Immune checkpoint expression on peripheral lymphocytes in cervical cancer patients: moving beyond the PD-1/PD-L1 axis

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Abstract

Abstract In recent years, immune checkpoint therapy to reverse NK and T cell exhaustion has emerged as a promising treatment in various cancers. Recently, the FDA has approved anti-PD-1 pembrolizumab for patients with recurrent or metastatic cervical cancer. Other checkpoint molecules, such as TIGIT and Tim-3 have yet to be fully explored in this disease. Here, we found that PD-1, TIGIT, and Tim-3 are over-expressed on some peripheral blood CD56dim and CD56bright NK cells and T cells in cervical cancer patients and women with premalignant lesions. However, we observed stronger significance and separation between groups when these three molecules were examined together. These cells, with an apparently "exhausted" phenotype, were significantly augmented in patients. Different PD-1 levels (PD1low, PD1int, PD-1hi) on T cells were used to further define checkpoint positive populations. Soluble PD-L1 was observed to be increased in cervical cancer. Within the cancer group, the highest levels of sPD-L1 and triple positive or double positive cells and tumor stage were found similarly within tumors of different stages. Our results might show an overview of what is happening in patients with precancerous lesions and cervical cancer, and may give an early clue as to whom to administer monoclonal checkpoint blocking therapies.

Introduction

In recent years the role of immune checkpoint molecules has emerged as one of the most promising avenues of biomolecular and clinical discovery [1]. The ability to regulate these molecular regulators represents a new paradigm in directed treatment. Fundamental to these advances are the ideas of exhausted effector cells, that is putatively cytotoxic cells that, for one reason or another, have lost their capacity to effectively participate in anti-tumor responses [2]. Increases in regulatory T cells, chronic inflammation, and myeloid and other

lineage suppressor cells have all been implicated in the suppression of immune response and evasion of tumor cells [3]. More recently, the so-called checkpoint molecules have risen to the fore. These include stimulatory molecules (not the subject of this paper), and inhibitory molecules such as CTLA-4, PD-1, TIGIT, LAG3, Tim-3, VISTA, Siglec-7, HHLA2, BTLA, and Adenosine A2aR [4, 5]. Together, or in various combinations, these molecules have been postulated to form patterns that describe dysfunctional or exhausted T and NK cells [6, 7]. The roles and distributions of these molecules may vary between diseases and disease models. In this sense, similar to other cancers, cervical tumors and advanced dysplasias have been characterized by various "dysfunctions" of the immune system, that is failure of immunosurveillance, loss of cytotoxic NK and T activity, loss of CD3 in TIL and failure to home to tumor sites [8]. Perhaps some of the most promising checkpoint markers of immune system dysfunction are PD-1, TIGIT and Tim-3. While these markers have been established on T cells, their role in NK cell activity is less well known. To date, PD-1 and Tim-3 have been found in cervical cancer tissues [9-11]. TIGIT has yet to be described in NK cells in cervical cancer or precursor lesions. The role of these three markers together on the same cells, or on different cell populations, has only begun to be described in certain models [12-15]. Recent works have begun to explore the role of different sub-populations within effector cells, that is populations that express sharply higher levels of PD-1 as a way of distinguishing the expected presence of "normal" checkpoint molecules (which may be physiologically expected in healthy populations) and pathogenic levels, that may be encountered in select patients [16]. The accurate distinction between these two groups may define the difference between tumors responsive to, or refractory to, current and future immunotherapy checkpoint blockades. The goal of this current paper is to characterize different NK and T cell populations with respect to checkpoint molecules in cervical cancer patients.

Material and methods

Patients and samples

We collected blood samples from 78 women: patients with cervical carcinoma (n=21; median age 44, age range 47.2 +/- 11.5 years), high grade lesions (n= 9; median age 37, age range 42.9 +/- 16.6 years), low grade lesions (n=24; median age 30, age range 30.2 +/- 9.6 years), and healthy donors (n=24; median age 38.5, age range 39.25 +/- 7.2 years). The women with premalignant lesions and cancer had their diagnosis confirmed with colposcopy and histopathological analysis. The cancer group included cervical adenocarcinoma and squamous cell carcinoma in situ (stage 0), and FIGO stages I, II, III and IV. These patients were attended at the Instituto Jalisciense de Cancerología, and the Hospital Civil Nuevo, Guadalajara, Jalisco, México. All study participants gave their written informed consent. This study was performed according to the guidelines of the local institutional ethics committees and in accordance with the 2013 Declaration of Helsinki.

Venous blood was collected from each donor (15 mL). Peripheral Blood Mononuclear Cells (PBMCs) were separated from the blood samples using Lymphoprep (STEMCELL Technologies, Vancouver, Canada) density gradient centrifugation. The PBMCs were stored frozen in liquid nitrogen at a temperature below –130degC until the cytometry analysis. The viability of the thawed cells was determined with trypan blue and cells were used when viability was greater than or equal to 90%. Serum from the blood of the same women was isolated and stored at -80 degC.

Flow Cytometry

A multicolor flow cytometry protocol was used to analyze the expression of immune checkpoint molecules (PD-1, TIGIT, and Tim-3) and activating receptors (DNAM-1, NKG2D, CD16) on study group PBMCs separated into the following populations: $CD3^{-}CD56^{dim}$and $CD3^{-}CD56^{bright}$ NK cells, and $CD56^{-}CD3^{+}$ T cells. We used the following antibodies to stain $5X10^5$ PBMCs: anti-CD3-ε (FITC, clone UCHT1), anti-CD56 (PE-Cy7, clone 5.1H11), anti-CD16 (Percp-Cy5.5, clone 3G8), anti-TIGIT (APC, clone A15153G), anti-Tim-3 (BV510, clone F38-2E2), and anti-PD-1 (BV421, clone EH12.2H7) (all antibodies purchased from BioLegend, San Diego, California, USA). Data acquisition was performed using the BD FACSCANTO II flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey, USA). First, for acquisition of each sample we derived an initial dot plot (FSC-A vs FSC-H) for singlets. This selection generated a dot plot with

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the combination of FSC-A vs SSC-A, after which we acquired 250,000 events from the lymphocyte gate. The results were analyzed with Kaluza software V2.1 (Beckman Coulter, Brea, California, USA). Isotype controls were used to adjust background fluorescence. To establish the parameters for gating we employed Fluorescence Minus One (FMOs) staining controls. $CD3^{neg}CD56^{dim}$ and $CD3^{neg}CD56^{bright}$ NK cells, and $CD56^{neg}CD3^{+}$ T cells data were visualized in t-Distributed Stochastic Neighbor Embedding (t-SNE) density plots generated in FCS express 7 Plus (De Novo Software). Normalized protein expression levels for CD3, CD56, CD16, PD-1, TIGIT, Tim-3, DNAM-1 and NKG2D in t-SNE fields were represented in red for high expression, and in blue for low expression (hot-to-cold heat map).

ELISA

Soluble PD-1L concentrations in the samples (female patient and healthy donor serum) were measured by an ELISA kit (ThermoFisher, Waltham, Massachusetts, USA), according to the manufacturer's protocol. Briefly, 50 µL of sample in duplicate was added to the sample wells. Wells were then incubated with the Biotin-Conjugated antibody for 2 h at room temperature. Streptavidin-HRP was next added to all the samples and incubated 1 h at room temperature. TMB Substrate Solution was added and the wells were incubated for about 30 min at room temperature. Finally, the reaction was stopped with Stop Solution and the color intensity was measured at 450 nm.

