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Activated Granulocytes and Granulocyte-derived Hydrogen Peroxide Are the Underlying Mechanism of Suppression of T-Cell Function in Advanced Cancer Patients[1]

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ABSTRACT

Impaired T-cell function in patients with advanced cancer has been a widely acknowledged finding, but mechanisms reported thus far are those primarily operating in the tumor microenvironment. Very few mechanisms have been put forth to explain several well-described defects in peripheral blood T cells, such as reduction in expression of signaling molecules, decreased production of cytokines, or increased apoptosis. We have closely examined the peripheral blood mononuclear cell (PBMC) samples derived from patients and healthy individuals, and we have observed an important difference that may underlie the majority of reported defects. We observed that in samples from patients only, an unusually large number of granulocytes copurify with low density PBMCs on a density gradient rather than sediment, as expected, to the bottom of the gradient. We also show that activating granulocytes from a healthy donor with *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine could also cause them to sediment aberrantly and copurify with PBMCs, suggesting that density change is a marker of their activation. To confirm this, we looked for other evidence of *in vivo* granulocyte activation and found it in drastically elevated plasma levels of 8-isoprostane, a product of lipid peroxidation and a marker of oxidative stress. Reduced T-cell receptor ζ chain expression and decreased cytokine production by patients' T cells correlated with the presence of activated granulocytes in their PBMCs. We showed that freshly obtained granulocytes from healthy donors, if activated, can also inhibit cytokine production by T cells. This action is abrogated by the addition of the hydrogen peroxide (H_2O_2) scavenger, catalase, implicating H_2O_2 as the effector molecule. Indeed, when added alone, H_2O_2 could suppress cytokine production of normal T cells. These findings indicate that granulocytes are activated in advanced cancer patients and that granulocyte-derived H_2O_2 is the major cause of severe systemic T-cell suppression.

INTRODUCTION

Despite increasing efforts, studies into tumor-induced immunosuppression have not provided plausible mechanisms that would explain the severe functional impairment in all circulating T cells seen in patients with advanced cancer (1). Although a growing number of tumor-specific antigens are being identified as potential targets that could be recognized by the immune system, cancer patients largely fail to mount an effective immune response against them. A failed T-cell response early in tumor development appears to be predominantly antigen specific (2). In some instances, tumor antigens are not sufficiently processed or presented to T cells because of the nature of the particular antigen (3) or defects in antigen-presenting cells (4). It has also been reported that proliferation of tumor-specific T cells could be hindered or the tumor-specific response could be switched to an unfavorable Th2[3] that does not support development of cytotoxic effector T cells from precursors stimulated at the tumor site (5). Even if competent T cells are made and capable of infiltrating the tumor, it has been reported that they can undergo apoptosis induced by Fas ligand expressed on the tumor cells (6). Although these mechanisms might explain tumor-specific immunosuppression, they are often restricted to the immediate tumor environment and fail to explain the more generalized immunosuppression that develops with tumor progression when patients stop responding even to common recall antigens (7). The first molecular mechanism described that potentially underlies these defects is the reduced expression of the TCRζ chain and TCR-associated protein tyrosine kinases in splenocytes from tumor-bearing mice (8). Subsequent studies with cancer patients indeed showed a reduced TCRζ chain expression that correlated with the tumor state of the patient (9–11). This defect was later correlated with antitumor responses in melanoma patients who underwent immunotherapy with IL-2 (12). Recently, one possible reason for these defects in TCR expression has been proposed to be the absence of L-arginine, known to be depleted in certain disease states (13) and accompanied by a decrease in T-cell proliferation (14, 15). A defect in the translocation of transcription factors in T cells, such as nuclear factor-κB, has been demonstrated and implicated in tumor-induced suppression (9, 16). As a likely consequence of both defects, an impaired cytokine production by T cells has been shown in patients with a variety of tumors (5, 17–21). Circulating suppressor cells or suppressor factors have been postulated as the cause for some of these defects in T-cell function. One type of candidate suppressor cells investigated have been the macrophages that can undergo activation in the vicinity of the tumor and produce factors, such as H_2O_2, that suppress various other immune effector cells (22). Macrophages, however, are not circulating cells, and their suppressive function is still expected to be limited to the effector cells in the tumor microenvironment.

