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Compartmentalized cytotoxic immune response leads to distinct pathogenic roles of natural killer and senescent CD8+ T cells in human cutaneous leishmaniasis

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Key Words: Cutaneous leishmaniasis, NK cells, CD8+ T cells, *Leishmania braziliensis,* cellular senescence, immunopathology.

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SUMMARY:

Cytotoxic activity mediated by $CD8^+$ T cells is the main signature of the immunopathogenesis of cutaneous leishmaniasis (CL). Here, we performed a broad evaluation of NK cells phenotypic and functional features during cutaneous leishmaniasis. We demonstrate for the first time that CL patients present the accumulation of circulating NK cells with multiple features of replicative senescence including low proliferative capacity and shorter telomeres, elevated expression of CD57, KLRG-1 but diminished CD27 stimulatory receptor expression. Moreover, they exhibited higher cytotoxic and inflammatory potential than age-matched controls. The accumulation of circulating senescent NK cells ($CD56^{dim}CD57^{brigh}$) correlated positively with skin lesion size in the same patients, suggesting that they, like circulating senescent $CD8^+$ T cells, may contribute to the immunopathology of CL. However, this senescent population had lower CLA expression and thus diminished skin-homing potential compared to total or senescent (EMRA) $CD8^+$ T cells. This was confirmed in CL skin lesions where we found a predominance of $CD8^+$ T cells (both senescent and non-senescent) that correlated with the severity of the disease. Although there was also a correlation between the proportions of senescent NK cells ($CD56^+CD57^+$) in the skin and lesion size, this was less evident. Collectively our results firsthand demonstrate that senescent cytotoxic cells may mediate the skin pathology during human cutaneous leishmaniasis. However, since senescent cytotoxic $CD8^+$ T cells predominate in the skin lesions, they may have a greater role than NK cells in mediating the non-specific skin damage in CL.

INTRODUCTION

Leishmania braziliensis is the main causal agent of American tegumentary leishmaniasis, a serious neglected tropical disease where destructive cutaneous lesions develop (WHO, 2015). In cutaneous leishmaniasis, a good prognosis is related to the predominance of a cellular Th1-type response with production of IFN-γ, TNF-α and activation of parasite-infected macrophages. On the other hand, the exacerbated Th1-immunity and cytotoxic response were identified as key elements in the immunopathogenesis of both murine and human cutaneous leishmaniasis caused by *L. braziliensis* (Faria et al. 2010; Novais et al. 2017; Novais et al. 2013; Santos et al. 2013). The gene expression analysis in CL skin lesions revealed that cytotoxicity-related genes are overexpressed compared to the cytokine pathway (Novais et al., 2015), representing the main signature of *L. braziliensis* infection. In CL-patients the accumulation of cytotoxic $CD8^+$ T cells or granzyme B producing cells were linked with the severity of CL (Faria et al. 2010). We showed recently that $CD8^+$ T cells are driven towards senescence acquiring high cytotoxic potential and skin homing capacity, which may promote the skin damage (Covre et al., 2018). Although the role of cytotoxic and senescent $CD8^+$ T cells in the immunopathology in CL is established, it is not clear if senescent NK cells also have a role in this process.

Natural killer (NK) compose 5% to 20% of peripheral blood mononuclear cells in humans and play a central role in the imunosurveillance through the cytotoxic and proinflammatory activities, without a requirement for prior sensitization (Ferraz et al., 2017). Similar to observations in the T cell pool, the differentiation state of NK cell modulates their functional capacity, which is still unknown in the context of *Leishmania* infection. NK cells may be divided into distinct phenotypic and functional subsets based on the relative expression of cell-surface CD56 and CD16 (FcRIIIa) (Lopez-Verges et al., 2010). The $CD56^{bright}$ NK subset has increased immunoregulatory and proliferative capacity after stimulation with cytokines, while the $CD56^{dim}$ cells (the majority population ~ 90%) represents the most differentiated subset. The protective role of NK cells during CL is demonstrated by the increased proliferative activity in cured individuals

compared to patients with active lesions (Maasho et al., 1998). Furthermore, higher numbers of $CD56^+$ cells are found in the peripheral blood and lesions of patients with diffuse cutaneous leishmaniasis (DCL) who have a positive response to immunotherapy (Pereira et al., 2009). Conversely, dysfunctional NK cell activity is linked to susceptibility and severity of human visceral leishmaniasis (VL) (Manna et al., 1993) and mucocutaneous leishmaniasis (Brodskyn et al., 1997).

The pivotal balance that regulates either the functional activity of senescent $CD8^+$ T cells or NK cytotoxic cells in blood and lesions of CL patients is poorly understood. Here, we characterized the phenotypic and functional profiles of circulating NK cell subsets in these individuals. We found that *L. braziliensis* infection induces the terminal differentiation of both CD8 and NK cells with a high cytotoxic and inflammatory potential that is related to the pathology of CL. We found that while senescent NK cells predominate in the blood compartment, senescent $CD8^+$ cells are preferentially localized in the cutaneous lesions and their presence is significantly associated with the tissue damage. Our results provide a broad understanding of the relationship between systemic and skin immunity and establish for the first time the relative roles of NK and $CD8^+$ T cells in the pathogenesis of CL.

MATERIALS & METHODS

Study subjects. Peripheral blood from 16 untreated cutaneous leishmaniasis (CL) patients attending University Hospital (HUCAM) of Universidade Federal do Espirito Santo, Brazil were investigated in this study. They consisted of 9 males and 7 females with illness duration ranging from 30 to 120 days and lesion sizes ranging from 200–600 mm^2. The diagnosis of CL was based on clinical and laboratory criteria and all patients in this study were positive for the PCR/restriction fragment length polymorphism of *L. braziliensis* and reported no prior infections or treatment. The control group consisted of 16 healthy age and gender matched individuals (HC) living in a non-endemic area without a history of leishmaniasis. All study participants (patients and healthy volunteers) were soronegative for HIV, HBV and HCV infections, had no history of chemotherapy,

radiotherapy or treatment with immunosuppressive medications within the last 6 months. They provided written informed consent, and study procedures were performed in accordance with the principles of the Declaration of Helsinki. This study was registered at HUCAM ethical committee under referential number 735.274.

PBMC isolation, cell sorting and culture: PBMC from CL and HC patients were isolated by centrifuging whole blood through a Ficoll-Hypaque (GE Healthcare) gradient followed by hemocytometry to determine absolute live cell number. Both NK and K562 cells were cultured in complete medium (RPMI-1640 supplemented with 10% heat-inactivated FCS, 100 U/mL penicillin, 100 mg/mL streptomycin, and 2 mM L-glutamine; Invitrogen). NK cells were negatively isolated from PBMC fraction using an NK Cell Isolation Kit/ VARIOMACS system (Miltenyi Biotec) according to the manufacturer's instructions.

Flow cytometric analysis. Flow cytometry was performed with the following antibodies: Live/Dead Blue Fixable Stain (L23105, Thermo Scientific), anti-CD3 (UCHT1), anti-CD16 (3G8), anti-CD27 (M-T271), anti-CD57 (NK-1), human cutaneous lymphocyte antigen (CLA) FITC (HECA-452), granzima B (GB11), perforina (∂G9), anti-CD107a and Ki67 (B56) from BD Biosciences. Anti-CD7 (M-T701), anti-CD56 (HCD56) and anti-KLRG1 (REA261) from Miltenyi Biotec. For surface markers, staining was performed at 4°C/ 30 min in the presence of saturating concentrations of antibodies. For intracellular analysis cells were fixed and permeabilized with the Fix & Perm® Kit (Invitrogen, Life Technologies, UK), before incubation with indicated antibodies. Intracellular staining for Ki67 was performed with Foxp3 Staining Buffer Set (Miltenyi Biotec, Bisley, UK). Samples were acquired in a Fortessa X-20 cytometer (BD Biosciences) and analysed using FlowJo software (Treestar).

