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PHAGOCYTES, GRANULOCYTES, AND MYELOPOIESIS

Mature CD10$^+$ and immature CD10$^-$ neutrophils present in G-CSF–treated donors display opposite effects on T cells

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Key Points

- CD10 as a marker discriminating mature from immature neutrophils within heterogeneous neutrophil populations in pathological settings.
- Immunosuppressive mature CD66b$^+$CD10$^+$ and immunostimulatory immature CD66b$^+$CD10$^-$ neutrophils coexist in G-CSF–treated donors.

The identification of discrete neutrophil populations, as well as the characterization of their immunoregulatory properties, is an emerging topic under extensive investigation. In such regard, the presence of circulating CD66b$^+$ neutrophil populations, exerting either immunosuppressive or proinflammatory functions, has been described in several acute and chronic inflammatory conditions. However, due to the lack of specific markers, the precise phenotype and maturation status of these neutrophil populations remain unclear. Herein, we report that CD10, also known as common acute lymphoblastic leukemia antigen, neutral endopeptidase, or enkephalinase, can be used as a marker that, within heterogeneous populations of circulating CD66b$^+$ neutrophils present in inflammatory conditions, clearly distinguishes the mature from the immature ones. Accordingly, we observed that the previously described immunosuppressive neutrophil population that appears in the circulation of granulocyte colony-stimulating factor (G-CSF)–treated donors (GDs) consists of mature CD66b$^+$CD10$^+$ neutrophils displaying an activated phenotype. These neutrophils inhibit proliferation and interferon γ (IFNγ) production by T cells via a CD18-mediated contact-dependent arginase 1 release. By contrast, we found that immature CD66b$^+$CD10$^-$ neutrophils, also present in GDs, display an immature morphology, promote T-cell survival, and enhance proliferation and IFNγ production by T cells. Altogether, our findings uncover that in GDs, circulating mature and immature neutrophils, distinguished by their differential CD10 expression, exert opposite immunoregulatory properties. Therefore, CD10 might be used as a phenotypic marker discriminating mature neutrophils from immature neutrophil populations present in patients with acute or chronic inflammatory conditions, as well as facilitating their isolation, to better define their specific immunoregulatory properties. (*Blood*. 2017;129(10):1343-1356)

Introduction

Over the past 2 decades, the interest in better clarifying the role of neutrophils in modulating immune responses has extraordinarily grown.[1-6] Research in this area has, for instance, uncovered the fact that, during systemic inflammation, autoimmune diseases, or cancer, distinct cell populations displaying neutrophil-like morphology and showing either immunosuppressive or proinflammatory functions may be found in blood.[7-10] Some of these neutrophil populations sediment within the peripheral blood mononuclear cell (PBMC) fraction after density gradient centrifugation of blood, and are thus generally defined as low-density neutrophils (LDNs).[7-8,10] Accordingly, the presence of immunosuppressive LDNs, also known as granulocytic myeloid-derived suppressor cells (G-MDSCs), has been identified in the peripheral blood from patients with cancer,[8,11-13] HIV-1 infections,[14,15] sepsis,[16] graft-versus-host disease,[17] as well as in pregnant women,[18] and, relevant to this study, in healthy granulocyte colony-stimulating factor (G-CSF)–treated donors (GDs) for stem cell mobilization.[19-20] Notably, immunosuppressive neutrophil populations have also been found in the normal-density neutrophil (NDN) fraction[14,21-22] or within the total leukocytes purified after red cell lysis of whole blood.[14,23-25] By contrast, subsets of LDNs displaying proinflammatory functions, more recently defined as low-density granulocytes, have been described in patients with autoimmune diseases, such as systemic lupus erythematosus (SLE) and psoriasis.[7]

The various LDN populations to date identified and described in pathological settings are heterogeneously composed by mixed populations

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of activated mature neutrophils, as well as neutrophils at different stages of differentiation.[7-8,10] Expression of both CD11b and CD16 is often used to determine the maturation status of LDNs,[8] but it is not always reliable as it can variably change upon activation or during the maturation process of neutrophils.[26] It is, therefore, necessary to identify more specific markers that could permit the precise identification, rapid separation, and functional characterization of mature neutrophils, as well as immature neutrophil populations detectable in disease states. In this regard, CD10 (also known as common acute lymphoblastic leukemia antigen, neutral endopeptidase, or enkephalinase) is a 100-kDa transmembrane glycoprotein whose expression, among granulocytes, is specifically displayed by mature neutrophils at their latest stages of differentiation.[26-28]

Given these premises, in this study, we investigated whether CD10 could represent a valid marker to discriminate mature neutrophils from immature neutrophil subsets under inflammatory conditions, and, if so, to eventually correlate its expression with cell-specific immunoregulatory properties. To address these issues, we mostly focused our studies on the neutrophil populations present in peripheral blood of GDs[19-20] to then validate our findings in SLE and cancer patients.

Methods

Study participants

Study participants included: peripheral blood stem cell donors receiving recombinant human G-CSF (Lenogastim; Italfarmaco) at a dose of 10 μg/kg per day for 5 days (GDs; n = 61), solid tumor patients (n = 17; supplemental Table 1, available on the *Blood* Web site), lymphoma patients (n = 28; supplemental Table 1), SLE patients (n = 9; supplemental Table 1). Criteria for study inclusion of cancer and SLE patients are reported in supplemental Methods and supplemental Table 1. GDs were selected according to the Italian Bone Marrow Donor Registry standards, based on their HLA compatibility with related or unrelated recipients. Eighty-five sex- and age-matched healthy donors (HDs) from the Blood Bank Unit of the Verona Azienda Ospedaliera Universitaria Integrata (AOUI) were also enrolled in the study. The study has been conducted according to the Declaration of Helsinki principles and approved by the ethic board of the Verona AOUI (protocol numbers: 2371, principal investigator [PI]: C.T.; 1826, PI: C.L.) and of the Spedali Civili di Brescia (NP2066-WV-H&NCancer; PI: W.V.). All human samples were obtained after informed consent. Participants were identified by number, not by name. Blood samples from study participants were collected in EDTA-treated tubes by venipuncture and processed within 1 hour.

Cell isolation

Mononuclear cells and granulocytes were isolated by density gradient centrifugation (Ficoll-Paque; GE Healthcare Life Sciences) of blood under endotoxin-free conditions.[29] Blood from GDs was typically diluted fivefold in phosphate-buffered saline prior to its centrifugation. LDNs were isolated from the mononuclear cell fraction, whereas NDNs were isolated from the granulocyte fraction, by either magnetic bead selection or cell sorting by flow cytometry. Isolation of total $CD66b^+$ or mature $CD10^+$ neutrophils by magnetic bead selection was performed by incubating mononuclear cells or granulocytes with fluorescence-conjugated anti-CD66b or anti-CD10 monoclonal antibodies (mAbs), respectively, followed by incubation with specific anti-fluorochrome microbeads (Miltenyi Biotec) according to the manufacturer's protocol. Immature $CD66b^+CD10^-$ LDNs were isolated by positive selection by anti-CD66b magnetic beads from the mononuclear cell fractions previously depleted of $CD10^+$ cells. For cell sorting by flow cytometry, mononuclear cells or granulocytes were incubated with specific anti-CD45, anti-CD66b, anti-CD11b, anti-CD16, and anti-CD10 mAbs, as described in the flow cytometry section of supplemental Methods, and sorted using a FACSAria II flow cytometer (Becton Dickinson). In selected experiments, $CD10^+$ neutrophils were directly isolated from total leukocytes obtained after dextran sedimentation of whole blood and hypotonic lysis of the remaining erythrocytes. NDNs were also isolated from the granulocyte fraction by negative selection using immunomagnetic beads as previously described.[30] Cell purity of all sorted populations was always >98%, as determined by flow cytometry.

Statistics

The comparison of variables was performed using an unpaired 2-tailed Mann-Whitney *U* test (for comparison between 2 groups) or a 1-way analysis of variance (ANOVA) with the Dunnett posttest (when multiple comparisons to control group were made). *P* values of <.05 were considered significant and asterisks indicate significant increases: $*P < .05$; $**P \leq .01$; $***P \leq .001$. Graphs were elaborated using GraphPad Prism version 5 software (GraphPad Software, Inc).

Results

$CD66b^+$ LDNs from GDs consist of heterogeneous populations of both mature and immature neutrophils

Initial findings confirmed that the frequency of $CD66b^+$ LDNs in GDs is significantly higher than in HDs (42.0% [16.5%-94.0%] vs 0.5% [0.1%-1.5%]; $P < .0001$) (Figure 1A).[19,20] Contaminating $CD66b^+$ eosinophils (<2%) were excluded from the calculation according to a gating strategy based on their $CD45^{high}CD16^{low/-}$ expression (shown in supplemental Figure 1A). Notably, by analyzing their CD16 and CD11b expression levels, we noticed that $CD66b^+$ NDNs from HDs appeared as a homogeneous population of mature $CD66b^+CD11b^+CD16^+$ neutrophils (Figure 1B). By contrast, $CD66b^+$ LDNs and $CD66b^+$ NDNs from GDs appeared very heterogeneous, as they contained cells at different stages of myeloid maturation, as revealed by their $CD11b^+CD16^+$ to $CD11b^{low/-}$ and/or $CD16^{low/-}$ phenotype (Figure 1C-D). $CD66b^+$ LDNs from HDs could not be investigated due to their very low abundance (Figure 1A; supplemental Figure 1A).