Here we propose a mechanism that likely underlies all of the others described thus far and that we consider the common cause for most of the observed defects in immune function in advanced cancer patients. This mechanism involves activation of granulocytes and oxidative stress mediated by H_2O_2 that they produce. Unlike macrophages that are primarily found in tissues, granulocytes are abundantly present in circulation. Phagocytosis by granulocytes is supported by an oxidative burst that releases reactive oxygen species from membrane-bound enzymes and facilitates killing of microorganisms. We offer evidence of *in vivo* granulocyte activation in cancer patients that correlates with the inhibition of TCRζ chain expression and cytokine production by their T cells. We show that coincubation of T cells with activated granulocytes *in vitro* leads to the inhibition of cytokine production and that the mediator of that inhibition is H_2O_2.

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[3] The abbreviations used are: Th1 and Th2, T helper types 1 and 2; TCR, T-cell receptor; IL, interleukin; PBMC, peripheral blood mononuclear cell; FMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; PMA, phorbol myristate acetate; TNF, tumor necrosis factor; PFA, paraformaldehyde.

MATERIALS AND METHODS

Blood Samples. Blood samples were obtained under an Institutional Review Board-approved protocol from patients with metastatic adenocarcinomas of the pancreas, colon, and breast, seen at the University of Pittsburgh Cancer Institute. All radiation or chemotherapy had been concluded at least 4 weeks prior to the blood drawing. PBMCs were purified on a density gradient using a lymphocyte separation medium (ICN, Aurora, OH) and stored at −80°C in fetal bovine serum containing medium with 10% DMSO.

Granulocyte and T-Cell Assays. Granulocytes were purified from peripheral blood with dextran sedimentation as described (23). PBMCs were collected by centrifugation in lymphocyte separation medium (ICN), and residual RBCs were lysed in a hypotonic saline solution. To obtain activated granulocytes, whole blood was incubated for 1 h with FMLP (Sigma Chemical Co., St. Louis, MO) prior to density gradient centrifugation. Cells were stained with anti-CD15 antibody (Becton Dickinson, Mountain View, CA) for flow cytometry or used for cytospins stained with Diff-Quick (Baxter, McGraw Park, IL). T cells were activated for 4 h with 20 μg/ml PMA and 1 μM ionomycin (Sigma Chemical Co.).

PBMC samples were analyzed for TCRζ expression and cytokine production by flow cytometry as described (24). For intracellular cytokine staining, 2 mM monensin (Sigma Chemical Co.) was added to T cells for 4 h. Cells were fixed with 2% PFA[3] and permeabilized with FACS buffer (PBS supplemented with 5% FBS and 0.1% sodium azide) containing 0.1% saponin (all from Sigma Chemical Co.). An anti-TCRζ chain antibody, TCR-ζ, TIA-2 (Coulter, Fullerton, CA) was used for indirect staining prior to a secondary goat antimouse *R*-phycoerythrin-conjugated antibody (Biosource, Camarillo, CA). Antibodies against the cytokines IFN-γ, TNF-α, IL-2, or IL-4 were obtained from PharMingen (San Diego, CA). Live cells were stained with antibodies against CD11b, CD14, and CD15 on granulocytes and monocytes and antibodies against CD3 and CD8 (Becton Dickinson) on T cells. Analysis was done on a FACS-Calibur (Becton Dickinson).

For granulocyte/T-cell coculture assays, both cell types were purified from the same blood sample. Granulocytes (4×10^6) were seeded into the lower compartment of a six-well transwell system (Costar, Cambridge, MA), and 2×10^6 PBMCs were either placed into the upper compartment or directly mixed with the granulocytes in a final volume of 5 ml. The experiment, involving freezing, fixation, or addition of bovine catalase (Sigma Chemical Co.), was performed with 7.5×10^5 T cells and granulocytes in a 24-well plate (Costar). Granulocytes were either frozen overnight in FBS with 10% DMSO at −80°C or fixed in 2% PFA/PBS for 20 min prior to the assay. For experiments involving H_2O_2, PBMCs were treated for 5 min, followed by an additional 5-min incubation with catalase at a final concentration of 1000 units/ml. Dose-response experiments with H_2O_2 were done in the absence of FBS.

