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Inflammation in chronic degenerative disorders: A novel $CD3^+CD56^+$ subset that regulates $CD8^+$ T cell effector function.

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ABBREVIATIONS:

- T1D: type 1 diabetes
- C-pep: C-peptide
- DCs: dendritic cells
- HLA: human leukocyte antigen
- MHC: The major histocompatibility complex
- HbA1c: glycated haemoglobin
- IAAs: autoantibodies against insulin
- GADA: autoantibodies against the 65-kDa isoform of GAD
- IA-2A: autoantibodies against the protein tyrosine phosphatase-related molecule IA-2
- ZnT8: autoantibodies against the pancreatic β-cell specific protein, zinc transporter 8
- Treg: T-regulatory cells
- NOD mice: non-obese diabetic mice
- NK cells: Natural Killer cells
- NKT cells: Natural Killer-T cells
- APCs: antigen-presenting cells
- CTLs: cytotoxic T lymphocytes
- IFN-γ: Interferon gamma
- TCR: T-cell receptor
- LAMP-1: lysosomal-associated membrane protein-1.
- PTPN22: protein tyrosine phosphatase no receptor type 22
- CTLA-4: Cytotoxic T-Lymphocyte Antigen 4
- IFIH1: Interferon Induced With Helicase C Domain
- IL-2Ra: Interlukin 2 receptor
- CCl1 -2 : CC-chemokine ligand-1 or ligand-2
- PAMP : pathogen-associate molecular pattern
- PRP: pattern recognition receptor

- TME: tumor microenviroment
- ROS: reactive oxygen species
- TNF-a: Tumor necrosis factor
- (TGF)-ß: transforming growth factor
- CBV: coxsackie B viruses
- NO: nitric oxide.

Abstract

It has been reported that a growing and heterogeneous group of regulatory cell modulate immune response. In particular, regulation of $CD8^+$ T lymphocyte effector functions is critical for tissue homeostasis and immune tolerance control. Here, we report that the co-expression of CD3 and CD56 molecules identify a novel human regulatory T cell population exerting suppressive activity on proliferation, cytotoxicity and IFN-γ production of TCR-activated human $CD8^+$ T lymphocytes. Regulatory functions of human circulating $CD3^+CD56^+$ T lymphocytes require cell-to-cell contact and are exerted in both autologous and allogeneic conditions. Of note, $CD3^+CD56^+$ T cells are reduced and functionally impaired in children affected by Type 1 Diabetes (T1D), at disease onset. Conversely the frequency of this cell subset is increased in patients with prostate cancer.

Taken together, our findings reveal that freshly isolated human $CD3^+CD56^+$ cells specifically control activation of human $CD8^+$ T lymphocytes. Perturbation of number and function of this cell subset may account for the deranged functions of $CD8^+$ T lymphocytes observed in autoimmune conditions, including T1D. Thus, therapeutic manipulation of $CD3^+CD56^+$ cells may represent an innovative approach to restore immune function in T1D.

1. BACKGROUND

1.1 Regulation of adaptive immune responses in immune mediated disease

The immune response is a complex phenomenon aimed to confer protection against pathogens and simultaneously maintain tissue homeostasis. Immune response also includes the establishment of immunologic memory and clearance of transformed cells. All of these functions share many common interactive pathways responsible for early triggers of the host immune response as well as modulate immune functions. It has been reported that alterations in these pathways can lead to a variety of both organ specific and systemic autoimmune syndromes, including Type 1 Diabetes (T1D). Aberrant regulatory activity also favors tumor escape from effector immune mechanisms. The key role of regulatory T lymphocytes in the prevention of autoimmunity and immune mediated diseases has been largely shown[1-2]. Fine-tuning of immune response is usually obtained by multiple regulatory processes, all belonging to the immune tolerance network, that are in place to prevent potentially deleterious immune responses against self tissues[3-5].

Regulatory cells represent a heterogeneous group of differentiated T cell subsets including the interleukin (IL)-10 producing Tr1, the transforming growth factor (TGF)-ß producing T helper 3 (Th3) and the $CD25^+CD4^+$ regulatory T (Treg) cells, constitutively expressing the forkhead box P3 (Foxp3) transcription factor[6-8]. $CD4^+CD25^+Foxp3^+$ Treg cells control the immune-effector response in terms of clonal expansion, differentiation, cytokine profile and tissue migration during immune response and are indispensable for the maintenance of immune self-tolerance[9]. $CD8^+$ T regulatory clones have been also described, but their role and phenotype features are still undefined[10-11]. It is not clear whether different $CD8^+$ regulatory cells represent an independent T subset or if they reflect the dynamic plasticity of a single population. Human $CD8^+$ Tregs have been implicated in various inflammatory disorders in association with autoimmunity, including T1D, infectious disease and cancer[12-15]. These $CD8^+$ cells may exert their activity through different mechanisms. Literature indicates that $CD8^+CD28^-$ T lymphocytes can suppress T helper (Th)-1 cells, induce $CD4^+$ T-cell anergy and regulate reactivation of T cells[11,16-17]. Furthermore, antigen-induced $CD8^+CD103^+$ regulatory T cells have been shown to suppress T cell effector functions in models of transplantation tolerance[18]. Moreover, it has been also observed that $CD8^+$ Treg cells modulate

immune response *in vivo* by suppressing activated $CD4^+$ T cells *via* cell-cell contact dependent mechanisms requiring CD11a/CD18 (LFA-1) expression[19]. Recent studies suggest that $CD8^+$ CD25hi (IL-2Rα chain) $Foxp3^+$ Treg cells, like $CD4^+$ CD25hi $Foxp3^+$ cells, suppress immunity via cytokine production including IL-10[20-21]. Conversely, $CD8^+$ CD122hi (IL-2Rβ chain) Treg cells produce IL-10 to suppress $CD8^+$ T cell effector functions[22]. $CD8^+$ T lymphocytes are one of the mayor adaptive immune cells, and play important role in maintaining immune homeostasis. Some different clinical studies have established that $CD8^+$ T cells can profoundly affect cancer progression. After stimulation, $CD8^+$ T cells exert functional activity through the release of cytotoxic granules that lyse target cells, as well as by the production of interferon (IFN)-γ[22-23]. When hyper-activated these cell release large amounts of pro-inflammatory cytokines, which are directly involved in disease pathogenesis. Importantly, so far, targeting of $CD8^+$ T cells by specific regulatory populations is unexplored.

Several evidence indicate that alteration of molecular mechanisms at the basis of immune response accelerate the course of chronic diseases, including cancer[5,24-25]. In the tumor microenvironment (TME), T cells have an altered phenotype and aberrant functions. The role of $CD4^+$ and $CD8^+$ Treg cells have been extensively elucidated in many different types of cancer[26-30]. Specifically, in tumor-infiltrating lymphocytes (TILs); the decreased ratios of intraepithelial $CD8^+$ T cells to $FOXP3^+CD25^+CD4^+$ Treg cells is associated with poor prognosis in ovarian, breast, and gastric cancers[30]. Furthermore, recent data indicate that $CD8^+Foxp3^+$Treg cells present in prostate cancer among TILs are able to suppress anti-cancer immune responses[31].

Over the past years, a number of observations indicate that normal peripheral blood of healthy individuals contains a small lymphocyte population co-expressing CD56 and CD3 surface markers that characterize human natural killer (NK) and T (CD3) cells, respectively[32].

Although the biological hallmarks and functional characteristics of $CD3^+CD56^+$ cells are not completely defined and experimental evidence associated these cells with different pathophysiological conditions. Indeed, significant decrease of hepatic recruitment of $CD3^+CD56^+$ cells has been found after liver transplant with high rejection activity, while high circulating number of $CD3^+CD56^+CD16^+$ cells has been described to predict a better *in vitro* fertilization

(IVF) treatment outcome[33-34]. Moreover, a positive correlation between $CD3^+CD56^+$ lymphocyte frequency and the serum hepatitis B viral DNA level was observed during early antiviral therapy[35-36]. Also, it was described a $Foxp3^+CD3^+CD56^+$ population with immunosuppressive function in human hepatocellular carcinoma[37-38]. Recently, it was been shown that the absolute number of circulating $CD3^+CD56^+$ T cells represent a valuable predictive marker of β-cell residual function up to one year after T1D diagnosis. Specifically, the high $CD3^+CD56^+$ T numbers, at disease onset, are associated with a higher β-cell activity in T1D one year later[39]. In addition, *Hu et al.* identified in human peripheral blood the $CD3^+CD56^+CD8^+CD161^-$ population able to kill autologous $CD4^+$ T cell upon T cell receptor (TCR) engagement[40]. However, the possible involvement of $CD3^+CD56^+$ cells in immune-tolerance control has been poorly investigated.

Here we describe that freshly isolated human $CD3^+CD56^+$ cells are an heterogeneous cell population, containing both $CD4^+$ and $CD8^+$ T cells, able to specifically modulate proliferation, cytotoxic function and cytokine production of T cell-receptor (TCR) activated $CD8^+$ T cells. Moreover, T1D children at diagnosis display reduced circulating number and impaired suppressive activity of $CD3^+CD56^+$ T cells. In contrast in subjects with prostate cancer, we observed an increase of circulating $CD3^+CD56^+$ that correlates with prognosis score.

1.2 Natural course of type 1 diabetes: genetic and environmental factors contribute in the damage of pancreatic β cells

T1D is a severe chronic disease characterized by the progressive autoimmune destruction of the insulin-secreting β-cells in the pancreas of genetically susceptible individuals. Insulin is a hormone that helps move sugar, or glucose, into body's tissues. When pancreatic cells are damaged, they are not able to produce insulin, glucose cannot be moved out of bloodstream into cell, and the blood sugar levels become high. The etiology of the disease is unclear but genetic, immunological and environmental factors act together to participate the disease pathogenesis. **(Fig. 1)**

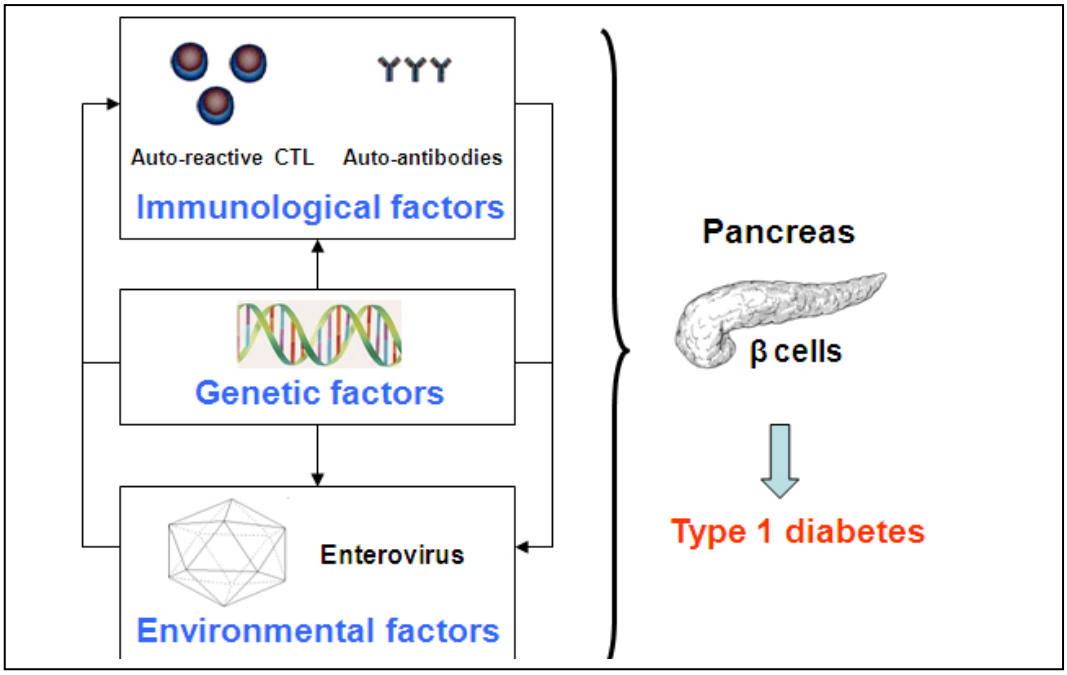


Fig.1 Environmental triggers, immunological and genetic factors influence the development of islet autoimmunity and progression to clinical diabetes. Discov Med. 2010

Genetic risk is largely conferred by strongest association between T1D and the human leukocyte antigen (HLA) class II haplotypes of HLA-DR and HLA-DQ located within the major histocompatibility complex (MHC) on chromosome 6p21[41-42]. The most common combinations of HLA genes present in children in whom the disease develops very early in life are DR4-DQ8 and DR3-DQ2, commonly present in 90% of children with T1D. A third haplotype, DR15-DQ6, is found in less than 1% of children with T1D, compared with more than 20% of the

general population, and is considered to be protective[43]. It has been reported that majority of genes associated to T1D risk encode proteins that are involved in immune function and regulation; for example, protein tyrosine phosphatase, non-receptor type 22 (PTPN22), Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4), Interferon Induced With Helicase C Domain 1(IFIH1) and Interleukin 2 receptor a (IL-2Ra) which regulate lymphocytes signalling and T cell tolerance[44-45]. Interestingly, recent evidences suggest that HLA genes primarily contribute to development of autoantibodies, while non-HLA genes and environmental factors may be more important in the progression from autoantibodies to clinically overt disease[46-47]. In T1D the progressive destruction of pancreatic β -cells begins before the clinical manifestation. This phase is usually asymptomatic or characterized by metabolic disturbances. The natural course of T1D is characterized by transient restoration of β-cell function. This phase is common called "honeymoon period" and it is characterized by a reduction of the daily exogenous insulin requirement while good metabolic control is maintained. When most of the β-cell mass is lost, clinical signs of chronic hyperglycemia become evident and consequently patients need frequent blood glucose testing and daily insulin replacement therapy. Different metabolic and clinical factor, including age of presentation, degree of metabolic decompensation at diagnosis and the presence of autoantibodies, are able to influence frequency and the duration of "honeymoon period".