Statistical analysis

Data were assessed for normality using the D'Agostino-Pearson normality test and comparison between the groups were performed using ANOVA with Dunnett's multiple comparisons test. A Pearson correlation analysis was performed to verify the linear associations between the groups. P values <0.05 were considered statistically significant. Prism version 7, GraphPad Software, was used for this analysis. Only significant p-values are displayed.

Results

$CD56^{dim}$ NK cells are increased in cervical cancer and precursor lesions while T cells are decreased

We first analyzed cytotoxic and potentially cytotoxic lymphocyte populations in our different study groups. We observed that the $CD56^{dim}$ NK cell population (Figure 1 A) was increased in cervical cancer patients (CC group), patients with high grade lesions (HG group), and patients with low grade lesions (LG group) in comparison with healthy donors (HD group). An inverse relationship was seen with $CD3^{+}$ T cells (Figure 1 C), with a decrease seen in all patient groups. In the $CD56^{bright}$ NK cell population, no significant changes were observed between groups (Figure 1 B).

When we analyzed the cell counts of individual samples, we found that $CD56^{dim}$ NK cell and $CD3^{+}$ T cell percentages (Figure 1 D) were often negatively correlated ($r = -0.6883; p < 0.0011$). That is, that the donors (from all groups, HD to CC) with higher $CD56^{dim}$ tended to have lower $CD3^{+}$ T cell percentages, and the those with higher T cell counts tended to have lower $CD56^{dim}$ counts. For this reason, we decided to focus our subsequent checkpoint analysis on both NK and T cells, with the idea that some patients might be more polarized towards either the NK or T cell responses. No significant correlation was found between $CD56^{bright}$ NK cells vs $CD3^{+}$ T cells or $CD56^{bright}$ NK cells vs $CD56^{dim}$ NK cells (data not shown).

Immune checkpoint profiling of $CD56^{bright}$ and $CD56^{dim}$ NK cells

In order to identify unique populations of cells in our patient samples, we utilized t-SNE analysis of our populations and markers (Figure 2 A). In $CD56^{dim}$ NK cells, we found that PD-1 was significantly increased in LG, HG and CC compared to controls (Figure 2 B). Here we observed that a subset of the patients had PD-1 levels much higher than the median of the controls, up to five and six and fold higher in the case of some of the cell populations. This distribution was not observed on the other immune checkpoints investigated. As PD-1 expression, albeit in T-cells, is already well characterized in cervical cancer patients, we used the t-SNE analysis to identify expression patterns that appeared linked with high PD-1 expression in cervical

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cancer. PD-1 and Tim-3 appeared to give similar t-SNE patterns, and it appeared a subset of the PD-1 positive cervical cancer cells might also be positive for TIGIT and perhaps other subsets might be positive for NKG2D and DNAM-1 (explored below). Focusing first on the inhibitory checkpoint molecules, we saw that, with the exception of an increase in Tim-3 in LG patients, changes in overall TIGIT and Tim-3 were not significantly different between patients and controls (Figure 2 C, D). However, when cells were gated (Supplemental Figure 1) on double and triple positive co-expression, i.e. PD-1^{+}TIGIT^{+}, PD-1^{+}Tim-3^{+} and PD-1^{+}TIGIT^{+} Tim-3^{+}populations, significant increases were observed (Figure 2 E-G). Co-expression of NKG2D, but not DNAM-1, was also increased in patient groups (Supplemental Figure 2).

While absolute percentages of the CD56$^{\text{bright}}$ NK cells were not changed between groups, a differential expression pattern of immune checkpoints in the CC, HG, and LG groups (Figure 3 A-G), in particular on PD-1 and Tim-3 molecules, was found, with an over 2.6 fold PD-1 and Tim-3 increase, and an approximately 3 fold triple positive increase compared to HD. No significant changes were observed on the co-expression of NKG2D or DNAM-1 (data not shown).

Immune checkpoint profiling of CD56^{-}CD3^{+} T cells

In order to visualize the co-expression of TIGIT, PD-1, Tim-3, DNAM-1 and NKG2D in T cells at the single-cell level within the CD56^{-}CD3^{+} T cell populations of the study groups, a high-dimensional t-SNE analysis was performed. The results showed a different pattern of immune checkpoint co-expression between CC, HG, LG and HD groups (Figure 4 A). In contrast with the single marker cytometry (Figure 1) that showed a loss of CD3+ T cells in patient groups, when we analyzed PD-1 expression in these cells, a significant increase was observed in all patient groups. That is, of the fewer T cells encountered in the patient groups, PD-1 expression was increased on these cells compared to T cells in HD. Next, analysis of the expression showed that, similar to the NK cells, expression of TIGIT and Tim-3 was not significant, but when double (PD-1^{+}TIGIT^{+}) and triple positive (PD-1^{+}TIGIT^{+}Tim-3^{+}) cells were analyzed, these populations were found to be significantly increased (up to 1.4 fold and 1.9 fold, respectively) in patient groups (Figure 4 B-G). A correlation test was performed to assess the association between PD-1 and TIGIT or PD-1 and Tim-3 in T cells from HD and CC. Those analyses did not show a significant correlation between the groups (data not shown).

Focusing on the activating receptors NKG2D and DNAM-1, our results were less clear. Here we found NKG2D (Figure 5 A) was significantly increased in LG and HG groups, while we did not observe any difference in the CC group. However, we did observe a population of PD-1^{+} T cells that were NKG2D positive and significantly (over 2 fold in HG) increased in all patient groups (Figure 5 B). Interestingly, when we evaluated PD-1 positive cells for lack of NKG2D expression, we saw that these PD-1^{+} NKG2D$^{\text{neg}}$ cells were also significantly increased in HG and CC patients versus healthy controls, though not to the same extent (Figure 5 C). Similar to what was observed with the NK cells, DNAM-1 was found to be expressed in about 95% of the T cells, thus expression of DNAM-1 showed no significant change between HD and HG or CC; there was a small but significant decreased in LG patients. However, we found a significant increase of cells that were positive for both DNAM-1 and TIGIT markers in CC (Figure 5 D-E).

Differing PD-1 expression identifies sub-populations with higher significance

Additionally, we assessed whether PD-1 positive T cells expressed PD-1 at different levels according to the PD-1 MFI (Figure 6 A). We found that PD-1 low T cells were not significantly increased between controls and patients, while two other gates that we labeled intermediate (PD-1$^{\text{int}}$) or high level (PD-1$^{\text{hi}}$) identified PD-1 positive populations that were significantly increased in patients with cancer and precursor lesions (Figure 6 B, F).