Detection of $F_{2\alpha}$-Isoprostane Plasma samples that had been stored at −80°C were used in an enzyme immunoassay that detects $F_{2\alpha}$-isoprostane (Cayman Chemical, Ann Arbor, MI). To measure total isoprostane, we hydrolyzed plasma samples with potassium hydroxide and performed detection after solid phase extraction with C_{18} columns (Millipore, Milford, MA) as recommended by the manufacturer.

RESULTS

Evidence of *in Vivo* Granulocyte Activation and Oxidative Stress Damage in Cancer Patients. Density gradient centrifugation has been traditionally used as a method to separate PBMCs, such as lymphocytes, from polymorphonuclear cells, such as granulocytes. Granulocytes are expected to sediment in the cell pellet because of their high buoyant density. We noticed that in the process of purification of blood from cancer patients (Fig. 1*A*) but not healthy controls (Fig. 1*B*), unusually large numbers of granulocytes sedimented with the same density as PBMCs. This was detected by flow cytometry analysis of the composition of purified PBMCs by cell size and granularity and confirmed by staining with anti-CD15 antibody specific for granulocytes (Fig. 2). We hypothesized that aberrant sedimentation of granulocytes could be a result of a change in their density attributable to activation. To reproduce these events *in vitro*, we treated whole blood from a healthy donor with FMLP, a peptide that induces receptor-mediated granulocyte activation (25). An hour of treatment indeed led to aberrant granulocyte sedimentation. Granulocytes copurified with PBMCs in a FMLP dose-dependent manner (Fig. 2*A*) and represented up to 12.5% of the population, as confirmed by CD15 expression (Fig. 2*B*) and by specific morphology observed in the cytospins (Fig. 2, *C* and *D*).

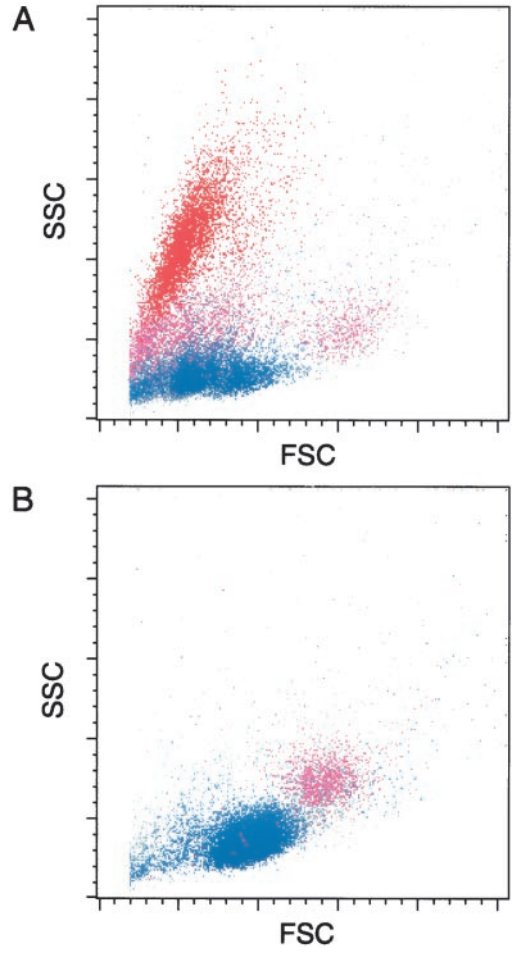

Fig. 1. In cancer patients but not in normal controls, granulocytes copurify with PBMCs on a density gradient. *A*, PBMC sample from a cancer patient. *B*, PBMC sample from a healthy control. *Blue*, $CD3^+$cells; *purple*, $CD14^+$ cells; *red*, $CD15^+$ cells; *gray*, other cells; *SSC*, side scatter; *FSC*, forward scatter.

Analysis of additional samples from healthy individuals and cancer patients confirmed that granulocytes are virtually excluded from purified PBMCs of healthy donors but appear in high percentages in PBMCs from cancer patients (Fig. 4*A*). The same PBMC samples that had large numbers of activated granulocytes had mainly normal neutrophil counts (Fig. 3*B*). The median lymphocyte count was decreased, however, and some patients were lymphopenic (Fig. 3*C*). Because we hypothesized that aberrant sedimentation of granulocytes was attributable to their *in vivo* activation, we sought another *in vivo* parameter to measure to further support this hypothesis. Because activation of granulocytes results in oxidative stress and production of short-lived free radicals, we looked for biomarkers for detection of damage caused by free radicals. Isoprostanes are products of lipid peroxidation (26), and we used an enzyme immunoassay to detect $F_{2\alpha}$-isoprostane in patient plasma samples. We found exceedingly high plasma levels of isoprostane (Fig. 3*D*), confirming that patients had been exposed to oxidative stress.