The natural history of T1D has improved through the combined use of genetic, autoantibody, and metabolic markers of the disease. Autoimmunity in T1D has typically been identified by the presence of autoantibodies to islet or β-cell antigens. The autoantibodies are not directly pathogenic but are biomarkers of the development of autoimmunity[46-49]. **(Fig. 2)**

The development of an autoimmune process against Langerhans islets is characterized by the appearance of islet autoantibodies, which can be measured in the serum of these subjects. **(Fig. 2)** Islet autoantibodies identified so far include those against insulin (IAAs), the 65-kDa isoform of GAD (GADA), the protein tyrosine phosphatase-related molecule IA-2 (IA-2A), and more recently autoantibodies against the pancreatic β-cell specific protein, zinc transporter 8 (ZnT8) [50-51]. The risk of developing diabetes is related to the number of detectable autoantibodies with different specificities, which suggests that spreading of the autoantigenic repertoire is part of the pathogenic process. Once islet autoantibodies

have developed, the progression to overt diabetes in autoantibody-positive individuals is determined by the age of antibody appearance and by the magnitude of the autoimmunity, in turn related to the age of the subject[52]. **(Fig. 2)**

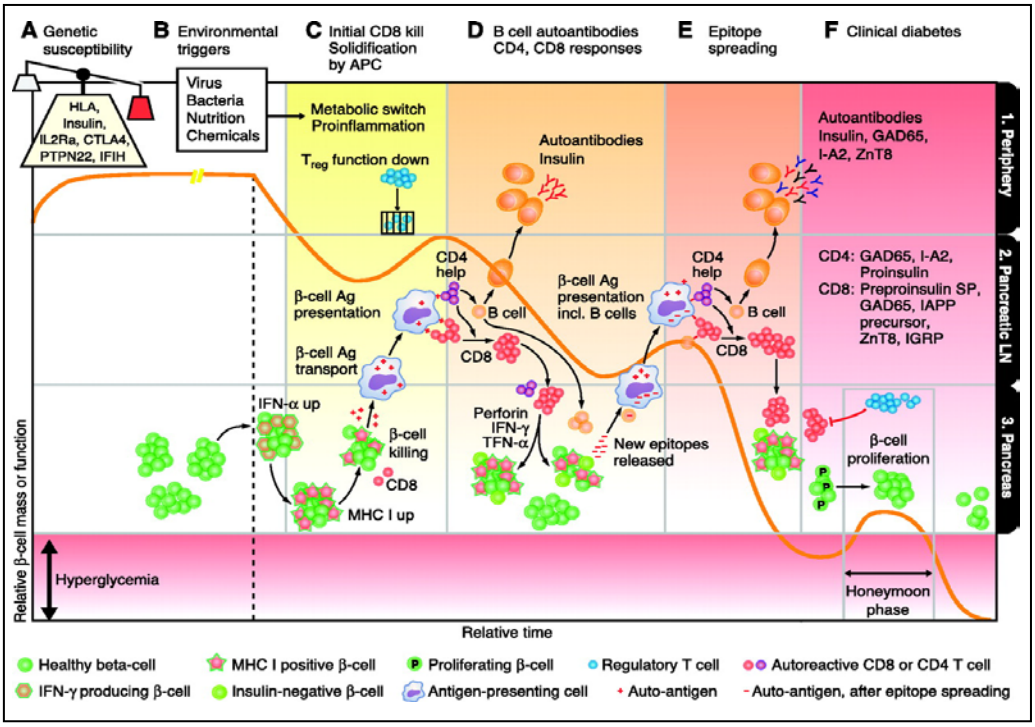


Fig. 2 Natural course of the immunopathogenesis of T1D. Physiol Rev. 2011

Candidate risk factors operating during infancy include those related to exposure to infectious agents, improved hygiene and mucosal exposure to dietary constituents. Enteroviruses, particularly coxsackie B viruses (CBV), are currently considered as the prime candidate among infectious agents by nature of their tropism for β-cells [56-56]. In addition, experiments using isolated human islets have shown that CBVs are able to infect and replicate in insulin-producing β-cells[57]. In contrast to the infectious hypotheses is the notion that improved hygiene is responsible for increased of T1D incidence[58-61].

Overall, several environmental factors seem to modify the risk of islet autoimmunity and T1D and have been considered to trigger autoimmune responses against pancreatic β-cells, eventually leading to β-cell destruction. The heterogeneity of T1D progression and clinical manifestations is likely a reflection of this complex multifactorial pathogenesis. Among environmental factors, several evidences point to infectious agents, components of early childhood diet and gut

microbioma. Fianlly, besides the “classical” triggers (infections, diet, gut), growing evidence points at inflammation and metabolic changes as significant cofactors in T1D pathogenesis[62].

1.3 Immunopathogenesis of type 1 diabetes: immune mechanisms underlying the selective destruction of pancreatic β-cells

Dysregulation of the immune system contributes to the breakdown of immune tolerance, leading to T1D. It is well known that initiation of T1D requires both $CD4^+$ and $CD8^+$ T cells, where autoreactive T cells differentiate into effectors by engaging β-cell antigens presented by antigen-presenting cells (APCs)[63]. In early onset diabetes, $CD8^+$ T cells are the most abundant pancreas-infiltrating cells during insulitis[64-66]. It has been reported that β-cell failure is caused by lymphocytic infiltration of the isle by dendritic cells (DCs), T lymphocytes, macrophages, B cells and natural killer cells[67-69]. Published evidences have been described a pathogenic additional role of macrophages in both the initiation and destruction phases of T1D. Recruitment of macrophages to islets is mediated chemokines produced mainly by $CD4^+$ T cells and pancreatic β-cells. Macrophages recruited to the pancreas produce IL-1β and Tumor Necrosis Factor (TNF)-α and reactive oxygen species (ROS) that can promote β-cell death. In particular, the TNF- α and IL-1β produced by macrophages and DCs have been observed in pancreatic islet infiltrates from patients with recent-onset T1D[70]. Finally, macrophages have been shown to produce IL-12 and to promote efficient differentiation of diabetogenic $CD8^+$ cytotoxic T lymphocytes (CTLs)[71-72]. Also, recruitment of macrophages to islets is mediated by the secretion of CC-chemokine ligand 1 (CCL1) by $CD4^+$ T and CCL2 produced by pancreatic β-cells[73]. Furthermore, insulin-autoreactive $CD4^+$ T cells have also been described in T1D patients and some studies indicated that high-avidity insulin-reactive thymocytes may evade central tolerance in patients[74]. $CD4^+$ T lymphocytes play an important role in the activation and proliferation of $CD8^+$ T lymphocytes and B cells[75-77]. B cells present MHC class I–peptide complexes to self-reactive $CD8^+$ T cells, that resulted in the proliferative expansion of self-reactive CTL in the pancreatic lymph nodes (PLNs) and the acquisition of a cytotoxic phenotype with a capacity to destroy β-cells[78-79]. NK cells are also involved in T1D pathogenesis; indeed, these cells are cytotoxic and produce cytokines, particularly IFN-γ, that could contribute to the destruction of β-cells[53,80-81]. Insulin-producing cells are sensitive to the action of this cytokine and IFN-γ can also promote cell death through NO synthesis and

induce cell-surface Fas receptor (Fas) expression on β-cells, favoring cell apoptosis. In addition, TNF-α can promote β-cell apoptosis through the Fas/Fas-Ligand (FasL) pathway, in addition it influences the production of the cytotoxic molecules by $CD8^+$ lymphocytes[82-85]. Normally, $CD8^+$ T cells effector function protect against viruses and contribute to eliminate tumor cells. In particular, in T1D this is achieved through TCR/CD8 recognition of peptide complex presented by major histocompatibility complex (MHC) class I molecules express on pancreatic β cells, resulting in the cytotoxic targeting of abnormal or infected cells. Upon recognition of antigen, naïve $CD8^+$ T cells were activated to proliferate and differentiate into cytokine producing effectors or CTL and undergo clonal expansion and respond to infection[86-89]. In addition, effective CTL immunity is associated with long-term protection against chronic or subsequent exposure to the virus or tumor, through the stable induction of antigen-specific $CD8^+$ T cell memory[90-91]. Activated effector cells then migrate to peripheral tissues and after recognition of antigen target the cells for destruction. Killing of target cells by CTLs is mediated through two major pathways, release of cytolytic granules containing granzyme B and perforin resulting in direct lysis of target cells, and induction of Fas signaling, leading to cytolysis via the activation of a death domain and a caspase apoptosis cascade. In patients with autoimmune hepatitis, FasL and granzyme B levels are elevated in the liver, suggesting a role for CTLs in hepatocyte apoptosis and liver damage[92-95]. Alterations of these pathways are observed in different autoimmune conditions **(Fig. 3)**.

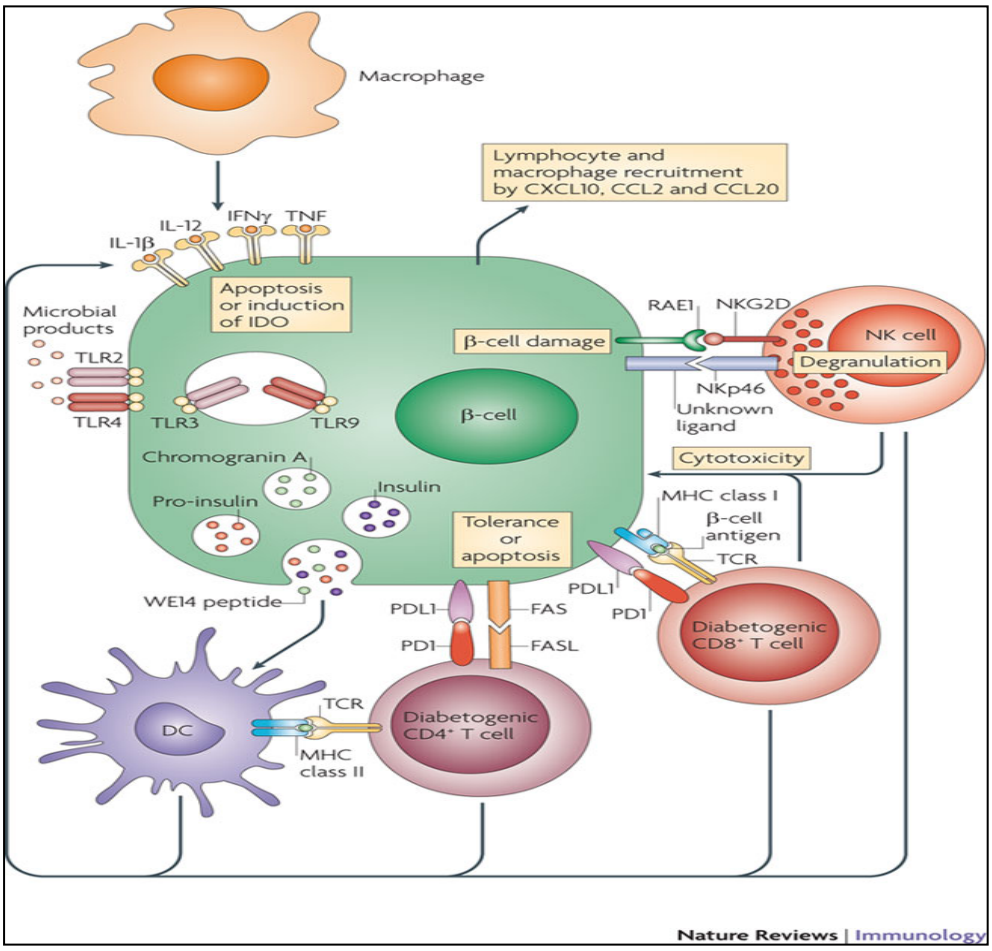


Fig. 3 Immunological mechanisms involved in the pathogenesis of T1D. Nat Rev Immunol. 2010

2. AIM

T1D results from autoimmune damage of insulin producing ß-cells in the islets of Langerhans, in the pancreas. The development of the T1D is determined by alteration of mechanisms that mediate immune tolerance, resulting in the expansion of islet-reactive $CD4^+$and $CD8^+$T effector (Teff) cells and subsequent β-cell destruction. In particular, autoreactive $CD8^+$ T cells play a central role in the destruction of pancreatic islet ß cells that leads to T1D.