We also investigated whether PD-1$^{\text{int}}$ (Figure 6 C-E) and PD-1$^{\text{hi}}$ (Figure 6 G-I) T cells co-expressed TIGIT and Tim-3. We saw a significant increase of the PD-1$^{\text{int}}$Tim-3^{+} population in the CC group in comparison with the HD group (Figure 6 D). Interestingly, we also found a significantly higher percentage of PD-1$^{\text{int}}$ and PD-1$^{\text{hi}}$CD3^{+} cells co-expressing TIGIT. The percentages of those triple positive (PD-1-TIGIT-Tim-3) PD-1$^{\text{int}}$ and PD-1$^{\text{hi}}$ CD3^{+} cells were also increased in cancer patients (Figure 6 E, F), which might suggest

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an exhausted T cell phenotype. It was notable that the stratification of the PD-1 positive T cells allowed visualization of more significantly separated populations of cells with checkpoint markers between HD and CC, and higher levels of significance. PD-1^{hi}, and both PD-1^{hi} TIGIT^{+} and PD-1^{hi}TIGIT^{+}Tim-3^{+}cell populations were also significantly increased in LG, and LG and HG respectively (Figure 6 F-I).

Checkpoint expression is enriched in CD8 versus CD4 T cells

We next investigated the distribution of the checkpoint markers on LG CD4 and CD8 T cells (Figure 7). As noted above, CD3^{+}percentages were decreased in all study groups when compared with HD. Examining just the LG samples, we observed that the CD4:CD8 ratio was 1.4:1, which is in line with what we have seen in healthy samples. When we analyzed the different checkpoint markers we began to see significant overrepresentation in CD8 cells. With respect to PD-1 we saw this ratio drop to 1.14:1; with TIGIT this dropped to 0.71:1 and with Tim-3 =0.65:1 (Figure 7 A). These were all significant changes compared to the raw CD4:CD8 ratios. Next, we evaluated the co-expression of PD-1 with TIGIT or Tim-3, and the co-expression of PD-1 with both TIGIT and Tim-3. We observed that the percentage of triple positive CD8 T cells was again significantly higher than CD4 cells (Figure 7 B). We also analyzed the expression of these markers selectively in PD-1 intermediate and high T cells. Here we found that the above observations were much more pronounced, with much higher ratios of CD8 versus CD4 T cells. The most notable effect was observed in PD-1 high cells that were also TIGIT positive: these cells exhibited about a 3:1 CD8:CD4 ratio, demonstrating an enrichment of CD8 about 4 fold over the ratio found in total T cells (Figure 7 C-H).

Soluble PD-L1 is increased in cervical cancer and is not correlated with disease stage

We performed an ELISA on serum samples from CC (n=21), HG (n=9), LG (n=24) and HD (n=24) donors and observed a significant increase between HD and CC and, HD and LG (Figure 8). Of note was the fact that a minority (6/24) of the cervical cancer patients had sharply higher sPD-L1 levels (1.5 fold to 2.5 fold increase over the median HD level). We next performed a correlation test to demonstrate the positive association between high sPD-L1, and T cell PD-1 expression. While these six patients also had some of the higher levels of T cell PD-1 expression, we did not find a significant correlation (data not shown).

As a final analysis, we evaluated our CC group according to stage, and attempted to associate our most "exhausted" T or NK cells (or sPD-L1) with stage of the tumor. We found no association. When we ranked our samples according to highest quartile of triple positive or double cells, we found equivalent numbers of highest soluble triple or double positive T cells or NK cells in both the patients with IB cancer, and those with invasive stage IV cancers.

We propose as a model that cytotoxic cells, either CD8 T cells, or CD56^{dim} NK cells or educated CD56^{bright} NK cells, begin to express different checkpoint molecules during the response to persistent HPV infection and cervical cancer. In the course of the progression of the disease the expression of these molecules will accumulate and identify the most afflicted or exhausted cells. Identification of patients with such characteristics may highlight those uniquely able to respond to combined antibody therapies.

Discussion

Immunological checkpoint–based anti-tumor therapy has attracted extensive attention. Reversing exhaustion by blocking PD-1 is a promising target for cervical cancer treatment. Although the blockade of PD-1 and its ligand may be of use in the treatment of cervical cancer, there also exist other checkpoint molecules, such as TIGIT and Tim-3 that play an important role in T and NK cell exhaustion [17, 18]. However, only a few studies have explored the expression of these immune checkpoints on T cells from cervical cancer patients [10, 11], and the expression of these three receptors on CD56^{dim} and CD56^{bright}NK cells from cancer patients is still unknown.

The expression of peripheral blood PD-1^{+} NK cells has already been reported in healthy donors and patients with ovarian carcinoma [19]. In that study, PD-1 expression was found to be restricted to the mature NK cell population (NKG2A^{neg}KIR^{+}CD57^{+}CD56^{dim} NK cells), which is characterized by high CD16, perforin, and granzyme expression [20]. However, and similar to our results, a more recent study Liu, Y et all observed

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PD-1 to be expressed in both $CD56^{bright}$ and also $CD56^{dim}$NK cells, from the blood or tumor infiltrates, in patients with digestive cancers including esophageal, liver, colorectal, gastric and biliary cancer [21].

In our study, we found a rare population of PD-$1^{+}CD56^{bright}$ NK cells increased in the blood of cervical cancer patients. This population also co-expressed TIGIT and or Tim-3. Although $CD56^{dim}$ NK cells are the typically cytotoxic NK cell subtype, some studies have shown that following activation, $CD56^{bright}$ NK cells are equally or even more cytotoxic [22-24]. This might suggest that the expression of different immune checkpoint molecules on $CD56^{bright}$ NK cells in cervical cancer patients might play an important role in the inhibition of cytotoxicity in some tumor environments.

Of course, the traditional role of $CD56^{bright}$ NK cells has to be taken into consideration. These cells are classically considered the cytokine producing subset of NK cells. With respect to this, the roll of immune checkpoint expression on IFN-γ production has been investigated in peripheral blood $PD1^{+}CD56^{bright}$ NK cells from asymptomatic pediatric thoracic transplant patients with lymphoproliferative disorders [25]. This group observed that those cells down-modulate the activating receptors NKp46 and NKG2D while up-regulating PD-1. These phenotypic changes were associated with decreased production of IFN-γ, an effect that was abrogated after disruption of the PD-1 inhibitory pathway. However, the authors suggest that these defects were partially PD-1 independent. This then suggests that it is important to evaluate the impact of other immune checkpoints such as TIGIT and Tim-3 in the functional activity of this subset of NK cells.

It is also important to note that great variety was observed in the controls with respect to the PD-1 population in NK cells; controls recovering from transient viral infections might explain this variation. Indeed, it has been noted that chronic asymptomatic HCMV infections may explain persistently high PD-1 levels in normal controls [19].

Returning to the $CD56^{dim}$ NK cells, our data showed both a significant increase in this population in cervical cancer patients in comparison with controls, and increases in PD-1 and TIGIT and/or Tim-3 co-expression. Here it is notable to observe that looking at only TIGIT or only Tim-3 we did not see significant increases. This might induce one to believe that these checkpoint markers are not important in cervical cancer and precursor lesions. However, when one examines double and triple expression of all three checkpoint molecules together the importance of all three become evident.

The increase of $CD56^{dim}$ NK cells in patients with cervical cancer was accompanied by a decrease in the percentage of T cells. We observed a significant negative correlation when we compared both lymphocyte populations in the cancer group. This suggests, that at the peripheral level at least, there is a compensatory relationship between cytotoxic cells; that is, that a loss of one cytotoxic population is balanced by an increase in another.