Expression of surface CD10 distinguishes mature from immature neutrophils among heterogeneous $CD66b^+$ LDNs and $CD66b^+$ NDNs from GDs

Expression of CD16 is known to be acquired at the band cell stage during neutrophil differentiation ($CD66b^+CD11b^{hi}CD16^{int}$ cells), and to be further upregulated in mature neutrophils ($CD66b^+CD11b^{hi}CD16^{hi}$ cells).[26] On the other end, CD16 expression in mature neutrophils is downregulated upon their activation.[26] Interestingly, both $CD66b^+CD11b^+CD16^+$ LDNs and $CD66b^+CD11b^+CD16^+$ NDNs from our cohort of GDs displayed lower CD16 expression than $CD66b^+CD11b^+CD16^+$ NDNs from HDs (Figure 1B-D). Therefore, based on their CD16 expression levels, discrimination of mature $CD66b^+CD11b^{hi}CD16^{hi}$ neutrophils and $CD66b^+CD11b^{hi}CD16^{int}$ band cells present within $CD66b^+CD11b^+CD16^+$ LDNs/NDNs from GDs could not be accomplished.

To better define the maturation/activation status of $CD66b^+$ LDNs/NDNs from GDs, we analyzed their CD10 expression. Accordingly, CD10 is specifically displayed only by mature neutrophils and not by band cells or more immature neutrophil precursors.[26-28] As revealed by both flow cytometry and morphological analyses (Figure 1E-G), both $CD66b^+CD16^+$ LDNs and $CD66b^+CD16^+$ NDNs from GDs mostly consisted instead of a mixed population of mature segmented $CD10^+$ neutrophils (Figure 1F-G, arrowheads in right panels) and $CD10^-$ band cells (Figure 1F-G, asterisks in right panels), whereas $CD66b^+CD16^+$ NDNs from HDs consisted of a homogeneous population of mature segmented $CD10^+$ neutrophils (Figure 1E, arrowheads in

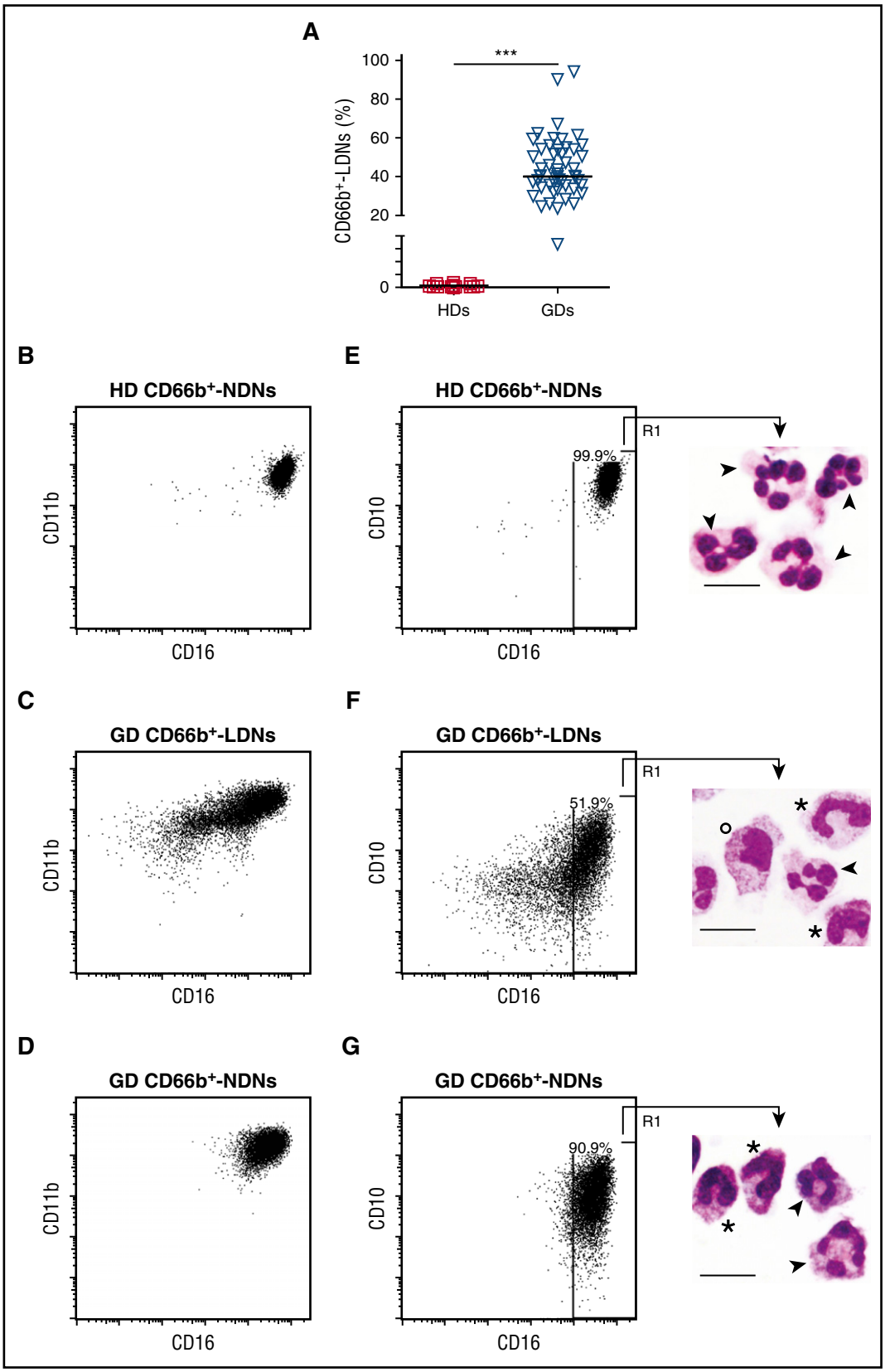

Figure 1. Frequency and phenotypic/morphologic characterization of CD66b$^+$ LDNs and CD66b$^+$ NDNs from GDs. (A) Frequency of CD66b$^+$ LDNs within CD45$^+$ PBMCs from HDs (n = 44) and GDs (n = 53). Graph values indicate medians from independent experiments. Each symbol stands for a single HD or GD. ***$P \leq .001$, by the Mann-Whitney *U* test. (B-D) Representative fluorescence-activated cell sorter (FACS) plots displaying CD16 and CD11b expression in CD66b$^+$ NDNs from HDs (B) or CD66b$^+$ LDNs (C) and CD66b$^+$ NDNs (D) from GDs. (E-G) Representative FACS plots (left column) displaying CD10 and CD16 expression in CD66b$^+$ NDNs from HDs (E) or CD66b$^+$ LDNs (F) and CD66b$^+$ NDNs (G) from GDs. Representative May-Grünwald Giemsa stained cytospins (right column; scale bar = 10 μm) of sorted CD16$^+$ (R1) cells from CD66b$^+$ NDNs from HDs (E) or CD66b$^+$ LDNs (F) and CD66b$^+$ NDNs (G) from GDs. Examples of segmented neutrophils (▼), band cells (*), and metamyelocytes (○) are reported.