Reduced TCRζ Expression and Cytokine Production in Peripheral Blood T Cells. We extended our earlier studies into tumor-induced T-cell suppression in cancer patients and tested T-cell function in 19

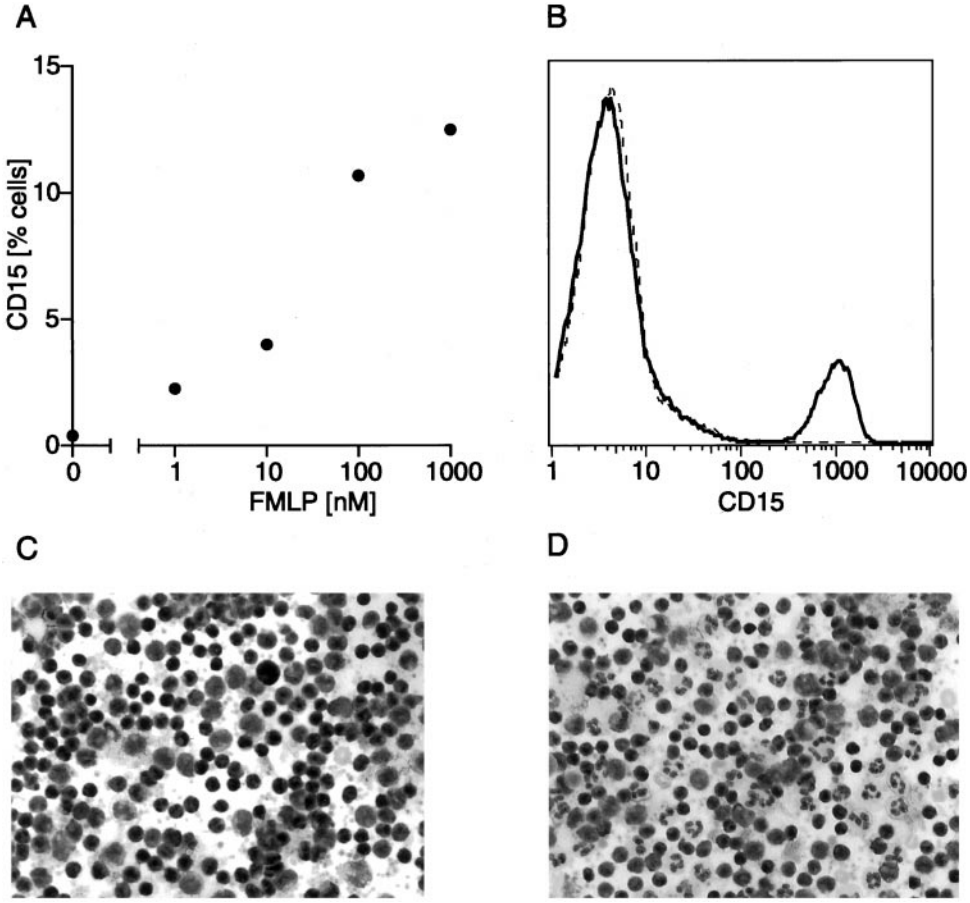

Fig. 2. Granulocytes that copurify with PBMCs from a healthy donor on a density gradient are activated. *A*, FMLP dose-dependent increase in percentage of granulocytes (CD15+) copurifying with PBMCs. *B*, peripheral blood incubated without (----) and with 1 μM FMLP (———) prior to density gradient centrifugation and tested for the presence of granulocytes (CD15). Cytospins of PBMCs purified before treatment (*C*) or after treatment (*D*) with FMLP.

pancreatic, 15 colon, and 1 breast cancer patient. TCRζ chain expression was significantly reduced in all these patients compared with healthy controls ($P < 0.0001$; Fig. 3*A*). We activated the same T cells with PMA and ionomycin, reagents that do not depend on the TCRζ chain for their function, and tested their ability to produce cytokines. All patients showed reduced numbers of IFN-γ-producing T cells compared with controls ($P < 0.0001$; Fig. 4*B*). To address the possibility that this defect may be only in the type 2 T cells, resulting in altered ratios of Th1 *versus* Th2 T cells, we also measured the type 2 cytokine IL-4. There was no compensatory increase in IL-4, the production of which was also reduced (data not shown). A similar reduction was observed in the production of TNF-α and IL-2 (data not shown).