T cells with regulatory properties have been shown to be invoved in the pathogenesis of immune mediated disorder[3-5]. However, to date, specific regulation of $CD8^+$ T cell function is still largely unexplored. Over the past years, a number of observations indicate that normal peripheral blood contains a small population of cells that express both CD56 and CD3 phenotypic markers (here defined as $CD3^+CD56^+$ cells) that characterize human natural killer (NK) and T (CD3) cells, respectively. Experimental evidence associated this subset with different pathophysiological conditions[36]. Indeed, significant decrease of hepatic recruitment of cells has been found after liver transplant with high rejection activity[32]. Recent data have shown that the absolute number of circulating $CD3^+CD56^+$ T cells represents a valuable predictive marker of β-cell residual function up to one year after T1D diagnosis[39]. Importantly, no study so far has examined the biological hallmarks and functional characteristics of $CD3^+CD56^+$ cells. In this study, we aim at analysing the possible immune regulatory role of $CD3^+CD56^+$ cells in healthy controls and in pathological conditions such as T1D. Possible results of our study may unveil an additional regulatory subset involved in the control of immune tolerance.

We hypothesized that $CD3^+CD56^+$ cells represent a novel regulatory population that control specifically the effector functions of $CD8^+$ cells, the main effectors for the destruction of ß-cells during T1D[65].

Perturbation of the number and function of $CD3^+CD56^+$ cells may account for the deranged functions of lymphocytes $CD8^+$ T observed in T1D. Therapeutic manipulation of $CD3^+CD56^+$cells may represent an innovative approach to restore immune tolerance in immune mediated disorders.

3. MATERIAL AND METHODS

3.1 Cell sorting, immune fluorescence and monoclonal antibodies

$CD3^+CD56^+$, $CD3^-CD56^+$, $CD3^+CD8^+$ and $CD3^+CD4^+$ cells were isolated, after Ficoll hypaque–gradient centrifugation (GE-Healthcare), from PBMCs of human healthy donors and T1D subjects by high-performance cell sorting (BD FACS-Jazz, BD Bioscience) in the IEOS-CNR Sorting Facility in Napoli, after staining with the following antibodies: FITC anti-human CD3 (BD PharMingen, clone UCHT1), PE-Cy7 anti-human CD56 (BD PharMingen, clone B159), FITC anti-human CD4 (BD PharMingen, clone RPA-T4), APC anti-human CD8 (BD PharMingen, clone RPA-T8) or by magnetic cell separation with microbeads $CD3^+CD56^+$ isolation Kit (Miltenyi Biotec). Sorted cells were 95%–98% pure by FACS analysis. Buffy coats from control healthy subjects were obtained after they signed an IRB-approved written informed consent. Samples were analyzed by immunofluorescence and Flow Cytometry by using a two laser equipped FACSCanto II (BD PharMingen). FITC, PE, PE-Cy7, PE-Cy5, APC-H7 and APC-labeled mAbs against CD3 (BD Pharmingen, clone UCHT1); CD4 (BD PharMingen, clone RPA-T4), CD8 (BD PharMingen, clone RPA-T8), CD16, (BD PharMingen, clone CLB/FcGran1), CD45 (BD Pharmingen, clone 2D1), CD25 (BD Pharmingen, clone M-A215), CD39 (BD Pharmingen, clone TU66), CD49d (BD Pharmingen, clone 9F10), CD45RA (BD Pharmingen, clone L48) CD45RO (BD Pharmingen, clone UCHL1), CD54 (BD Pharmingen, clone HA58), CD56 (BD Pharmingen, clone B159), CD57 (BD Pharmingen, clone HNK-1), CD62L (BD Pharmingen, clone SK11), CD69 (BD Pharmingen, clone L78), CD107/LAMP-1 (BD Pharmingen, clone H4A3), CD94 (BD Pharmingen, clone HP-3D9), CCR7 (BD Pharmingen, clone 15503), CTLA-4 (BD Pharmingen, clone BNI3) CXCR4 (BD Pharmingen, clone 12G5) Foxp3 (ebioscience, clone 259D/C7), GITR (Myltenyi, clone DTA-1), DNAM-1 (BD Pharmingen, clone DX11), PD-1 (BD Pharmingen, clone EH12.1), IFN-γ (BD Pharmingen, clone B27), NKG2A (Beckman Coulter, clone Z199), NKp30 (Beckman Coulter, clone Z25), NKp46 (Beckman Coulter, clone BAB281), CD1d:Ig fusion protein (BD Pharmingen), Vα24 (Beckman Coulter, clone C15) and isotype-matched controls

all from BD Pharmingen. FITC and PE labeled mAbs against TCR Vβ epitopes, namely anti-Vβ1, Vβ2, Vβ3, Vβ4, Vβ5.1, Vβ5.2, Vβ5.3, Vβ7.1, Vβ8 Vβ9, Vβ11, Vβ13.1, Vβ13.2, Vβ13.6, Vβ14, Vβ16, Vβ17, Vβ20, Vβ21.3 Vβ22, Vβ23. To analyze the production of Interferon (IFN)-γ purified cells intracellular staining with the specific mAb was performed by using the fixing/permeabilization (Caltag 554722), following the manufacturer's instructions. To avoid extracellular cytokine export, the cultures were performed in the presence of 5 μg/ml of Brefeldin-A (Sigma-Aldrich), as described[50]. Analysis was performed by using FlowJo Software (Tree Star). The control 345.134 IgG2a mAb, recognizing a glycoprotein widely expressed on human leucocytes[51] was a kind gift of Dr. S. Ferrone; recombinant human soluble NCAM-1/CD56 molecule was purchased from R&D Systems, Inc.

3.2 Cell culture, CD107/LAMP-1 expression and cytokine production

To obtain IL-2PBMC or IL-2CD8$^+$ cells, PBMC or flow sorted CD8$^+$ T cells were cultured for 48 hours in RPMI-1640 (Life Technologies) supplemented with 5% AB human serum in the presence of recombinant human IL-2 (Sigma) at 100UI/ml. IL-2PBMC or IL-2CD8$^+$ cells were incubated for 4 hours with anti-CD3 plus anti-CD28 mAb-coupled microbeads (Life Technologies) at the cell/bead ratio of 1:1 or with the K562 cell line (ATCC) at 1:1 ratio. CD107/LAMP-1 expression and IFN-γ production were evaluated in flow cytometry gated CD3$^+$CD56$^-$, CD3$^+$CD56$^+$, CD3$^-$CD56$^+$ (NK cells), CD4$^+$ and CD8$^+$ T cells, as indicated. When indicated IL-2PBMC or IL-2CD8$^+$ cells were co-cultured with fresh isolated with CD3$^+$CD56$^+$, CD3$^-$CD56$^+$ (NK cells) and CD3$^+$CD8$^+$ lymphocytes at different ratio. Brefeldin-A (BFA) at 5 μg/ml (Sigma) was added in the last three hours of culture for CD107/LAMP-1 staining or for the whole culture period for IFN-γ production[30,50]. Short term-cell lines were obtained culturing flow sorted CD3$^+$CD56$^+$ or CD3$^+$CD56$^-$, in the presence of anti-CD3 plus anti-CD28 mAb-coupled microbeads (0.2 beads/cell) with regular supplementation of hrIL-2 (20 UI/ml) for 10 days. To avoid cell-cell contact, coculture of CD3$^+$CD56$^+$ with IL-2CD8$^+$ lymphocytes was performed by using transwell inserts (Corning Life Sciences).

3.3 Proliferation assay

For the assessment of cell proliferation, cells were cultured in the presence of microbeads coated with anti-CD3 plus anti-CD28 (Life Technologies) at the cell/bead ratio of 0.1:1, as previously indicated[96]. Cultures were incubated for 72 hours at 37°C in a humidified atmosphere containing 5% CO_2 and pulsed with 0.5 μCi/well [^{3}H] thymidine for the final 16 hours. The incorporation of the labeled nucleotide was determined by scintillation counting after automatic cell harvesting. All tests were performed in the presence of RPMI 1640 Medium supplemented with 5% heat inactivated 5% AB human serum (Invitrogen).

3.4 Seahorse experiments

Metabolic profile was evaluated in $CD3^+CD56^+$, $CD3^-CD56^+$ and $CD3^+CD4^+$, in the absence or in the presence of anti-CD3 plus anti-CD28 microbeads (0.1 beads/cell) (Invitrogen). Real time measurements of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were made by an XF-96 Extracellular Flux Analyzer (Seahorse Bioscience). Specifically, cells were plated in XF-96 plates (Seahorse Bioscience) at the concentration of 2 x 10^5 cells/well and cultured with RPMI-1640 medium supplemented with 5% AB human serum. OCR was measured in XF media (non-buffered DMEM medium containing 10 mM glucose, 2 mM L-glutamin, and 1 mM sodium pyruvate), under basal conditions and in response to 5 μM oligomycin, 1.5 μM of carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP) and 1 μM of antimycin and rotenone (Sigma Aldrich). ECAR was measured in XF media in basal condition and in response to 10 mM glucose, 5 μM oligomycin and 100 mM of 2DG (all from Sigma Aldrich). Experiments with the Seahorse were done with the following assay conditions: 3 minutes mixture; 3 minutes wait; and 3 minutes measurement.

3.5 Statistical analysis

Statistical evaluation of data, by *InStat 3.0* software (GraphPad Software Inc., San Diego, California, USA), was performed by Mann-Whitney test, as indicated. Two-sided p values less than 0.05 were considered significant.

3.6 T1D subjects

The study was approved by the Institutional Review Board (IRB) of the Università degli Studi di Napoli "Federico II". Prot N. 200/16.

Recruitment of T1D patients (8 ± 7 years age, in a females:males ratio of 1:1), with glucose values between 80-180 mg/dl, after glycemic stabilization on exogenous insulin, achieved in 5 days. Diagnosis of T1D was defined according to the Global International Diabetes Federation/International Society for Pediatric and Adolescent Diabetes Guidelines for Diabetes in Childhood and Adolescence[97]and included symptoms of diabetes in addition to casual plasma glucose concentration ≥ 11.1 mmol/L (200 mg/dl), or fasting plasma glucose ≥ 7.0 mmol/l (≥ 126 mg/dl), or 2 hours postload glucose ≥ 11.1 mmol/l (≥ 200 mg/dl) during an oral glucose tolerance test, and glycated hemoglobin (HbA_{1c}) ≥ 6.5.[97] The criteria used to select healthy control subjects for T1D studies were the following: fasting blood glucose of < 5.5 mmol/L (< 100 mg/dl), negative personal and familial history of autoimmune disorders, and negativity for islet autoantibodies at the 99th percentile. The T1D children and control subjects (matched for age, sex and BMI) were recruited at the Dipartimento di Scienze Mediche Traslazionali, Sezione di Pediatria Università di Napoli ''Federico II'', after the IRB of the Università degli Studi di Napoli "Federico II" approved the study and parents gave their written informed consent.

