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MOLECULAR CHARACTERIZATION OF HUMAN CD4+ IL-10-PRODUCING REGULATORY CELLS

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1. INTRODUCTION

1.1. ADAPTIVE IMMUNITY

Adaptive Immunity, also called specific or acquired immunity, is mediated by lymphocytes and stimulated by exposure to infectious agents. In contrast to innate immunity it is characterized by specificity for distinct macromolecules and memory, which is the ability to respond more vigorously to repeated exposure to the same microbe [1, 2].

There are two types of adaptive immune responses called humoral and cell-mediated immunity, that are mediated by different component of the immune system.

Humoral Immunity is mediated by antibodies that are secreted by B lymphocytes. It is the principal defense mechanism against extracellular microbes and their toxins because secreted antibodies can bind to these microbes and toxins and assist in their elimination. Different classes of antibodies may activate different effector mechanisms: different types of antibodies promote the ingestion of microbes by host cells (phagocytosis) or bind to and trigger the release of inflammatory mediators from cells [2].

Cellular Immunity is mediated by T lymphocytes. Intracellular microbes, such as viruses or some bacteria, survive and proliferate inside phagocytes and other host cells where they are not detectable by circulating antibodies. Cell mediated immunity promotes the destruction of microbes residing in phagocytes or the killing of infected cells to eliminate reservoirs of infection [2].

1.2. T LYMPHOCYTE DEVELOPMENT AND FUNCTION

T cells precursors originate from a common lymphoid hematopoietic stem cell in the bone marrow and successively migrate to the thymus for their maturation. These progenitors can differentiate into T cells but there are not committed yet to the T cell lineage since they still don't possess a mature T-cell receptor (TCR) [3].

During the maturation process in the thymus T cells are instructed to discriminate between self and non-self acquiring the capacity to recognize foreign antigens in association with self-MHC without attacking self components. This process is called "central tolerance" and consists of two phases termed positive and negative selection respectively.

During the positive selection T cell precursors are pre-selected according to their TCR compatibility with self-MHC presented by thymic epithelial cells. This process ensures that TCRs in any given individual have affinity for the MHC alleles present in that individual. T cell precursors with no affinity, which are not "self-restricted", are committed to die by apoptosis.

During the "negative selection" instead, T cell precursors are selected for their abilities to bind self-antigens displayed by self-MHC. T cells showing a too high affinity for self-antigen are induced to die by apoptosis ensuring the elimination of auto-reactive T cells. This step of T cell maturation is associated the CD4 or CD8 lineage commitment. CD4 and CD8 are proteins called co-receptors that bind the invariant part of MHC class II and MHC class I respectively and play an important role in TCR signal transduction interacting with membrane-associated signaling molecules [3].

These proteins define two major T cell lineages which are different not only for MHC specificity but also for their function [4].

CD4+ MHC class II-restricted T cells function mainly as T helper (T_H) cells. These cells help the activity of other immune cells, such as B cells or cytotoxic T cells, secreting specific cytokines to maximize immune response.

CD8+ MHC class I-restricted T cells instead, after activation, acquire cytotoxic properties that enable them to kill cells presenting their target antigens [4].

After thymic maturation T cells are in the "naive" state and continuously circulate through the blood to secondary lymphoid organs where they can encounter antigens specific for their TCR, displayed by Antigen Presenting Cells (APC).

The term naïve refers to both CD4 and CD8 T cells and derives from the idea that these cells are immunologically inexperienced because they have not encountered antigen.

These cells are characterized by the expression of CD45RA and they express L-selectin (CD62L) and the chemokine receptor CCR7 to migrate to lymphoid organs [5-7].

1.3. CD4+ T LYMPHOCYTE ACTIVATION

Naïve CD4+ T cells exported from the thymus express a highly diverse TCR repertoire that enables them to potentially recognize all kind of pathogens [8].

Migration to lymphoid organs is the first step for their activation because there they can physically encounter their specific antigens, which are presented by professional APC like Dendritic Cells (DC).

In lymphoid organs CD4+ T cells recognize peptide-MHC class II complexes displayed by APC and, after a massive expansion named clonal expansion, are able to differentiate into several subsets of effector cells [9].

Lineage-specific differentiation depends on the cytokine milieu of the microenvironment, as well as on the concentration of antigens, type of APCs, and co-stimulatory molecules. The initial sources of cytokines are APCs as well as other members of the innate immune cells. Subsequently, some of the cytokines produced by the differentiating cells can create a positive feedback loop, whereby the differentiation and response are enhanced [10].

T cells with different phenotypic and functional properties exert different effector functions.

The CD4+ T cells carry out multiple functions, ranging from activation of the cells of the innate immune system, B-lymphocytes, cytotoxic T cells, as well as non immune cells, and also play critical role in the suppression of immune reaction.

Different subsets of CD4+ cells besides the classical T-helper 1 (Th1) and T-helper 2 (Th2) cells have been identified; these include T-helper 17 (Th17), follicular helper T cell (Tfh), induced T-regulatory cells (iTreg), and the regulatory type 1 cells (Tr1).

Th1, as described in detail later, are required to fight intracellular pathogens like intracellular bacteria and viruses [11]. Th2 are important to fight extracellular parasites like worms and are involved in allergic diseases like asthma [12]. Th17 are important for the immune response against fungi and extracellular bacteria and play a pathogenic role in chronic inflammation associated to autoimmune diseases [13].

Tfh, originally thought to be Th2 cells, are present in the B cell follicles and in the germinal centers of secondary lymphoid organs and provide help to B cells [14].

Regulatory T cells (Tregs and Tr1 cells) instead have the task to prevent unwanted immune reaction to limit tissue damage and to maintain tolerance to self [15-17].

Once the pathogen is cleared the most of T effector cells die but some survive and become long-lived memory T cells that can mediate secondary immune when host is re-exposed to the same infectious agent. Memory T cells are heterogeneous and can be divided into two main categories: central memory T cells (T_{CM} cells) and effector memory T cells (T_{EM} cells).

T_{CM} cells express the lymph node homing receptor CCR7 and CD62L typical of naïve cells. These cells show reduced effector functions but a high expansion potential when re-exposed to antigen.

The majority of T_{CM} cells is non-polarized and responsive to IL-2 but some are pre-committed to the Th1 or Th2 lineage. Pre-Th1 express CXCR3 which is a chemokine receptor expressed on virtually all the *in vivo* occurring Th1 cells and secrete IFN-γ which is the main effector cytokine of activated Th1 cells.

Pre-Th2 express CCR4 which is a chemokine rector highly enriched in Th2 cells and secrete IL-4 which is considered the Th2 effector cytokine.

T_{EM} cells instead have lost CCR7 and CD62L and perform efficiently and rapidly their effector functions producing a variety of microbicidal cytokines, including IFN-ɣ, IL-4 and IL-5 within several hours of TCR stimulation.

These cells are characterized by the expression of CCR5 or CCR2 that mediate homing to non-lymphoid tissues.

1.4. TH1 DIFFERENTIATION

Th1 cells are involved in the elimination of intracellular pathogens [11], but an overshooting Th1 response can cause lethal immunopathology [18]. They mainly secrete IFN-*γ* which his essential for the activation of mononuclear phagocytes, including macrophages, microglial cells, thereby resulting in enhanced phagocytic activity. Interleukin 12 (IL-12) and IFN-*γ* are the critical cytokines initiating the downstream signaling cascade to develop Th1 cells. IL-12 is secreted in large amounts by APCs after their activation through the pattern recognition receptors.

IFN-γ and IL-12 stimulate Th1 differentiation by activating the transcription factor T-bet, STAT1 and STAT4 [19]. The T-box transcription factor T-bet, is the “master regulator” of Th1 differentiation. Master regulators are transcription factors that are important not only for their ability to activate the set of genes to promote differentiation of a particular T cell subset, but also for being able to suppress the development of opposing cell lineages [19, 20].

T-bet significantly enhances the production of IFN-*γ*, and plays important role in suppressing the development of Th2 and Th17 targeting and repressing IL4 gene and RORγt respectively.

T-bet also induces the chemokine receptors CXCR3 that allow Th1 effector cells to enter peripheral tissues. CXCR3 is expressed on virtually all *in vivo* occurring human Th1 cells and allows the isolation of high numbers of resting Th1 memory T cell subsets [21].

T-bet expression is strongly dependent on signal transducer and activator of transcription 1 (STAT1) which is in turn activated by IFN-*γ* [22]. IL-12-induced STAT4 is another

important transcription factor involved in the Th1 cell differentiation: STAT4 in fact induces IFN-*γ* production creating a positive feedback loop for further T-bet [22].

Runt-related transcription factor family members also participate in the differentiation process. Runx1 and Runx3 were found to promote Th1 cell differentiation. Runx3, in coordination with T-bet, binds to the IFN*γ* promoter and silences the genes encoding IL4, leading to the Th1 lineage differentiation [23]. Moreover, Runx3, through interaction with GATA3, leads to the inhibition of Th2 differentiation [24]. Runx1 together with T-bet inhibits Th17 development by interfering with the ROR*γ*t master regulator [25].

1.5. REGULATORY T CELL DIFFERENTIATION

Early evidence for the presence of CD4+ with immunoregulatory function derive from observations in severe combine immunodeficient (SCID) mice model in which a specific CD4+ T subset was shown to protect against intestinal inflammation. In this work two distinct CD4+ populations with opposing function were isolated: one $CD45^{high}$ subset with immunosuppressive potential and one $CD45^{low}$ with proinflammatory function [26].

Successively other groups clearly demonstrated the dependence of the immunological self-tolerance by a specific subset of CD4+ T cells, which expressed high levels of IL-2 receptor alpha (CD25) [27]. They showed also that depletion of this specific subset could lead to severe immunopathology [27].

Different groups then identified a thymically derived CD4+CD25+FOXP3+ population with regulatory function and called them natural Tregs (nTreg) describing the pivotal role of the master transcription factor FOXP3 in their function and development [28-30].

The evidence that patients with mutations in the gene coding for FOXP3 lack the Treg compartment and developed a fatal autoimmune disease called IPEX, X-linked syndrome of immune dysfunction, polyendocrinopathy and enteropathy further confirmed the importance of this transcription factor in immunopathology [31].

nTregs differentiate from thymocytes which express TCRs with high affinity for self-peptide MHC complexes, are resistant to thymic deletion [32] and can suppress several immune cell types including CD4+ T cells, CD8+ T cells, NKT cells, NK cells and macrophages [32, 33].