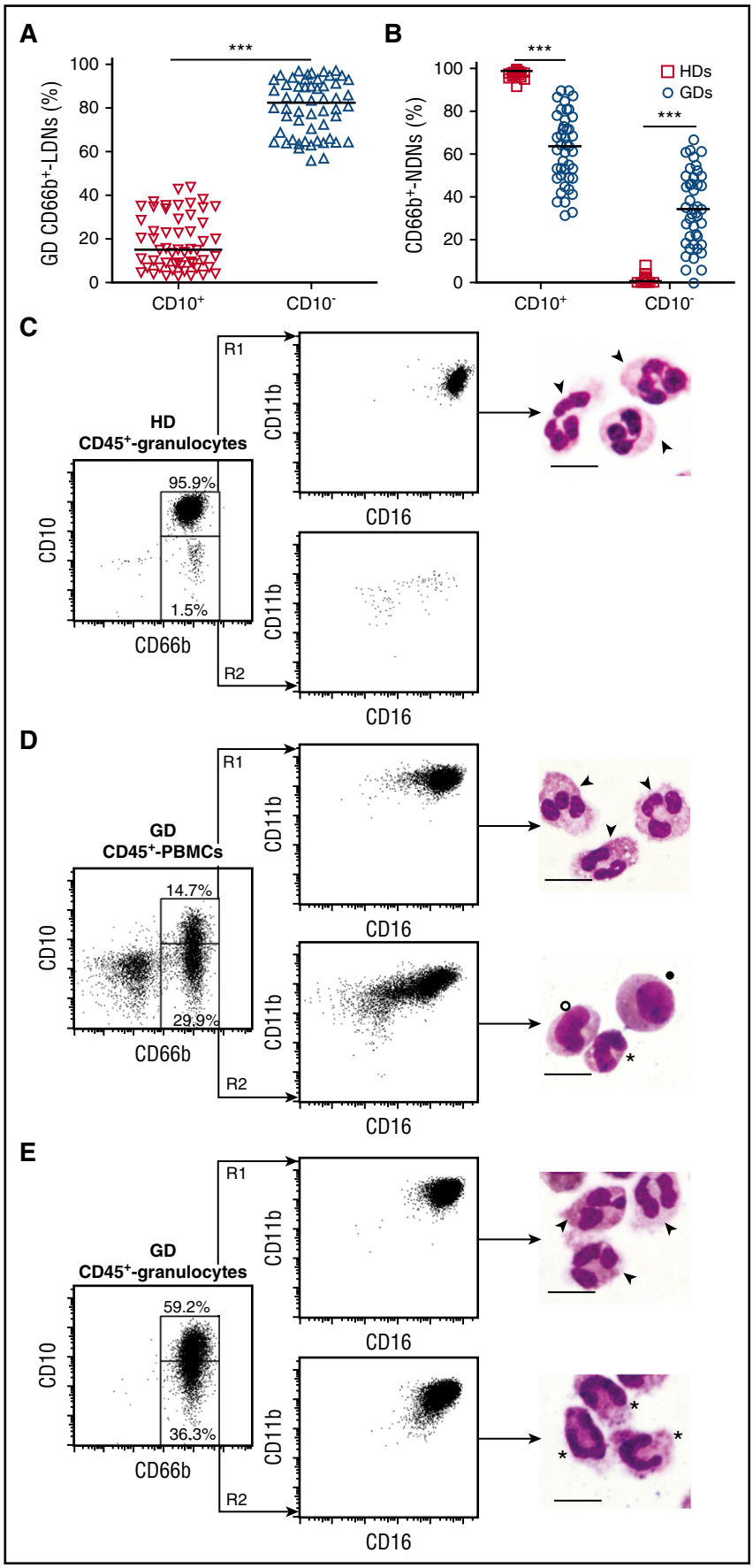

Figure 2. Frequency and phenotypic/morphologic characterization of mature CD10$^+$ and immature CD10$^-$ neutrophils within CD66b$^+$ LDNs and CD66b$^+$ NDNs from GDs. (A-B) Frequency of CD10$^+$ and CD10$^-$ cells within CD66b$^+$ LDNs in PBMCs from GDs (n = 53) (A) or within CD66b$^+$ NDNs from HDs (n = 44) and GDs (n = 53) (B). Graph values indicate medians from independent experiments. Each symbol stands for a single HD or GD. ***$P \leq .001$, by the Mann-Whitney U test. (C-E) Representative FACS plots of CD11b and CD16 expression and May-Grünwald Giemsa stained cytospins (scale bar = 10 μm) of CD66b$^+$CD10$^+$ (R1) or CD66b$^+$CD10$^-$ (R2) cells within CD45$^+$ granulocytes from HDs (C) or GDs (E) and CD45$^+$ PBMCs (D) from GDs. Examples of segmented neutrophils (▼), band cells (*), metamyelocytes (○), and myelocytes (●) are reported.

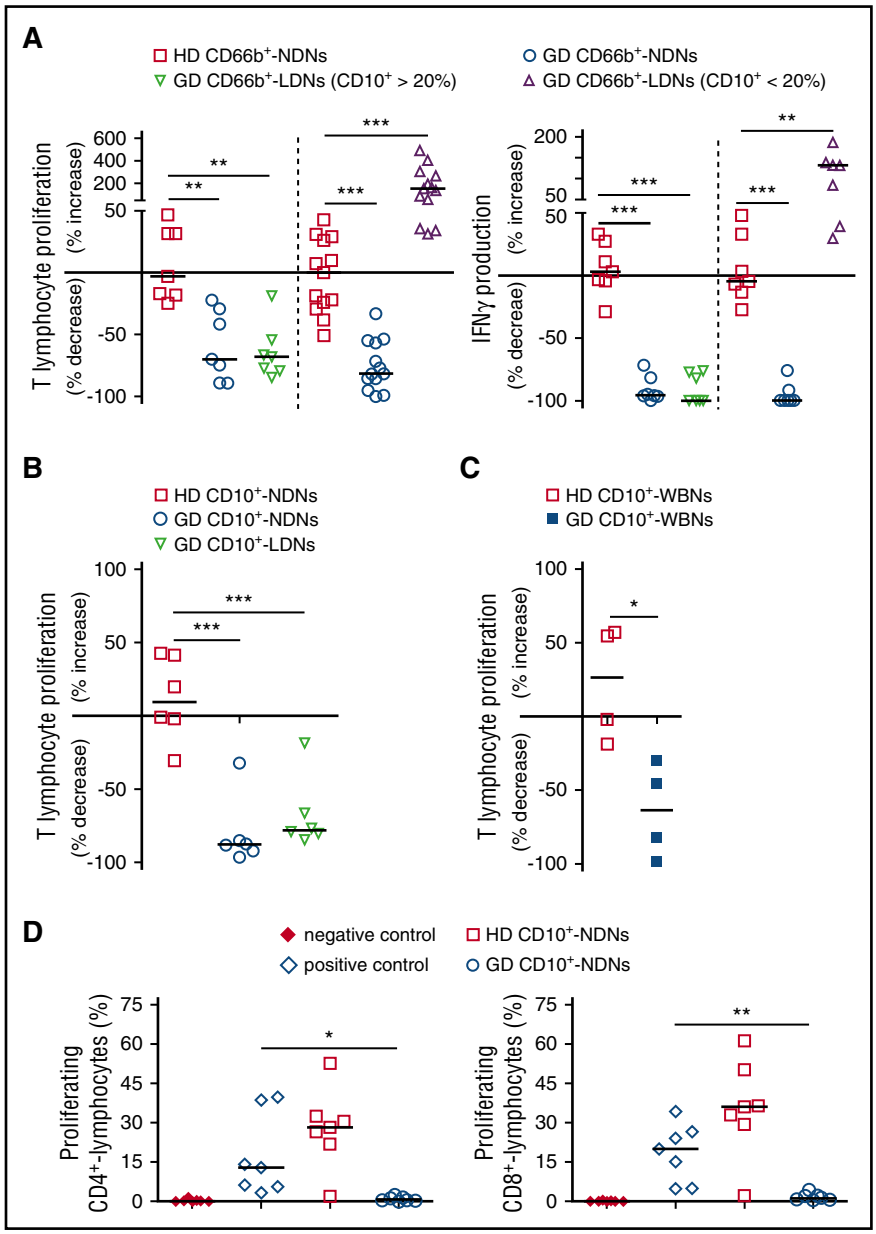

Figure 3. CD10$^+$ LDNs and CD10$^+$ NDNs from GDs inhibit proliferation and IFNγ production by T cells. (A-C) CD3/CD28-stimulated T cells were cultured for 96 hours in the absence or presence of CD66b$^+$ or CD10$^+$ LDN or NDN populations at a 5:1 neutrophil-to-T-cell ratio. T-cell proliferation was measured by 5-bromo-2′-deoxyuridine (BrdU) incorporation (see supplemental Methods), whereas T-cell–derived IFNγ was measured in coculture supernatants by enzyme-linked immunosorbent assay (ELISA). (A) The percentages of increase/decrease of proliferation (left panel) or IFNγ production (right panel) by T cells induced by: CD66b$^+$ NDNs from HDs (□), CD66b$^+$ NDNs from GDs (○), CD66b$^+$ LDNs from GDs (containing >20% mature CD10$^+$ neutrophils; represented on the left part of the graphs) (▽) or CD66b$^+$ LDNs from GDs (containing <20% mature CD10$^+$ neutrophils; represented on the right part of the graphs) (△). Graph values indicate medians from independent experiments (n = 7-13). $^{**}P \leq .01$; $^{***}P \leq .001$, by 1-way ANOVA with the Dunnett posttest. (B) Percentage of increase/decrease of T-cell proliferation induced by CD10$^+$ NDNs from HDs (□), CD10$^+$ NDNs from GDs (○), or CD10$^+$ LDNs from GDs (▽). Graph values indicate medians from independent experiments (n = 6). $^{***}P \leq .001$, by 1-way ANOVA with the Dunnett posttest. (C) Percentage of increase/decrease of T-cell proliferation in the presence of total CD10$^+$ neutrophils isolated from whole blood (WBNs) of HDs (□) or GDs (■). Graph values indicate medians from independent experiments (n = 4). $^*P \leq .05$ by the Mann-Whitney *U* test. (D) CD3/CD28-stimulated T cells were cultured for 96 hours in the presence or absence of CD10$^+$ NDNs from HDs or GDs, at a 5:1 neutrophil-to-T-cell ratio. The percentages of proliferating CD4$^+$ and CD8$^+$ T cells, either left unstimulated (negative control) or stimulated with anti-CD3/CD28 mAbs, in the absence (positive control) or in the presence of CD10$^+$ NDNs from HDs or GDs, were revealed by the CFSE dilution assay (see supplemental Methods). Graph values indicate medians from independent experiments (n = 7). $^*P \leq .05$; $^{**}P \leq .01$, by 1-way ANOVA with the Dunnett posttest.