Granulocyte-derived Hydrogen Peroxide Inhibits Cytokine Production. The observation that suppression of the TCRζ chain expression and T-cell cytokine production coincided with signs of granulocyte activation and elevated isoprostane plasma levels in cancer patients encouraged us to seek a direct proof that activated granulocytes can be responsible for inhibition of T-cell function. We purified granulocytes from healthy donor blood by density sedimentation and obtained a >90% pure population as confirmed by CD15 expression (data not shown). We mixed these granulocytes with lymphocytes from the same donor and coincubated them under different conditions (Fig. 5*A*). In the absence of granulocytes, >30% of T cells produced IFN-γ when activated with PMA and ionomycin. Coactivation of lymphocytes and granulocytes had a strong inhibitory effect on IFN-γ production by T cells. Coactivation in a transwell system, where granulocytes and lymphocytes were separated by a membrane, showed that inhibition may be caused by soluble factors. The integrity of the granulocytes was important for this inhibition inasmuch as either fixation with paraformaldehyde or freezing greatly reduced their inhibitory effect (Fig. 5*B*).

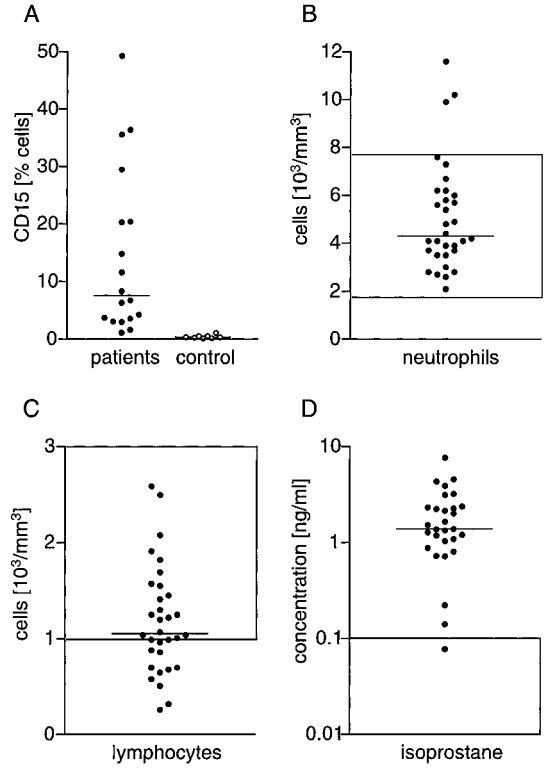

Fig. 3. Copurification of activated granulocytes with PBMCs, white blood count, and signs of oxidative stress. In *A*, $CD15^+$ cells copurified with PBMCs in patients compared with healthy controls. *B*, absolute neutrophil count in peripheral blood of patients. *C*, absolute lymphocytes count in peripheral blood of patients. *D*, plasma levels of isoprostane in patients. Normal ranges are marked with a *rectangle*.

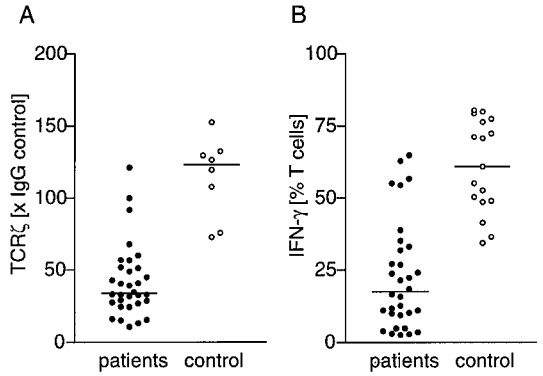

Fig. 4. TCRζ chain expression and IFN-γ production by T cells from cancer patients compared with healthy controls. *A*, mean fluorescence intensity of staining of all T cells from TCRζ chain expression shown as multiples of antibody isotype control. *B*, percentage of IFN-γ-producing T cells among all T cells. *Horizontal bars*, median values.