Their main effector cytokines include the suppressive cytokines IL-10 and TGF-β [34, 35]. IL-10 and TGF-β are potent inhibitory cytokines and their important role to suppress pro-inflammatory responses has been demonstrated.

Foxp3+ Tregs can also be induced from naive T cells in the periphery, and low level of antigenic stimulation and TGF-β were identified as critical factors for the differentiation of these "induced" Tregs (iTreg) [36-38].

In vitro model systems allowed isolating a lot of molecules and mechanisms that contribute to Treg cell suppressive activities including IL-2 consumption, cytolysis of the target cells or secretion or surface exposure of inhibitory molecules.

IL-2 consumption mechanism derives from the evidence that Treg express high level of CD25 and have the capacity to compete with effector T cells for IL-2; this competition results in IL-2 deprivation of the effector cells and in consequent apoptosis.

Another potential mechanism for Treg mediated suppression of T cells is the cytolysis of the target cells. Tregs cells can in fact be *in vitro* activated to produce the cytotoxic enzyme granzyme A and kill CD4+ and CD8+ activated cells in perforin dependent manner

[39]. *In vivo* studies also demonstrated that the presence of Treg in tumor microenvironments were lytic for NK cells and CTLs in granzyme B and perforin-dependent manner [40].

One of the major effects of Tregs activity is the inhibition of T cells priming and obviously APC are their main target. The surface molecules CTLA-4,and LAG-3 were shown to be main players in this process.

CTLA-4 on the surface of Treg was shown to prevent the upregulation of CD80 and CD86, which are the main co-stimulatory receptors on APCs [41]. LAG-3 on Treg was shown to interact with MHC class II on APCs and this binding resulted in a suppression of DC maturation and consequently T cell stimulatory capacities [42].

1.6. TR1 CELLS

T regulatory type 1 cells are CD4+ lymphocytes characterized by their ability to secrete high amounts of IL-10, considerable levels of TGF-β, low levels of IFN-γ, but no IL-4 upon TCR-mediated activation [43].

These cells have a peculiar biological function since they are suppressive and suppress via IL-10 and TGF-β [44].

The absence of IL-10 producing Tr1 cells has been described in several different immune-mediated diseases including diabetes [45] and celiac disease [46] while their presence was associated with infectious agents persistence in cases of chronic viral infections and in bacterial infections [47, 48].

In vitro they show low proliferative capacity when activated by specific antigens or by anti-CD3/28 and, in unstimulated conditions, they preferentially expand in presence of IL-15 [49].

Tr1 transiently express FOXP3 upon activation but this is not maintained after activation and it is not required for their induction or function [50].

To date these cells lack a defined cells surface signature and are usually identified by IL-10 production, often after extensive in vitro culture. Several groups found consistent correlation between IL-10 producing CD4+ lymphocytes and surface markers like Lymphocyte activation gene-3 (LAG-3) [51], inducible co-stimulatory molecule (ICOS) [52] and programmed cell death receptor-1 (PD-1) [53] but the most of them were not specifically expressed in Tr1 and as consequence were not good makers to distinguish Tr1 from other Treg subsets.

In addition to the cytokine-mediated suppression, Tr1 cells can inhibit T cell response by other defined mechanisms such as a cell contact-dependent mechanism involving the inhibitory receptors CTLA-4 and PD-1 and by cytolysis with the release of the cytotoxic enzyme Granzyme B.

Most of the functional studies regarding human Tr1 cell suppressive activity have been performed *in vitro* but the relative importance of each mechanism *in vivo* remains to be defined.

1.7. CD8 T LYMPHOCYTES

Cytotoxic lymphocytes are characterized by the expression of the CD8 glycoprotein on their surface, as well as by the expression of a TCR that specifically recognize peptides loaded on MHC class I molecules [4].

CD8 T cells play an important role in fighting intracellular microbes such as viruses and tumors and are activated by all the potentially infected cells exposing antigens derived by infectious agent or tumor antigens on their membrane through MHC Class I complexes.

Some Dendritic cells have the ability to capture and ingest virus-infected cells or tumor cells and present the viral and tumor antigens to naïve CD8+ lymphocytes to start the primary response in lymphoid organs [54]. In this pathway termed “cross-presentation”, the ingested antigens are transported from the vesicles into the cytosol, from where peptides enter the class I pathway. This permissiveness for protein traffic from endosomal vesicles to cytosol is unique to dendritic cells and allows virus antigen presentation without dendritic cell infection or functional impairment [54].

When a naïve CD8+ T cell encounters antigen, it undergoes vigorous clonal expansion and differentiation into a population comprised primarily of short-lived, cytotoxic effectors that undergo cell death after the pathogen is cleared. The elimination of the antigen-experienced progeny of a selected T cell clone, however, is generally incomplete, ensuring preservation of a portion of daughter cells to provide a “memory” of the pathogen encounter. At least two functionally distinct classes of memory cells have been described, based on their pattern of tissue homing in the absence of antigen: effector-memory and central-memory CD8+ T cells [55]. Effector–memory CD8+T cells provide protection against reinfection by patrolling peripheral tissues, but have a poor capacity for

homeostatic renewal and secondary proliferation. Central–memory CD8+ T cells, in contrast, recapitulate the surveillance behavior of their naive predecessor by migrating through secondary lymphoid organs; they are distinguished by efficient homeostatic renewal and rapid secondary proliferative responses to generate cytotoxic effectors upon re-encounter with pathogen [56].

During the differentiation process, CD8 T cells express effector proteins such as the cytokine IFN-γ, the pore-forming protein perforin, and a family of serine esterases known collectively as granzymes, which are essential for cytolytic activity of CTLs [57].

IFN-γ, perforin, and granzymes are each induced at transcriptional level after activation, but distinct regulatory mechanisms appear to be most, if not all, antigen-specific.

CD8+T cells express IFN-γ and granzyme B during the course of an infection, but only a fraction of these express perforin and IFN-γ [57].

The T-box transcription factors expressed in T cells, T-bet and Eomesodermin (Eomes), have been described as master regulators of CD8 T cell differentiation and function [58].

During the early stages of CD8 T cell activation, T-bet and Eomes cooperate to promote cytotoxic lymphocyte formation by inducing the expression of the cytolytic molecules perforin and granzyme B.

T-bet and Eomes have cooperative and redundant roles in CD8 T cells, but they also have unique influences on CD8 T cell function. In murine models, early effector CD8 T cells are characterized by high levels of T-bet, which gradually decline as cells progress toward a memory phenotype.

In contrast, although Eomes is upregulated in early effectors, its expression increases as cells progress from an effector to a memory cell [56, 59].

In the context of CD8 memory T cells, both T-bet and Eomes sustain memory phenotypes by stabilizing the expression of IL-2Rβ, thus promoting IL15 signaling and continued proliferation of memory cells [56].

1.8. MicroRNAs BIOGENESIS AND SYTHESIS

MicroRNAs (miRNAs) are small (approximately 21 nucleotides in length) endogenously expressed non coding RNAs. MicroRNA genes are transcribed into primary miRNAs (pri-miRNAs) by RNA polymeraseII. Pri-miRNAs are bound by the microprocessor subunit DCGR8 and processed by the ribonuclease III (RNase III) activity of Drosha into hairpin structures known as precursor miRNA (pre-miRNAs).

Pre-miRNAs are shuttled by Exportin5 from the nucleus into the cytoplasm where the RNase III Dicer cleaves off the hairpin loop of the pre-miRNA [60].The resulting duplex segregates and the mature single-stranded miRNA associate with Argonaute (AGO) protein and other accessory proteins to form the miRNA-induced silencing complex (RISC), which mediates the translational repression and the increased degradation of its mRNA targets [60-62]. The miRNA provide specificity through complementary base pairing with the target mRNAs and their expression can be regulated at different stages during their biogenesis. The AGO protein recruits other proteins that deadenylate the target mRNA, a process that ultimately lead to mRNA decapping and degradation [60].

miRNA deficient CD4+ T cells showed a dramatic increase in effector T cell differentiation and cytokine production suggesting a direct role in naïve T cell maintenance [63, 64].

It has been clearly demonstrated that microRNAs play an important role in CD4 T cell activation and differentiation and can act at different levels on these processes [65].

Moreover specific miRNA signatures have been associated to the different T cell subsets [66]. Two well-documented examples of the importance of microRNAs in CD4 T cell differentiation are miR-125b and the miR-29 family.

MiR-125b has been recently identified as a CD4+ naïve T cell specific microRNA and its expression in naïve T cells favors the "naïve state" blocking T cell differentiation upon TCR activation. miR-125b is highly expressed in human naïve T cells compared to various memory T cell populations and exert its function inhibiting the expression of several genes involved in T cell differentiation, including *IFNG, IL2RB, IL10RA* and *PRDM1* [66].

MiR-29a and miR-29b are the most potent inhibitor of Th1 cell differentiation and IFN-γ expression [67]. MiR-29 is highly expressed in naïve T cells and is progressively down-regulated during Th1 cell differentiation; it inhibits Th1 differentiation through multiple targets including *TBX21 (T-bet), EOMES* and *IFNG* itself [67-69].

2. AIM OF THE PROJECT

IL-10 is a pleiotropic cytokine that is produced by different subset of T cells [70]. It has been shown that T cells that produce IL-10 can exert regulatory functions in human pathologies and in animal disease models. The best-characterized IL-10 producing cells are the Regulatory T cells.

They express the master transcription factor Foxp3, are auto-reactive and express the tolerogenic cytokines IL-10 and TGF-β.

IL-10 and TGF-β are also the marker cytokines of additional T cell subsets with regulatory function. Thus, IL-10 producing T cells have been originally proposed to represent an independent lineage and were called type 1 regulatory cells (Tr1) [44]. They are exclusively generated in vitro with different tolerogenic protocols, express low levels of other cytokines like IL-2, IFN-γ and IL-4, but high levels of Granzyme B and exert cytotoxic functions [17, 44].