Telomere hybridisation. Isolated NK cells were fixed on poly-L-lysine coated glass slides and processed for fluorescence in situ hybridization (FISH), as described previously (Agaram et al., 2014). Samples were hybridized with peptide nucleic acid probes for the telomere (TelC-Cy3 probe, 5′- CCCTAACCCTAACCCTAA-3′; (Cambridge Research Biochemicals) and the nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes). Images were acquired using a Leica SPE2 confocal microscope using LAS X software. Images corresponded to a full Z-stack with 0.5µm step size and were projected at maximum intensity and analysed using ImageJ version 2.0.0-rc-43/1.51q.

Calcein-release cytotoxicity assay. The cytotoxic activity was assessed using the K562 cells (human erythroleukemia cell line) as target. Briefly, 20,000 K562 cells were labelled with Calcein-AM (Sigma-Aldrich) at 10 µM for 1 hour and plated in a 96-well flat-bottom and co-culture with NK cells in complete medium containing 500 IU/mL rhIL-2 (Miltenyi Biotech). Effector and target cells were combined at a ratio of 40:1 in triplicate. After 4 hours of co-culture fluorescence was measured in 75 µl of cell culture supernatant using a Spectramax Gemini spectrofluorimeter. Specific lysis was calculated as % killing = (test release–spontaneous release) / (max release–spontaneous release) x 100.

CD107a degranulation assay. Isolated NK cells were incubated at 37°C for 4h with target K562 cells, at a effector to target (E:T) ratio of 2:1, in the presence of APC-conjugated CD107a antibody (BD Biosciences), as previously described (Aktas et al., 2009). After incubation, cells were stained for surface markers and CD107a expression and analyzed by flow cytometry.

Immunofluorescence analysis. For confocal microscopy, 8mm punch biopsies from the edge of lesional skin were embedding in OCT and freezing on liquid nitrogen and preserved at −80 °C until use. Healthy (uninfected) skin samples were taken from volunteers using a 4 mm punch. The

frozen skin samples were longitudinally sectioned at 8µm thickness and placed on microscopy glass slides. Immunofluorescence staining was performed and images were analysed on the SlidePath Digital Image Hub (Leica) with Definiens Tissue Studio 3.6 (Definiens AG). Multiple primary staining of nuclei with DAPI (1 µg/mL), granzyme B (Abcam-4059), CD57 (187274-Abcam), CD8 (MCA352-BioRad) and CD56 (56C04-Thermo) was performed. The secondary staining was carried out using the following antibodies anti-rat (A11006), anti-mouse (A21124) or (A21046) and anti-rabbit (A21244) all from Thermo Scientific. Analysis was performed in manually selecting regions of interest (ROI) using Chromoplex Staining Detection system (Leica Biosystems). The cell and markers frequencies were defined according to the evaluated marker and cell nuclei of each ROI. Imaged with a 20× objective (200 × magnification) on an upright confocal microscope with a 710 Metahead (Zeiss) by z-stacktile-scans.

Granzyme B and cytokines determination. Granzyme B, IL-2, IFN-γ and TNF-□ were determined in cell culture supernatant of NK-cytotoxicity assays using cytokine bead array (CBA) (Flex Set from BD Biosciences) according to the manufacturer's instruction.

Proliferation assay. Sorted NK cells were cultured in the presence rhIL-2 (100 U/mL) for 48h, followed by intracellular staining for the cell cycle related nuclear antigen Ki67 Alexa Fluor 647 (B56) from BD Bioscience and analysed by flow cytometry. Supplemented culture medium without rIL-2 was used as control.

tSNE analysis. Unbiased representations of multi-parameter flow cytometry data were generated using the t-distributed stochastic neighbour embedding (tSNE) algorithm. The R package 'Rtsne' available on CRAN (github.com/jkrijthe/Rtsne) was used to perform the Barnes Hut implementation of tSNE on flow cytometry data, where a similar number of events from each

sample were analyzed in parallel based on the fluorescent parameters and grouped near to each other based on the similarity expression levels.

Statistics: GraphPad Prism (version 7) was used to perform statistical analysis. Statistical significance was evaluated using the paired Student t-test. Mann-Whitney test was performed for all continuous, nonparametric variables and correlations were calculated using Pearson's correlation coefficient. Differences were considered significant when p was <0.05.

RESULTS

Patients with localized cutaneous leishmaniasis have an increase in the number of circulating NK cells that present a mature/senescent phenotype.

First, we investigated the heterogeneity of circulating NK cell populations among healthy control (HC) and cutaneous leishmaniasis patients (CL) groups. The percentage of total NK cells identified as $CD3^{-}CD7^{+}CD56^{+}$ (Supplementary figure 1) was similar in PBMC from CL compared to HC group (Figure 1A). However, compared to HC group, NK cells from CL patients have a decreased frequency of $CD56^{bright}$ and increased frequencies of $CD56^{dim}$ subset (Figure 1B) as well higher frequencies of mature $CD16^{+}CD56^{dim}$ subset (Figure 1C), both constituting the most mature NK populations.

The simultaneous expression of CD57 and KLRG1 and loss of CD27 co-stimulatory receptor have been used to define highly differentiated T and NK cells that exhibit characteristics of cellular senescence (Lopez-Verges et al., 2010; Luetke-Eversloh et al., 2013). CL patients demonstrate increased frequencies of CD57 and KLRG1 in the total NK pool compared to HC group that was mainly found in the mature $CD56^{dim}$ subset (Figure 2A and B). The acquisition of CD57 expression is gradual and we observed an accumulation on $CD57^{bright}$ cells in CL patients (Figure 2C and D). This supports that circulating NK cells are driven towards senescence during CL.

NK cells from cutaneous leishmaniasis patients exhibit increased effector function but low proliferative capacity and telomere erosion

We therefore next assessed the pro-inflammatory cytokine secretion, degranulation and the killing potential of circulating NK cells against MHC class I deficient- K562 cells. Compared to controls, CL- NK cells displayed both higher degranulation and greater killing capacity of the target cells (Figure 3A). Moreover, they secreted significantly increased levels of IFN-γ, TNF-α and granzyme B (Figure 3A) but showed less proliferation after IL-2 activation (Figure 3B), which was accentuated within the $CD56^{dim}$ $CD57^{bright}$ subset (Figure 3C). Moreover, QFISH analysis in isolated NK cells from CL patients showed shorter telomeres that were significantly lower (1.7 to 1.9- fold) than those observed in the HC group (Figure 3D),

We next evaluated the *ex vivo* capacity of senescent NK cells to produce cytotoxic mediators. Thus, we found that $CD57^{dim}$ and $CD57^{bright}$ cells spontaneously produce a higher amount of granzyme B and perforin compared to the less mature phenotype ($CD57^{neg}$) (Figure 4A and B). Thus, senescent NK and T cell have increased cytotoxic and pro-inflammatory capacity (Pereira and Akbar, 2016), which may potentially contribute to the pathogenesis of CL.