Confirming that activated granulocytes can suppress *in vitro* IFN-γ production by T cells, we went back to the patients' samples to determine the correlation of these two parameters *in vivo*. We show that low numbers of granulocytes, predominantly found in PBMC samples from healthy individuals, correlate with high percentages of IFN-γ-producing T cells in those individuals. Similarly, high numbers of granulocytes, predominantly found in PBMC samples from cancer patients, correlate with low percentages of IFN-γ-producing T cells (Fig. 5*C*).

Granulocytes limit tissue destruction by releasing protecting enzymes such as superoxide dismutase and catalase. Superoxide dismutase converts superoxide anion to H_2O_2, which in turn is degraded to water by catalase. When we added increasing concentrations of catalase to the cocultures of granulocytes and lymphocytes, most of the inhibition of IFN-γ production was abrogated (Fig. 5*D*). This identified H_2O_2 as the effector molecule mediating inhibition of this T-cell function. Adding H_2O_2 directly to T cells resulted in a reduced number of cytokine-producing cells, not only for IFN-γ but also TNF-α, IL-2, and IL-4, in a dose-dependent manner (Fig. 5, *E* and *F*).

DISCUSSION

We have provided evidence here for one mechanism that underlies several well-described defects in T-cell function in patients with advanced cancer. We show that cancer patients respond to the presence of the tumor with activation of granulocytes. This is likely an attempt on the part of the innate immune system to protect the organism. The chronic