Previous studies in the group led to the identification of CD4+FOXP3- effector-like cells in human blood that coproduce IL-10 and IFN-γ and suppress T cell activation *via IL-10* [71]. These cells do not belong to the Treg cell lineage since they are Foxp3- but they show some similarities with Th1 cells since they express CCR5, T-bet and produce high levels of IFN-γ. These in vivo occurring "Th1-10" cells thus share some relevant features with *in vitro* generated Tr1 cells, but also differ from the latter because they have characteristics of Th1 effector cells. In order to be able to characterize these cells more in detail, surface markers that are associated with IL-10 production and suppressive functions were identified. In particular we found that the costimulatory receptor CD27, which is downregulated in CD8+ T cells upon differentiation to cytotoxic effector cells [72], is a good marker to distinguish between IL-10 positive (Th1-10

cells) and negative cells (that contain conventional CTL [73]) among CD4+Foxp3- effector-like cells.

The aim of my thesis is to provide a comprehensive molecular characterization of Th1-10 cells in order to understand their relationship to conventional Th1, Th1-10 and CD4+ CTL subsets, focusing in particular on gene expression and microRNA profiling.

3. RESULTS

3.1. PURIFICATION OF HUMAN PRIMARY LYMPHOCYTES SUBSETS

Human blood primary lymphocyte subsets were purified >95% by cell sorting using different combinations of surface markers. A specific combination of surface marker (Table 1) was used to sort four different subsets: Th1 Central Memory cells (Th1-CM), Th1 Effector Memory cells (Th1-EM), CTLs and Th1-10.

Table 1 Sorting phenotype

SUBSETS	DONORS	SORTING PHENOTYPE
Th1 CENTRAL MEMORY	4	CD4+IL7R+CD25-CCR6-CXCR3+CCR5-
Th1 EFFECTOR MEMORY	4	CD4+IL7R+CD25-CCR6-CXCR3+CCR5+
CTL	4	CD4+IL7R-CD25-CCR6-CCR5+CD27-
Th1 10	4	CD4+IL7R-CD25-CCR6-CCR5+CD27+

Since these populations produce different levels of IFN-γ and IL-10, they were first tested for IL-10 and INF-γ expression both at mRNA and protein level as control. To detect IL10 and IFNG proteins they were first activated with PMA/Ionomycin while gene expression was performed directly *ex vivo*. As shown in Fig.1, *IL10* mRNA was almost selectively expressed in Th-10 compared to all the other subsets (Fig. 1a) while *IFNG* was highest in CTLs and Th1-10, poorly expressed in Th1-EM and almost absent in Th1-CM (Fig. 1c). Protein stainings performed on CTLs and Th1-10 cells confirmed PCR observations: IL10 was selectively expressed in Th1-10 cells (Fig. 1b) while IFNG was comparable between CTLs and Th1-10 cells (Fig. 1d).

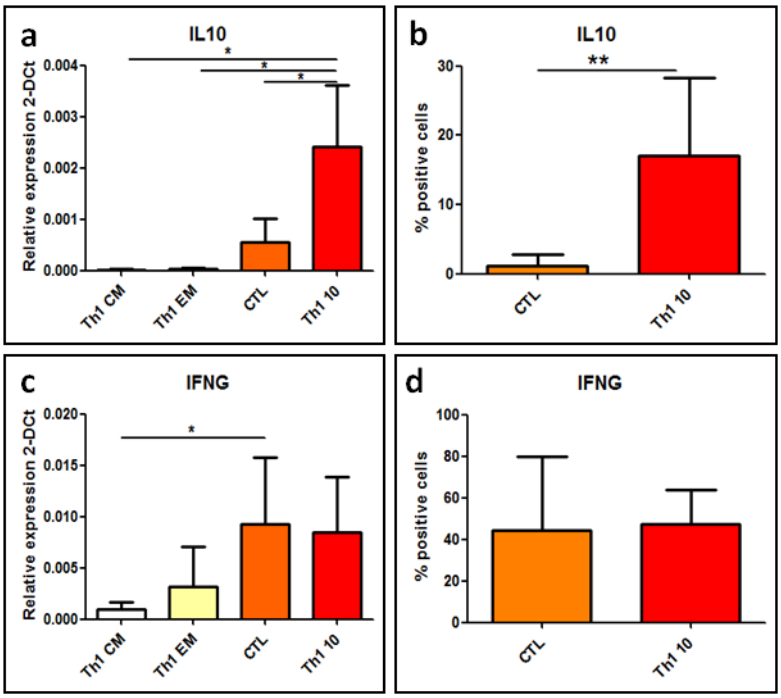

Fig.1 Quantitative Real Time PCR analysis of the Th1-10 cytokine genes: *IL10* **(a)** and *IFNG* **(c)**. Their expression was confirmed at protein level by intracellular staining and FACS analysis after PMA/Ionomycyin treatment for 4h IL10 **(b)** and IFNG **(d)**. PCR and staining data are representative of four independent experiments. For PCR data the statistical analysis was performed on relative quantities (2-ΔCt) using a One-way ANOVA and Tukey post test (* = $p < 0.05$). For staining data a t-test between two groups (CTL and Th1-10) was performed . (* = $p < 0.05$). The t-test was performed on the percentage of IL10 and IFNG positive cells among Th1-10 cells and CTLs.

3.2. GENE EXPRESSION PROFILING IN HUMAN LYMPHOCYTE SUBSETS

RNA from Th1 Central Memory cells, Th1 Effector Memory cells, CTLs and Th1-10 cells was collected to perform a gene expression analysis (Illumina platform).

The gene expression profiling of the four subsets were compared and the differentially expressed miRNAs were selected by One-way ANOVA test ($p < 0.01$).

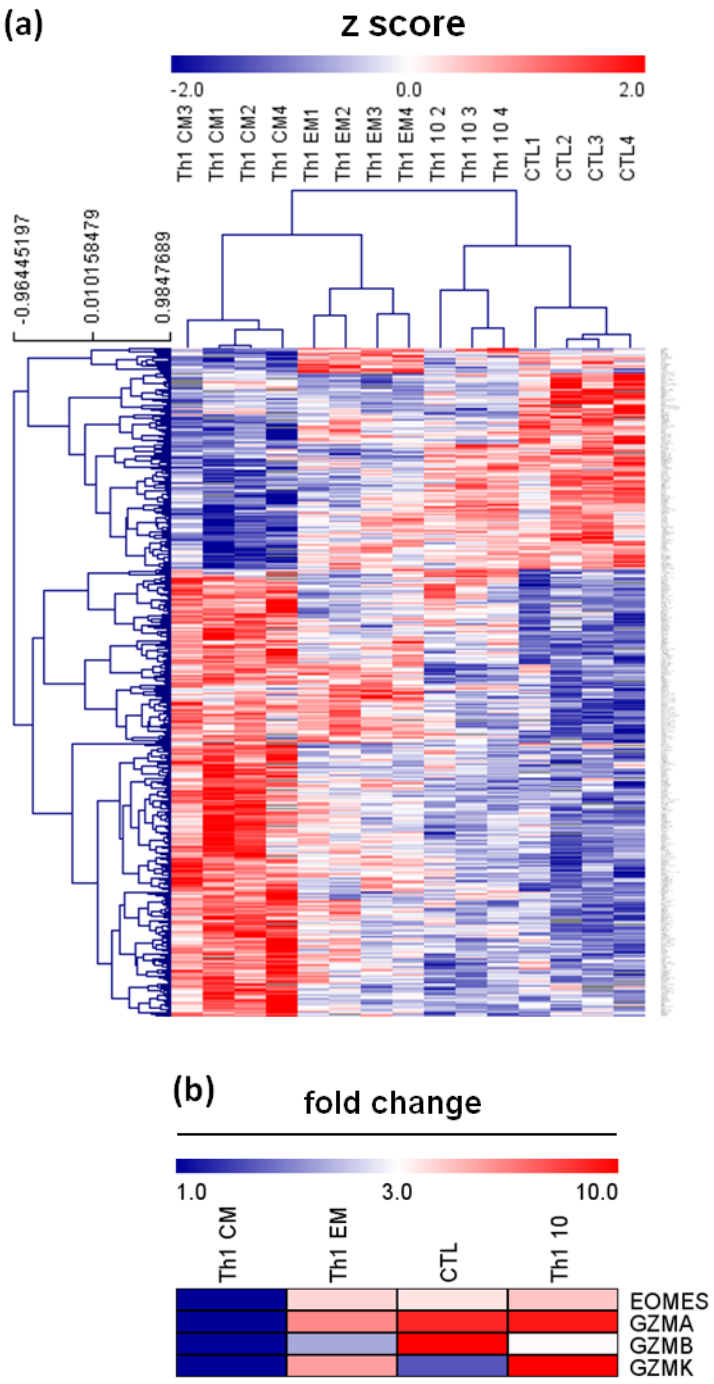

Fig.2 In **(a)** a hierarchical clustering of 120 gene selected by one-way ANOVA ($P < 0.01$) distance: Pearson. The analysis of the differentially expressed gene allowed distinguishing among two major clusters: one defining Th1-CM and Th1-EM cells and one including Th1-10 cells and CTLs. In **(b)** a heat map of a selected group of genes, positive to ANOVA and involved in cytotoxicity. Values are plotted as fold change relative to Th1-CM cells.

A hierarchical clustering of the significantly modulated genes clearly distinguished among two major clusters: one including TH1-CM and Th1-EM cells and one including Th1-10 cells and CTL (Fig. 2a).

Among the modulated genes we focused our attention on a subset of genes, enriched in Th1-10 cells and CTLs, known in literature to be involved in cytotoxicity: *EOMES*, a transcription factor known to induce cytotoxicity in CD8+ T cells, and the cytotoxic effector proteins *GZMA*, *GZMB* and *GZMK* (Fig. 2b).

The modulation of these genes was validated by Real Time PCR on RNA samples obtained from 4 unrelated healthy donors. This step enabled us also to confirm the accuracy of the Gene Expression Array used for the profiling. In detail, *EOMES* gene was up-regulated in Th1-EM, CTLs and Th1 10 compared to Th1-CM (Fig. 3a). *GZMB* was poorly expressed in Th1-CM, showed a higher expression in Th1-EM and was highly enriched in CTLs compared to all other subsets (Fig. 3c).

GZMK instead was poorly expressed in Th1-CM, higher in Th1-EM and CTL and highly enriched in Th1-10 cells (Fig. 3e). The expression of the modulated genes was confirmed also at protein level on Th1-10 cells and CTLs by intracellular staining and FACS analysis.