right panel). A few metamyelocytes could be also observed within CD66b$^+$CD16$^+$ LDNs from GDs (Figure 1F, open circle in right panel). These observations were confirmed by performing a detailed analysis of nuclear morphology in sorted CD66b$^+$CD16$^+$ LDNs/NDNs from 8 GDs (Figure 1E, G right panels; supplemental Figure 1B). Because even purified CD66b$^+$CD16^{hi} LDNs/NDNs from GDs (sorted on the basis of the CD16 expression levels displayed by mature CD66b$^+$CD11b$^+$CD16^{hi} NDNs from HDs) included about 20% band cells (supplemental Figure 1C-F), altogether, data demonstrate that mature neutrophils from GDs cannot be isolated via CD16. To calculate the precise frequency of mature and immature neutrophils within CD66b$^+$ LDNs and CD66b$^+$ NDNs from GDs (Figure 2A-B), we therefore relied on CD10 expression (as evaluated according to the gating strategy shown in Figure 2C-E, left panels, and supplemental Figure 2A). We found that CD10$^+$ cells from GDs, present at median frequencies of 15% (Figure 2A) and 63% (Figure 2B) within, respectively, CD66b$^+$ LDNs and CD66b$^+$ NDNs, appeared as homogeneous populations of mature segmented CD11b$^+$CD16$^+$ neutrophils (Figure 2C-E, R1, top panels). The mature phenotype of sorted CD10$^+$ LDNs/NDNs from 20 GDs was also confirmed by their typical nuclear morphology (Figure 2C-E, R1, top right panels; supplemental Figure 2B). By contrast, CD66b$^+$CD10$^-$ LDNs from GDs displayed a heterogeneous composition mostly consisting of CD11b$^+$CD16$^+$ band cells (median frequency of 50.8%) and CD11b$^+$CD16$^{+/dim}$ metamyelocytes (34.5%), only a small fraction of CD11b^{dim}CD16$^-$ myelocytes (9.2%; Figure 2D, R2, black circle in the bottom right panel), and almost no CD11b$^-$CD16$^-$ promyelocytes (1.4%) (supplemental Figure 2C-D). Finally, CD66b$^+$CD10$^-$ NDNs from GDs mostly consisted of CD10$^-$CD11b$^+$CD16$^+$ band cells (Figure 2E, R2, bottom panels). Again, a detailed analysis of the nuclear morphology in sorted CD66b$^+$CD10$^-$ LDNs/NDNs from 20 GDs confirmed their nature of immature neutrophils (Figure 2D-E, R2, bottom right panels; supplemental Figure 2B). Taken together, data show that CD10 expression identifies mature neutrophils among the heterogeneous populations of CD66b$^+$ cells present in GDs at different levels of maturation/activation.

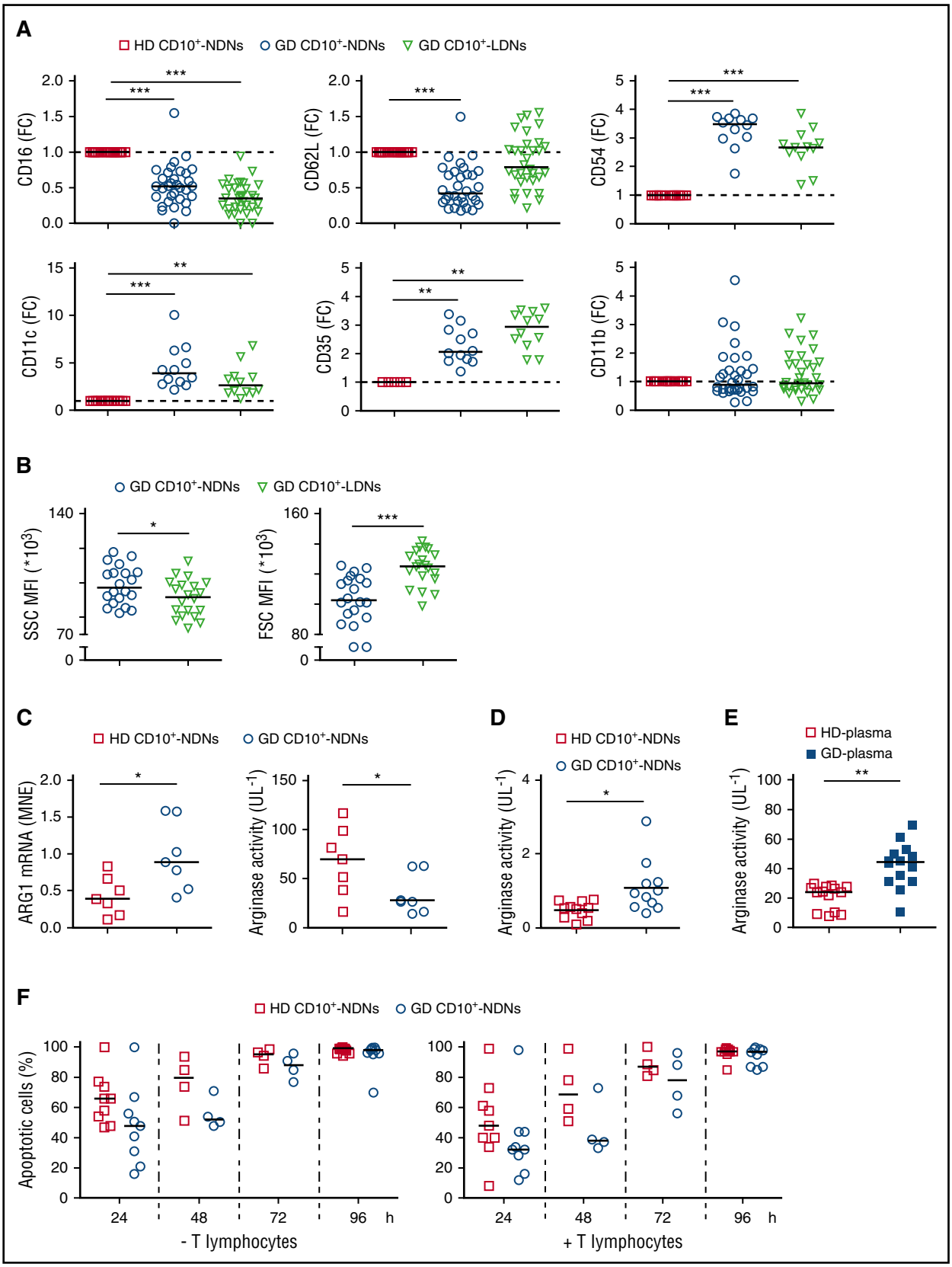

Figure 4. CD10$^+$ LDNs and/or CD10$^+$ NDNs from GDs display an activated phenotype, high levels of ARG1 mRNA, and low intracellular arginase activity. (A) Panels display the levels of CD16, CD62L, CD54, CD11c, CD35, and CD11b expression in CD10$^+$ NDNs and CD10$^+$ LDNs from GDs, as evaluated by flow cytometry. For each antigen, data are expressed as median fold change (FC) of its mean fluorescence intensity (MFI) in CD10$^+$ NDNs or CD10$^+$ LDNs from GDs over CD10$^+$ NDNs from HDs. Graph values indicate medians from independent experiments. Each symbol stands for a single HD or GD (n = 12-32). $^{**}P \leq .01$; $^{***}P \leq .001$, by 1-way ANOVA with the Dunnett posttest. (B) Panels display the MFI of the side scatter (SSC) (left panel) and the forward scatter (FSC) (right panel) of CD10$^+$ NDNs and CD10$^+$ NDNs from GDs

CD10$^+$ LDNs and CD10$^+$ NDNs from GDs inhibit proliferation and IFNγ production by T cells

Although CD66b$^+$ LDNs from GDs have been previously reported to be immunosuppressive,[19,20] whether immature and/or mature neutrophil populations are responsible for such immunosuppressive capacity has never been investigated. It also remains unknown whether NDNs from GDs manifest immunosuppressive capacities. To address these issues, total CD66b$^+$ LDNs and CD66b$^+$ NDNs from GDs and/or HDs were isolated either by magnetic bead selection or by cell sorting (>99.0% purity; see supplemental Figure 3A; "Methods"), and then examined for their capacity to affect T-lymphocyte functions. To our surprise, CD66b$^+$ NDNs from all GDs tested were found to potently inhibit both CD3/CD28-induced proliferation (Figure 3A left panel; supplemental Figure 3B) and interferon γ (IFNγ) production (Figure 3A right panel) by T cells. Also, CD66b$^+$ LDNs from GDs were found to inhibit T-cell responses but only if they contained >20% mature CD10$^+$ neutrophils; if not, they were instead found to enhance T-cell responses (Figure 3A; supplemental Figure 3B). Given that the degree of both the inhibitory and stimulatory effects mediated by CD66b$^+$ LDNs and CD66b$^+$ NDNs from GDs were dependent on their ratio with T cells (supplemental Figure 3C-D) in all subsequent experiments we used the 5:1 neutrophil-to-T-cell ratio (eg, the most effective one). Data obtained so far lead us to hypothesize that mature CD10$^+$ neutrophils present within CD66b$^+$ LDNs and CD66b$^+$ NDNs from GDs were the putative cell populations responsible for the observed T-cell immunosuppression. To validate this hypothesis, we directly isolated CD10$^+$ LDNs and CD10$^+$ NDNs from GDs, as well as CD10$^+$ NDNs from HDs (as controls), either by positive bead selection or by cell sorting (see supplemental Figure 4A; "Methods") and then examined their capacity to modulate CD3/CD28-induced T-cell responses. In line with our assumption, both CD10$^+$ LDNs and CD10$^+$ NDNs from GDs, but not CD10$^+$ NDNs from HDs, were found to strongly inhibit CD3/CD28-induced T-cell proliferation (Figure 3B). The fact that CD10$^+$ NDNs, isolated either as CD10$^+$ or CD66b$^+$ cells from GDs, inhibited T-cell proliferation at equivalent levels (Figure 3A left panel, 3B) excluded the possibility that potentially contaminating CD10$^-$ eosinophils[31] and/or CD10$^-$ band cells (Figure 2B,E) could exert immunosuppressive functions. Moreover, none of the isolation methods caused an activation of neutrophils (as revealed by eventual changes in CD11b, CD16, and CD62L expression), and/or an alteration of their vitality, as compared with the negative selection purification procedure (supplemental Figure 4B-C).[30] Finally, unfractionated CD10$^+$ neutrophils from GDs (eg, directly isolated from total leukocytes purified from whole, unprocessed blood) inhibited T-cell proliferation to a similar degree of CD10$^+$ LDNs and CD10$^+$ NDNs (Figure 3C).