The role that checkpoint molecules may play in complementary, but not identical cytotoxic populations is still not very well understood. Here we observed in cervical cancer patients that while $PD1^{+}$ NK cells were less than 50% of the total NK cells (Range= 4.21-47.8%), PD-1 expression on T cells could reach 77% (Range=39.01-77.48%) of the CD3 cells. In contrast, we observed that TIGIT or Tim-3 is expressed much more highly on NK cells (Range= 62.02-95.51%; 11.19-88.97%, respectively) in comparison with T cells (Range= 32.97-71.92%; 2.69%-51.74%) in cervical cancer patients. These results are consistent with the experiments performed on checkpoint molecules in NK and T cells from tumor-bearing mice and patients with colon cancer [18], where TIGIT expression was observed to be higher on NK cells and was associated with NK cell exhaustion. This same paper also found that PD-1 and CTLA-4 were expressed mainly by tumor-infiltrating T cells and were barely expressed by tumor-infiltrating NK cells. Thus, in accordance with that report, it is possible that peripheral NK cell activity might be mainly regulated by TIGIT and Tim-3 in cervical cancer patients.

When comparing the percentage of immune checkpoints on T cells from cervical cancer patients and healthy donors, we found a significant increase in the percentage of PD-1^{+} and $TIGIT^{+}$ T cells in the cancer group. We also observed a greater proportion of T cells in cervical cancer patients with a double (PD-$1^{+}TIGIT^{+}$ and PD-1^{+}Tim-3^{+}) or triple positive (PD-$1^{+}TIGIT^{+}$Tim-3^{+}) co-expression of immune checkpoint molecules. As

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noted above with NK cells, focusing on double and triple positive cells allowed us to see significant populations that were not visible when focusing on single positive groups.

In our T cell samples, we observed a wide range PD-1 expression levels on individual cells. Other groups have reported on this diversity of expression, and based on those works we began by subdividing our PD-1 positive T cells into six different groups, which we eventually condensed down to three broad gates: PD-1 low, PD-1 intermediate (int) and PD-1 high (hi). Our results show that the difference between patients and controls is most stark and significant when comparing PD-1 intermediate or PD-1 high expressing cells. Others have also noted that there might exist a difference between low or basal PD-1 expression, and higher levels, indicating more complete exhaustion[26, 27] [16]. For example, different levels of PD-1^+ have been reported on CD8 T cell subsets expressing LAG-3 and PD-1 after chronic stimulation within tolerizing environments [28]. In this work, the authors found that CD8 T cells could differentiate into either LAG-3+PD-1^{int}, LAG-3^{neg}PD-1^{int}, or PD-1^{high}. These subsets were found to show distinct phenotypes and functional properties as well; CD8 T cells expressing the highest levels of PD-1 (PD-1^{hi}) were not capable of producing IFN-γ, while in contrast, cells expressing an intermediate level of PD-1 could produce IFN-γ.

Therefore, we focused our analysis of the PD-1^+ T cells into two PD-1 population: high and intermediate; as the PD-1^{low} population was very common in controls, we did not continue our analysis with this population. Our data demonstrated, for the first time, to our knowledge, that in cervical cancer patients the percentage of PD-1^{int} and PD-1^{high} $CD3^+$ cells is significantly higher in patients as compared with healthy donors. Interestingly, and also not reported elsewhere, we also saw a greater percentage of $CD3^+$ cells with intermediate and high expression of PD-1 co-expressing TIGIT and Tim-3 as well. Together these results suggest that in peripheral blood from cervical cancer patients it is possible to find T cell populations expressing PD-1 at different levels and co-expressing other immune checkpoint molecules (TIGIT and Tim-3). These types of cells show a pattern of immune checkpoint receptor expression previously observed in tumor-infiltrating CD8 T cells from patients with non-small-cell lung cancer, where distinct expression levels of PD-1 show a different spectrum of exhaustion [16].

It is important to note that the DNAM-1 receptor plays a role in T cell and NK cell-mediated cytotoxicity against tumors via its interaction with CD155 and CD112 [29]. When comparing healthy donors and cervical cancer patients we did not see significant changes in the expression of DNAM-1 on NK cells and T cells (DNAM-1 expression was uniformly high, above 90% of cells -we should note, however, that a decreased percentage of T cells expressing this receptor was observed in the LG group, but this same group also had lower T cell expression of TIGIT, suggesting lower activation of these cells in these patients). It has been reported that the expression of DNAM-1 is downregulated during chronic HIV infection [30]. It is also known that the same ligands that activate cells via DNAM-1 (CD155 and CD112) may also lead to inhibition via binding with TIGIT [29]. For this reason, we investigated the co-expression of both these receptors on NK cells and T cells. What we found was a significant increase in the percentage of DNAM-1^+ TIGIT$^+$$CD3^+$ cells (and a decrease of the DNAM-1^+ TIGIT$^{\text{neg}}$$CD3^+$ population, data not shown) in our cervical cancer group. This might indicate that emerging TIGIT is playing a dominant role in some of these previously activated DNAM-1^+ T cells. For CD8 T cells, the TIGIT / DNAM-1 axis has emerged as an inhibitory / stimulatory receptor pair of importance for CD8 T cell function, similar to CTLA-4 and CD28 [31].

It also must be noted that the analysis of these cells in the tumor environment, not just peripheral blood, is absolutely essential. In a recent study [27], Pesce, Silvia et all. compared the NK cell phenotype in patients affected by peritoneal carcinomatosis versus healthy donors and observed that the features of the tumor-associated PD-1+ NK cell subset are different from those of the subset present in healthy donors. Another study showed an increase in PD-1 expression on infiltrating T cells from CIN grade II-III patients versus CIN 0 patients [10]. A recent study on multiple checkpoint molecules in T cells from blood and tumors from different samples found PD-1, TIGIT and Tim-3, among other receptors, to be markedly increased in tumor infiltrating cells compared to peripheral blood [32].

The increase in soluble PD-L1 in CC was notable. We observed a sharp increase in only 6/24 CC patient samples. This suggests that liberation of soluble PD-L1 may be a phenomenon seen only in certain most

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advanced patients where escape from immuno surveillance has been modulated by the PD-1/ PD-L1 system, and that these patients are those most likely to respond positively to PD-1 or PD-L1 blockade. When we evaluated the levels of sPD-L1 with respect to T cell PD-1 percentages we noted that these six patients also were among the highest of the PD-1 positive patients, and also tended to have elevated double and triple positive cells. It is also possible that the presence of sPD-L1 may indicate patients that would not respond well to some forms of PD-L1 blockade antibody therapy as it has been reported that secreted PD-L1 variants can mediate resistance to PD-L1 blockade by acting as a decoy [33]. Continuing with the idea that sPD-L1 or the presence of highest PD-1 or TIGIT and Tim3 levels might serve as a marker for advancement of the tumor development, we classified our samples with respect to tumor subtype and grade. We did not find any association, thus indicating that the expression of these cell types and sPD-L1 is not directly related with the development of the tumors, but instead may identify select patients with tumors at different stages that would be uniquely amenable to current and emerging therapies targeting PD-1, TIGIT and Tim-3.