The intracellular staining confirmed the expression of EOMES, GZMB and GZMK both in Th1 10 and CTLs and the differential expression of GZMB and GZMK in the two subsets, with GZMB and GZMK respectively enriched in CTL and Th1-10 (Fig 3b-d-f). . Thus, Th1-10 cells co-express IL-10 and Granzyme K, but are distinct from conventional CTLs that express Granzyme B and lack IL-10 production.

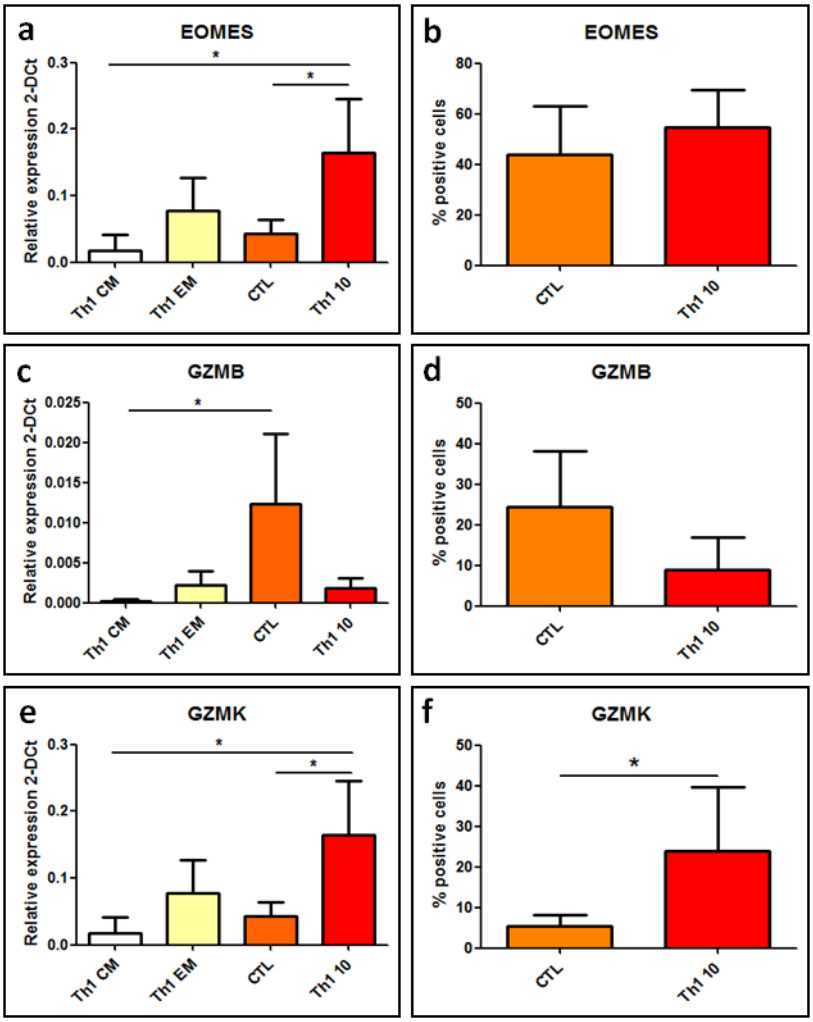

Fig.3 Quantitative Real Time PCR analysis of the genes involved in cytotoxicity*: EOMES* **(a)**, *GZMB* **(c)** and *GZMK* **(e)**. mRNA expression was confirmed at protein level by cellular staining and FACS analysis : EOMES **(b)**, GZMB **(d)** and GZMK **(f)**. PCR and staining data are representative of four independent experiments. For PCR data the statistical analysis was performed on relative quantities (2-DCt) using a One-way ANOVA and Tukey post test (* = $p < 0.05$). For staining data a t-test was performed on the percentage of EOMES, GZMB and GZMK positive cells in CTL and Th1-10. (* = $p < 0.05$).

3.3. LENTIVIRUS-MEDIATED *EOMES* GENE TRANSFER IN PRIMARY T CELLS

As a proof of principle to demonstrate Eomes involvement in the modulation of cytotoxicity related genes in CD4+ T cells, we primed CD4 naïve T cells under Th1 conditions and transduced them with a lentiviral vector coding for EOMES. As negative control, the same cells cultured under the same conditions, were transduced with an empty vector (MOCK).

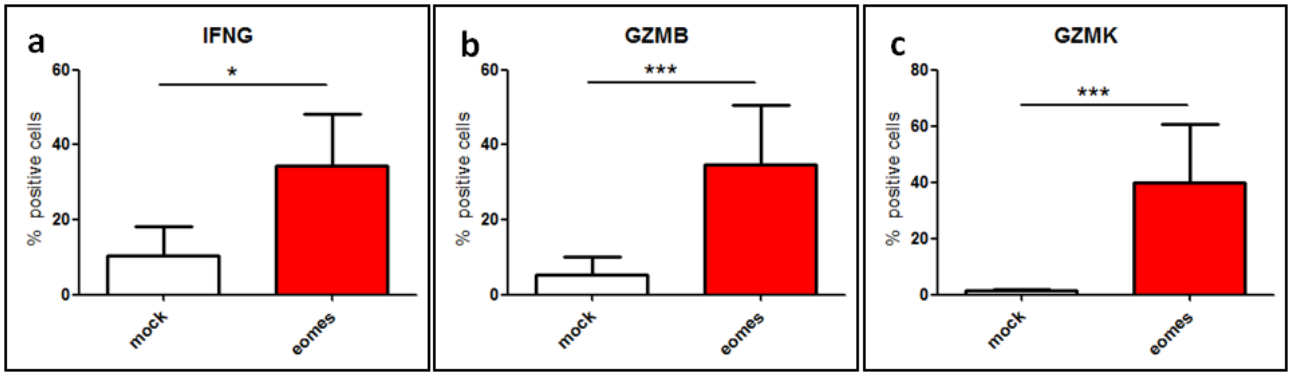

Fig.4 Naïve T cells were primed for 14 days under Th1 conditions and were transduced with a lentiviral vector coding for EOMES or with an empty control vector (MOCK). Cells expressing EOMES were analyzed for IFNG **(a)**, GZMB **(b)** and GZMK **(c)** expression after PMA-Ionomycin activation (4h). A t-test was performed on the percentage of INFG, GZMB and GZMK positive cells in order to compare EOMES-transduced cells and MOCK control cells (* = $p < 0.05$; *** = $p < 0.001$).

Intracellular stainings for EOMES and GZMs were performed after 14 days and transduction efficiency was assessed by the reporter gene GFP (not shown).

EOMES overexpression induced a significant up-regulation of GZMB and GZMK in developing Th1 cells (Fig. 4b-4c). Thus EOMES could determine the cytotoxic potential of Th1-10 cells and CTLs. As functional positive control these cells were tested for INF-γ expression, which is a known Eomes transcriptional target (Fig. 4a).

3.4. EVALUATION OF CYTOTOXICITY POTENTIAL OF TH1-10 CELLS AND CTLs

In order to evaluate the cytotoxicity potential of CTLs and Th1-10 cells we performed a "degranulation assay" based on CD107a detection.

Naïve, CTLs and Th1-10 cells were isolated *ex vivo* by FACS sorting and co-cultured with myeloid Dendritic Cells (mDC) of the same donor and the superantigen Staphylococcal Enterotoxin B (SEB). Superantigens are bifunctional molecules that simultaneously bind the MHC Class II on APCs and the T Cell Receptor on T Cells, mimicking thus an antigenic stimulation of T cells by APC.

After 4 days of co-culture T cells were collected, fixed and stained with anti-CD107 antibody for FACS analysis. As internal control of SEB stimulated cells, T cells co-cultured with mDC only were used.

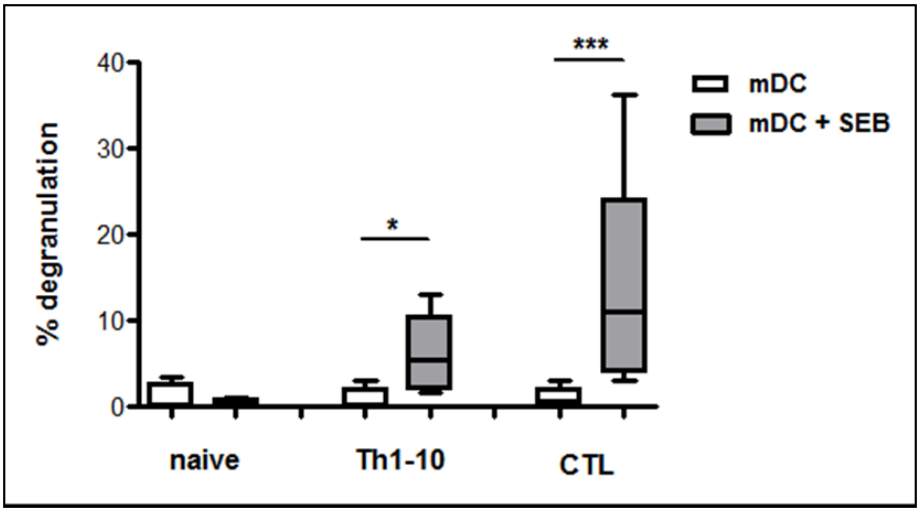

Fig.5 Naïve, CTL and Th1 10 cells were isolated *ex vivo* and co-cultured with mDC alone (blank) and mDC + SEB (grey). After 4 days T cells were collected, fixed and stained with anti-CD107 for FACS analysis. A t-test was performed on the percentage of CD107 positive cells to compare control and stimulated cells in each group (naïve cells, CTL and Th1-10). * = $p < 0.05$; *** = $p < 0.001$.

In Fig.5 the mean percentage of de-granulating cells of three independent experiments is shown.

The results clearly showed that both Th1 10 cells and CTLs had cytotoxic functions but CTLs had a higher cytotoxic potential than Th1-10 cells.

3.5. EVALUATION OF SUPPRESSIVE POTENTIAL OF TH-10 CELLS AND CTLs

Considering that Th1-10 cells produce high amounts of IL10 that is known for their immunoregulatory properties, we tested their suppressive potential. To this aim, we used a non-radioactive *in vitro* suppression assay.