Given that both CD10$^+$ LDNs and CD10$^+$ NDNs from GDs manifested similar immunosuppressive functions, and because CD10$^+$ LDNs could usually be recovered in very limited numbers, we used CD10$^+$ NDNs from either GDs or HDs in all functional assays. By doing so, we could demonstrate that the suppressive effect mediated by CD10$^+$ NDNs from GDs was exerted, at comparable levels, on both CD4$^+$ and CD8$^+$ T cells (Figure 3D; supplemental Figure 4D) and was displayed even if T cells were stimulated with anti-CD28 mAbs plus hOKT3γ1, Ala-Ala (modified anti-CD3 mAbs avoiding a nonspecific triggering of neutrophil responses via FcγRs) (supplemental Figure 4E).[32] We could also observe that CD10$^+$ NDNs from GDs inhibited the proliferation of T cells that are already committed to polyclonal proliferation (supplemental Figure 4F).[33] However, differently from what was previously reported,[20] CD10$^+$ NDNs from HDs did not acquire any capacity to inhibit T-cell proliferation when exposed to elevated doses of G-CSF in vitro (up to 5×10^3 U mL^{-1}, approximately corresponding to the daily dose administered to GDs) (supplemental Figure 4G).

CD10$^+$ LDNs and CD10$^+$ NDNs from GDs display an activated phenotype

An in-depth characterization by flow cytometry revealed that both CD10$^+$ LDNs and CD10$^+$ NDNs from GDs exhibited not only lower CD16 (Figures 2D-E and 4A; supplemental Figure 5), but also higher CD54 (α-ICAM-1), CD11c, and CD35 (complement receptor 1) expression than CD10$^+$ NDNs from HDs (Figure 4A; supplemental Figure 5). A significant reduction of CD62L expression, with respect to CD10$^+$ NDNs from HDs, was also observed in CD10$^+$ NDNs, but not in CD10$^+$ LDNs, from GDs (Figure 4A; supplemental Figure 5). Consistent with their lower buoyancy, and with previous findings on LDNs from trauma patients,[34] CD10$^+$ LDNs also appeared emptier of granules and more swollen than CD10$^+$ NDNs from GDs, as revealed by, respectively, their lower side scatter (Figure 4B left panel), and their higher forward scatter (Figure 4B, right panel). By contrast, no significant differences in CD11b and CD11a expressions were found among the different cell populations analyzed (Figure 4A; supplemental Figure 5). Moreover, CD10$^+$ NDNs from GDs expressed higher levels of Arginase 1 (ARG1) messenger RNA (mRNA) (Figure 4C left panel) and reduced intracellular arginase activity (Figure 4C right panel) than CD10$^+$ NDNs from HDs, which was accompanied by elevated arginase activity in the culture medium harvested from GD CD10$^+$ NDN/T-cell cocultures (Figure 4D) and in GD plasma (Figure 4E). These findings imply an increase in both production and release of ARG1 by CD10$^+$ NDNs in GDs, similarly to what was previously attributed to G-MDSCs of cancer patients.[35] In the latter study, the increase in transcription was interpreted as a compensatory mechanism to sustain their enhanced ARGI release.[35] Finally, the survival rate of CD10$^+$ NDNs from GDs did not significantly differ from that of CD10$^+$ NDNs from HDs, whether it is cultured in medium alone or in the presence of T cells (Figure 4F). Altogether, data demonstrate that both CD10$^+$ LDNs and CD10$^+$ NDNs from GDs display an activated phenotype.

CD10$^+$ NDNs from GDs inhibit proliferation and IFNγ production by T cells via CD18-mediated contact-dependent ARG1 release

Under some experimental conditions, the immunosuppressive functions of either LDN or NDN subsets have been ascribed to phenomena

Figure 4 (continued) (n = 20), as evaluated by flow cytometry. Graph values indicate medians from independent experiments. Each symbol stands for a single GD. *$P \leq .05$; ***$P \leq .001$, by the Mann-Whitney *U* test. (C) Panels display ARG1 mRNA expression (left panel) and intracellular arginase activity (right panel), as evaluated by reverse transcription polymerase chain reaction (RT-PCR) and specific enzymatic assay, respectively, in CD10$^+$ NDNs from HDs or GDs. Graph values indicate medians from independent experiments (n = 7). *$P \leq .05$, by the Mann-Whitney *U* test. (D-E) The panels display arginase activity in the culture medium harvested from cocultures of T cells and CD10$^+$ NDNs from HDs (n = 11) or GDs (n = 11) (D) or in plasma obtained from HDs (n = 14) or GDs (n = 14) (E). Graph values indicate medians from independent experiments. Each symbol stands for a single HD or GD. *$P \leq .05$; **$P \leq .01$, by the Mann-Whitney *U* test. (F) The frequency of apoptotic CD10$^+$ NDNs from HDs or GDs, cultured, for the time indicated, alone (left panel) or in the presence of CD3/CD28-stimulated T cells at a 5:1 neutrophil-to-T-cell ratio (right panel) as revealed by flow cytometry. Graph values indicate medians from independent experiments (n = 4-9). MNE, mean normalized expression.

occurring through contact-dependent mechanisms.[21,23,36] Consistently, we found that the addition of anti-CD18 mAbs (either as full immunoglobulin Gs [IgGs] or as $F(ab)_2$ fragments), but not isotype-matched control mAbs, significantly reversed the inhibition of proliferation and IFNγ production by T cells induced by $CD10^+$ NDNs from GDs (Figure 5A-B). Furthermore, the capacity of $CD10^+$ NDNs from GDs to inhibit both $CD4^+$ and $CD8^+$ T-cell proliferation was almost completely lost if neutrophils were separated from T cells by the use of transwells (supplemental Figure 6A). In line with the increased arginase activity in the culture medium harvested from GD $CD10^+$ NDN/T-cell cocultures (Figure 4D), the addition of L-arginine to the $CD10^+$ NDN-T-cell cocultures also reversed the immunosuppressive effects mediated by $CD10^+$ NDNs from GDs (Figure 5C; data not shown). The latter findings support the notion that $CD10^+$ NDNs from GDs exert T-cell–immunosuppressive functions by depleting L-arginine from the environment. Accordingly, we found that the addition of anti-CD18 mAbs (either as full IgGs or as $F(ab)_2$ fragments), but not isotype-matched control mAbs, also significantly reduced the levels of ARG1 present in the culture medium harvested from cocultures of T cells and $CD10^+$ NDNs from GDs (Figure 5D). Moreover, we excluded the fact that the suppressive effect by $CD10^+$ NDNs from GDs could be caused by an induction of T-cell apoptosis. In fact, both $CD4^+$ and $CD8^+$ T cells underwent similar levels of apoptosis when stimulated with anti-CD3/CD28 antibodies, regardless of the presence or absence of $CD10^+$ NDNs from GDs or HDs (supplemental Figure 6B). Finally, we found that catalase (an H_2O_2 scavenger) did not affect the immunosuppressive functions of $CD10^+$ NDNs from GDs, although it significantly reversed the immunosuppressive functions of phorbol 12-myristate 13-acetate–stimulated NDNs from HDs (Figure 5E; supplemental Figure 6C). Taken together, data suggest that the inhibitory effects on T-cell proliferation by $CD10^+$ NDNs from GDs involve ARG1 release and cell-cell interactions via CD18, but not an induction of T-cell apoptosis or the production of reactive oxygen species.

Immature $CD66b^+CD10^-$ LDNs from GDs promote T-cell survival and increase proliferation and IFNγ production by T cells

Our observations that $CD66b^+$ LDNs from GDs enhanced CD3/CD28-induced proliferation and IFNγ production by T cells when containing <20% mature $CD10^+$ neutrophils (Figure 3A), suggested that immature $CD66b^+CD10^-$ LDNs from GDs might have immunostimulatory functions. To verify this hypothesis, we isolated $CD66b^+$ $CD10^-$ LDNs from PBMCs of GDs to a >99% purity by either magnetic bead selection or cell sorting by flow cytometry (see "Methods"; supplemental Figure 7A) and confirmed that they strongly enhanced CD3/CD28-induced $CD4^+/CD8^+$ T-cell proliferation (Figure 6A; supplemental Figure 7B). By contrast, autologous $CD66b^+CD10^-$ NDNs (mostly band cells, Figure 2E; supplemental Figure 2B) did not significantly affect T-cell proliferation (Figure 6B). Consistent with their immature status, we also observed that the survival rate of $CD66b^+CD10^-$ LDNs from GDs was much higher than that of $CD10^+$ NDNs from HDs when cultured either alone (Figure 6C, left panel) or in the presence of T cells (Figure 6C, right panel). However, despite their enhanced survival, $CD66b^+CD10^-$ LDNs from GDs neither divided nor proliferated (supplemental Figure 7C). Finally, we observed that $CD66b^+CD10^-$ LDNs from GDs promoted T-cell survival (especially at later time points of incubation; Figure 6D) and that contact-dependent mechanisms (supplemental Figure 7D), mediated by CD18 (Figure 6E-F), were necessary for their immunostimulatory action.