3.7 Subjects with prostate cancer

In this study, we analysed the frequency of human of $CD3^{+}CD56^{+}$ T lymphocytes, isolated from peripheral blood of subjects with prostatic cancer are higher (n=10) compared to normal blood collected from healthy individuals as the control group, (n=12). PBMC were isolated using Ficoll-Paque density gradient centrifugation, from subjects with prostatic cancer. The cell suspension were then used for flow cytometry analysis. PBMc were stained with anti-CD3 BV510, anti-CD56 PE-CY7 and anti CD45 APC-H7. All antibodies were purchased from BD eBiosciences, (BD eBioesciences, USa). Samples were analyzed on BD FACS Canto II and the frequency of $CD3^{+}CD56^{+}$ T lymphocytes were calculated and compared with healthy controls. Actually, the Gleason grading system for prostatic carcinoma is the dominant method around the world in research and in daily practice. Here we correlate the frequency of $CD3^{+}CD56^{+}$ T lymphocytes with Gleason grade prostate cancer.

4. RESULTS

4.1 Phenotype of $CD3^+CD56^+$ T lymphocytes

In order to evaluate the phenotype of human $CD3^+CD56^+$ T cells, we analyzed the expression of several T and NK cell-associated markers in $CD3^+CD56^+$, $CD3^+CD56^-$ and $CD3^-CD56^+$ (NK) cells. As shown, $CD3^+CD56^+$ cells expressed, at different levels, T cell markers and the analysis of CD45 isoforms shows that $CD3^+CD56^+$ subset preferentially expressed CD45RA molecule, while they not showed surface molecules usually associated with NK, including NKG2A, CD94, Nkp46, DNAM-1 and TCR-Vα24 chain. (**Fig. 1)**. The absence of TCR-Vα24/Vβ11 chains indicate that $CD3^+CD56^+$ T lymphocytes did not belong to the NKT cell subset (**Fig. 1**). In addition, $CD3^+CD56^+$ cells expressed the TCR α and β chains, with a heterogeneous repertoire of Vβ gene families, similar to that observed in $CD3^+CD56^-$ T cell population (**data not shown)**. Moreover, $CD3^+CD56^+$ cells did not express the common Treg cell-specific markers, including CD25, Foxp3, GITR, CTLA-4, CD39 and PD-1 (**data not shown)**. The expression of CD54, CD57, CD62L CD16, CD49d, HLA-DR, and of the chemokine receptors CCR7 and CXCR4 were also analyzed in $CD3^+CD56^+$ cells (**data not shown).**

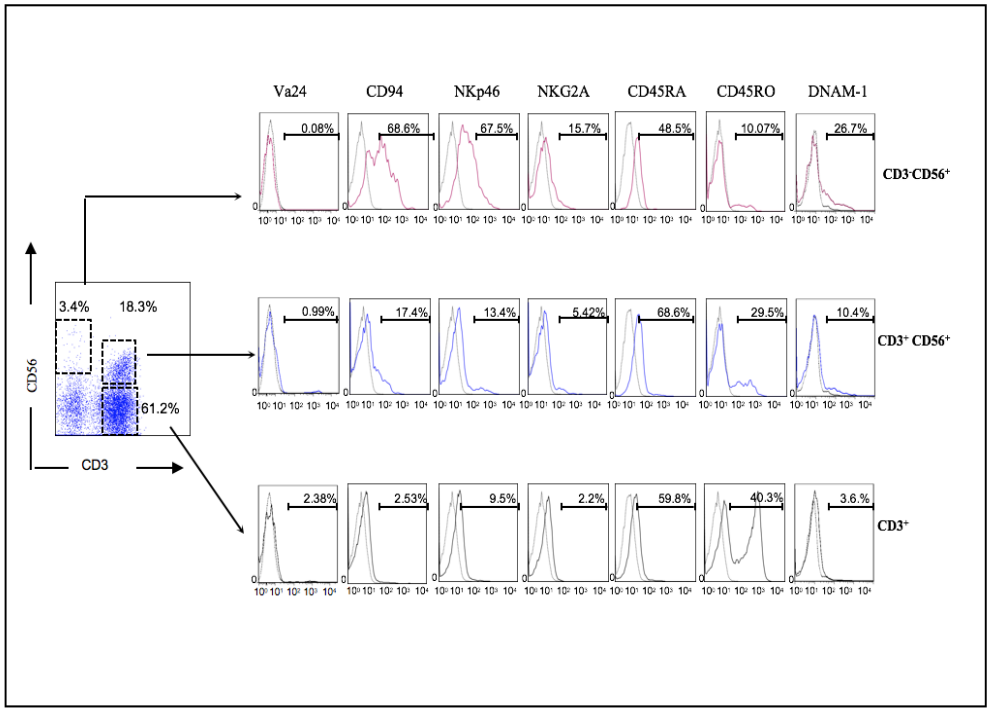
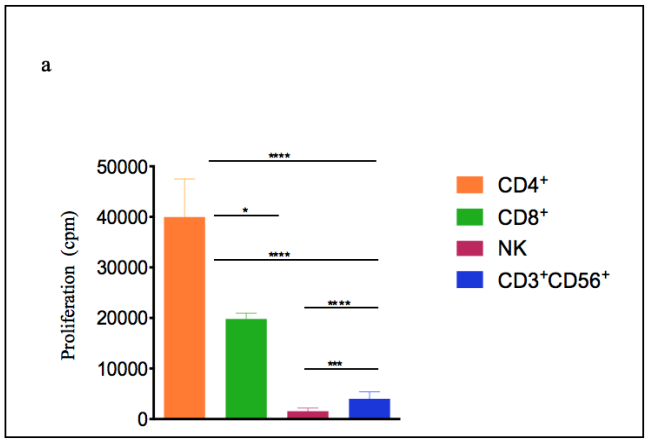


Fig. 1 Phenotype of $CD3^{+}CD56^{+}$ human lymphocytes. (**a**) Flow cytometry of peripheral blood from one representative healthy control. Upper panel indicates the gating strategy to define NK, $CD3^{+}CD56^{+}$ and $CD3^{+}CD56^{-}$ cell subsets, showing the characteristic phenotype of these cell subsets. Expression of several molecules usually associated with NK, T and (i)NKT cells are reported.

4.2. Functional properties of $CD3^+CD56^+$ T lymphocytes

To investigate the functional properties of $CD3^+CD56^+$ cells, we evaluated their proliferation, IFN-γ production and cytotoxic ability through the expression of Lysosomal-Associated Membrane Protein-1 (LAMP-1/CD107a), after stimulation with either TCR or NK-dependent stimuli. To determine the proliferative response of this cell subset, flow cytometry sorted $CD3^+CD56^+$ cells were stimulated for 72 hours with anti-CD3 plus anti-CD28 mAbs. We found that these cells had reduced proliferative ability compared with $CD4^+$ and $CD8^+$ conventional T (Tconv) cells. (**Fig. 2a)** To evaluate the functions of $CD3^+CD56^+$ subset, we cultured PBMCs for 48 hours in the presence of human recombinant IL-2 (hrIL-2) to obtain IL-2PBMC. This condition has been described to optimize both cytotoxic activity and IFN-γ production[98]. We observed that, co-culture for 4 hours of IL-2PBMC with K562 cell line, that represents a prototypic NK-target, was unable to induce CD107a/LAMP-1 expression and IFN-γ production in both $CD3^+CD56^+$ and $CD3^+CD56^-$ cells (**Fig. 2b**). As expected, CD107a/LAMP-1 expression and IFN-γ production were observed in NK lymphocytes. In contrast, after TCR activation of IL-2PBMC with microbeads coated with anti-CD3 plus anti-CD28 mAbs, cytotoxicity and IFN-γ production were detected in both $CD3^+CD56^+$ and $CD3^+CD56^-$ cells[99] (**Fig. 2c**). As expected, NK cells did not respond to TCR stimulation.

Together, these results indicated that human $CD3^+CD56^+$ cells showed phenotypic and functional profile similar to that observed in $CD3^+$ T lymphocytes.



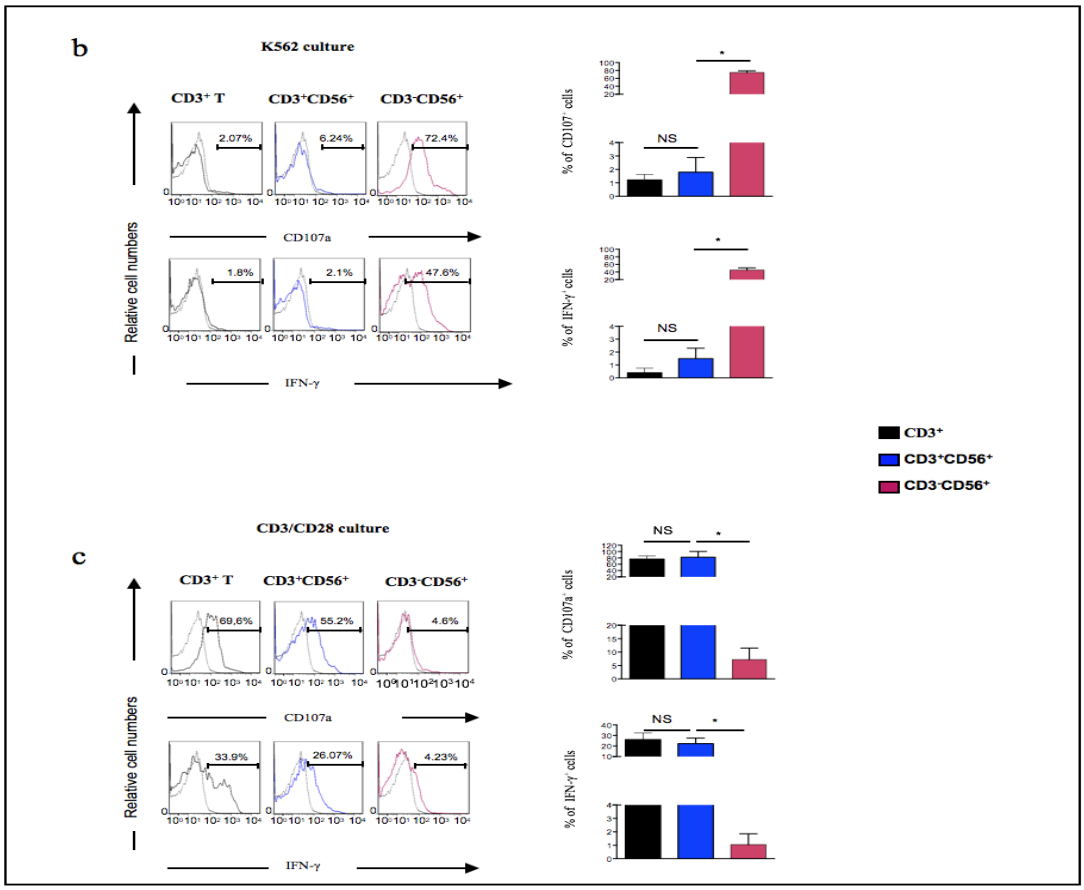
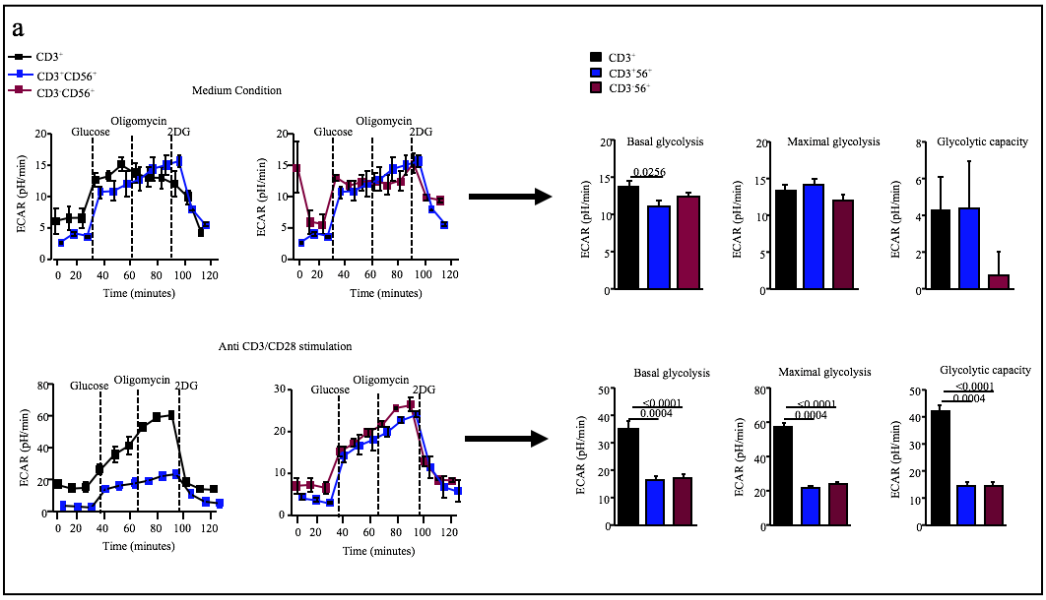