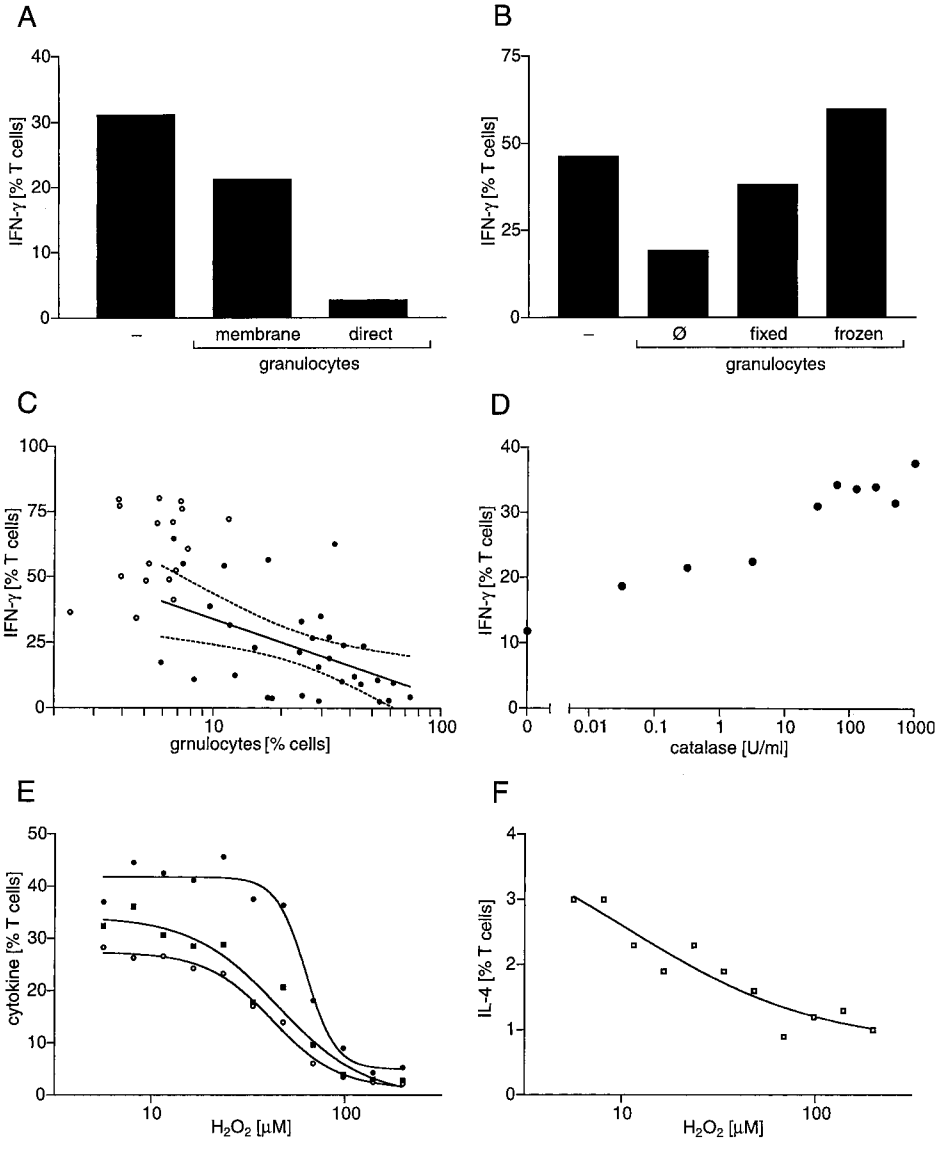

Fig. 5. Granulocyte-mediated inhibition of cytokine production *in vitro*. *A*, IFN-γ-producing T cells in the absence or presence of granulocytes in a transwell system (*membrane*) or in direct contact (*direct*). *B*, IFN-γ-producing T cells in the absence or presence of untreated (Ø), PFA-fixed (*fixed*), or frozen and thawed (*frozen*) granulocytes. *C*, percentage of IFN-γ-producing T cells after activation, in PBMCs from patients (●) and healthy controls (○), plotted against the percentage of contaminating granulocytes. The regression line for patient samples is shown with the 95% confidence interval ($P = 0.006$; slope, -29.6 ± 9.9). *D*, increase in the percentage of IFN-γ-producing T cells in the presence of catalase. Dose-dependent inhibition by hydrogen peroxide of IFN-γ (●), TFN-α (■), and IL-2 (○) (*E*) and IL-4 (□) producing T cells (*F*) is shown.

presence of the tumor and thus chronic activation of granulocytes with the accompanying production of H_2O_2 results, ironically, in the suppression of the adaptive immune functions. Here we show specifically that the release of reactive oxygen species during the oxidative burst of granulocytes, *i.e.*, H_2O_2, is the major contributor to a systemic T-cell dysfunction. This is supported by the observation that cancer patients showed signs of extensive granulocyte activation with massive elevation of plasma levels of 8-isoprostane, a product of lipid oxidation and a marker for oxidative stress, that exceeded measurements found in other diseases with documented oxidative stress (26–28).

Our *in vitro* experiments demonstrated directly that coincubation of granulocytes and lymphocytes could inhibit cytokine production but required the functional integrity and activation of granulocytes. The observation that granulocyte-mediated inhibition of cytokine production could be abrogated by the addition of catalase to inhibit H_2O_2 identified this product as the major soluble mediator of activated granulocyte effects. We confirmed this by adding H_2O_2 to T cells and inducing potent inhibition of cytokine production in a dose-dependent manner. Analyzing *ex vivo* cytokine production by patients' T cells, we found that the inhibition was most pronounced for IFN-γ and, although not significant, was also detectable for IL-4. We did not detect a switch from Th1 cytokines, which promote cell-mediated cytotoxicity, toward an antibody-mediated Th2-dominated responses. A decrease in cytokine production of T cells significantly correlated with the number of circulating activated granulocytes.

The activation of granulocytes could be induced by an inflammatory response or cytokines produced by the tumor. Tumor cells are known to secrete cytokines, among them are cytokines that affect granulocytes (29, 30). Possible candidates are TNF-α and IL-8, which could be elevated in the serum of cancer patients (30, 31) and stimulate the oxidative burst (32, 33). Although we have concentrated on documenting immunosuppressive effects on T cells, it is highly probable that other cells of the immune system could be equally affected by the same mediators of oxidative stress. A decrease in the function of NK cells mediated by H_2O_2 has been documented (22), whereas causes of defects in the function of dendritic cells are unknown (4). Little is known about the functional competence of B cells in cancer patients, but B cells might be affected in a similar manner to the T cells. Our data suggest that inhibition of the oxidative burst of granulocytes might be a useful treatment of cancer patients prior to attempting any form of immunotherapy.

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