$CD66b^+$ LDNs from either cancer or SLE patients include mature and immature neutrophil subsets that are clearly distinguishable based on their CD10 expression levels

In a final series of experiments, we verified whether analysis of CD10 expression could also be used to characterize the maturation status of both LDNs and NDNs in other diseases. For this purpose, we enrolled patients carrying solid tumors or lymphoma in whom the presence of immunosuppressive $CD66b^+$ LDNs (generally known as G-MDSCs) is well ascertained (Dumitru et al,[8] Moses and Brandau,[13] and Marini et al[37]; supplemental Table 1) or patients with SLE because they have circulating $CD66b^+$ LDNs (called low-density granulocytes) displaying "proinflammatory" properties[7] (supplemental Table 1). As shown in Figure 7A-E, we could confirm that the frequency of $CD66b^+$ LDNs in all these patients is significantly increased as compared with that of HDs. We also found that within $CD66b^+$ LDNs from solid tumor (Figure 7C), lymphoma (Figure 7D), or SLE (Figure 7E) patients, it was possible to clearly distinguish mature $CD10^+CD11b^+CD16^+$ neutrophils (Figure 7C-E, R1, top panels) from $CD10^-CD11b^+$ $CD16^+$ band cells or other more immature neutrophil subsets (Figure 7C-E, R2, bottom panels), similarly to what was observed in GDs. The same findings were obtained by analyzing the nuclear morphology of $CD10^+$ LDNs/NDNs and $CD66b^+CD10^-$ LDNs isolated from solid tumor, lymphoma, or SLE patients (supplemental Figure 8A-B). The overall frequencies of mature $CD10^+$ neutrophils in $CD66b^+$ LDNs and $CD66b^+$ NDNs from these groups of patients is reported in Figure 7F-G. Although no difference in the frequencies of $CD10^+$ cells within $CD66b^+$ NDNs from patients or HDs was observed (Figure 7G), immunosuppressive $CD66b^+$ LDNs from solid tumor and lymphoma patients,[8,13,37,38] contained a significantly higher frequency of mature $CD10^+$ neutrophils than $CD66b^+$ LDNs from SLE patients (Figure 7F). And in fact, we found that $CD66b^+$ LDNs from SLE patients, but not autologous NDNs, enhanced T-cell proliferation when containing <20% $CD10^+$ neutrophils (Figure 7H; data not shown), similarly to their GD counterpart and consistent with their more immature nature. Altogether, data confirm that within heterogeneous populations of $CD66b^+$ cells, CD10 can be used to discriminate mature neutrophils from immature neutrophil populations even in cancer and SLE patients.

Discussion

Heterogeneous populations of both immature and activated mature neutrophils have been shown to coexist in the peripheral blood of patients with cancer, infections, or autoimmune diseases,[7-8,10,13] and even in GDs.[39] Such a neutrophil heterogeneity occurs from either a systemic activation of neutrophils and/or an "emergency granulopoiesis."[40] Under inflammatory conditions, both immature and in vivo activated neutrophils may display altered buoyancy properties, in turn causing their recovery as LDNs after blood density gradient centrifugation.[8,10,13] However, to date, little effort has been made to characterize the immunoregulatory properties of mature vs immature neutrophils contained within LDNs.

In this study, we demonstrate that, in GDs, mature neutrophils can be clearly distinguished from immature neutrophil populations, and, in turn, isolated from the blood, based on their selective expression of CD10. By doing so, we show that mature $CD10^+$ LDNs and $CD10^+$ NDNs from GDs inhibit proliferation and IFNγ production by T cells via CD18-mediated contact-dependent release of ARG1, likely

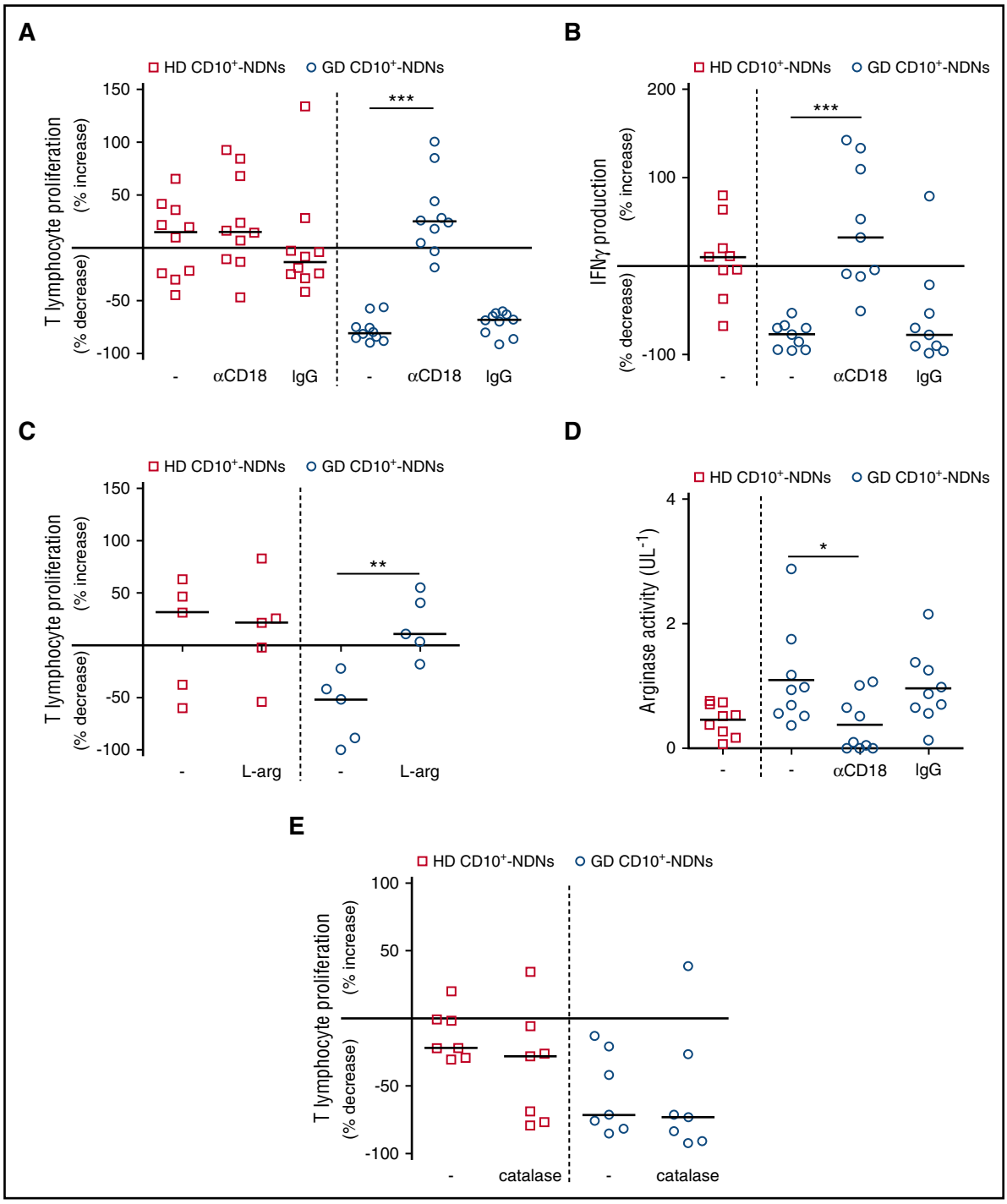

Figure 5. CD10$^+$ NDNs from GDs inhibit proliferation and IFNγ production by T cells via CD18-mediated contact-dependent ARG1 release. (A-C,E) CD3/CD28-stimulated T cells were cultured for 96 hours in the absence or the presence of CD10$^+$ NDNs from HDs or GDs, added at a 5:1 neutrophil-to-T-cell ratio, with or without: 10 μg mL^{-1} anti-CD18 or isotype control mAbs (A-B), 200 μg mL^{-1} L-arginine (C), 250 U mL^{-1} catalase (E). The percentages of increase and decrease of proliferation, as measured by BrdU incorporation (A,C,E) or IFNγ production (B) by T cells, are reported. Graph values indicate medians from independent experiments (n = 5-10). $^{**}P \leq .01$; $^{***}P \leq .001$, by the Mann-Whitney *U* test. (D) The panel displays arginase activity, as evaluated by specific enzymatic assay, in supernatants from CD3/CD28-stimulated T cells cultured for 96 hours in the absence or the presence of CD10$^+$ NDNs from HDs or GDs (with or without 10 μg mL^{-1} anti-CD18 or isotype control mAbs) at a 5:1 neutrophil-to-T-cell ratio. Graph values indicate medians from independent experiments (n = 9). $^{*}P \leq .05$, by the Mann-Whitney *U* test.

from the gelatinase granules.[41] Considering the fact that, in our hands, CD10$^+$ NDNs from HDs treated in vitro with G-CSF did not acquire immunosuppressive functions, the properties of CD10$^+$ LDNs and CD10$^+$ NDNs from GDs should reflect a complex maturation and activation process. The latter might be caused by their in vivo exposure to G-CSF, likely in combination with other G-CSF–dependent or –independent factors. By contrast, we show that immature CD66b$^+$CD10$^-$ LDNs from GDs manifest the opposite behavior because they promoted T-cell survival and enhanced proliferation and IFNγ production by T cells via CD18-mediated contact-dependent