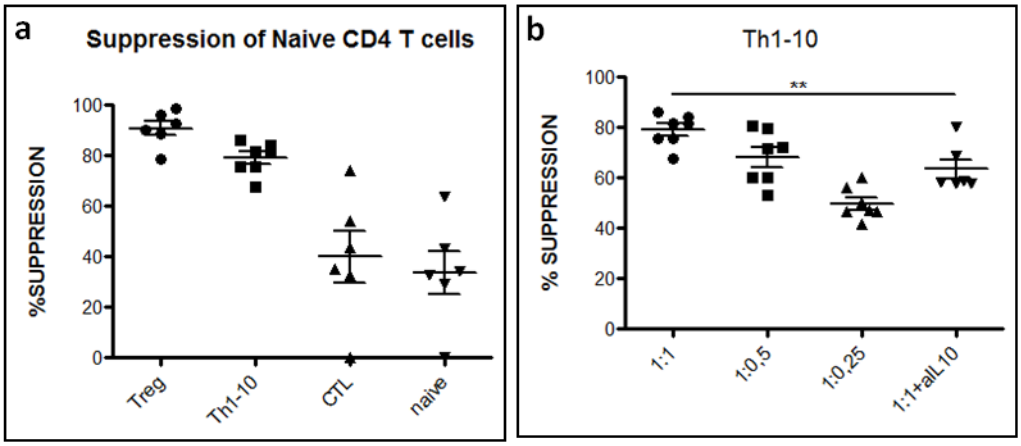

Fig.6 Non-radioactive *in vitro* suppression assay on naïve, CFSE-labelled, responder T cells, co-cultured with different subsets of suppressor cells (Treg, CTLs, Th1-10 and naïve T cells as negative control) in the presence of anti-CD3-loaded Dendritic Cells. Proliferation of responder cells in the presence of suppressor cells was analyzed after 5 days and % suppression calculated. **(a)** Th1-10 suppressive potential was comparable to Treg and significantly higher than in CTLs. Statistical analysis was performed using a One-way ANOVA test with Tukey post test (*** = $p < 0.001$). **(b)** Th1 10 suppressive potential was partially abolished by anti IL-10 antibody. Statistical analysis was performed using a t-test (*** = $p < 0.001$).

The mean suppressive potential of Treg, CTLs and Th1-10 cells isolated from the same donors were compared. The results showed that Th1-10 potently suppressed T cell proliferation in a dose-dependent manner and nearly as efficiently as control CD25+ Tregs (Fig. 6a). Moreover, suppression by Th1-10 cells was, at least in part, IL-10-dependent, since it was significantly reduced when neutralizing anti-IL10 antibodies were added to the medium (Fig. 6b). Conversely, CTLs had only a low suppressive potential. Suppressive potential was calculated as percentage of proliferating responder T cells. In summary, Th1-10 but not CTLs have high suppression capacity because they secrete high levels of IL-10.

3.6. MicroRNAs EXPRESSION IN HUMAN LYMPHOCYTE SUBSETS

To further characterize the molecular asset of the different subsets, we performed a microRNA profiling starting from the same total RNA used for gene expression profiling.

Expression analysis of 667 mature microRNAs was assessed by Real Time PCR using TaqMan Low Density Arrays (AppliedBiosystems).

MiRNOMEs of the four subsets were compared and the differentially expressed miRNAs were selected by One-way ANOVA test with a $p < 0.01$. As shown in Figure 7a, a hierarchical clustering of the significantly modulated genes clearly distinguished among two major clusters: one including Th1-CM and Th1-EM and one including Th1-10 and CTLs. Interestingly that profile perfectly reproduced what already observed for the gene expression.

Among the modulated miRNAs we decided to validate by single assay real time PCR miR-31, miR-150, miR-125a-5p and miR-92a.

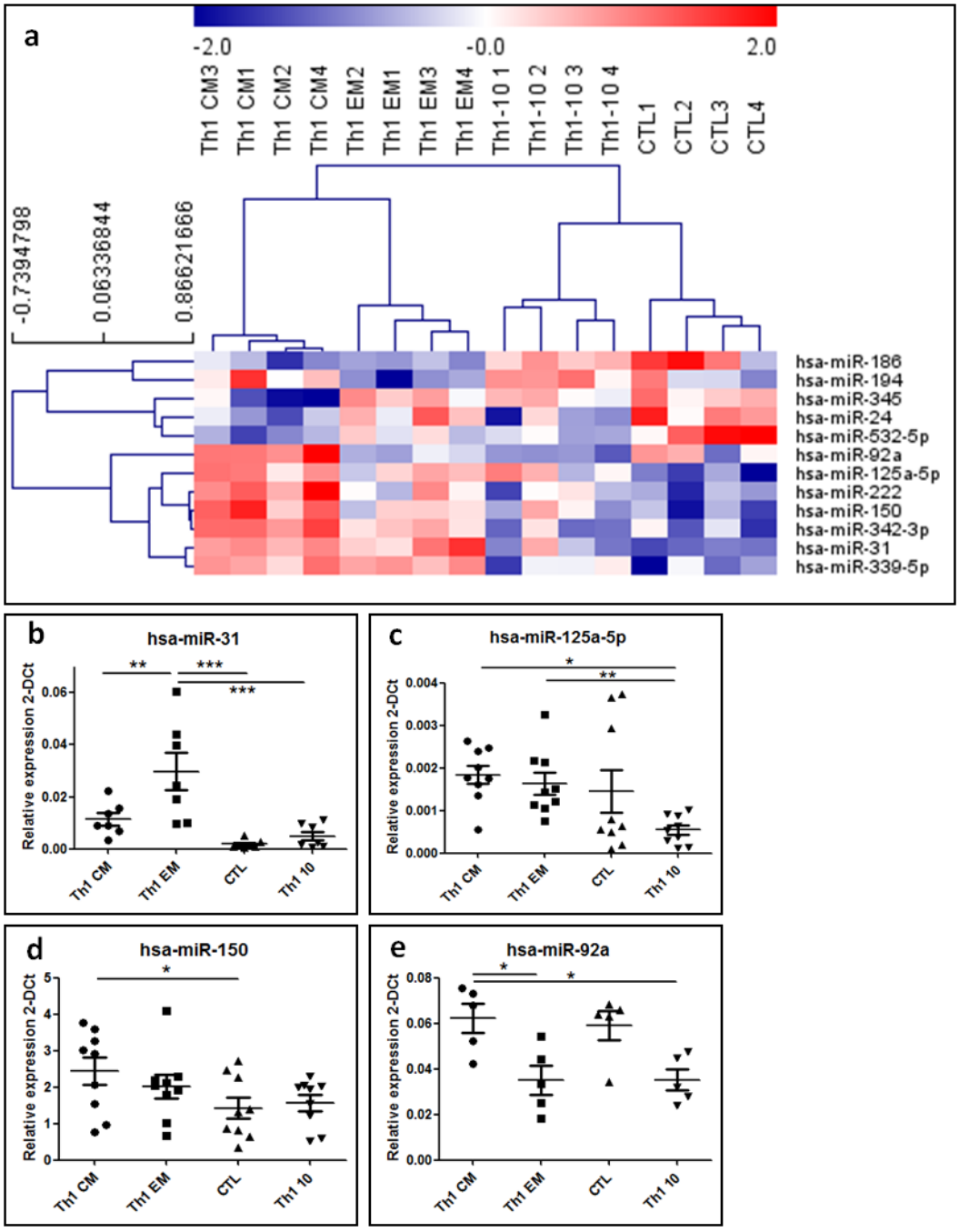

Fig.7 (a) Hierarchical clustering of 12 miRNAs co-expressed in Th1-CM, Th1-EM, CTL and Th1-10 subsets, selected by one-way ANOVA ($P < 0.01$). Data, normalized on global mean, are presented as z-scores calculated on ΔCt. **(b-e)** Validation of a subset of significantly modulated microRNAs (miR-31, miR-125a-5p, miR-150 and miR-92a) by single assay Real Time PCR. PCR data are presented as relative quantities (2-DCt). The statistical analysis was performed using a One-way ANOVA and Tukey post test among four groups: Th1-CM, Th1-EM, CTLs and Th1-10 cells. (* = $p < 0.05$; ** = $p < 0.01$; ***= $p < 0.001$).

The choice was mainly driven by the integration of the state of the art of microRNAs in lymphocytes subsets, microRNA expression data set, the gene expression profiling data set and bioinformatic tools for microRNA target prediction such as TargetScan. In particular, considering that microRNA can influence gene expression by RNA degradation or blocking translation, we focused on predicted target genes that in our gene expression profiling showed an inverse correlation to the expression of miRNAs of interest. Subset-specific miRNAs modulation was confirmed by Real Time PCR.

MiR-31 was highest in Th1-EM cells and clearly down-modulated in all the other subsets (Fig. 7b). MiR-125a showed a selected down-regulation in Th1-10 cells. Statistical significance was reached in the comparisons Th1-10 vs. Th1-CM and Th1-10 vs. Th1-EM (Fig. 7c). Mir-150 expression was highest in Th1-CM and lower in Th1-EM, CTLs and Th1-10 cells. Statistical significance was reached in the comparison of Th1-CM and CTLs (Fig. 7d). Finally miR-92a was higher in Th1-CM than in Th1-EM, CTL and Th1-10 cells and statistical significance was reached in the comparisons Th1-CM vs. TH1-EM and Th1-CM vs. Th1-10 (Fig. 7e).

3.7. *EOMES* IS A TARGET GENE OF miR-92a. DUAL-LUCIFERASE ASSAY

We then focused our attention on miR-92a, because it showed an inverse correlation with *EOMES* gene expression, being down-regulated in Th1-10 (Fig. 8b), and because *EOMES* was described as a predicted target of miR-92a (TargetScan)(Fig. 8a). To establish whether *EOMES* gene was a direct target of miR-92a we performed a Dual Luciferase Reporter Assay on *EOMES* 3' Untranslated Region (UTR).