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Conflict of interest:

The authors state no commercial or financial conflict of interest

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Figure legends

Figure 1. Percentages of peripheral blood NK and T cells in healthy donors (HD group), low grade lesions (LG group), high grade lesions (HG group), and cervical cancer patients (CC group). (A) Frequency of $CD56^{dim}$ NK cells, (B) $CD56^{bright}$ NK cells, and (C) $CD56^{-}$ $CD3^{+}$T cells were evaluated in PBMCs from HD (n=17), LG (n=19), HG (n=8) and CC (n=19) groups by flow cytometry. (D-F) A correlation analysis between $CD56^{dim}$ NK cells and $CD3^{+}$ T cells was also performed for LG, HG, and CC groups. Frequency data are shown as individual percentages of expression and their mean. Comparison between the groups were performed using ANOVA with Dunnett's multiple comparisons test. A Pearson correlation analysis was performed to verify the linear associations between $CD56^{dim}$ NK cells and $CD3^{+}$ T cells. The correlation coefficient, r, and*p* values are shown on the figures. *p [?] 0.05, **p [?] 0.01, ***p [?] 0.001.

Figure 2. Expression of PD-1, TIGIT and Tim-3 on peripheral blood $CD56^{dim}$ NK cells from healthy donors (HD group), low grade lesions (LG group), high grade lesions (HG group) and cervical cancer patients (CC group). (A) t-SNE analysis was used to visualized expression distribution within the $CD3^{-}CD56^{dim}$ NK cell population of DNAM-1, CD16, NKG2D, TIGIT, PD-1 and Tim-3 in HD (n= 17), LG (n= 19), HG (n= 8) and CC (n= 19) groups. PD-1 expression patterns for all 4 groups are highlighted in red and common staining regions in the CC group for PD-1, TIGIT, Tim-3, and NKG2D are outlined in black. Flow cytometry gating of the data was performed to analyze the single expression of the immune checkpoints: (B) PD-1, (C) TIGIT, (D) Tim-3; double expression (E) $PD\text{-}1^{+}TIGIT^{+}$, (F) $PD\text{-}1^{+}Tim\text{-}3^{+}$; and triple expression (G) of $PD\text{-}1^{+}TIGIT^{+}Tim\text{-}3^{+}$on $CD56^{dim}$ NK cells. Data are shown as individual percentages of expression and their mean. Comparisons between the groups were performed using ANOVA with Dunnett's multiple comparisons test. *p [?] 0.05, **p [?] 0.01, ***p [?] 0.001.

Figure 3. Expression of PD-1, TIGIT and Tim-3 on peripheral blood $CD56^{bright}$ NK cell from healthy donors

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(HD group), low grade lesions (LG group), high grade lesions (HG group) and cervical cancer patients (CC group). (A) t-SNE analysis was used to visualized expression distribution within $CD3^{-}CD56^{bright}$ NK cell population of DNAM-1, CD16, NKG2D, TIGIT, PD-1 and Tim-3 in HD (n=13), LG (n=20), HG (n=7) and CC (n=19) groups. PD-1 expression patterns for all 4 groups are highlighted in red and common staining regions in the CC group for PD-1, TIGIT, Tim-3, and NKG2D are outlined in black; the red box in the HD group indicates the TIGIT high region in those samples. Flow cytometry gating of the data was performed to analyze the single expression of the immune checkpoints: (B) PD-1, (C) TIGIT, (D) Tim-3; double expression (E) $PD\text{-}1^{+}TIGIT^{+}$, (F) $PD\text{-}1^{+}Tim\text{-}3^{+}$; and triple expression (G) of $PD\text{-}1^{+}TIGIT^{+}Tim\text{-}3^{+}$on $CD56^{bright}$ NK cell. Data are shown as individual percentages of expression and their mean. Comparisons between the groups were performed using ANOVA with Dunnett's multiple comparisons test. *p [?] 0.05, **p [?] 0.01, ***p [?] 0.001.

Figure 4. Expression of PD-1, TIGIT and Tim-3 on peripheral blood T cells from from healthy donors (HD group), low grade lesions (LG group), high grade lesions (HG group) and cervical cancer patients (CC group). (A) t-SNE analysis was used to visualized expression distribution within $CD56^{-}CD3^{+}$ cells of DNAM-1, CD16, NKG2D, TIGIT, PD-1 and Tim-3 in HD (n=13), LG (n=19), HG (n=8) and CC (n=19) groups. PD-1 and TIGIT expression patterns for all 4 groups are highlighted in red and common staining regions in the CC group for PD-1, TIGIT, Tim-3, DNAM-1 and NKG2D are outlined in black. Flow cytometry gating of the data was performed to analyze the single expression of the immune checkpoints: (B) PD-1, (C) TIGIT, (D) Tim-3; double expression of (E) $PD\text{-}1^{+}TIGIT^{+}$, (F) $PD\text{-}1^{+}Tim\text{-}3^{+}$; and triple expression (G) of $PD\text{-}1^{+}TIGIT^{+}Tim\text{-}3^{+}$on CD3+ T cells. Data are shown as individual percentages of expression and their mean. Comparisons between the groups were performed using ANOVA with Dunnett's multiple comparisons test. *p [?] 0.05, **p [?] 0.01, ***p [?] 0.001.

Figure 5. NKG2D and DNAM-1 expression on peripheral blood $CD3^{+}$ cells from healthy donors (HD group), low grade lesions (LG group), high grade lesions (HG group) and cervical cancer patients (CC group). (A) NKG2D expression alone and (B) with PD-1 co-expression was assessed. (C) PD-1 was also evaluated on $NKG2D^{neg}$ $CD3^{+}$ cells. (D) DNAM-1 expression and (E) co-expression with its antagonist receptor, TIGIT, was evaluated in the same groups: HD (n=13), LG (n=19), HG (n=8) and CC (n=19). Data are shown as individual percentages of expression and their mean. Comparisons between the groups were performed using ANOVA with Dunnett's multiple comparisons test. *p [?] 0.05, **p [?] 0.01, ***p [?] 0.001.

Figure 6. PD-1 intermediate ($PD\text{-}1^{int}$) and PD-1 high ($PD\text{-}1^{hi}$) expression on peripheral blood T cells from healthy donors (HD group), low grade lesions (LG group), high grade lesions (HG group) and cervical cancer patients (CC group). PD-1 expression was measured by flow cytometry and staining was characterized as high or intermediate depending on MFI expression. (A) Examples of high and intermediate PD-1 expression in HD versus CC. In (B-E) $PD\text{-}1^{int}$ and (F-I) $PD\text{-}1^{hi}$, double positive and triple positive checkpoint marker populations were evaluated. Data are shown as individual percentages of expression and their mean from HD (n=13), LG (n=19), HG (n=8) and CC (n=19) groups. Comparisons between the groups were performed using ANOVA with Dunnett's multiple comparisons test. *p [?] 0.05, **p [?] 0.01, ***p [?] 0.001.

