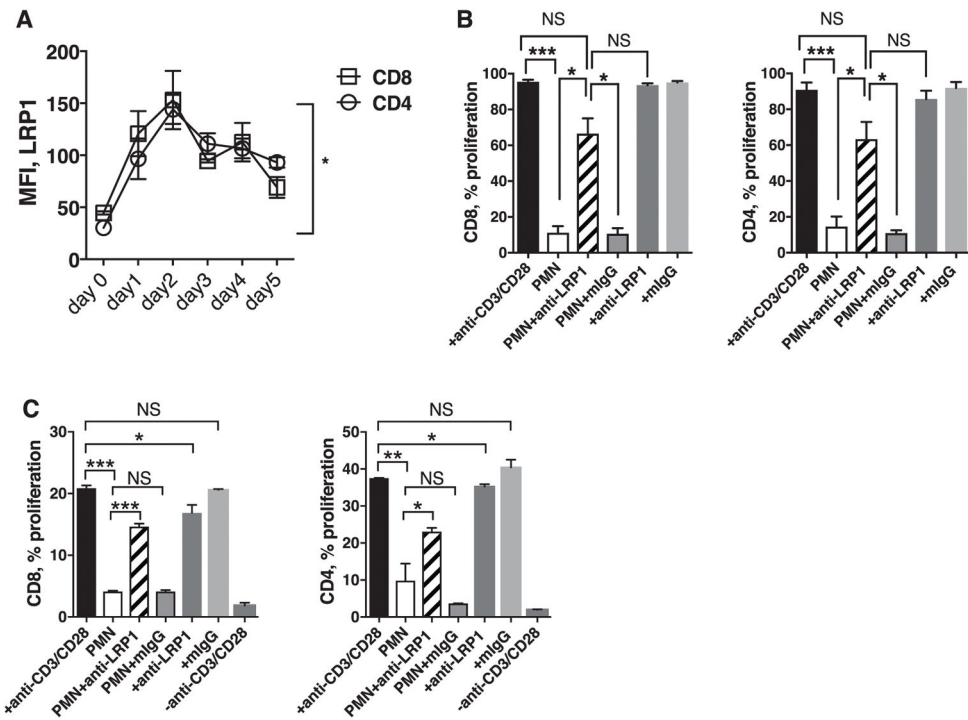
**Figure 4. PMN-mediated T cell inhibition is prevented by anti-P3 blocking antibody and requires P3 enzyme activity**

CFSE-labeled PBMC stimulated with anti-CD3/CD28 mAbs were co-cultured with PMN at the indicated PMN: PBMC ratio. (A) PMN were treated with anti-P3 mAb (clone WGM2) or mIgG (clone 11711). The PMN to PBMC ratio is 3:1. Results from five different donors ( $n=5$ ) are shown as mean  $\pm$  SEM. (B) PMN were pre-incubated with or without PMSF, Elafin or  $\alpha$ 1-Antitrypsin (AAT) before adding to PBMC cultures. The PMN to PBMC ratio is 4:1. Results shown as mean  $\pm$  SEM are representative of three different donors performed in triplicates. \*\*\*\* $P < 0.0001$ , \*\*\* $P < 0.0005$ , \*\* $P < 0.005$ , \* $P < 0.05$ .  $P=NS$  (not significant). Fixed PMN were used.