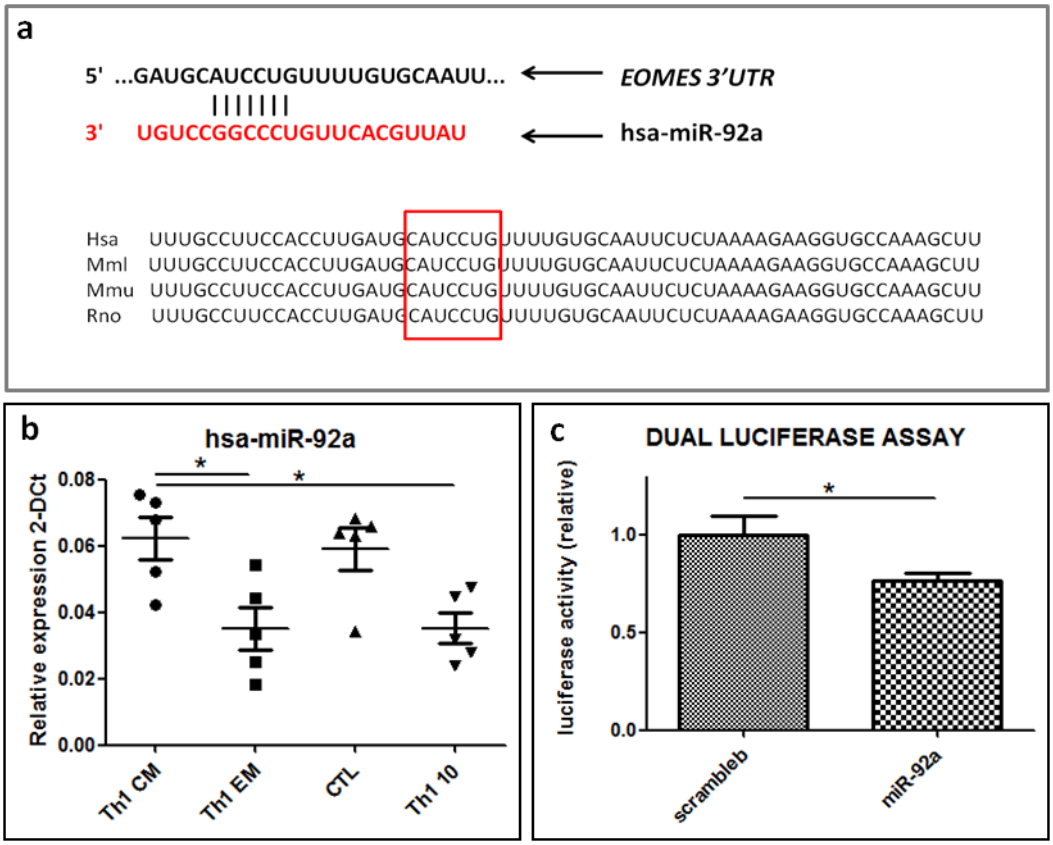

Fig.8 (a) Comparison of miR-92a-responsive elements in human, macaca, mouse and rattus in *Eomes* transcript by TargetScanHuman, release 5.1. **(b)** Real Time PCR for miR-92a performed on ex vivo isolated Th1CM, Th1-EM, Th1-10 and CTL cells. Data are presented as relative quantities (2-DCt). The statistical analysis was performed using a One-way ANOVA and Tukey post test between four groups: Th1-CM, Th1-EM, CTLs and Th1-10. (* = $p < 0.05$; ** = $p < 0.01$; ***= $p < 0.001$). **(c)** Dual-Luciferase Assay performed on HEK-293T cells cotransfected with psiCHECK Vector containing *EOMES* 3'UTR together with a synthetic mature miR-92a or a synthetic control miRNA with a scrambled sequence. The statistical analysis was performed using a t-test (* = $p < 0.05$). In graph the mean of luciferase activity values are shown.

In this assay Renilla luciferase (hRluc) was used as the primary reporter to monitor mRNA regulation and Firefly luciferase (luc2) was used as a control reporter for normalization. We found that miR-92a significantly down-regulated EOMES 3'UTR-specific luciferase activity, indicating that *EOMES* can be a direct Target of miR-92a (Fig.8c).

3.8. IL2RB IS UPREGULATED IN TH1-10 CELLS AND HAS AN IMPORTANT ROLE IN THEIR SURVIVAL AND PROLIFERATION

Considering that interleukin 15 (IL-15) plays an important role in regulating homeostasis of cytotoxic lymphocytes, and that *IL2RB* gene emerged from the profiling as selectively up-regulated in Th1-10 cells (not shown), we validated its expression in Th1-CM, Th1-EM, CTLs and Th1-10 cells both at mRNA and protein level.

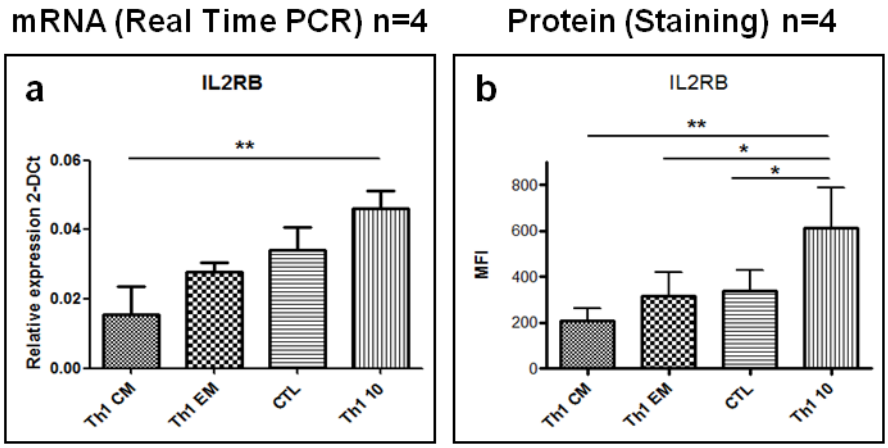

Fig. 9 (a) Quantitative Real Time PCR analysis of *IL2RB* in Th1-CM, Th1-EM, CTLs and Th1-10. Statistical analysis was performed on relative quantities (2-ΔCt) using a One-way ANOVA and Tukey post test (** = $p < 0.01$). **(b)** *IL2RB* expression was confirmed at protein level by cellular staining and FACS analysis. Statistical analysis was performed on MFI values (Mean Fluorescence Intensity) using a One-way ANOVA and Tukey post test (* = $p < 0.05$; ** = $p < 0.01$). PCR and staining data are representative of four independent experiments.

As shown in Fig.9, *IL2RB* mRNA showed an increase in Th1-10 cells compared to Th1-CM, Th1-EM, and CTLs, and was significantly higher in Th1-10 compared to Th1-CM (Fig. 9a). Protein levels showed the same trend and the receptor was significantly increased in Th1-10 cells compared to all the other subsets (Fig.9b).

To assess the role of IL2RB in Th1-10 cell homeostasis, I sorted Th1-CM, Th1-EM, CTLs and Th1-10 cells *ex vivo*, labeled them with CFSE, and performed a proliferation assay using different

stimuli. Sorted populations were stimulated with IL15 alone (10ng/ml) for 7 days and in parallel with anti-CD3 anti-CD28 for 3 days with or without IL15 at the same concentration.

In Fig. 10 one of three independent experiments with different donors is shown.

When cultured in IL15 alone (green) Th1-CM and Th1-EM showed a modest proliferation, while CTL and Th1-10 did not (Fig. 10a). Interestingly, in these culture conditions Th1-CM, Th1-EM and Th1-10 cells showed a good viability while almost all the CTLs underwent cell death (Fig. 10b).

When cultured under TCR activating conditions (orange), all subsets were capable to proliferate but while IL-15 potently promoted proliferation of Th1 10 cells (red) it had only a modest effect on the other subsets (Fig. 10a).

As observed in the absence of TCR stimulation, Th1-CM, Th1-EM and Th1-10 cells showed a good viability while almost all CTLs underwent cell death (Fig.10b).

This experiment confirms a functional role of the high IL2Rβ expression in Th1-10 cells suggesting an important role in their survival and proliferation *in vivo*.

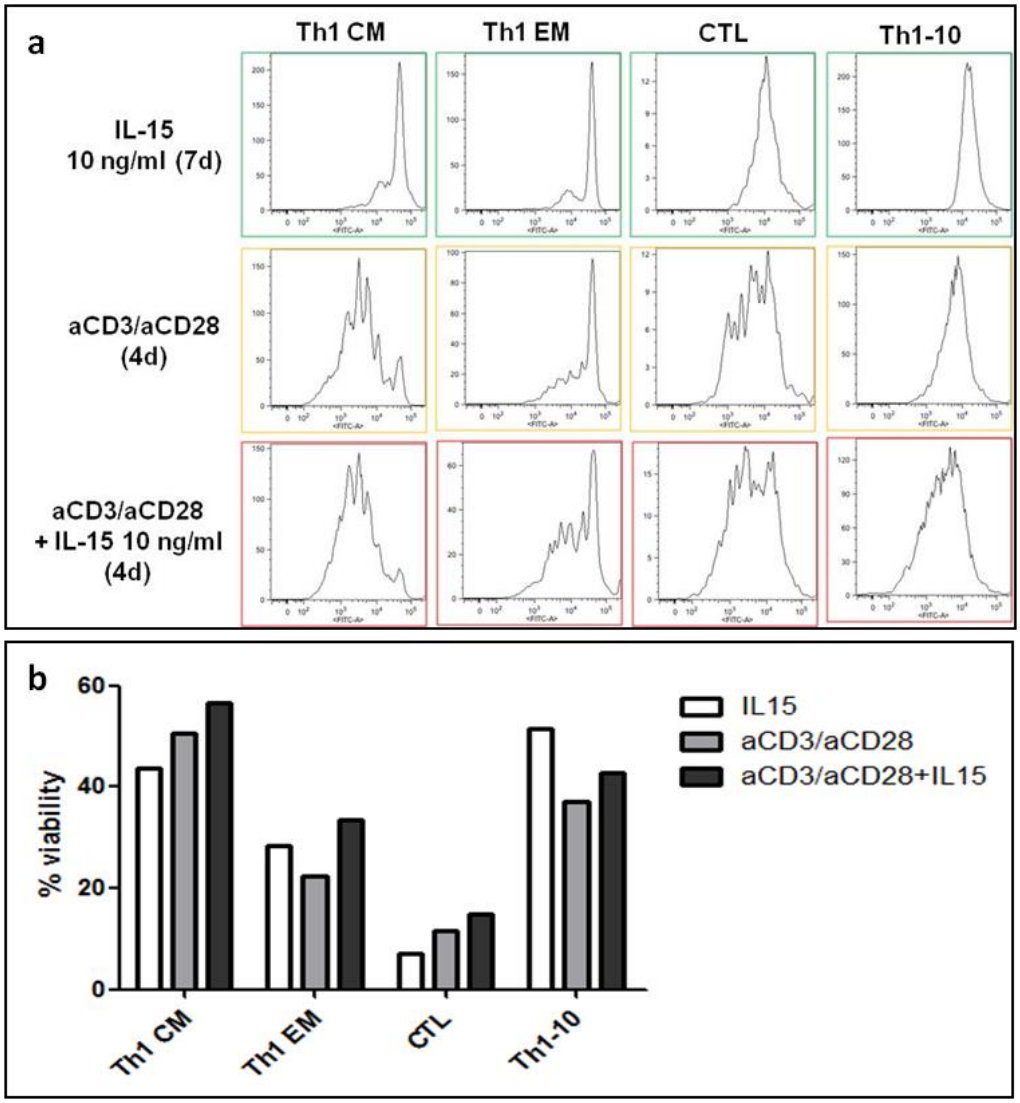

Fig. 10. (a) Th1-CM, Th1-EM, CTLs and Th1-10 cells were sorted *ex vivo*, were labelled with CFSE and tested for their proliferation ability in response to IL-15 alone or in combination with TCR stimulation. Sorted populations were stimulated with IL15 (10ng/ml) alone for 7 days (green) or with anti-CD3 anti-CD28 for 4 days without (orange) or with (red) IL15 at the same concentration. **(b)** For each cell type, viability in the different culture conditions is shown.