Fig. 2 (a) Thymidine incorporation (cpm) of $CD3^+CD56^+$ cells compared with $CD4^+$ (orange line), $CD8^+$ (green line) T cells, and NK (magenta line). Data are from seven independent experiments (mean ± S.E.M) **(b)** Upper panels show results of CD107a/LAMP-1 and IFN-γ staining profiles of $CD3^+CD56^-$ (black line), $CD3^+CD56^+$ (blue line), NK (magenta line), after four hours of culture in the presence of K562 cell line (plain line). **(c)** Lower panels indicate CD107a/LAMP-1 and IFN-γ staining profile of $CD3^+CD56^-$ (black line), $CD3^+CD56^+$ (blue line), NK (magenta line), after a four hour culture in the presence anti-CD3 plus anti-CD28 microbeads (plain line); * P <0.05; **** P <0.0001; (two-tailed t-test).

4.3 Metabolic programs of freshly isolated $CD3^+CD56^+$ T cells

To define the metabolic profile of $CD3^+CD56^+$ subset, flow cytometry sorted human $CD3^+CD56^+$ T, NK and $CD3^+$ T cells were cultured for 1 hour alone or in the presence of anti-CD3 plus anti-CD28 microbeads. Glycolysis and mitochondrial respiration were evaluated by measuring the extra cellular acidification rate (ECAR) and oxygen-consumption rate (OCR), respectively. Unstimulated $CD3^+CD56^+$ cells showed similar rate of basal and maximal glycolysis compared with $CD3^+$ T and NK cells. In addition, $CD3^+CD56^+$ evidenced a significant higher glycolytic capacity than NK cells (**Fig. 3a, upper left panels**). TCR stimulation of $CD3^+CD56^+$ cells, similarly to NK, did not increase their basal and maximal glycolysis (**Fig. 3a, upper right panels**). Conversely, the analysis of OCR revealed that unstimulated $CD3^+CD56^+$ T subset had higher basal, ATP-linked and maximal respiration compared to $CD3^+$ T cells; maximal respiration was similar to that observed in NK cells (**Fig. 3b, lower left panels**). Upon TCR stimulation $CD3^+CD56^+$ cells showed higher basal, ATP-linked and maximal respiration compared to $CD3^+$ T lymphocytes and NK cells (**Fig. 3b, lower right panels**).

These data suggested that $CD3^+CD56^+$ T cells preferentially engage mitochondrial respiration as cellular bio-energetic pathway after TCR triggering.



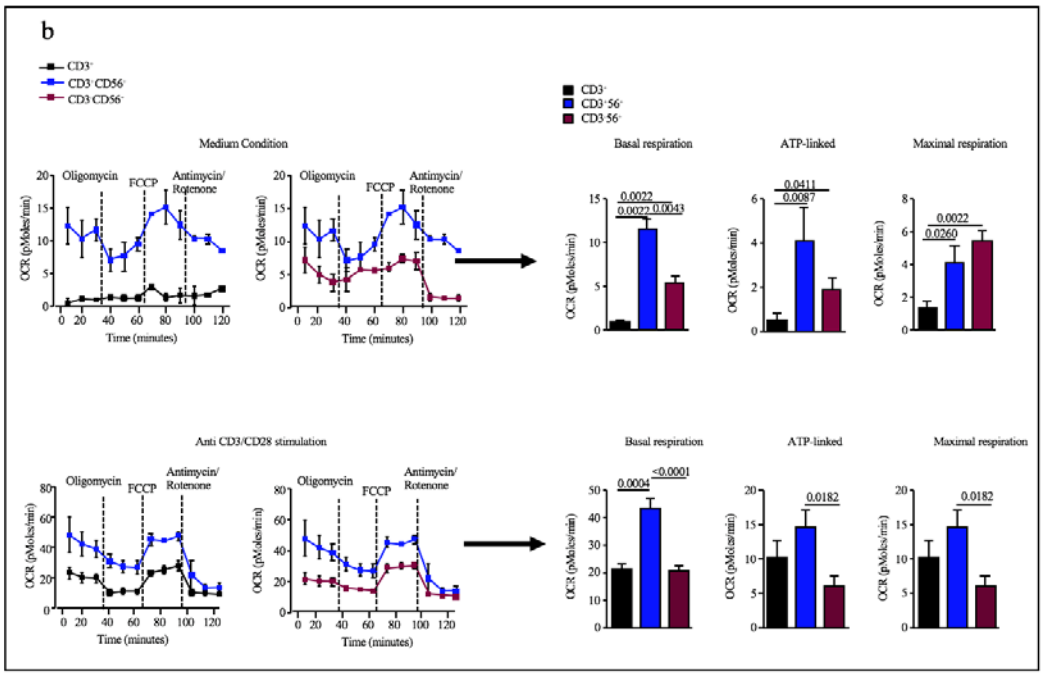


Fig. 3 Metabolic profiles of $CD3^+$, $CD3^+CD56^+$ and NK cell subsets in the presence or absence of stimulation with anti-CD3 plus anti-CD28 microbeads. **(a)** Upper left panels indicate glycolytic profile of flow sorted $CD3^+$ (black squares), $CD3^+CD56^+$ (blue squares) and NK (magenta squares) subsets cultured in the presence of Medium or with anti-CD3 plus anti-CD28 microbeads, while the upper right panels show basal, maximal glycolysis and the glycolytic capacity. Mean ± S.E.M. **(b)** Lower left panels indicate oxygen-consumption rate (OCR) profile of flow sorted $CD3^+$ (black squares), $CD3^+CD56^+$ (blue squares) and $CD56^+CD3^-$ (magenta squares) subsets cultured in the presence of Medium or with anti-CD3 plus anti-CD28 microbeads, lower right panels show basal, ATP-linked and maximal respiration. (two-tailed *t*-test) Mean ± S.E.M.

4.4 $CD3^+CD56^+$ cells modulate TCR-dependent cytotoxicity and the production of IFN-γ in $CD8^+$ T cells

To explore the possibility that human $CD3^+CD56^+$ cells contribute to immune regulatory networks, we investigated the capability of this subset to modulate the cytotoxic activity and the IFN-γ production of TCR triggered IL-2PBMC. As shown, IL-2PBMC were stimulated for 4 hours with anti-CD3 plus anti-CD28 microbeads in the presence of autologous freshly isolated $CD3^+CD56^+$, NK or $CD3^+CD8^+$ T cells. To specifically analyze IL-2PBMC, these cells were labeled before co-culture with anti-CD45 mAb. We observed that $CD3^+CD56^+$ lymphocytes significantly impaired both the expression of CD107a/LAMP-1 and IFN-γ production of CD45-labelled $CD8^+CD3^+$ cells gated on IL-2PBMC **(Fig. 4a)**. These effects were maintained up to a 0.2:1 $CD3^+CD56^+$/IL-2PBMCs ratio (**data not shown**). As control, the NK or $CD3^+CD8^+$ T cells were not able to modulate CD107a/LAMP-1 expression and IFN-γ production **(Fig. 4a)**. In addition, $CD3^+CD56^+$ T cells were unable to significantly modulate CD107a/LAMP-1 expression and IFN-γ production in TCR activated $CD3^+CD4^+$ cells **(data not shown)**. We next, investigated whether $CD3^+CD56^+$ lymphocytes exert their regulatory activity also in allogeneic condition. We found that $CD3^+CD56^+$ subset modulated CD107a/LAMP-1 expression and IFN-γ production also when co-cultured with allogeneic $CD8^+CD3^+$ IL-2PBMC stimulated for four hours with anti CD3 plus anti CD28 microbeads (**Fig. 4b**). Conversely, no significant effects on CD107a/LAMP expression and IFN-γ production were observed when allogeneic NK effectors were co-cultured with TCR triggered $CD8^+CD3^+$ IL-2PBMc cells **(data not shown)**.

To confirm the regulatory activity of human $CD3^+CD56^+$ cells on isolated $CD8^+$ T cells, we cultured flow cytometry sorted $CD8^+CD3^+$ lymphocytes with rhIL-2 (100UI/ml), for 48 hours to obtain purified and sorted IL-2$CD8^+$ cells. We found that, also in these experimental conditions, freshly isolated human $CD3^+CD56^+$ cells strongly suppress TCR dependent CD107a/LAMP-1 expression and IFN-γ production of purified IL-2$CD8^+$ compared to $CD3^-CD56^+$ (NK) and $CD3^+CD8^+$ T cells as controls (**Fig. 5**). Together these data indicated that human $CD3^+CD56^+$ cells exert regulatory activity on TCR-dependent cytotoxic activity of $CD8^+$ T cells.

Then, we evaluated the ability of $CD3^+CD56^+$ cells to affect the proliferation of $CD4^+$ and $CD8^+$ T lymphocytes stimulated with anti-CD3 plus anti-CD28 microbeads *in vitro*. We observed that flow cytometry sorted $CD3^+CD56^+$ cells reduced [^{3}H] thymidine incorporation $CD3^+CD8^+$ purified cells and have not significant effect on proliferation of cytometry sorted $CD3^+CD4^+$ (**Fig. 6**). In addition, cell division was assessed by CFSE staining, confirming an inhibition on the $CD3^+CD8^+$ cells as compared with the $CD3^+CD4^+$ counterpart **(data not shown).**

Finally, to investigate whether $CD3^+CD56^+$ cells maintain their phenotype and regulatory properties overtime, we generated short-term cell lines culturing freshly isolated $CD3^+CD56^+$ cells in the presence of anti-CD3 plus anti-CD28 microbeads for 10 days with regular supplementation of hrIL-2. We observed that from 8 to 10% of cultured $CD3^+CD56^-$ cells acquired CD56 molecule expression. Intriguingly, such induced $CD3^+CD56^+$ maintain their ability to suppress CD107a/LAMP-1 expression of IL-2$CD8^+$ (**Fig. 7**).

Taken together, these data indicate that $CD3^+$ $CD56^+$ co-expression identifies a plastic human T cell subset regulating the effector functions of TCR activated $CD8^+$ T cells.