Accumulation of senescent cytotoxic NK cells correlates with lesion size observed in CL patients

We found that CL patients who had the highest proportion of senescent/cytotoxic $CD57^{bright}$ NK cells (Figure 2C) had the largest cutaneous lesions (Figure 4C) suggesting that they have a role in the cutaneous immunopathology as was shown previously for senescent (EMRA) CD8+ T cells in these patients (Covre et al., 2018). Furthermore, we found a significant correlation between the increase in senescent NK and senescent $CD8^{+}$ T cells in the same patients (Figure 4D) suggesting that related mechanisms induce the expansion of both populations in the circulation of CL patients.

Senescent CD8 T and NK cells in the circulation have different cytotoxic capacity and migratory potential into the skin of CL pathogenesis

We next compared the spontaneous skin-homing capacity, proliferative and cytotoxic potential of circulating senescent NK ($CD57^{bright}$) and senescent $CD8^+$ (EMRA) T cells from the same donors. We found that senescent NK cells ($CD57^{bright}$) produced increased amount of perforin, granzyme B and CD107 compared to senescent $CD8^+$ T cells (EMRAs) in the same individuals. Furthermore, although the senescent NK subset exhibits a high proliferative potential ($Ki67^+$), they showed a lower capacity for skin homing, as demonstrated by the CLA expression compared to $CD8^+$ EMRA T cells (Figure 5D-E). The total $CD8^+$ T and NK cell populations from the patients showed higher levels of perforin, granzyme B and CD107 expression compared to healthy controls (Suplementary Figure 2A-C). In contrast, the circulating $CD8^+$ T cells exhibited increased skin homing but decreased proliferative potential compared to the NK cells from the same CL patients (Supplementary Figure 2D,E). In addition, no differences in total frequencies of both NK^+ and $CD8^+$ cells pool were observed in patients and controls (data not show).

We confirmed the functional profile of cytotoxic senescent cells by the t-distributed Stochastic Neighbor Embedding (t-SNE) algorithm (Amir et al., 2013). We arbitrarily identified 2 different clusters of $CD8^+$ EMRA (Green) and $CD57^{bright}$ (Red) subsets on the basis of population boundaries distinguishable on the t-SNE density plots (Figure 5F) and the expression intensity of markers in each cluster were displayed using a heat map. As expected, NK $CD57^{bright}$ had increased Ki67, CD107a, and perforin while expressing lower levels of CLA compared to $CD8^+$ EMRA T cells. Granzyme B expression overlap across the distinct clusters defined in the t-SNE map and show a similar distribution in both subsets (Figure 5F).

Senescent cytotoxic cells accumulate in the skin and correlate with lesion size of CL patients.

We next investigated the presence and function of NK and $CD8^+$ T cells and their senescent subsets in the skin lesions to determine their relative contribution to the observed immunopathology. Representative immunofluorescence micrographs demonstrate a significant accumulation of granzyme-producing cells in the lesions (Figure 6A, C and E) with a predominance of $CD8^+$ T cells compared to NK cells (Figure 6F). Interestingly, there was a significantly greater accumulation of senescent $CD8^+$ T compared to senescent NK cells in the skin of CL patients (Figure 6G). This suggests that the increased potential for skin homing through the higher expression of CLA leads to a preferential accumulation of senescent $CD8^+$ T cells in the skin lesions of CL patients that extends previous observations (Covre et al., 2018).

We correlated the size of the skin lesions in patients with cutaneous leishmaniasis with the extent of CD8+ T cells or NK cell infiltration. We found that there was a positive correlation between the accumulation of total $CD8^+$ T cell but not NK cells in the skin and lesion size (Figure 6H). Interestingly, the accumulation of senescent $CD8^+$ T cells strongly correlate with lesion size while a weak correlation is linked to the differentiated NK cells (Figure 6I). This suggests that $CD8^+$ T cells in the skin have a relatively greater impact that NK cells on the immunopathology in the skin of patients with CL.

DISCUSSION

$CD8^+$ T cells play a key role in CL immunopathology through cytotoxicity, however the contribution of NK cells to this process is unresolved. NK cell function changes from immature $CD56^{bright}$ and mature but naïve $CD56^{dim}CD57^-$ population that produce high amounts of cytokines to terminally differentiated/ senescent $CD56^{dim}CD57^+$ subset exhibiting strong cytotoxic activity (Lopez-Verges et al., 2010; Nielsen et al., 2013). Moreover, the acquisition of senescence characteristics in NK cells, acquired through persistent antigen exposure and/or chronic infection,

is associated with the expression of CD16, CD57, KLRG1 and loss of CD27 (Müller-Durovic et al., 2016; Tarazona et al., 2001), as demonstrated in our findings.

Given the pathogenic potential of both T and NK cytotoxic compartments, we tested their potential for skin-homing. Although circulating NK cells exhibited potent cytotoxicity and inflammatory function compared to $CD8^+$ T cells they showed reduced CLA expression and thus capacity to home to the skin. This raised the possibility that NK cells in CL may exert their function preferentially in the circulation of the patients while $CD8^+$ T cells may exhibit their function in the skin of the patients.

CD8 T cells expressing higher levels of CLA are found in patients with cutaneous disorders (Ferran et al., 2008; Koelle et al., 2002) including cutaneous leishmaniasis (Covre et al. 2018; Mendes-Aguiar et al. 2009; Santos et al. 2013) compared to healthy subjects. This raises the question of how CLA expression is induced on these cells. Although proinflammatory cytokines have been shown to induce CLA expression on T cells (Akdis et al., 1999), both T cells and NK cells in CL patients are exposed to the same inflammatory environment *in vivo* yet there is a difference in expression of this molecule between both subsets. Alternatively, it is possible that specific $CD8^+$ memory T lymphocytes are programmed to express CLA after antigenic stimulation. For example, the expression of CLA by memory $CD8^+$ T lymphocytes increases during CL and is seen after *Leishmania* antigen recall *in vitro* (Covre et al. 2018; Mendes-Aguiar et al. 2009; Santos et al. 2013). Furthermore, CLA-negative precursor cells can be induced from CLA negative $CD8^+$ T cells populations after TCR stimulation with viral and bacterial antigens (Koelle et al., 2002; Sieling et al., 2007). Therefore the increase in CLA expressing $CD8^+$ T cells that harbour senescence characteristics during CL may arise from extensive TCR activation and proliferation resulting from stimulation with *L. braziliensis* antigenic epitopes. In contrast as NK cells are not activated by the TCR, CLA expression is not induced by them.

Analysis of gene expression in CL skin lesions has shown that cytotoxicity-related genes are overexpressed compared to the Th1 cytokine pathway and this represents the main signature

associated with the promotion of the tissue damage and skin ulceration during cutaneous leishmaniasis caused by *L. braziliensis* (Novais et al., 2015). Despite this, not much is known of the overall effector mechanisms including cytotoxicity mediated by NK cells in human cutaneous leishmaniasis. It has been previously shown that the pathogenic role of $CD8^+$ T cells in both human and murine CL caused by *L. braziliensis* infection occurs through cytotoxic and pro-inflammatory activity that correlates with lesions size in patients (Faria et al. 2010; Melby et al. 1994; Novais et al. 2013; Santos et al. 2013). Our results show that cytotoxic (and senescent) $CD8^+$ T cell preferentially accumulate in the skin lesions of CL patients compared to NK cells suggesting that a functional compartmentalization of these cells occurs in CL. While senescent NK cells preferentially exert their function in the circulation, senescent $CD8^+$ T cells preferentially exert their functional activity in the skin.