4. DISCUSSION

Previous studies in the group led to the identification of CD4+FOXP3- effector-like cells in human blood that coproduce IL-10 and IFN-γ and suppress T cell activation *via IL-10*. These cells do not belong to the Treg cell lineage since they are Foxp3- but they show some similarities with Th1 cells since they express CCR5, T-bet and produce high levels of IFN-γ. Thus, they share relevant characteristics with both Tr1 and Th1 cells.

Molecular studies allowed a better characterization of these cells, finding surface marker strictly associated with IL10 production and suppressive functions. In particular we found that the surface marker CD27 is a good marker to distinguish between IL10 positive (that we called Th1-10 cells) and negative cells (that we called cytotoxic T lymphocytes or CTLs) among CD4+CD25-IL-7R- effector like cells.

The aim of my thesis was to provide a comprehensive molecular characterization of Th1-10 cells in order to understand their relationship to Tr1 cells and different Th1 subsets.

To this extent a Gene Expression profiling and a microRNA profiling were performed on purified Th1-CM, Th1-EM, and CTL and Th1-10 cells from peripheral blood of healthy donors. Hierarchical clustering of the modulated genes and microRNAs expression levels suggested that Th1-CM and EM cells were closely related and it also indicated that Th1-10 cells and CTLs were quite similar. Interestingly, Th1-EM and Th1-10 cells seemed to be less different than TH1-CM and CTLs, suggesting a progressive differentiation model where Th1-CM are the least differentiated cells that can give rise to Th1EM cells as proposed previously. Th1-EM might further differentiate to Th1-10 cells, while CTL are the most differentiated subset that might be either derived directly from Th1-EM or differentiate from Th1-10 cells. Consistent with progressive Th1 differentiation, Th1-10 and CTLs showed higher levels of *IFNG* compared to

Th1-CM and Th1-EM *ex vivo,* while *IL-10* expression was mainly restricted to Th1-10 cells, confirming what we observed at protein level upon activation.

In order to confirm that IL-10 production contributed to the regulatory properties of Th1-10 cells, we performed a suppression assay (shown in detail in the results section). We showed that Th1-10 cells had a high regulatory potential being able to block the proliferation of activated CD4 naïve T cells at the same extent of conventional Treg. This suppression capacity was partially abolished by the anti-10 antibody suggesting that this mechanism is at least partially mediated by secreted IL10. Conversely, CTLs suppressed poorly or not at all, suggesting that CTLs and Th1-10 cells have different functions in immune responses.

One of the most striking evidences derived from the profiling was that Th1-10 and CTL cells shared the expression of specific genes involved in cytotoxicity including the transcription factor *EOMES, GZMA, GZMB* and *GZMK*; the expression and modulation of these genes was also evaluated and confirmed at protein level. What clearly distinguished between Th1 10 cells and CTLs was the level of expression of these genes: while *GZMB* was expressed at higher levels in CTLs, compared to Th1 10, *GZMK* showed the opposite pattern.

EOMES and *GZMA* instead were expressed at comparable levels in both the subsets.

Both CTLs and Th1 10 cells showed an increase in the release of cytotoxic granules upon stimulation, demonstrating that they both possessed cytotoxic functions. Thus CTLs and Th1-10 cells are two distinct CD4+ cytotoxic T cell subsets.

The existence of CD4+ T cells with cytotoxic potential has already been reported in literature. At first cytolytic T cells were considered a potential artifact of *in vitro*-generated T cell lines but more recent reports provided unambiguous evidence that *in vivo*, some antigen-specific CD4+ T cells possess direct MHC class II restricted cytotoxic activity. *In vivo* the presence of CD4 CTL is

usually associated to chronic activation such as chronic viral infections (CMV, EBV and HIV) or to autoimmune conditions like rheumatoid arthritis; their cytotoxic activity is usually mediated by GzmB [74]. The similarity of these cells with the ones we described was the reason why we called CTLs the cells expressing high amount of GzmB.

In summary, conventional cytotoxic CD4+ T cells are similar to $CD8^+$ CTL since they are CD27- and express GzmB; they fail to secrete IL-10 and are poorly suppressive. Conversely, Th1-10 cells, although they belong to the cytotoxic T cell lineage, are at a different stage of differentiation, because they express CD27 and GZMK, but are largely GZMB negative.

A recent paper showed the presence of two distinct subsets of CD8+ T cells in CMV infected patients: one expressing low levels of T-bet and enriched for GZMB and one with low T-bet, high levels of Eomes and enriched for GZMK. Interestingly GZMB enriched CD8+ cells killed with high efficiency virus infected cells while GZMK+ cells did not [75]. This evidence suggests that Th1-10 cells and CTLs might have different target cells such as dendritic cells and virus-infected APC respectively.

We also investigated the possible role of Eomes in Th1-10 and CTL cytotoxicity considering that its role has been well described in CD8+ T cells. Eomes in fact is induced in effector CD8+ T cells *in vitro* and *in vivo* and its ectopic expression is sufficient to invoke attributes of effector CD8+ T cells, including interferon-gamma (IFN-γ), perforin (PRF1), and GZMB [56].

We transduced CD4 naïve T cells culture under Th1 polarizing conditions them with a lentiviral vector coding for EOMES and demonstrated that EOMES was able to induce both GZMK and GZMB in CD4+ T cells.

While the role of EOMES in GZMB induction in CD8+ T cells has been already demonstrated, but is also discussed, this was the first evidence that EOMES regulates GZMK. It remains to be determined which transcription factor regulates the CTLs versus the Th1-10 fate.

Validation of the microRNA signature emerged from the profiling led us to identify miR-92a, selectively down-regulated in Th1-10 cells, as a potential mediator of EOMES upregulation: *EOMES* expression in fact was anti-correlated to miR-92a. Using dual luciferase assay we demonstrated that miR-92a directly targeted the 3'UTR of *EOMES*. These findings identify miR-92a as a possible mediator of Th1-10 cytotoxicity.

The identification of a cytotoxic signature in Th1-10, which are cells with regulatory function, was not completely surprising also because it is well established that also conventional regulatory CD4+ T cells, including FOXP3+ Tregs and Tr1 cells can use granzymes to kill cells [76]. Upon activation, FOXP3+Treg express GZMA and can lyse target cells of both myeloid and lymphoid origin. Also *in vitro* generated-Tr1 cells express upon activation GZMB in association with PRF1 , allowing Ag-non-specific Tr1 mediated killing of myeloid APC [76]. However, we showed here that *in vivo* CD4+ occurring cytotoxic T cells that express GZMB have only low regulatory functions, but rather represent cytotoxic effector cells that might eliminate transformed or virus-infected APC. Conversely, Th1-10 cells that have Tr1-like suppressive functions express high levels of GZMK, but not of GZMB. Thus GZMK, but not GZMB is a suitable marker to identify cells with Tr1-like functions *in vivo*.

Interestingly Th1-10 showed also a selective up-regulation of *IL10RA* and *IL2RB* both at the mRNA and the protein level. Preliminary results suggested an important role of *IL2RB* upregulation in the TCR-dependent expansion of Th1-10 cells. Thus, Th1-10 cells retained some proliferative capacity when sorted *ex vivo* and activated *in vitro* with anti-CD3 and anti-CD28

antibodies, and this effect was markedly enhanced when cells are cultured in medium with IL-15. IL-15 also had a pro-survival effect on Th-10 cells. This effect was not observed for CTLs that did not expand when stimulated but rather showed a massive cell death even in the presence of IL-15.

These results marked many interesting analogies with cytotoxic CD8+ T cells: Th1-10 in fact were sorted as CD27+, which is known to be required for generation and long-term maintenance of CD8+ T cell immunity and expressed EOMES, that sustain CD8 memory phenotypes by stabilizing the expression of IL-2Rβ and promoting IL-15 signaling and proliferation. These analogies lead us to conclude that Th1-10 are not terminally differentiated cells, since they retain proliferative capacity; CTLs instead show cytotoxic properties but resembles terminally differentiated cells with have an “exhausted phenotype” and low expansion potential.

T cell populations secreting both IFN-γ and IL-10 were found in both mouse and man [52, 77, 78] and have been implicated in regulating the immune response to persistent infections with intracellular pathogens. The common feature of these infections is the development of a chronic stage of disease characterized by IL-10 secretion whereby low levels of the pathogen prevail and provide protection against subsequent reinfection. Under these circumstances, IL-10 plays a role in protecting against excessive inflammation-associated pathology; in this scenario Th1-10 cells could be involved in a negative feedback loop of Th1 responses.

Our findings advance our understanding of the molecular mechanisms involved in peripheral tolerance but also offer possible tools to expand Th1-10 cells to be used for adoptive transfer therapy in the treatment of autoimmune diseases.

5. MATERIALS AND METHODS

5.1. PURIFICATION OF PRIMARY IMMUNOLOGICAL CELL SUBSETS

Buffy-coated blood of healthy donors was obtained from the Ospedale Maggiore in Milan and peripheral blood mononuclear cells were isolated by Ficoll-hypaque density gradient centrifugation. Human blood primary lymphocyte subsets were purified >95% by cell sorting using different combinations of surface markers (Table 1).