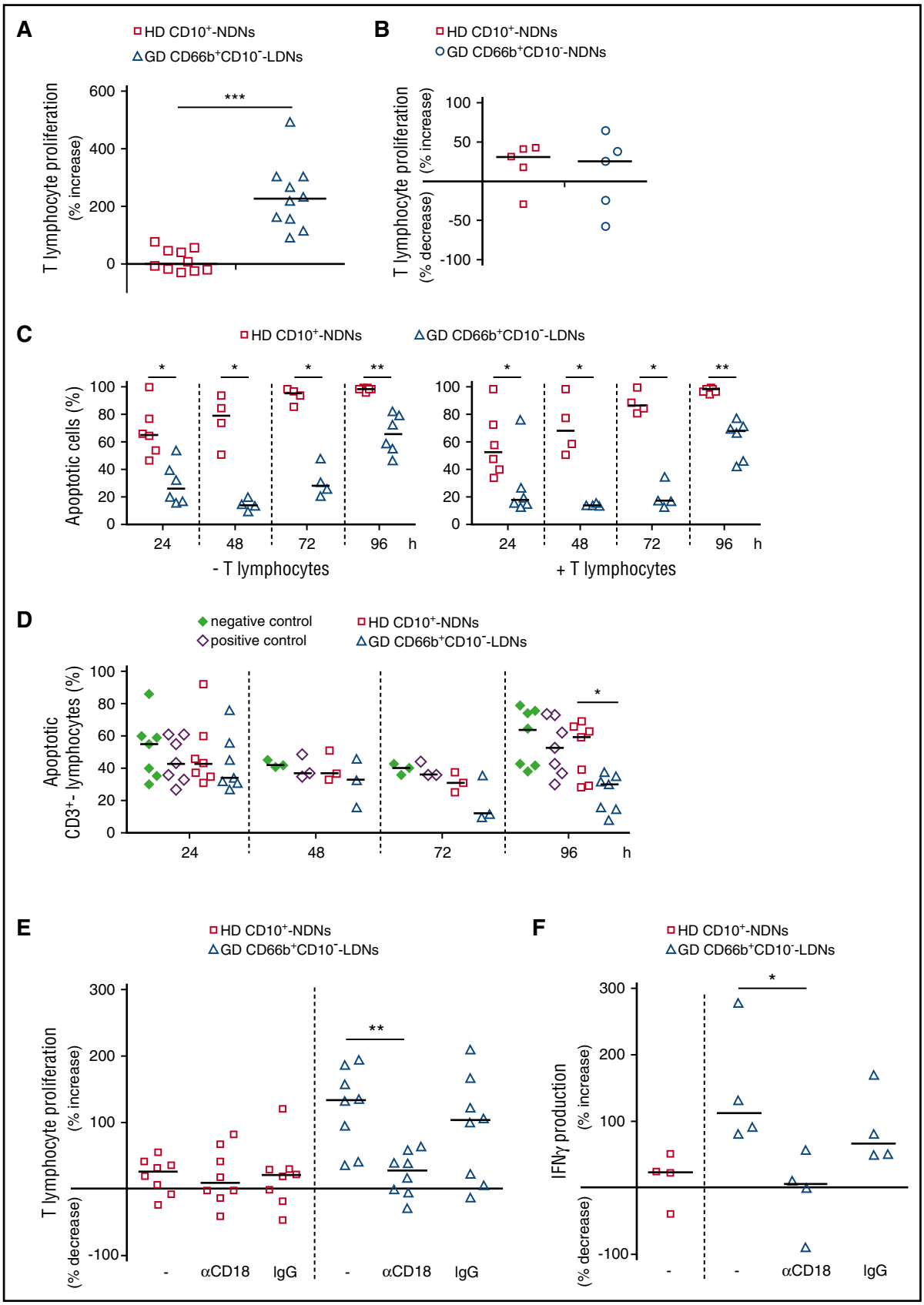

Figure 6. CD66b$^+$ CD10$^-$LDNs from GDs increase proliferation and IFNγ production by T cells. (A-B,E-F) CD3/CD28-stimulated T cells were cultured for 96 hours in the absence or the presence of either CD10$^+$ NDNs from HDs and CD66b$^+$CD10$^-$ LDNs from GDs (A,E-F), or CD10$^+$ NDNs from HDs and CD66b$^+$CD10$^-$ NDNs from GDs (B), at a 5:1 neutrophil-to-T-cell ratio and with or without 10 μg mL^{-1} anti-CD18 or isotype control mAbs (E-F). The percentages of increase/decrease of proliferation, as measured by BrdU incorporation (A-B,E) or IFNγ production (F), by T cells is reported. Graph values indicate medians from independent experiments (n = 4-10). *$P \leq .05$;

mechanisms. Notably, we found that it is possible to clearly distinguish mature neutrophils from immature neutrophil populations also within CD66b$^+$ LDNs from cancer and SLE patients, again relying on their CD10 positivity/negativity.

A series of remarkable information emerges from our work. One is the demonstration that CD10 functions as a better marker than CD16 to identify and isolate mature neutrophils in individuals with acute or chronic inflammatory conditions in whom CD16 is downregulated. Accordingly, under situations in which in vivo cell activation occurs, such as in cancer,[21,35] HIV-1 infection,[42] pregnancy,[43] and G-CSF treatment (as shown in this study), the levels of CD16 expression in mature neutrophils and band cells may become indistinguishable (this manuscript and Hübl et al[44]), unlike those of CD10. In fact, we confirmed that, in line with previous findings,[26-28] CD10 is exclusively displayed by mature neutrophils at their segmented, but not at earlier maturation, stage(s), even in patients. Nonetheless, even though the CD10 expression patterns in mature neutrophils from HDs, GDs, cancer, and SLE patients was, in our hands, comparable, our data need to be further validated. In fact, only 1 study has reported that CD10 expression may slightly increase in neutrophils incubated with lipopolysaccharide (LPS), but this was observed to occur in vitro.[45] Similarly, CD10 enzymatic activities have been shown to either decrease or increase upon in vitro stimulation of neutrophils with, respectively, phorbol 12-myristate 13-acetate,[46] or formyl-methionyl-leucyl phenylalanine, C5a, LPS, or granulocyte-macrophage colony-stimulating factor.[47] Second, we show that the relative frequencies of mature vs immature neutrophils, present within the heterogeneous CD66b$^+$ cell populations of GDs, determine the type of immunoregulatory properties ultimately displayed by them. The latter might also occur in SLE and cancer patients. Accordingly, we show that LDNs from cancer patients, which usually display immunosuppressive properties,[13,37-38] contain high frequencies of CD10$^+$ neutrophils, whereas LDNs from our small cohort of SLE patients displayed immunostimulatory actions when containing a high frequency of immature CD66b$^+$CD10$^-$ neutrophils. Third, we noticed that activated CD10$^+$ neutrophils from GDs resemble, in terms of phenotype and immunosuppressive functions, the previously described CD16^{bright}CD62L^{dim} neutrophil population isolated from healthy volunteers administered with LPS or trauma patients.[23] The latter cells, however, displayed their immunosuppressive properties via CD11b-mediated cell contacts and reactive oxygen species production but not ARG1 release. Together with our findings, these observations indicate that in vivo–activated, mature neutrophils may use multiple mechanisms to exert immunosuppression.[8-10] Fourth, we show that activated mature CD10$^+$ LDNs and CD10$^+$ NDNs, as well as unfractionated CD10$^+$ neutrophils purified from whole blood of GDs, equally suppressed T-cell responses, arguing that the immunosuppressive properties of these cell populations are independent of their buoyancy. This latter finding resembles that described by Pillay et al, again in relation to immunosuppressive CD16^{bright}CD62L^{dim} neutrophils.[23] Altogether, data support the concept that, at least under certain inflammatory conditions in which a strong systemic activation may occur, the switch of mature neutrophils into immunosuppressive subsets is not restricted to the LDN populations, but it is rather acquired by the entire pool of circulating neutrophils.[9-10,14,48] The establishment of whether density-gradient centrifugation of blood effectively separates specialized mature LDNs that have acquired specific immunoregulatory properties necessitates a careful comparison among LDNs, NDNs, and unfractionated neutrophils from the same diseased individuals. It is also unknown which is/are the factor/s promoting the development of suppressive neutrophils in vivo. In this context, G-CSF has been shown to play a pivotal role in mouse tumor models,[49,50] but a controversial one in tumor patients with elevated G-MDSCs.[51-53] On the other hand, G-CSF might be critical in patients with septic shock, a condition in which subsets of immunosuppressive mature LDNs were described,[16] and in which G-CSF plasma levels are often elevated.[54,55]

As mentioned, the presence of immature CD66b$^+$CD10$^-$ neutrophils in the circulation (known as "left shift"), for instance in sepsis or severe systemic inflammatory response syndrome,[40,56] typically derives from an active release of myeloid cells from the bone marrow. However, apart from the diagnostic and prognostic value of the immature neutrophil counts in sepsis and related inflammatory conditions,[57-60] very little is known of their capacity to modulate immune responses. To date, the study by Pillay et al has shown that CD16^{dim}CD62L^{bright} band cells are unable to affect T-cell proliferation.[23] Along the same line, Guerin et al reported that CD14$^-$CD24$^+$ immature neutrophils (likely band cells) isolated from LDNs of septic patients manifest killing properties toward T cells,[57] whereas Singhal et al have recently reported that bone marrow–derived CD66b$^+$CD10$^-$ band cells may generate antigen-presenting cell–like hybrid neutrophils displaying T-cell stimulatory properties.[61] Herein, we report that CD66b$^+$CD10$^-$ LDNs (mostly composed of band cells and metamyelocytes), but not autologous CD66b$^+$CD10$^-$ NDNs (consisting of pure band cells), from GDs enhance proliferation and IFNγ production by T cells. All of these apparently controversial results might be explained by different cell isolation methods used or the relative composition and maturation/activation statuses of the immature neutrophil populations under investigation. Nonetheless, they highlight how crucial the necessity to define the specific immunoregulatory properties of band cells, as well as of more immature neutrophil progenitors, in diseases is.