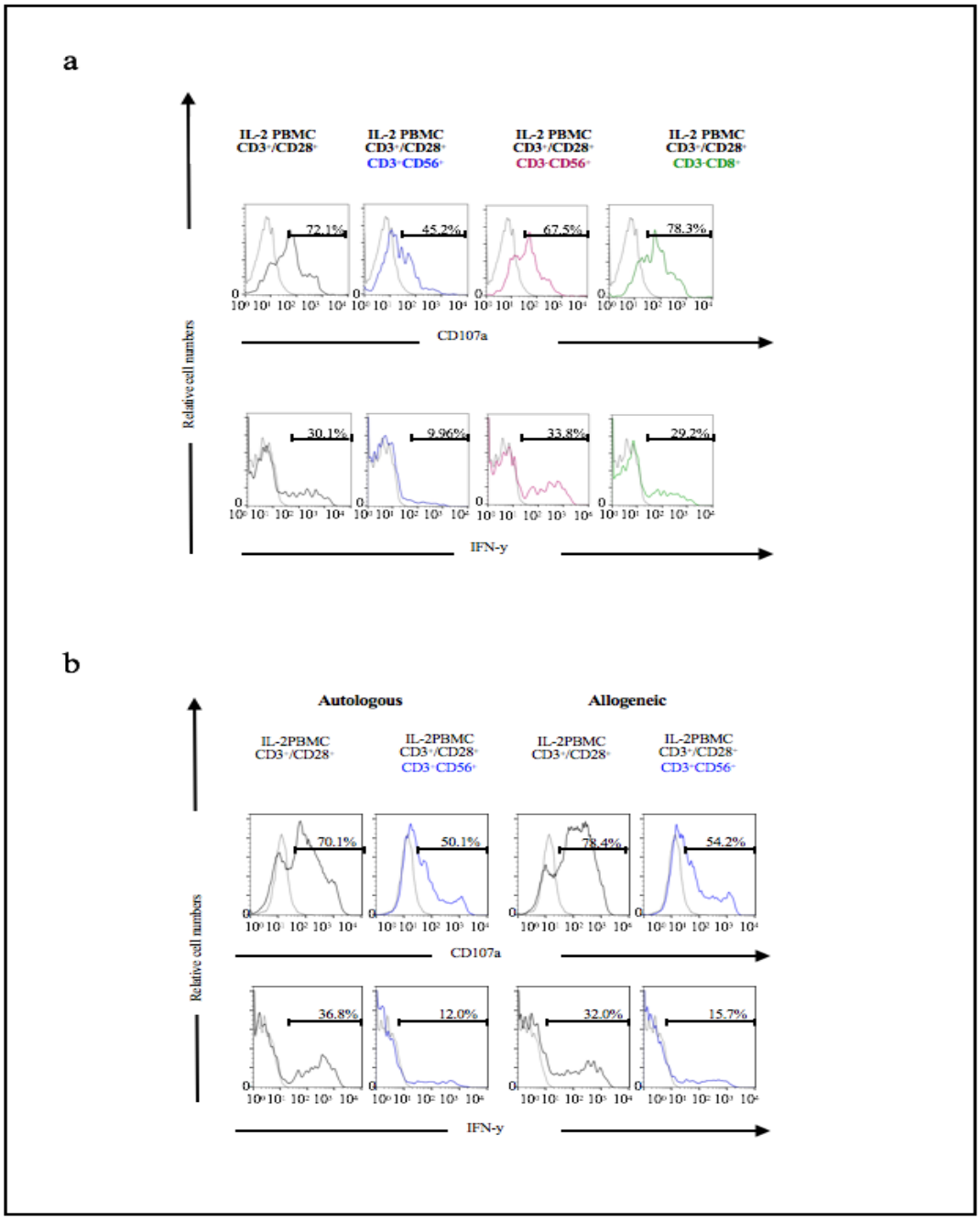


Fig. 4 Upper and lower panels show CD107a/LAMP-1 and IFN-γ staining profiles of flow cytometry gated $CD8^+$ IL2-PBMC T cells after a 4 hours culture with anti-CD3 plus anti-CD28 microbeads; **(a)** Upper panels show CD107a/LAMP-1 and IFN-γ staining of flow cytometry gated $CD8^+$ T cells after a 4 hour culture of IL2-PBMC stimulated with anti-CD3 plus anti-CD28 microbeads; IL2-PBMC (black line) and co-cultures with $CD3^+CD56^+$ (blue line), $CD3^-CD56^+$ (magenta line), $CD3^+CD8^+$ cells (green line) are shown; dotted lines indicate medium culture. **(b)** Lover panels indicate that $CD3^+CD56^+$ lymphocytes exert their regulatory activity both in autologous or allogeneic conditions. IL2PBMC (black line) and co-cultures with $CD3^+CD56^+$ (blue line) are shown.

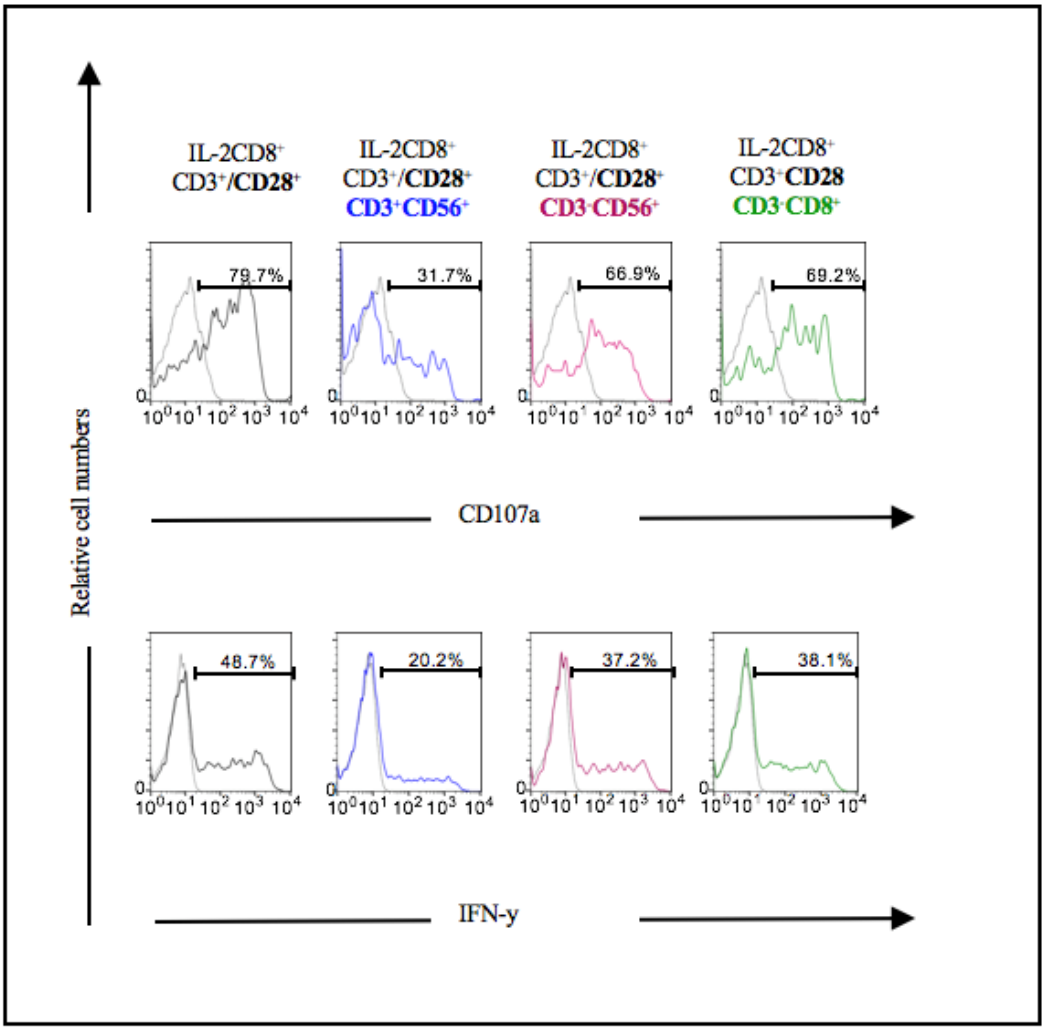


Fig. 5 Regulatory activity of $CD3^+CD56^+$ cells on sorted purified IL2-$CD8^+CD3^+$ cells. This panels show CD107a/LAMP-1 and IFN-γ staining profiles of IL2-$CD8^+$ sorted cells after a 4 hour stimulation with anti-CD3 plus anti-CD28 microbeads; data are from one of three independent experiments; IL2-CD8 (black line) and co-cultures with $CD3^+CD56^+$ (blue line), $CD3^-CD56^+$ (magenta line) and $CD3^+CD8^+$ cells (green line) are shown; dotted lines indicate medium culture.

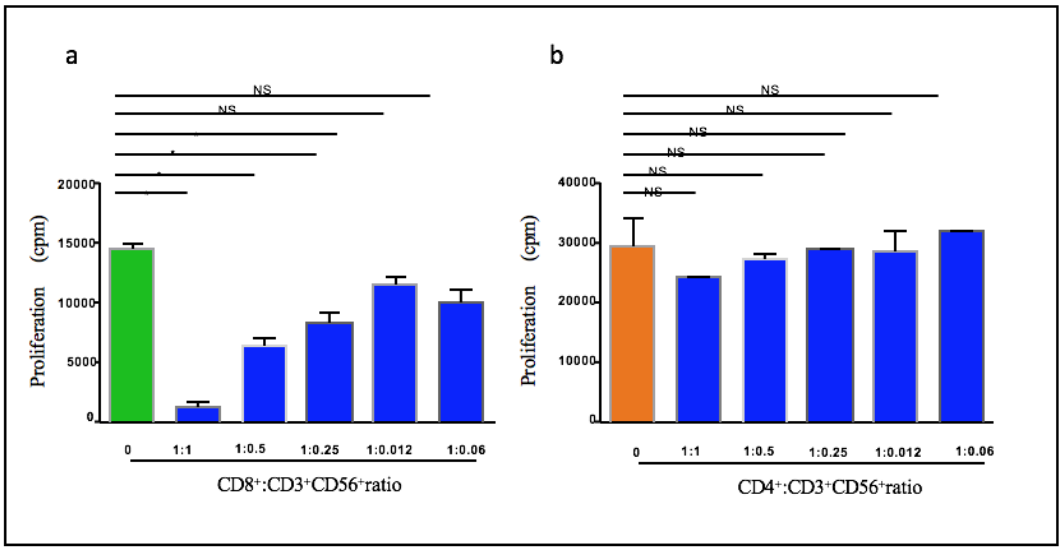


Fig. 6 $CD3^+CD56^+$ lymphocytes suppress proliferation of TCR activated $CD8^+$ T cells. **(a)** Left panels show thymidine incorporation of flow cytometry sorted $CD8^+$ T lymphocytes cultured for 72 hours with anti-CD3 plus anti-CD28 microbeads, alone (green column) or in the presence of $CD3^+CD56^+$ cells at the indicated different ratios (blue columns) Mean ± S.E.M. **(b)** Right panels show thymidine incorporation of flow cytometry sorted $CD4^+$ T lymphocytes cultured for 72 hours with anti-CD3 plus anti-CD28 microbeads alone (orange column) or in the presence of $CD3^+CD56^+$ cells at the indicated ratios (blue columns). Cell proliferation was evaluated as ^3H thymidine incorporation in the last 6 hours Left and Right panels indicate results obtained in eight independent experiments (n=8). Mean ± S.E.M. * P <0.05; (two-tailed Mann Whitney test).