Table 1: sorting strategy

SUBSETS	DONORS	SORTING PHENOTYPE
Th1 CENTRAL MEMORY	4	CD4+IL7R+CD25-CCR6-CXCR3+CCR5-
Th1 EFFECTOR MEMORY	4	CD4+IL7R+CD25-CCR6-CXCR3+CCR5+
CTL	4	CD4+IL7R-CD25-CCR6-CCR5+CD27-
Th1 10	4	CD4+IL7R-CD25-CCR6-CCR5+CD27+

Antibodies used: anti-human CD4 OP, clone OKT4 (Biolegend); anti-human CD25 FITC , clone BC96 (Biolegend); anti-human CD127 biotin, clone HIL-7R-M21 (BD Pharmingen); streptavidin-Allophycocyanin (APC), (Biolegend); anti-Human CD195 (CCR5) PE-Cy7, clone 2D7/CCR5 (BD Pharmingen); anti-Human CD196 (CCR6) PE, clone R6H1 (eBioscience) and anti-human CD183 (CXCR3) PE-Cy5, clone 1C6/CXCR3 (BD Pharmingen).

5.2. RNA ISOLATION AND microRNA-mRNA EXPRESSION PROFILING

Total RNA was isolated using mirVana miRNA Isolation Kit (Ambion) following the standard protocol. Briefly, the lysates were extracted once with Acid-Phenol Chloroform and further purified to yield total. Extracted RNA was quantified with RiboGreen Quantitation Kit

(Molecular Probes) on an Infinite F200 plate reader (Tecan Trading AG). All extracted RNA samples were quality controlled for integrity with 2100 Bioanalyzer (Agilent Technologies) and samples with RIN lower than 8 were discarded.

Gene expression of whole transcriptome was performed, with Illumina Direct Hybridization Assays according to the standard protocol (Illumina Inc.).

For each sample 80 ng of total RNA were reverse transcribed according to the Illumina TotalPrep RNA Amplification kit (Ambion) and cRNA was generated after a 14 hours of *in vitro* transcription. Washing, staining and hybridization were performed according to the standard Illumina protocol: briefly, 750 ng of cRNA of each sample in a final volume of 15 µl were hybridized onto Illumina HumanHT-12 v3 Expression BeadChip arrays. Hybridization and scanning were performed according to the manufacturer's indications on an Illumina iScan System and data were processed with BeadStudio v.3;

For microRNA analysis, 10 ng of total RNA were reversed transcribed using the Megaplex RT stem-loop primers in a 7.5 µl reaction volume through the protocol's default 40 cycles run; 2.5 µl of each RT product were pre-amplified in a 25 µl reaction volume with Megaplex PreAmp primers to increase detection sensitivity according to manufacturer specifications. Both A and B pools were used in all steps, enabling specific cDNA synthesis of 664 human miRNAs (mirBase v.10.1) and 3 small RNA controls common to all plates (RNA44, RNA48, MammU6). 9 µl of 4-fold diluted pre-amplified RT product were 100 fold diluted in the PCR reaction mix and amplified using TaqMan Low Density Arrays (TLDAs) on a 7900HT (Applied Biosystems) according to the standard protocol.

5.3. DATA FILTERING AND STATISTICAL ANALYSIS

Gene expression profiling

Gene expression arrays were quantile normalized, with background subtraction, and average signals were calculated on gene-level data for genes whose detection p-value was lower than 0.001 in at least one of the cohorts considered (Th1-CM, Th1-EM, CTLs and TH1-10). Normalized data were log2 transformed and presented as z-scores. A one-way ANOVA (p-value< 0.001) was used to select microRNA classifiers for all 4 cell subsets profiled.

Unsupervised hierarchical clustering on significant genes were performed with Pearson distance and complete linkage. Statistical test and hierarchical clustering were performed on MeV software version 4.5.

miRNA profiling

Raw CT values were calculated using the SDS software v.2 and exported as "Amplification Data" text files for each plate. TLDAs affected by globally poor or anomalous amplification were discarded. After this quality control step, 16 samples belonging to 4 distinct cell subsets entered the final dataset. Data were normalized using the global mean method and ΔCts were used to calculate z-scores.

A one-way ANOVA (p-value< 0.001) was used to select microRNA classifiers for all 4 cell subsets profiled. In order to minimize the biological variability for the classifiers selection we considered miRNAs expressed in at least 2/3 (66%) of the profiled samples analyzed in each cell subset. Unsupervised hierarchical clusters on significant miRNAs were performed with Pearson distance and complete linkage. Statistical test and hierarchical clustering were performed on MeV software version 4.5.

5.4. TaqMan microRNA AND GENE EXPRESSION ASSAYS

For assessment of *IL10*, *IFNG*, *IL2RB*, *IL10RA*, *GZMK, GZMB, PERFORIN, EOMES* and *18S* (as normalizer) gene expression levels, TaqMan Gene Expression assays (Applied Biosystems) were used. 200 ng of total RNA were used for reverse transcription with VILO Reverse Transctiptase (Invitrogen). Diluted cDNA was then used as input for RT-qPCR.

For assessment of miR-31, miR-125a-5p, miR-92a and RNU48 levels on specific human lymphocyte subsets, single Taqman MicroRNA assays were used (Applied Biosystems). 5 ng of total RNA were used for reverse transcription with specific primers for miR-31, miR-125a-5p, miR-92a and RNU48 (as normalizer) with Taqman MicroRNA Reverse Transcription Kit and RT was followed by real time PCR reaction on a 7900HT Real Time PCR System according to the manufacturer's standard protocol.

5.5. LENTIVIRUS-MEDIATED *EOMES* GENE TRANSFER IN PRIMARY T CELLS.

Naive CD4+ T lymphocytes were purified (>98%) by negative selection with magnetic beads and were activated at a density of 1×10^5 cells per well on 96-well MaxiSorp plates (Nunc) coated with anti-CD3 (0.1 μg/ml; UCHT1; BD) and anti-CD28 (1 μg/ml; CD28.2; BD) and grown in complete RPMI medium and IL-2 (20 IU/ml; Novartis).

Lentiviral particles were produced according to a standard protocol (System Biosciences User Manual). Naïve CD4+ T cells were simultaneously activated as described above and were transduced with either control lentiviral vector or lentiviral vector encoding the transcription factor EOMES at a multiplicity of infection of 1×10^7 transducing units per ml in the presence of polybrene (8 μg/ml). Cells were detached on day 3, and transduction efficiency was assessed by flow cytometry as the frequency of cells positive for green fluorescent protein. Transduced cells

were then re-plated on uncoated wells and were cultured in complete RPMI medium + IL12 (20 ng/ml, Th1 polarizing conditions).

After 14 days, cells were stimulated for 6 h with PMA (P8139; Sigma) and ionomycin (I0634; Sigma), and GZMB and GZMK expression was assessed by intracellular staining.

5.6. DEGRANULATION ASSAY

CD107a, also known as lysosome-associated membrane protein 1 (LAMP-1), is a glycoprotein present in the membrane of cytotoxic granules. As a result of the release of intracellular vesicles, CD107 is exposed on the cell surface. Thus surface-exposed CD107 enables rapid assessment of cell mediated cytotoxicity and detection of functional cytotoxic T cells

Naïve, CTLs and Th1-10 cells were isolated *ex vivo* and co-cultured with autologous myeloid dendritic cells (mDCs) alone (control) or with mDCs + SEB (TCR stimulated).

Staphylococcal Enterotoxin B (SEB) is a superantigen. Superantigens are bifunctional molecules that simultaneously bind the MHC Class II on APCs and the T Cell Receptor on T Cells, mimicking thus an antigenic stimulation of T cells by APC. After 4 days T cells were collected, fixed and stained with anti-CD107 for FACS analysis.

5.7. CFSE LABELLING AND PROLIFERATION/SUPPRESSION ASSAYS

Cell division was measured by labeling with CFSE dye, which is a non-fluorescent, highly membrane-permeable diacetate taken up readily by cells. Once it is inside the cell, intracellular esterases cleave the diacetate groups and the resultant fluorescent moiety, 5,6-carboxyfluorescein succinimidyl ester (CFSE), is retained within the cell. CFSE, which is

partitioned in DNA, is divided between daughter cells in each successive division, and the mean fluorescence intensity of the cells decreases with each successive division. Hence, discrete populations can be detected on the basis of decreasing fluorescence intensity, identifying cells that have undergone up to 10 successive divisions. In these studies, sorted cells were incubated at 37°C for 15 min with CFSE diluted in PBS at a concentration of 2 μg/ml. In order to stop the reaction, an equal amount of of foetal calf serum (FCS) was added; cells were washed twice in RPMI-FCS medium and then re-suspended in RPMI-FCS medium.

For proliferation assays, ex vivo labeled Th1-CM, Th1-EM, CTLs and Th1-10 cells were cultured in complete medium (RPMI) in the presence of IL15 (10ng/ml, BD Recombinant) for 7 days or stimulated on anti-CD3 (1 μg/ml; UCHT1; BD) and anti-CD28 (1 μg/ml; CD28.2; BD) coated plates and cultured in complete medium (RPMI) with or without IL15 (10ng/ml, BD Recombinant). CFSE-labeled cells were analyzed on a FACSCalibur (Becton Dickinson).

For suppression assays, *ex vivo* labeled naïve CD4 T cells (responder cells) were co-cultured with different subsets of suppressor cells isolated from the same donors (Treg, CTL, Th1-10 and naïve T cells as negative control) in the presence of allogenic myeloid Dendritic Cells and activated on anti-CD3-coated plates. Proliferation of responder cells in the absence or presence of suppressor cells was analyzed after 5 days and percentage of suppression was calculated using the FlowJo Version 8.7.

5.8. DUAL-LUCIFERASE ASSAY

HEK293T cells were transfected with 10 ng of each psiCHECK-2 construct along with 20 nM miR-92a duplex or scrambled control miRNA (Qiagen) with Lipofectamine2000 (Invitrogen). After 24

h, cells were lysed and firefly and renilla luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega).

The 3'UTR of Eomes gene was cloned in psiCHECK-2 immediately downstream of the gene encoding renilla luciferase. Results are presented as the ratio of renilla luciferase activity to firefly luciferase activity.

6. REFERENCES

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