A key unanswered question is the mechanism by which senescent $CD8^+$ T cells induce the immunopathology in the skin of CL patients. It is well recognized that there are very few parasite in CL lesions indicating that it is unlikely that there is extensive activation of these cells via the T cell receptor. One possibility is that the CD8 T cells in the lesions are inducing non- specific killing of healthy tissue. Previous studies have shown that senescent $CD8^+$ T cells acquire the expression of functional NK receptors (Kurioka et al., 2018; Pereira et al., 2019). Furthermore, inflammation and also the development of senescence induces the expression of NK ligands in tissue cells (Pereira et al., 2019). Therefore skin homing $CD8^+$ T cells in CL may mediate non-specific pathology by killing NKR-ligand expressing cells such as fibroblasts and keratinocytes in the skin. Supporting this possibility, both NKG2D and Rae1γ (an NKG2D ligand in the mouse) are expressed by a large number of leukocytes within skin lesions induced by *L. major* infection in mice (Crosby et al., 2014) regardless of the parasitic load of the lesion. In addition, the blockade of NKG2D in *L. major*-infected mice prevents lysis of target cells and reduces lesion development *in vivo* (Crosby et al., 2014). In preliminary studies we have also observed increased expression of both NKG2D on $CD8^+$ T cells and MICA/ MICAB on stromal cells within CL patient lesions (Covre

et al., unpublished observations). This suggests that *Leishmania*-specific $CD8^+$ T cells in the skin may participate in the development and severity of cutaneous leishmaniasis through non-antigen driven bystander cytotoxicity mechanisms.

Collectively our results underpin a broad understanding of systemic and local changes in both blood and skin of patients with CL identifying the discrete compartmentalization of NK activity in the blood and CD8+ T cell activity in the skin of the patients. Furthermore we suggest that $CD8^+$ T cells and not NK cells are mainly responsible for the non-specific skin lesional pathology. This is the first demonstration of an immunopathogenic role for senescent T cells *in vivo*.

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CONFLICT OF INTEREST. The authors state no conflict of interest.

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Legend to figures

Figure 1. Patients with cutaneous leishmaniasis present increased frequency on circulating NK cells within a $CD56^{dim}$ phenotype. (A) Frequency of total NK cells from healthy control donors (HC n=16) and patients with cutaneous leishmaniasis patients (CL n=16). (B) Frequency of $CD56^{dim}$ and $CD56^{bright}$ subsets within total NK cells and (C) cumulative frequencies of $CD56^{dim}$ and $CD56^{bright}$ subsets within $CD16^{+}$ cells. The graphs or dot plots show median with 95% of confidence. The p values were calculated using Mann Whitney test. *p <0.05, **p <0.01.

Figure 2. Circulating NK cells from CL patients increase the expression of differentiation markers. (A) Representative histogram and (B) cumulative data of percentage of CD57, KLRG1 and CD27 receptors in total NK cells and $CD56^{dim}$ and $CD56^{bright}$ subsets. (C) Representative histogram and (D) cumulative data of NK subsets frequencies expressing CD57. The analyses were performed by flow cytometry using samples from HC (n=10-16) and CL (n=10-16) groups. The graphs show median with 95% of confidence. The p values were calculated using Mann Whitney test. *p <0.05, ** p <0.01, *** p <0.001.

Figure 3. NK cells from CL patients present pronounced inflammatory and functional profile and display features of cellular senescence. Purified NK cells (over 95% of purity) from HC (n=6) and CL (n=6) groups were co-cultured with K562 target cells (E:T ratio of 10:1) for 4h. (A) The cytotoxic activity assessed by calcein-release lysis assay; degranulation capacity or production of granzyme B, IFN-γ and TNF-α were determined after co-culture. (B) Representative histogram and cumulative data of the proliferative capacity assessed by Ki67 staining of purified NK cells or (C) their subsets stratified by CD57 expression. Cells were cultured with IL-2 (100U/mL) for 48h. Results show the relative fold change normalized to the unstimulated control. (D) Telomere FISH

image in NK cells hybridized with telomere probe (TelC Cy3) and fold change of quantitative fluorescence intensity levels normalized with CL group. The graphs show median with 95% of confidence. The p values were calculated using Mann Whitney test. *p <0.05, **p <0.01, p <0.001***, p <0.0001***.

Figure 4. Highly differentiated NK cell subset is cytotoxic and correlates with lesion size in patients. Cumulative data of basal production of (A) granzyme B and (B) perforin in NK cell subsets stratified by CD57 expression. The analyses were performed by flow cytometry using samples from HC (n=7) and CL (n=7) groups. Results show the relative fold change normalized to the respective early differentiated- $CD57^{neg}$ subset. (C) Spearman's correlation test between frequencies of CD57 subsets within $CD56^{dim}$ NK cells and lesions size (mm^2) of CL patients (n= 16) or (F) between frequencies of NK $CD57^{bright}$ and $CD8^+$ EMRA subsets.

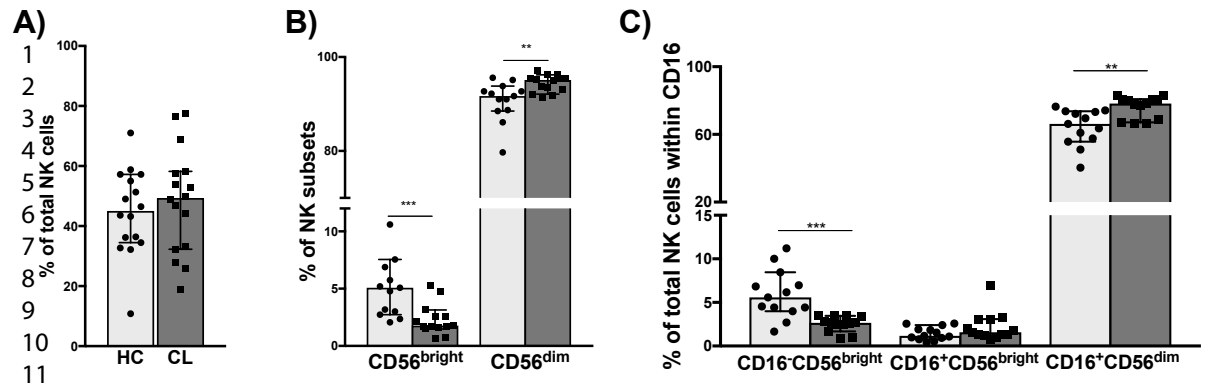
Figure 5. Cytotoxic granules production, proliferative and migratory capacity are highly diverse across senescent $CD8^+$ T and NK cells from HC and CL patients. (A-E) PBMC from healthy donors (HC) (n=7) and cutaneous leishmaniasis patients (CL) (n=7) were directly stained for surface and intracellular markers and analysed by flow cytometry. (F) *t*-SNE was performed gating on $CD8^+$ EMRA and NK $CD57^{bright}$ cells from HC and CL groups. The level of expression of Ki67, CLA, CD107a, Perforin and granzyme B were evaluated separately on live cells generating the expression levels of the hierarchical clusters represented in red for high expression, whereas blue represents low expression (cold-to-hot heat map).