In sum, our study demonstrates that it is mandatory to separate mature from immature circulating neutrophils, if one wants to precisely uncover their specific immunoregulatory capacities in diseases. In this context, there is currently an increasing interest in clarifying the role of immunosuppressive myeloid cell populations in patients undergoing unrelated donor allogeneic stem cell transplantation, for the final purpose of preventing the occurrence of acute graft-versus-host disease.[62-64] In this regard, our work encourages prospective studies aimed at carefully investigating the frequencies of mature CD66b$^+$CD10$^+$ and immature CD66b$^+$CD10$^-$ neutrophil subsets present in grafts obtained from GDs, and what their role is in determining the occurrence of acute graft-versus-host disease. Similarly, studies aimed at verifying whether CD66b$^+$CD10$^+$ and CD66b$^+$CD10$^-$ neutrophil populations isolated from patients with inflammatory diseases other than GDs display opposite effects on T-cell functions are eagerly awaited. Finally, considering the interest in CD10 as a prognostic/diagnostic marker in oncology,[65] particularly on the correlation

Figure 6 (continued) $^{**}P \leq .01$; $^{***}P \leq .001$, by the Mann-Whitney *U* test. (C) CD10$^+$ NDNs from HDs and CD66b$^+$CD10$^-$ LDNs from GDs were cultured for the times indicated either alone (left panel) or in the presence of CD3/CD28-stimulated T cells (right panel) at a 5:1 neutrophil-to-T-cell ratio. The frequency of apoptotic neutrophils is reported. Graph values indicate medians from independent experiments (n = 4-6). $^{*}P \leq .05$; $^{**}P \leq .01$, by the Mann-Whitney *U* test. (D) Untreated (negative control) or CD3/CD28-stimulated T cells were cultured for the times indicated either alone (positive control) or in the presence of CD10$^+$ NDNs from HDs or CD66b$^+$CD10$^-$ LDNs from GDs added at a 5:1 neutrophil-to-T-cell ratio. The percentage of apoptotic T cells is reported. Graph values indicate medians from independent experiments (n = 3-7). $^{*}P \leq .05$, by the Mann-Whitney *U* test.

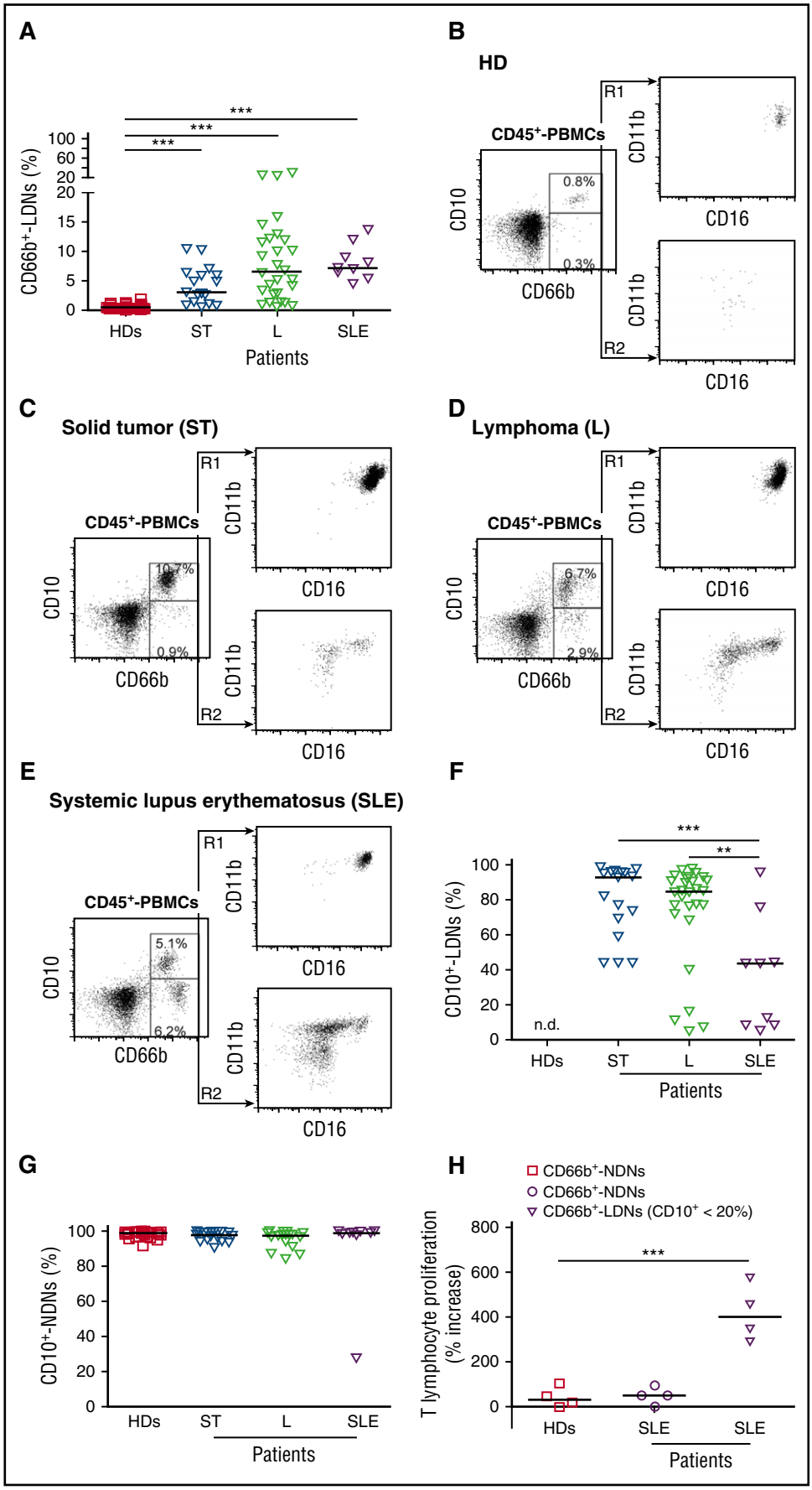

Figure 7. Frequency and phenotypic characterization of CD66b$^+$ LDNs from solid tumor, lymphoma, or SLE patients. (A) Frequency of CD66b$^+$ LDNs in PBMCs from HDs (n = 33) or patients with solid tumors (ST) (n = 17), lymphoma (L) (n = 28), or SLE patients (n = 9). Graph values indicate medians from independent experiments. Each symbol stands for a single HD or ST, L, or SLE patient. ***$P \leq .001$, by 1-way ANOVA with the Dunnett posttest. (B-E) Representative FACS plots of CD11b and CD16 expression in CD10$^+$ (R1) or CD10$^-$ (R2) cells within CD66b$^+$ LDNs from PBMCs of HDs (B), ST patients (C), L patients (D) or SLE patients (E). (F-G) Frequency of CD10$^+$ LDNs within CD66b$^+$ LDNs (F) or CD66b$^+$ NDNs (G) from HD (n = 33), ST patients (n = 17), L patients (n = 28), or SLE patients (n = 9). Graph values indicate medians from independent experiments. Each symbol stands for a single HD, ST, L, or SLE patient. **$P \leq .01$; ***$P \leq .001$, by 1-way ANOVA with the Dunnett posttest. (H) CD3/CD28-stimulated T cells were cultured for 96 hours in the presence or absence of CD66b$^+$ NDNs from HDs or CD66b$^+$ NDNs and CD66b$^+$ LDNs from SLE patients at a 5:1 neutrophil-to-T-cell ratio. The percentage of increase of T-cell proliferation, as measured by BrdU incorporation, is reported. Graph values indicate medians from independent experiments (n = 4). ***$P \leq .001$, by 1-way ANOVA with the Dunnett posttest.

between CD10 expression in malignancy and tumor progression and metastasis potential,[65] it would be intriguing to investigate whether cancer-infiltrating $CD10^+$ neutrophils, eventually committed to an immunosuppressive phenotype, could be involved in determining tumor progression.

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Authorship

Contribution: O.M., C.T., P.S., D.D.S., A.V., M.C., G.G., E.T., C.L., I.T., A.M., A.G., A.R., F.M., W.V., and C.S. designed the research study and performed data analysis; O.M., S.C., D.B., F.C., E.Z., C.C., M.T.S., and N.T. performed experiments; D.D.S., A.V., M.C., and G.G. provided GD blood samples; E.T., C.L., I.T., and A.M. provided SLE patient blood samples; A.G., A.R., F.M., W.V., C.S., and C.T. provided tumor patient blood samples; and M.A.C. and P.S. wrote the paper.

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