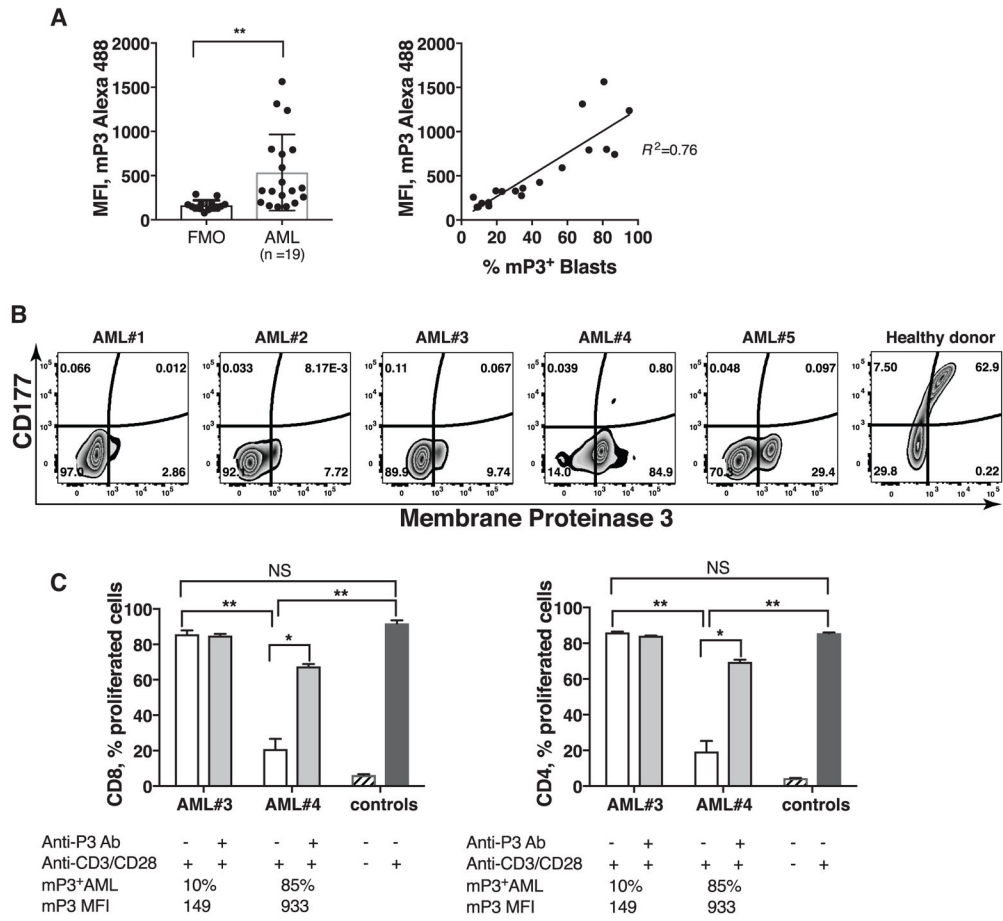
**Figure 5. mP3<sup>+</sup> PMN mediate the inhibition of T cell proliferation**

CFSE labeled PBMC were activated with anti-CD3/CD28 mAbs in all cell cultures. Sort-purified PMN were fixed with 0.5% PFA. **(A)** Sorted CD177-expressing and CD177-negative PMN were co-cultured with PBMC at a ratio of 3:1. T cells alone were used as control. Data from three different donors ( $n=3$ ) are shown as mean  $\pm$  SEM. \*\*\* $P < 0.001$ , \*\* $P < 0.003$ , \* $P < 0.05$ ,  $P=NS$  (not significant). **(B)** Representative histogram plots of three different donors are shown. Numbers on histograms represent the percentage of proliferating T cells. **(C)** Nonlinear regression analysis with results from seven different donors ( $n=7$ ) shows the correlation between % inhibition and the relative mP3-expressing PMN /PBMC ratio.  $R^2 = 0.72$  for CD8 and  $R^2 = 0.62$  for CD4, respectively.



**Figure 6. LRP1 blockade prevents PMN-mediated inhibition of T cell proliferation**  
**(A)** PBMC from healthy donors were stimulated with anti-CD3/CD28 mAbs for up to 5 days and LRP1 surface expression was determined by flow cytometry. Graph shows the median fluorescence intensity (MFI) of LRP1 on CD8 and CD4 T cells, respectively. Representative results from 1 of 2 different donors performed in duplicates are shown. Error bars represent mean  $\pm$  SEM. \* $P$ <0.05. **(B)** Fixed PMN were co-cultured with activated PBMC at a ratio of 3:1 for 5 days in the presence or absence of anti-LRP1 mAb (clone 8G1) or mIgG isotype control. Data from three different donors ( $n=3$ ) are expressed as mean  $\pm$  SEM. **(C)** Fixed PMN were co-cultured with sort-purified CD8 or CD4 T cells activated with anti-CD3/CD28 mAbs at 3:1 ratio for 5 days in the presence or absence of anti-LRP1 mAb (clone 8G1) or mIgG isotype control. Results shown as mean  $\pm$  SEM are representative of three different donors performed in duplicates. \*\*\* $P$ < 0.001, \*\* $P$ < 0.01, \* $P$ < 0.05,  $P$ =NS (not significant).





**Figure 7. mP3<sup>+</sup> AML mediates the inhibition of T cell proliferation, and proliferation is restored via P3 blockade**

(A) Flow cytometric analysis of CD177 and mP3 expression on AML blasts and healthy donor PMN were performed. FMO, fluorescence minus one. (B) Representative plots of AML blasts from AML patients ( $n=19$ ) and PMN of healthy donor ( $n=20$ ) are shown. (C) Following fixation with 0.5% PFA, allogeneic AML were pre-incubated with or without anti-P3 Ab for 30 minutes before they were co-cultured with PBMC at a 5:1 ratio (experiment was performed in duplicates) for up to 5 days. PBMC stimulated with or without anti-CD3/CD28 mAbs were used as positive and negative controls, respectively. Error bars represent mean  $\pm$  SEM. \*\* $P < 0.01$ , \* $P < 0.05$ ,  $P = NS$  (not significant).