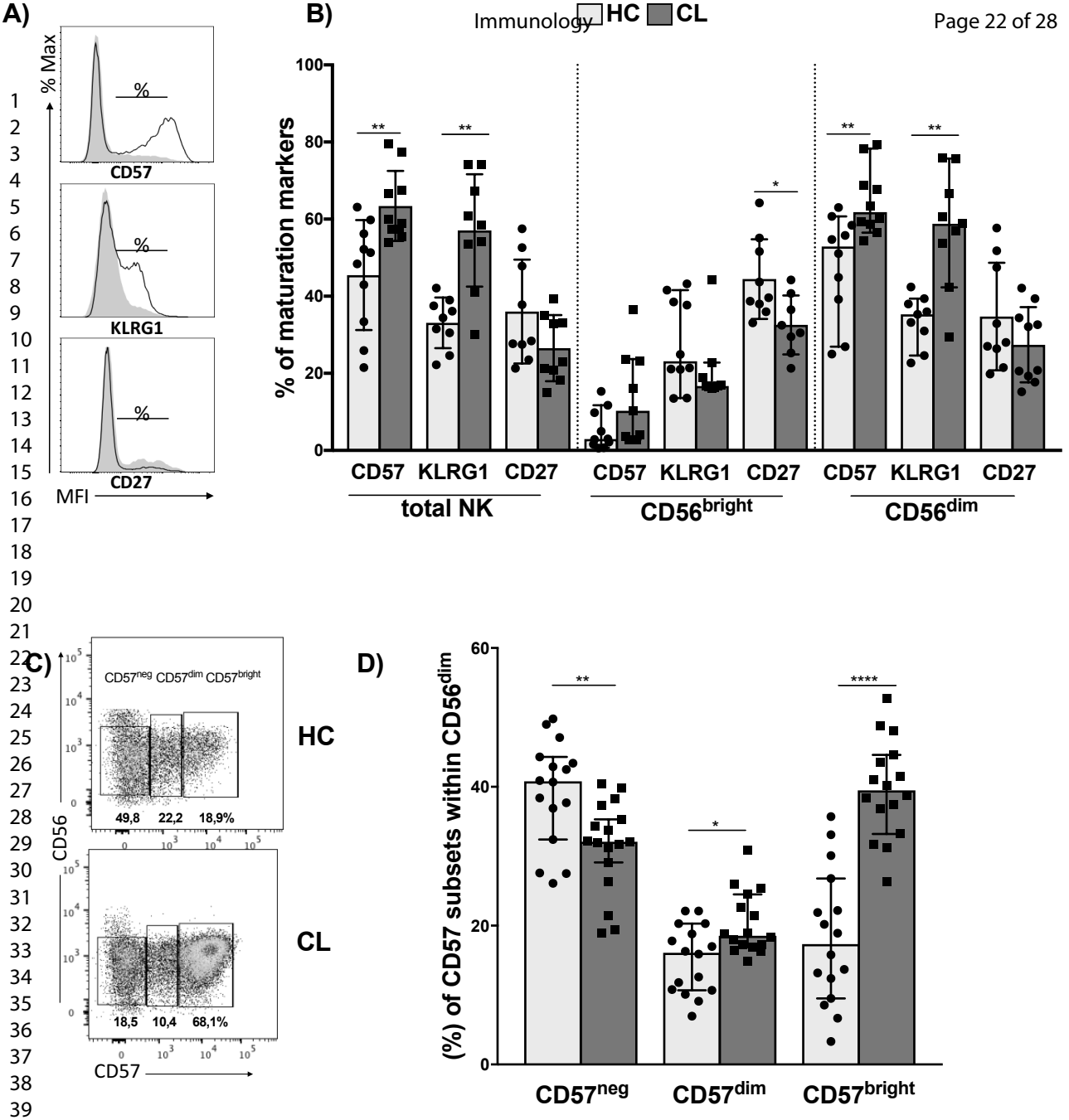
Figure 6. Senescent cytotoxic cells accumulate in the skin and correlate with lesion size of CL patients. Histological sections of human skin punch-biopsy from CL patients (n=6) stained for DAPI (blue); NK or $CD8^+$ T cells (green); granzyme B (red) and CD57 (grey). The yellow arrows indicate senescent cytotoxic cells. Representative images (Top panels A-D) and cumulative data of total

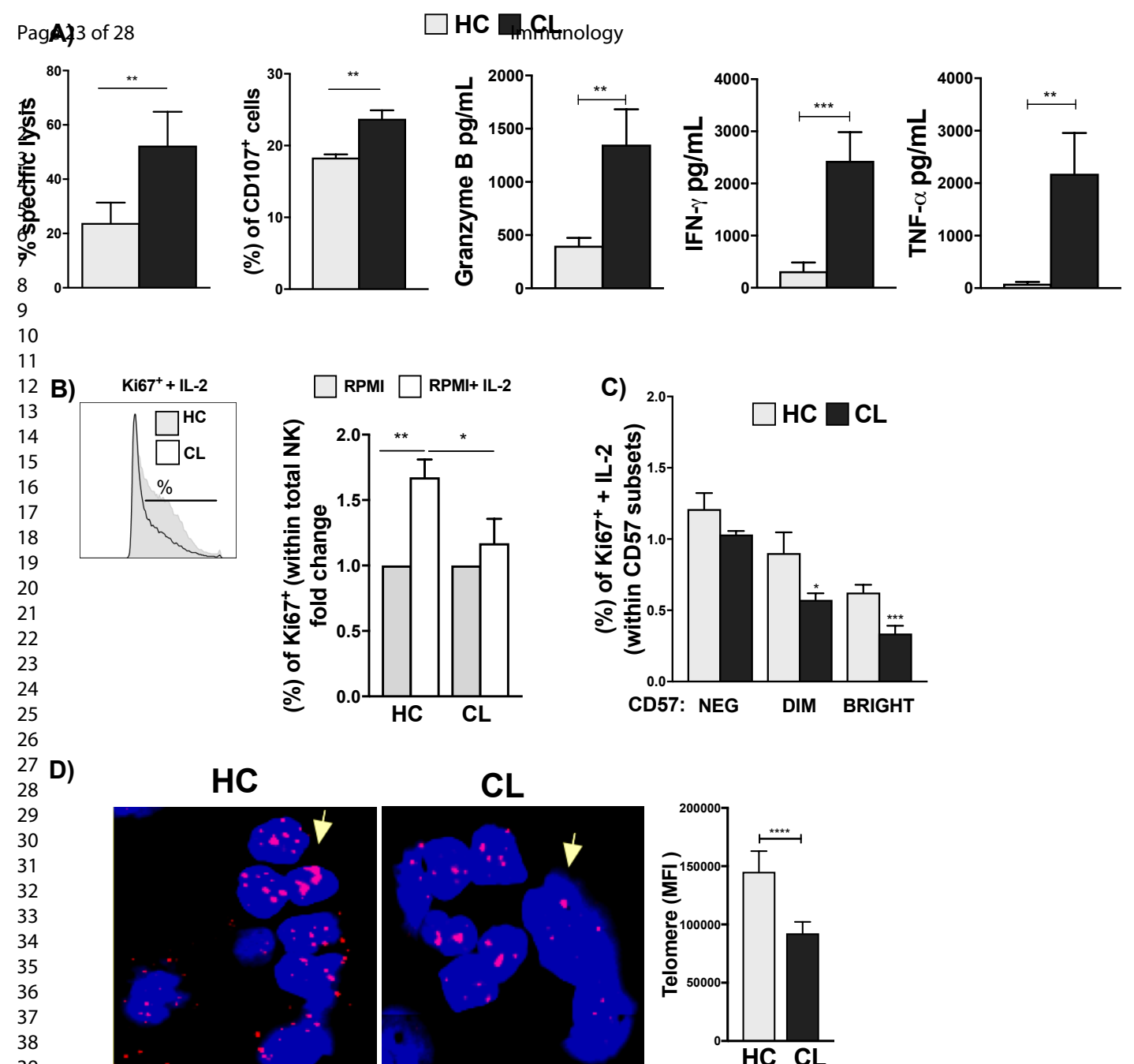
cytotoxic cells and granzyme-producing senescent cells frequencies (Bottom graphs E-G). Scatterplot showing the Spearman's correlation test relationship between frequencies of NK and $CD8^+$ T cells and lesion size (H) or NK^{CD57} and $CD8^+$ T EMRA and lesion size. The graphs show median with 95% of confidence. The p values were calculated using Mann Whitney test. *p <0.05, **p <0.01, ***p <0.001, ****p <0.0001.