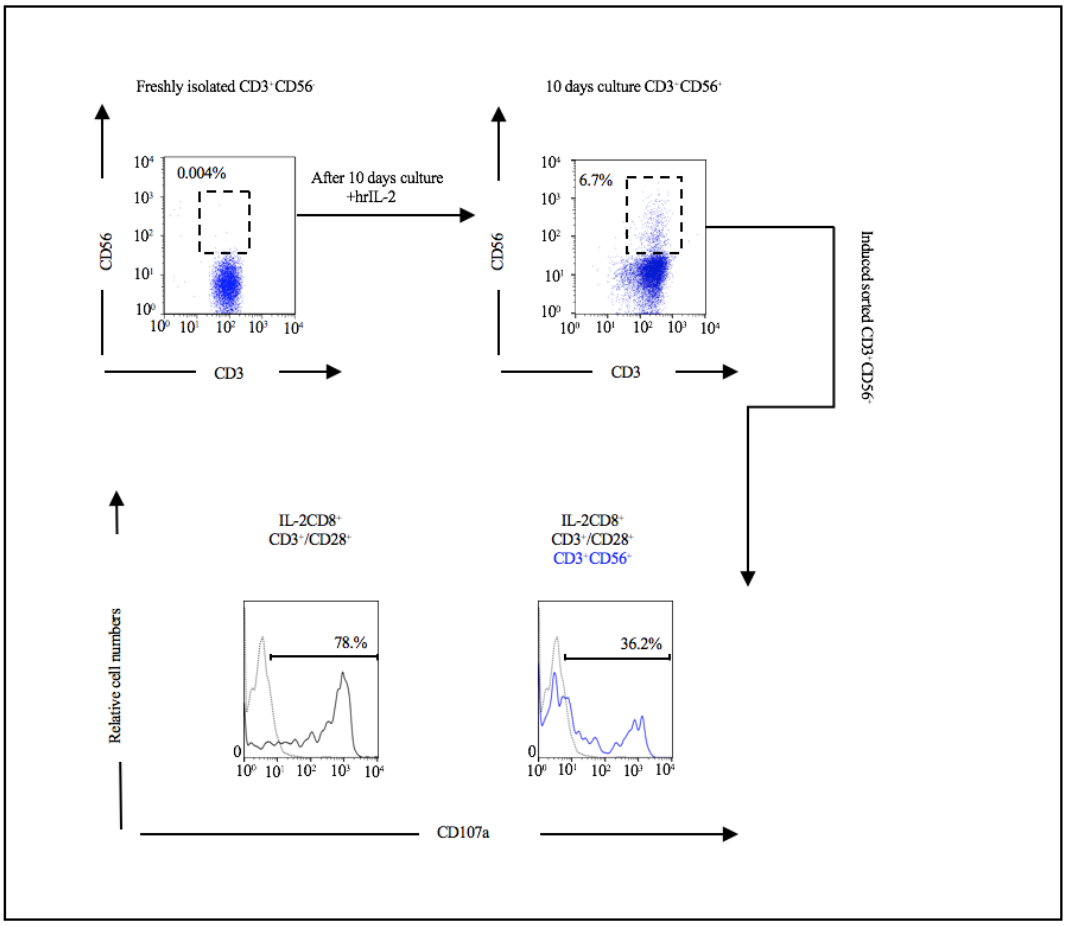


Fig. 7 Freshly isolated, sorted $CD3^{+}CD56^{-}$ cells (purity >99%) were cultured for 10 days in the presence of anti-CD3 plus anti-CD28 microbeads and regular rhIL-2 supplementation (upper left and right panels respectively). As shown, after 10 days culture; these "induced" $CD3^{+}CD56^{+}$ cells were isolated and co-cultured with TCR activated IL2-$CD8^{+}$. Low panels show CD107a/LAMP-1 expression by IL2-$CD8^{+}$ stimulated for 4 hours with anti-CD3 plus anti-CD28 microbeads alone (left lower panel) or in the presence of "induced" $CD3^{+}CD56^{+}$ (right lower panel). Data are from one representative experiment.

4.5 $CD3^+CD56^+$ regulatory activity requires cell to cell contact and is independent on CD56-mediated interactions

In order to investigate on the mechanisms underlying $CD3^+CD56^+$ regulatory properties, we first evaluated whether suppressive activity of $CD3^+CD56^+$ cells relied on direct cell-cell contact with TCR target cells or depends on soluble molecules. As shown $CD3^+CD56^+$ cells lost their suppressive capacity when physical separated from $CD8^+$ cells in a transwell assay. This results were obtained when anti-CD3 plus anti-CD28 microbeads were added to IL-2$CD8^+$ cells (low chamber) or to both IL-2$CD8^+$ and $CD3^+CD56^+$ cells (low and upper chambers) (**Fig. 8)**. To investigate the direct involvement of CD56 in delivering contact-dependent suppressive signals, either $CD3^+CD56^+$ and IL-2$CD8^+$ cells were cultured with saturating concentration of soluble human recombinant neural cell adhesion molecule (hrNCAM/CD56) or anti-CD56 mAb, (30min) and successively they were tested for their ability to suppress activity of TCR stimulated Il-2$CD8^+$ T. We found that in these experimental conditions, $CD3^+CD56^+$ lymphocytes retained their capability to modulate effector functions of TCR activated $CD8^+$ IL-2PBMc in term of CD107a/LAMP-1 expression and IFN-γ production by TCR activated IL-2CD8 cells. Some results were obtained when $CD3^+CD56^+$ lymphocytes were pre-treated with hrNCAM/CD56 or anti-CD56 mAbs before culture. Thus, CD56 dependent contact was not relevant for $CD3^+CD56^+$ regulatory activity (**Fig. 9**).

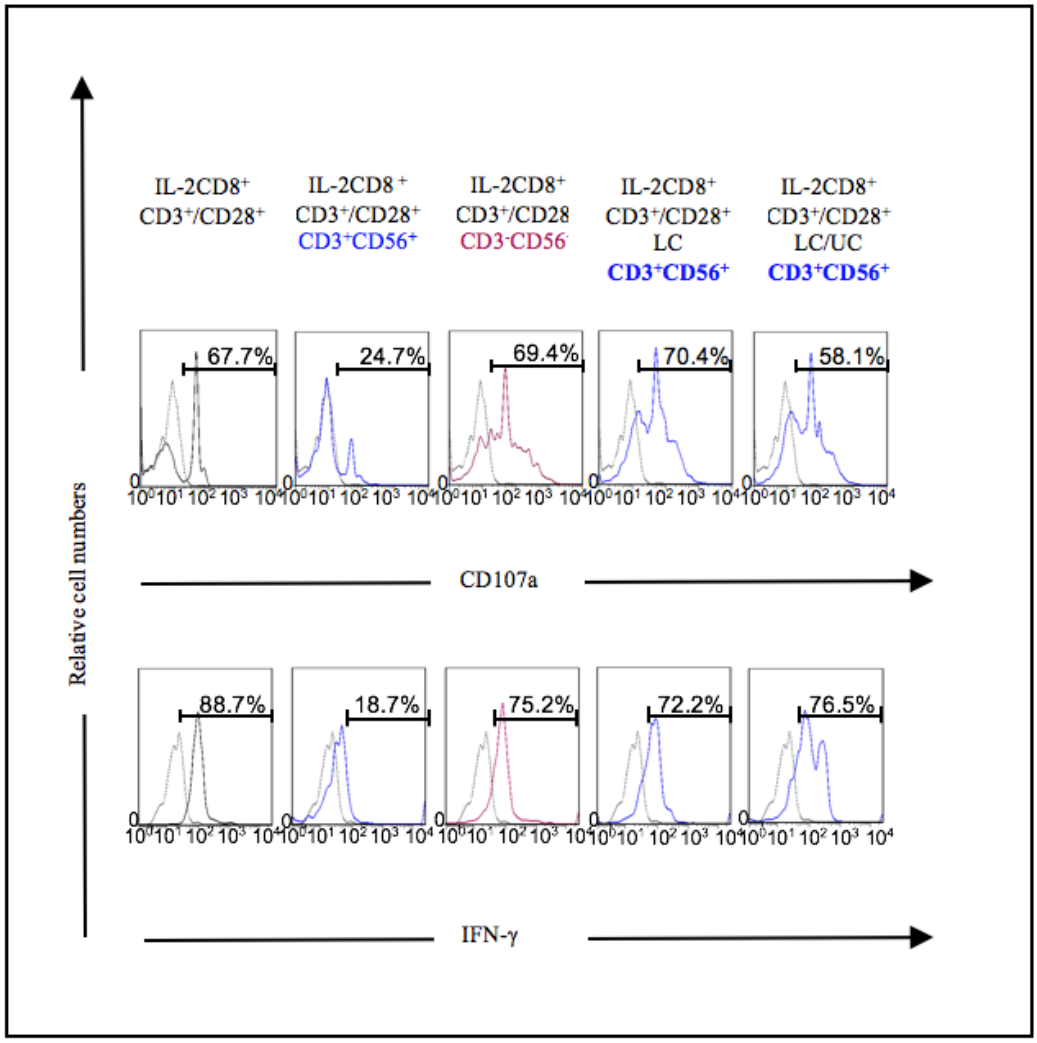


Fig. 8 $CD3^+CD56^+$ regulatory functions require cell to cell contact. This panel indicates CD107a/LAMP-1 and IFN-γ staining of $IL\text{-}2CD8^+$ after a 4 hour culture with anti-CD3 plus anti-CD28 microbeads; $IL\text{-}2CD8^+$ (black line) or co-cultures with $CD3^+CD56^+$ cells (blue line) are shown; as indicated, $CD3^+CD56^+$ co-cultures were performed using transwell device in order to prevent cell to cell contact. These results were obtained when anti-CD3 plus anti-CD28 microbeads were added to $IL\text{-}2CD8^+$ cells (low chamber) or to both $IL\text{-}2CD8^+$ and $CD3^+CD56^+$ cells (low and upper chambers).

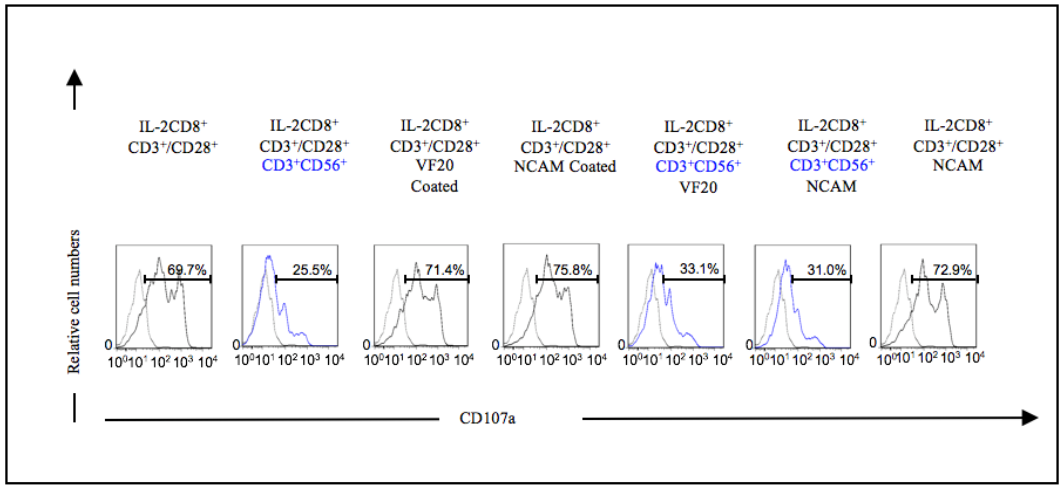


Fig. 9 $CD3^+CD56^+$ regulatory functions do not involve CD56-dependent interactions. CD107a/LAMP-1 staining of IL2-$CD8^+$ after a 4hour culture with anti-CD3 plus anti-CD28 microbeads; IL2-$CD8^+$ (black line) or co-cultures with $CD3^+CD56^+$ cells (blue line) are shown; as indicated, $CD3^+CD56^+$ co-cultures were performed in the presence of anti-CD56 mAb, NCAM/CD56 recombinant molecule and the control 345.134 IgG2a mAb; data are from one representative experiment out of three.

4.6 T1D subjects show a reduced number and impaired suppressive activity of $CD3^+CD56^+$ cells

Previously data from our laboratory have showed that circulating $CD3^+CD56^+$ cell number correlate with β-cell residual function up to one year after diabetes diagnosis. In fact, high number of $CD3^+CD56^+$ cells directly correlated with pancreatic β-cell activity one year after diagnosis[39]. In this study we analyzed both number and suppressive capability of $CD3^+CD56^+$ cells in a large cohort of T1D affected children at disease onset, in comparison with age- and sex-matched healthy subjects. We found that, at diagnosis, both percentage and absolute number of this cell subset were significantly lower in T1D children (n=100) than healthy subjects (n=70) (**Fig. 10 a, b**). To better understanding the role of $CD3^+CD56^+$ cells in the pathogenesis of T1D, we tested the ability of flow sorted $CD3^+CD56^+$ lymphocytes from T1D individuals to modulate TCR dependent CD107a of autologous $CD8^+$ cells gate on IL-2PBMC.

As shown, $CD3^+CD56^+$ cells from T1D subjects (n=13) had impaired suppressive ability to modulate CD107a expression when co-cultured with TCR engaged $CD8^+$ IL-2PBMc, cells compared with age and sex-matched healthy individuals (n=17) (**Fig. 10c**). Moreover, to discriminate whether impaired suppression observed in $CD3^+CD56^+$ from T1D subjects may be ascribed to resistance of their $CD8^+$ T cells, we co-cultured healthy donor $CD3^+CD56^+$ cells with T1D-derived IL-2PBMC lymphocytes. $CD3^+CD56^+$ cells from healthy subjects suppressed cytotoxic activity of IL-2PBMC cells from T1D subjects; however, autologous $CD3^+CD56^+$ cells showed impaired suppressive capability **(data not shown)**.

These findings indicated a specific defect in numbers and function of human $CD3^+CD56^+$ cell compartment in autoimmune diabetes.