Figure 7. Expression of PD-1, TIGIT and Tim-3 on peripheral blood CD4 and CD8 T cells from low grade lesions (LG group). (A) Expression of PD-1, TIGIT and Tim-3 in CD4 versus CD8 T cells from the low grade lesion patient group. (B) double and triple co-expression: $PD\text{-}1^{+}TIGIT^{+}$, $PD\text{-}1^{+}Tim\text{-}3^{+}$ and $PD\text{-}1^{+}TIGIT^{+}Tim\text{-}3^{+}$in CD4 versus CD8 T cells from the low grade patient group. PD-1 expression was divided according to the MFI in two populations, PD-1 intermediate ($PD\text{-}1^{int}$) and PD-1 high ($PD\text{-}1^{hi}$). The co-expression of PD-1 TIGIT, Tim-3 and both receptors in CD4 versus CD8 cells was also evaluated on (C-E) $PD\text{-}1^{int}$ and (F-H) $PD\text{-}1^{hi}CD3^{+}$ cells. Data are shown as individual percentages of expression and their mean. Comparisons between the groups were performed using ANOVA with Dunnett's multiple comparisons test. **p [?] 0.01, ***p [?] 0.001, ****p [?] 0.0001.

Figure 8. Soluble PD-1L (sPD-1L) in serum from healthy donors (HD group), low grade lesions (LG group), high grade lesions (HG group) and cervical cancer patients (CC group). The concentration of soluble PD-1L in the serum of the different groups was measured by ELISA. Data are shown as pg/ml of sPD-1L and their

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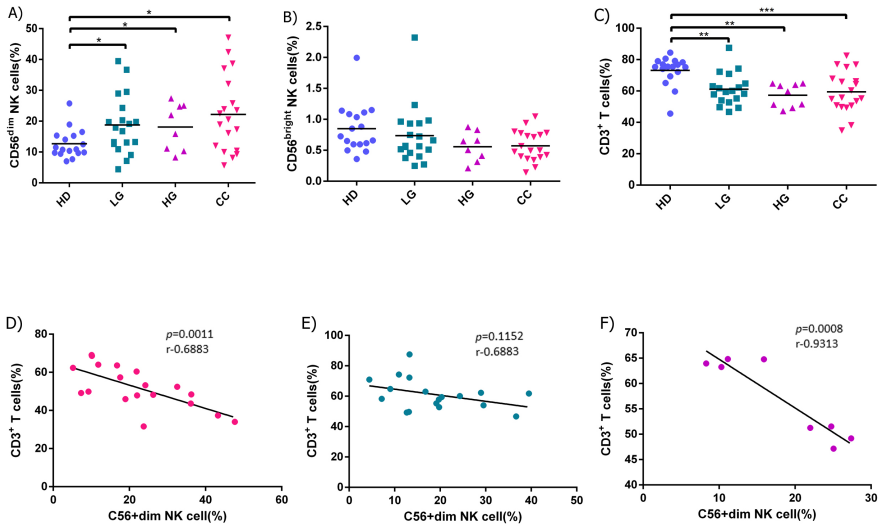
mean. Comparisons between HD (n=24), LG (n=24), HG (n=9) and CC (n=21) groups was performed using ANOVA with Dunnett's multiple comparisons test. *p [?] 0.05, **p [?] 0.01, ***p [?] 0.001.

Supplemental Figure 1. Gating strategy for the flow cytometric analysis of PBMCs from patients and controls. (A) representative workflow showing identification of NK and T cell populations. (B) representative $CD56^{dim}$ NK cells single antibody staining and FMO controls; left healthy donor, right cervical cancer. (C) representative $CD56^{dim}$ NK cells double and triple staining examples, the fifth plot in the row shows PD-1^{+}TIGIT^{+}Tim-3^{+}cells derived from the double positive populations; left healthy donor, right cervical cancer. (D) representative $CD56^{bright}$ NK cells single antibody staining and FMO controls; left healthy donor, right cervical cancer (E) representative $CD56^{bright}$ NK cells double and triple staining examples, the fifth plot in the row shows PD-1^{+}TIGIT^{+}Tim-3^{+}cells derived from the double positive populations; left healthy donor, right cervical cancer. (F) representative $CD3^{+}$ T cells single antibody staining and FMO controls; left healthy donor, right cervical cancer. (G) representative $CD3^{+}$ T cells double and triple staining examples, the fifth plot in the row shows PD-1^{+}TIGIT^{+}Tim-3^{+}cells derived from the double positive populations; left healthy donor, right cervical cancer.

Supplemental Figure 2. NKG2D and DNAM-1 expression on peripheral blood $CD56^{dim}$ NK cells from healthy donors (HD group), low grade Lesions (LG group), high grade lesions (HG group) and cervical cancer patients (CC group). (A) NKG2D expression alone and (B) with PD-1 co-expression was assessed. (C) PD-1 was also evaluated on $NKG2D^{neg}$ $CD3^{+}$ cells. (D) DNAM-1 expression and (E) co-expression with its antagonist receptor, TIGIT, was evaluated in the same groups: HD (n=17), LG (n=20), HG (n=7) and CC (n=19). Data are shown as individual percentages of expression and their mean. Comparisons between the groups were performed using ANOVA with Dunnett's multiple comparisons test. *p [?] 0.05, **p [?] 0.01, ***p [?] 0.001.



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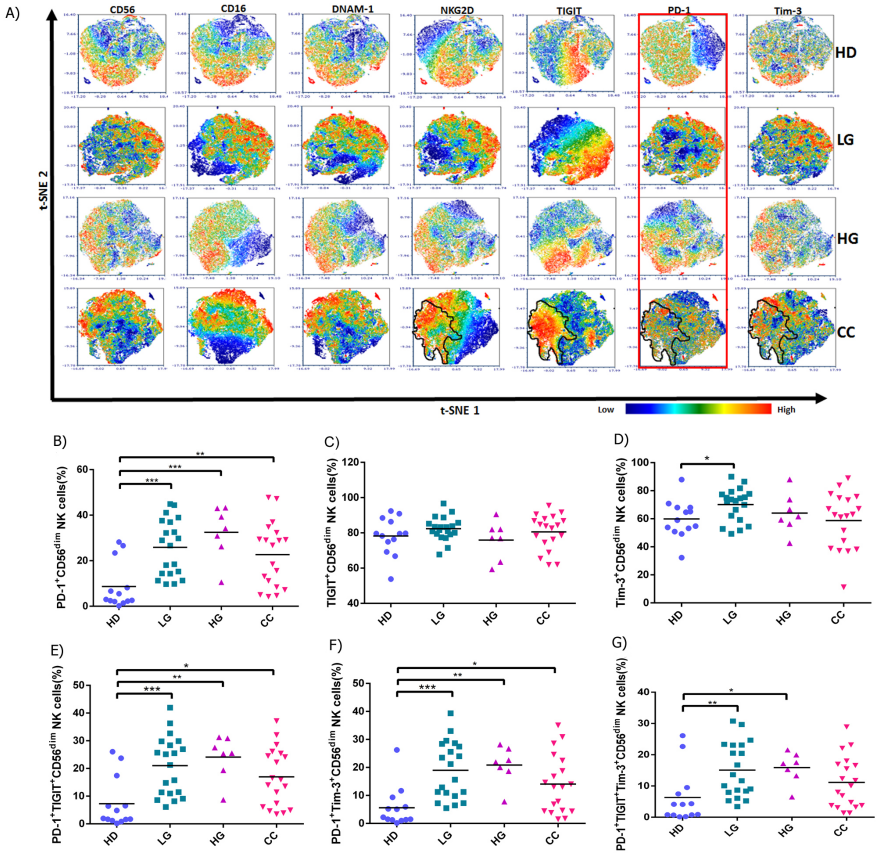