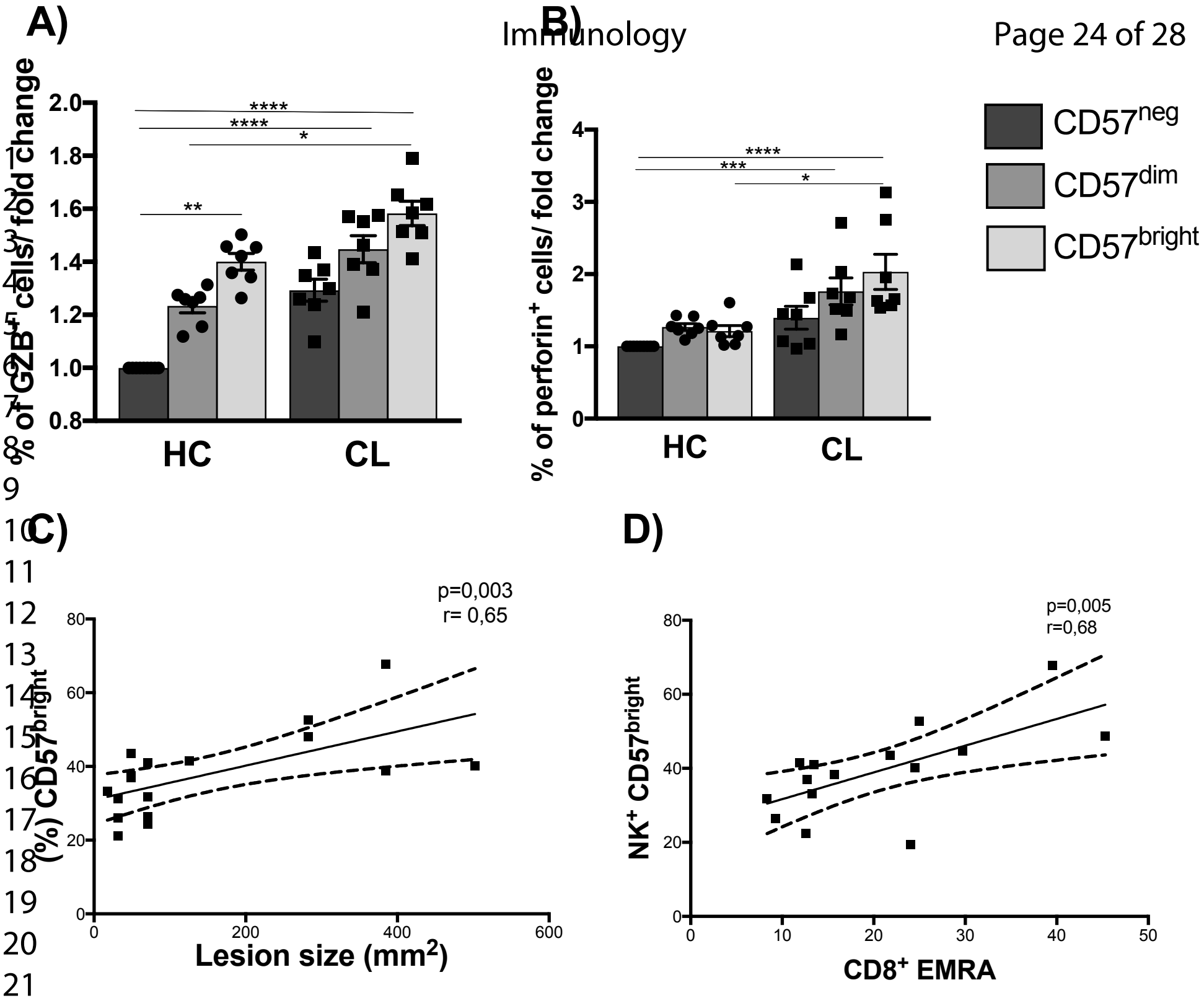
Supplementary figure 1. Representative gate strategy. (A) NK cells were identified from PBMC by gating on lymphocytes, single cells, live cells, CD3-negative and CD7-positive cells (B) Total NK cells stratified into 3 subsets accordingly CD56 and CD16 expression.

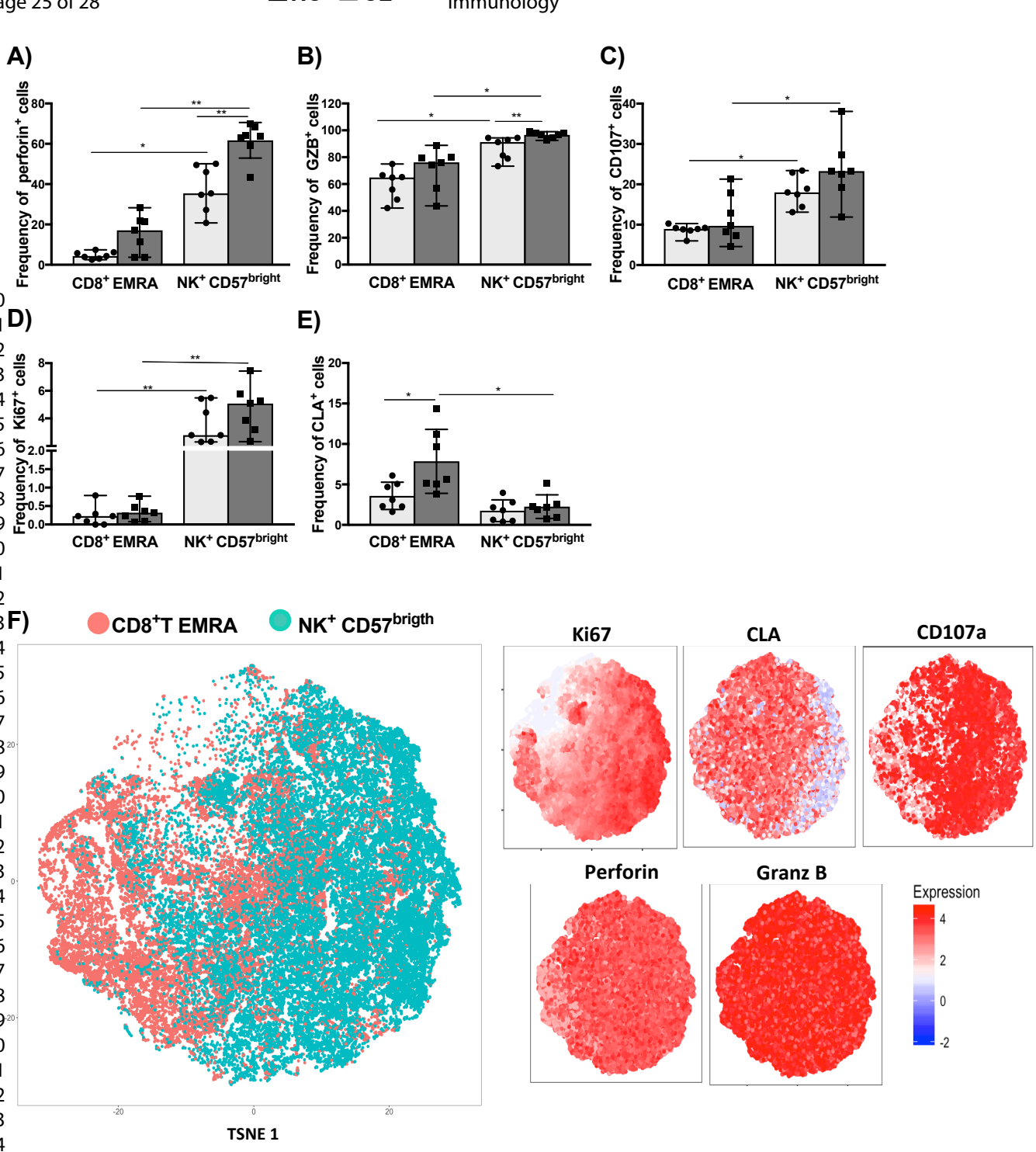
Supplementary 2. Circulating cytotoxic cells in LC patients. (A) PBMC from healthy donors (HC) (n=7) and cutaneous leishmaniasis patients (CL) (n=7) were directly stained for surface and intra-cellular markers. Data show circulating total NK and T $CD8^+$ cells-expressing (A) perforin; (B) granzyme B; (C) CD107; (D) Ki67 and (E) CLA. The graphs show median with 95% of confidence. The p values were calculated using Mann Whitney test. *p <0.05, **p <0.01, ***p <0.001.