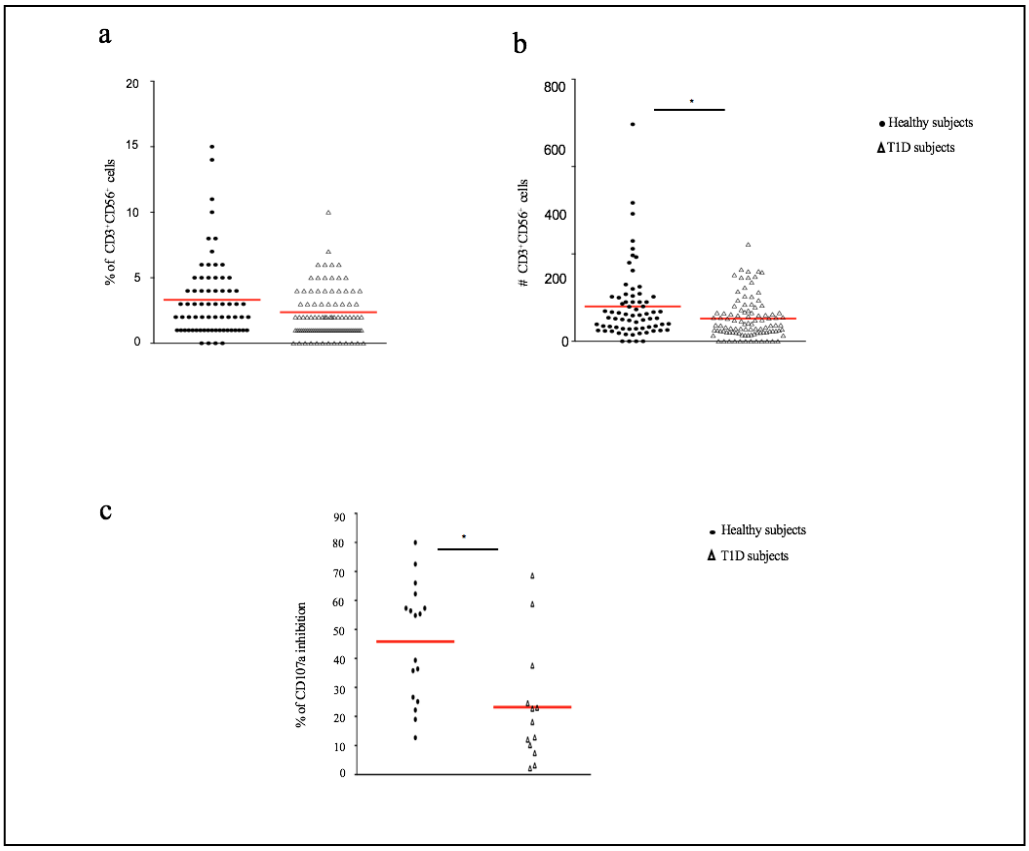


Fig. 10 T1D children at diagnosis show a reduced percentage and absolute number and impaired suppressive activity of $CD3^{+}CD56^{+}$ cells**.** (a and b). Left and right upper panels indicate respectively the percentage and absolute number of peripheral $CD3^{+}CD56^{+}$ lymphocytes in T1D children (n=100) at disease onset, as compared with age/sex matched healthy controls (n=70); (**c**) Cumulative data of CD107a/LAMP-1 inhibition by $CD3^{+}CD56^{+}$ cells, staining of $CD8^{+}$ gated IL2-PBMC, from T1D compared (n=13), to healthy donors, (n=17); * *P* <0.0005; ***P* <0.0001; (two-tailed Mann Whitney test).

4.7 Subjects with prostate cancer show a higher number of $CD3^+CD56^+$ cells in peripheral blood.

Subjects with prostate cancer show a higher number of $CD3^+CD56^+$ cells in peripheral blood (n=10) compared to normal blood collected from healthy individuals (n=12). PBMCs were isolated using Ficoll-Paque density gradient centrifugation, from subjects with prostate cancer. The cell suspension was then used for flow cytometry analysis **(Fig. 11a)**. Furthermore, the percentage of $CD3^+CD56^+$ T lymphocytes in the peripheral blood of patients with prostate cancer positive correlation with the Gleason grades **(Fig. 11b)**.

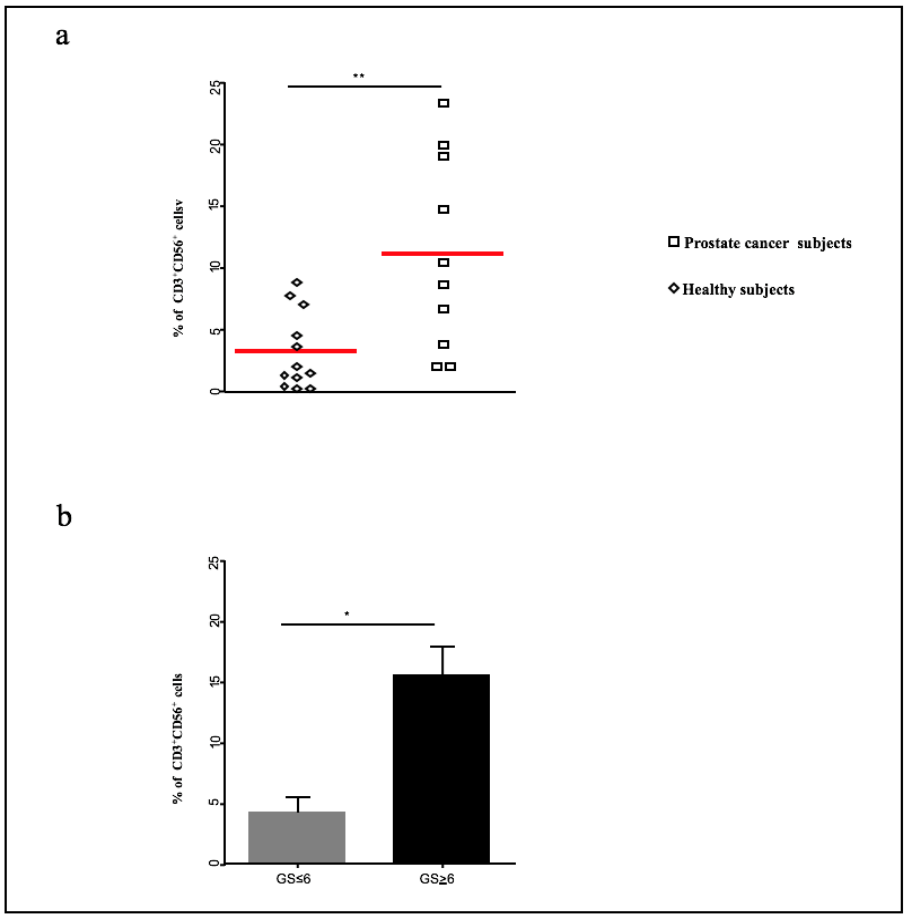


Fig. 11. a) frequency of $CD3^+CD56^+$ lymphocytes in peripheral blood of prostate cancer patients (n=10), compared with age/sex matched healthy controls (n=12); **b**) $CD3^+CD56^+$ T lymphocytes in the peripheral blood of patients with prostate cancer positively correlates with the Gleason grades**,** (GS). Mean ± S.E.M. * *P* <0.05; ** *P* <0.01 (two-tailed Mann Whitney test).

5. DISCUSSION AND CONCLUSION

This study reveals that $CD3^+CD56^+$ co-expression identifies a T cell subset with previously unexplored regulatory properties able to modulate TCR-dependent effector functions of human $CD8^+$ T cells. Here we found that human freshly isolated $CD3^+CD56^+$ cells suppress cytotoxicity and IFN-γ production of *in vitro* activated $CD8^+$ T lymphocytes in both autologous and allogeneic conditions. Decreased number and impairment of suppressive activity of $CD3^+CD56^+$ cells were observed in a large cohort of children affected by autoimmune diabetes. This strongly suggests the involvement of $CD3^+CD56^+$ cells in control of immune tolerance and in the development of autoimmune condition.

Human $CD3^+CD56^+$ cells represent a distinct T cell subset preferentially expressing CD8 co-receptor with a heterogeneous αβ–TCR repertoire, clearly divergent from NKT, as testified by the lack of binding with CD1d tetramer and the absence of TCR-Vα24/Vβ11 chains. $CD3^+CD56^+$ cells up-regulate cytotoxic molecules, produce IFN-γ and display a low proliferative rate upon TCR stimulation *in vitro*; they are unresponsive to NK-dependent stimuli, in terms of cytotoxicity and cytokine production. A specific metabolic signature, engaging the mitochondrial respiration rather than glycolysis upon TCR activation, characterizes this cell subset.

Freshly isolated human $CD3^+CD56^+$ cells specifically suppress cytotoxicity and IFN-y production of co-cultured human $CD8^+$ T cells *in vitro*. These modulating effects are exerted in both autologous and allogeneic conditions. We also show that $CD3^+CD56^+$ cells affect TCR-dependent proliferation of $CD8^+$ but have not significant effect on proliferation $CD4^+$ subsets. Overall this data suggest that $CD3^+CD56^+$ cells represent a T cell subset with specific phenotypic profile, functional properties and metabolic requirements. These data are in line with previous evidences suggesting a possible regulatory role of this population[34-36,100]. These findings are also in agreement with observations found by our laboratory showing that absolute numbers of $CD3^+CD56^+$ circulating cells associate with higher residual β-cell function in subjects affected by T1D[39].

$CD3^+CD56^+$ cells represent a plastic regulatory T cell subset. Indeed, in our experimental system TCR stimulation (in the presence of low doses of hrIL-2) of $CD3^+CD56^-$ cells gave rise to a fraction of "induced" $CD3^+CD56^+$ cells able to

suppress effector functions of $CD8^+$ T cells activated *via* TCR. These reflect the behavior of well-characterized induced $Foxp3^+$ Treg (iTreg) cells[101-102]. However, suppressive capability of $CD3^+CD56^+$ cells is not related to the expression of common regulatory cell lineage markers, such as Foxp3, CTLA-4, GITR and CD49d.

Furthermore, regulatory ability of $CD3^+CD56^+$ cells strictly relied on cell-to-cell contact and is independent on CD56-mediated interactions[8,103-105]. However, the precise mechanism and surface molecules mediating this suppressive function remain to be explored.

Apoptosis induction has been reported to represent a key regulatory mechanism required to maintain immune homeostasis[106-107]. In our experimental conditions, we found that apoptosis was dispensable for regulatory activity of human $CD3^+CD56^+$ subset. Indeed, $CD3^+CD56^+$ lymphocytes significantly suppress CD107a/LAMP-1 expression and IFN-γ production of TCR activated non apoptotic (Annexin V negative) $CD8^+$ T cells.

There is growing evidence showing the key role of $CD8^+$ T cells in the pathogenesis of several autoimmune disorders including multiple sclerosis (MS) and T1D[108-111]. Here we report that in a large cohort T1D child, at diagnosis, $CD3^+CD56^+$ cells were reduced in comparison with healthy individuals and that fail to suppress $CD8^+$ effector functions *in vitro*. These data extend our previous observation showing that $CD3^+CD56^+$ cell number at diagnosis predicts residual β-cell function in T1D one year later[39]. Remarkably, our results are conceivable with the hypothesis that altered $CD3^+CD56^+$ number and function may account for the deranged effector function of $CD8^+$ T lymphocytes, typical of T1D[109-111].

The study of the role of host immune system in the tumour microenvironment is crucial for understanding how they affect the development and course of human tumors[112-113]. In the present study, we investigated the involvement of $CD3^+CD56^+$ cells in the pathogenesis of prostate cancer. Here we observed that $CD3^+CD56^+$ cells were higher in prostate cancer patients and directly correlated with Gleason grade, indicating that activity of $CD3^+$ $CD56^+$ cells could be involved in prostatic tumours progression.

In summary, human $CD3^+CD56^+$ cells represent a plastic T cell subset, involved in the control of $CD8^+$mediated immune response. Dysregulation in regulatory

functions of this cell subset could determine an altered immune response, that lead to disorder such as T1D and cancer. Obtained result may pave the way to new experimental approaches aimed to expand $CD3^+CD56^+$ cells in vitro that may reshape $CD8^+$ T cell hyper-activation in autoimmunity. Conversely, targeting of this regulatory cell subset in human cancer could help improve anti-cancer immune response during tumor progression. In perspective, the therapeutic manipulation of $CD3^+CD56^+$ and their monitoring may represent a novel target for immune interventions upon alteration of immune response.

6. ACKNOWLEDGMENTS

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8. List of publications production during PhD course:

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