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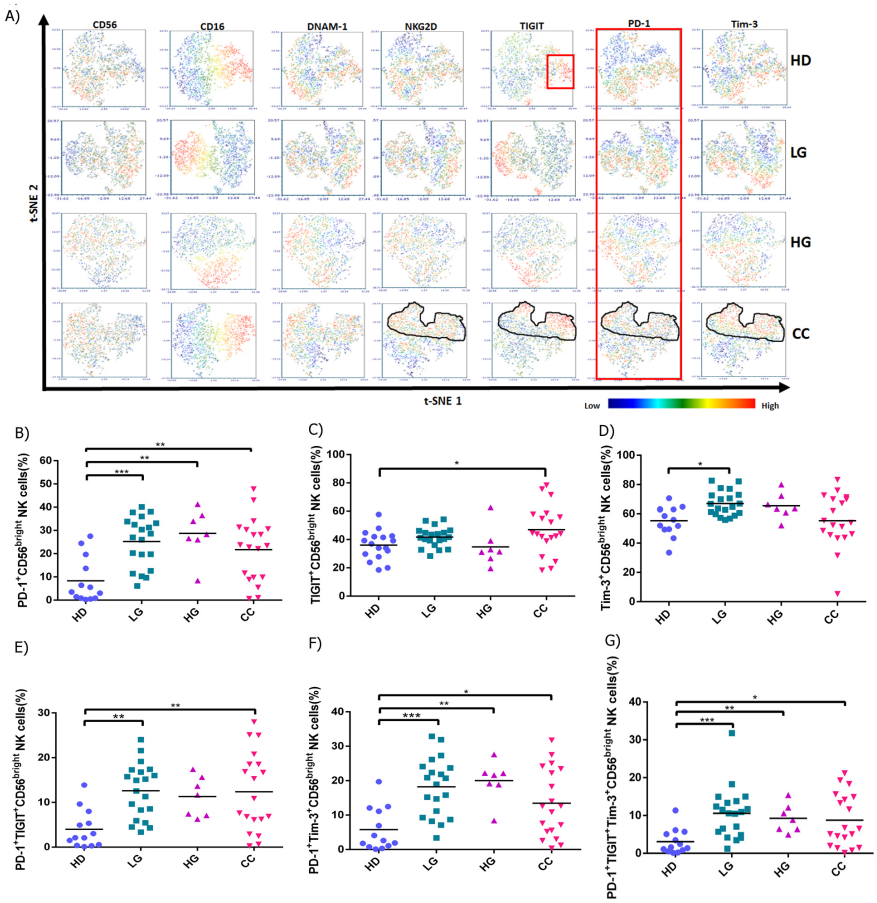

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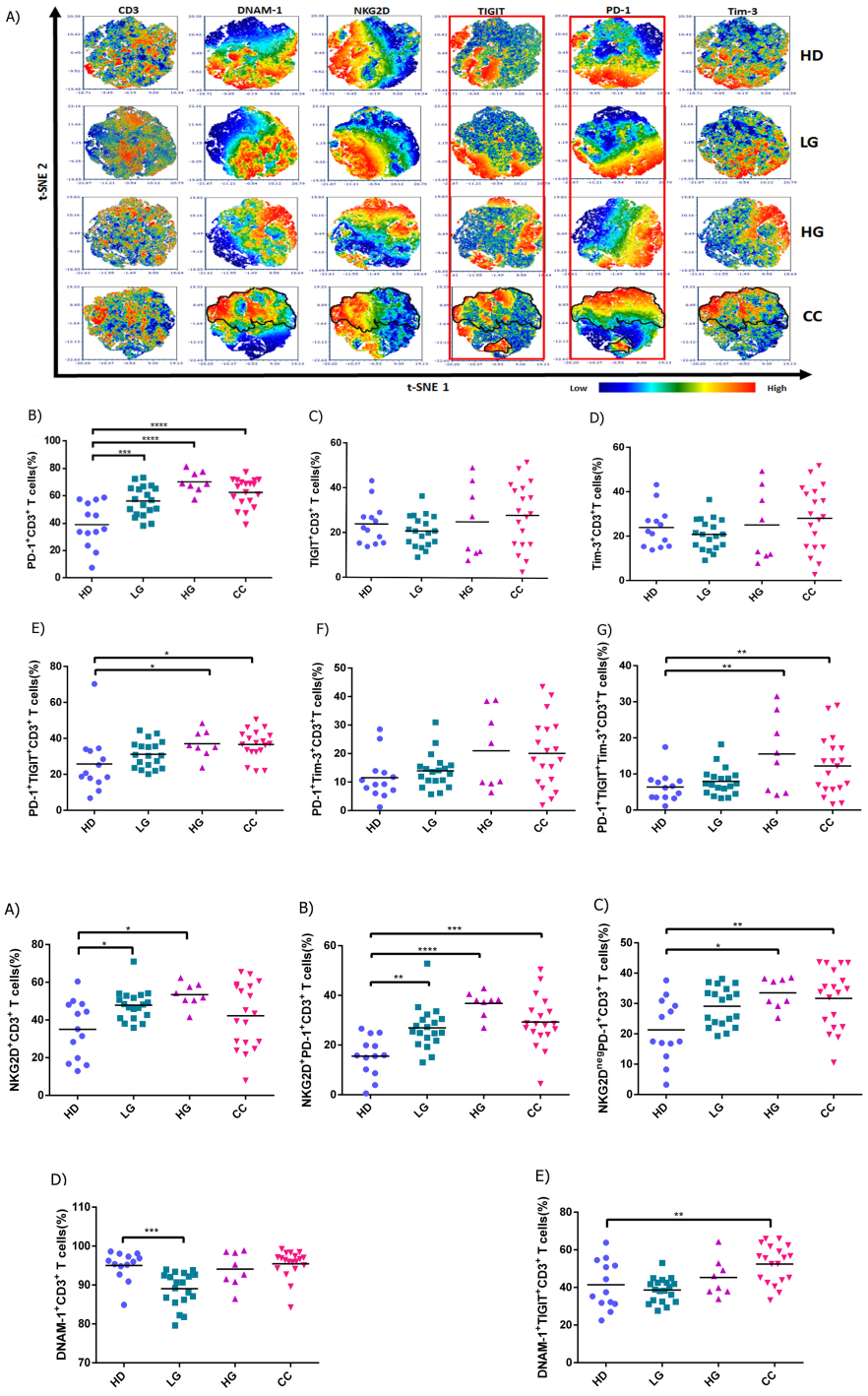