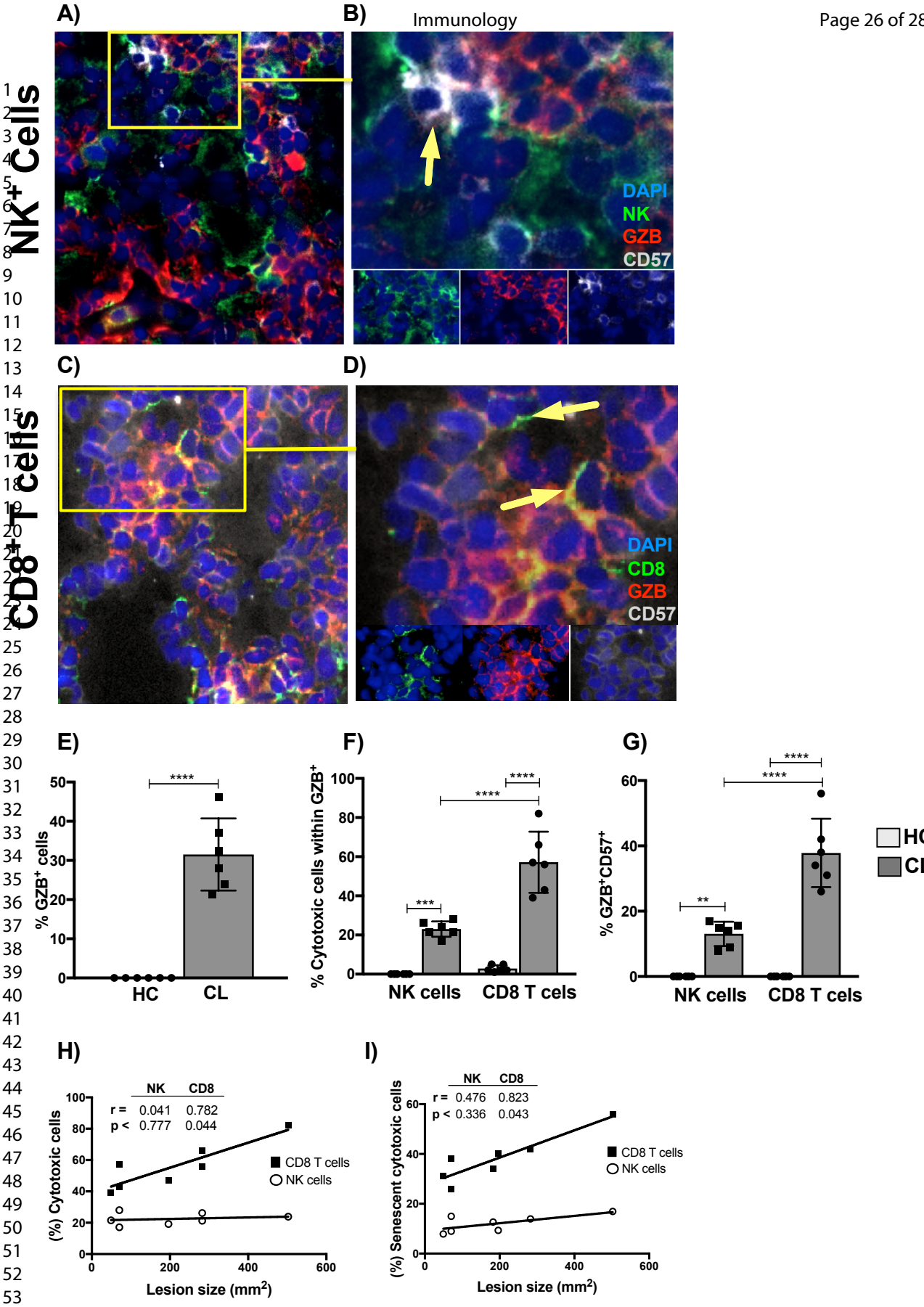

HC
CL
A)
% of total NK cells
HC
CL
B)
% of NK subsets
CD56bright
CD56dim
**
***
C)
% of total NK cells within CD16
CD16-CD56bright
CD16+CD56bright
CD16+CD56dim
**
***

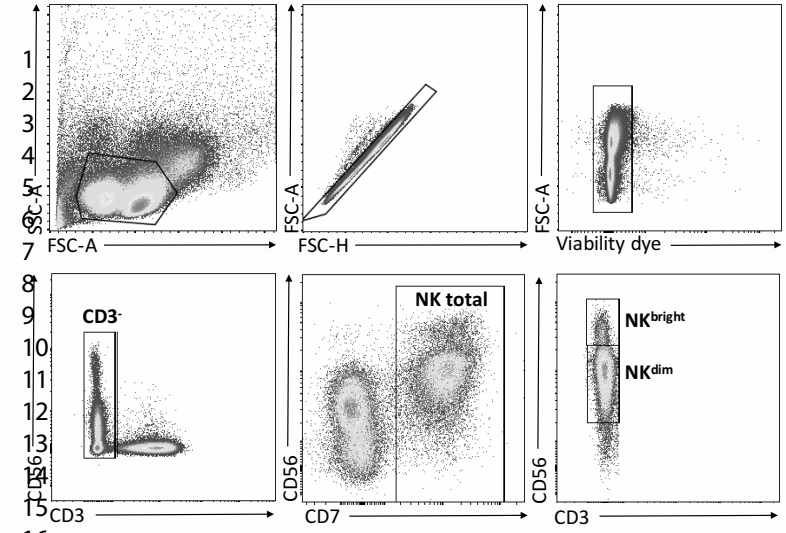

A)
% Max
%
CD57
%
KLRG1
%
CD27
MFI
B)
HC
CL
% of maturation markers
100
80
60
40
20
0
**
**
*
**
**
CD57
KLRG1
CD27
CD57
KLRG1
CD27
CD57
KLRG1
CD27
total NK
CD56bright
CD56dim
C)
CD57neg CD57dim CD57bright
HC
49,8
22,2
18,9%
CD56
CL
18,5
10,4
68,1%
CD57
D)
(%) of CD57 subsets within CD56dim
60
40
20
0
**
*
****
CD57neg
CD57dim
CD57bright

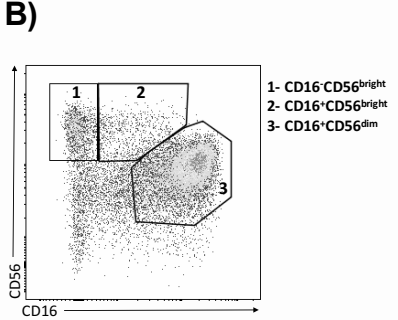

A)
HC
CL
% specific lysis
(%) of CD107+ cells
Granzyme B pg/mL
IFN-γ pg/mL
TNF-α pg/mL
B)
Ki67+ + IL-2
HC
CL
%
RPMI
RPMI+ IL-2
(%) of Ki67+ (within total NK) fold change
HC
CL
C)
HC
CL
(%) of Ki67+ + IL-2 (within CD57 subsets)
CD57: NEG
DIM
BRIGHT
D)
HC
CL
Telomere (MFI)
HC
CL

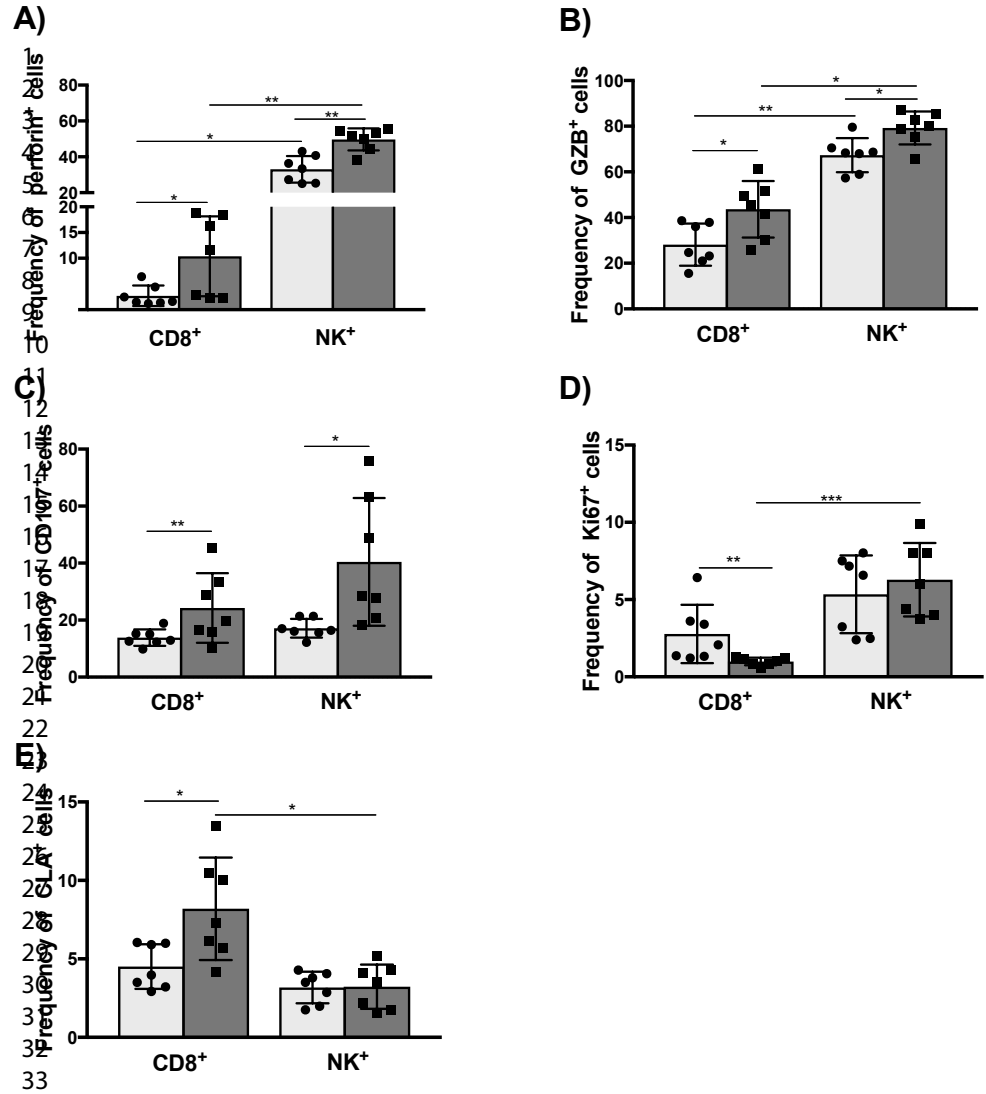

A)
% of GzB+ cells/ fold change
2.0
1.8
1.6
1.4
1.2
1.0
0.8
****
****
*
**
HC
CL
B)
% of perforin+ cells/ fold change
4
3
2
1
0
****
***
*
HC
CL
CD57neg
CD57dim
CD57bright
C)
(%) CD57bright
p=0,003
r= 0,65
80
60
40
20
0
0
200
400
600
Lesion size (mm2)
D)
NK+ CD57bright
p=0,005
r=0,68
80
60
40
20
0
0
10
20
30
40
50
CD8+ EMRA


HC
CL
A)
Frequency of perforin+ cells
B)
Frequency of GZB+ cells
C)
Frequency of CD107+ cells
D)
Frequency of Ki67+ cells
E)
Frequency of CLA+ cells
CD8+ EMRA
NK+ CD57bright
F)
CD8+T EMRA
NK+ CD57brigth
TSNE 1
TSNE 2
Ki67
CLA
CD107a
Perforin
Granz B
Expression
4
2
0
-2


A)
B)
NK+ Cells
DAPI
NK
GZB
CD57
C)
D)
CD8+T cells
DAPI
CD8
GZB
CD57
E)
% GZB+ cells
****
HC
CL
F)
% Cytotoxic cells within GZB+
***
****
NK cells
CD8 T cels
G)
% GZB+CD57+
**
HC
CL
H)
NK CD8
r = 0.041 0.782
p < 0.777 0.044
(%) Cytotoxic cells
CD8 T cells
NK cells
Lesion size (mm2)
I)
NK CD8
r = 0.476 0.823
p < 0.336 0.043
(%) Senescent cytotoxic cells
CD8 T cells
NK cells
Lesion size (mm2)


A)
SSC-A
FSC-A
FSC-A
FSC-H
FSC-A
Viability dye
CD3-
CD3
CD56
CD7
NK total
CD56
CD3
NKbright
NKdim


B)
1
2
3
CD56
CD16
1- CD16-CD56bright
2- CD16+CD56bright
3- CD16+CD56dim


A)
Frequency of perforin+ cells
CD8+
NK+
B)
Frequency of GZB+ cells
CD8+
NK+
C)
Frequency of CD107+ cells
CD8+
NK+
D)
Frequency of Ki67+ cells
CD8+
NK+
E)
Frequency of CLA+ cells
CD8+
NK+