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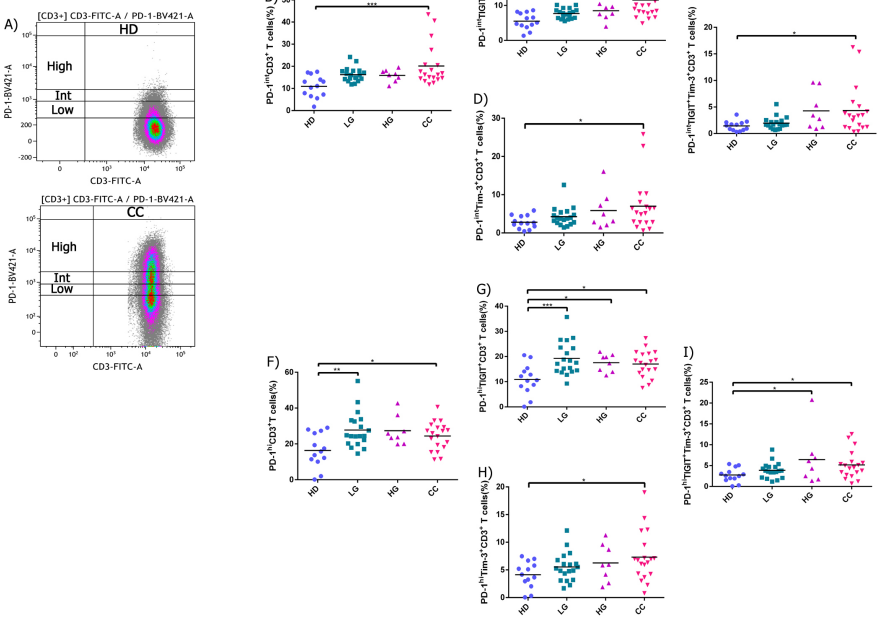

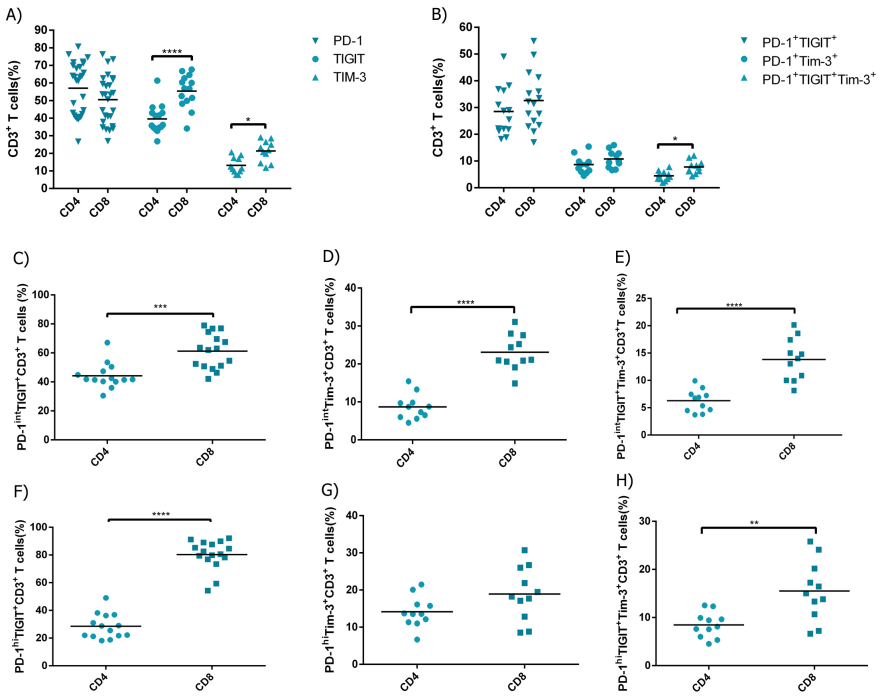

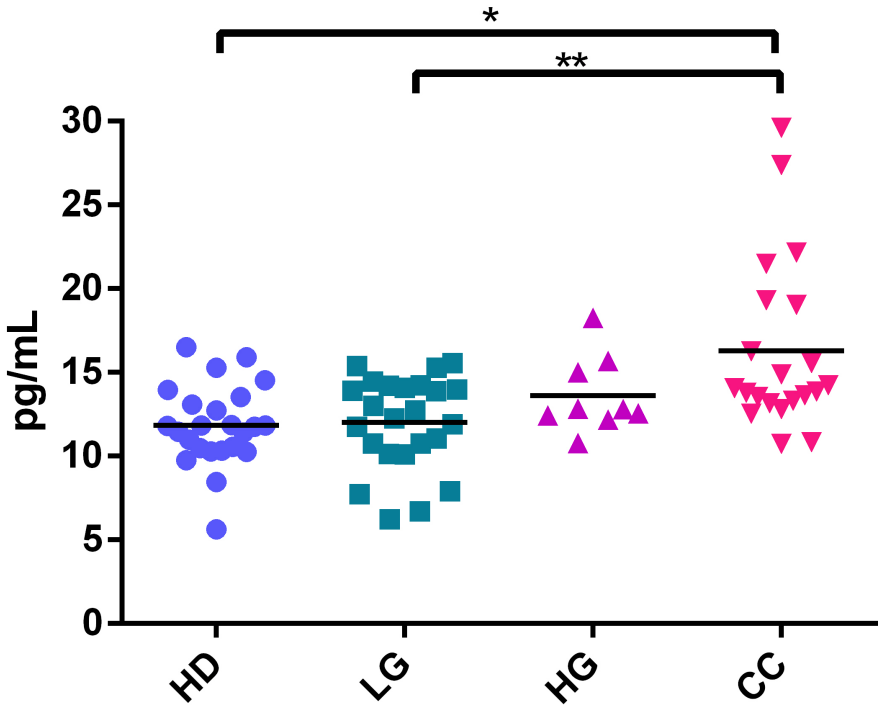

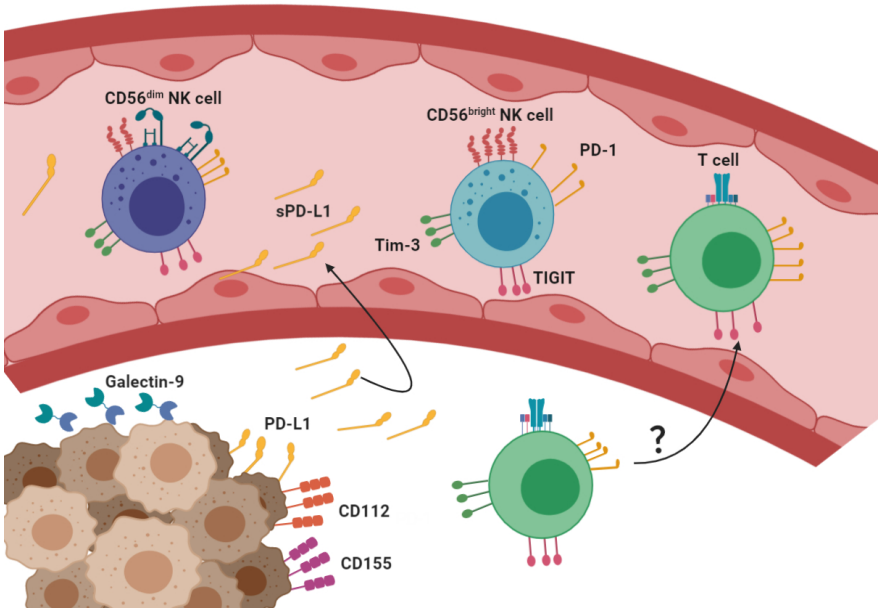

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