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Characterization and functional relevance of a newly disclosed subset of NCR^{pos} γδ T cells naturally resident in human intestine.

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Table of Contents

Abstract

γδ T cells display a broad array of anti-tumor functions by combining their rapid innate-like cytotoxic response with secretion of immunoregulatory cytokines, such as IFN-γ. Intestinal intraepithelial lymphocytes (IELs) are particularly enriched of γδ T cells; however, data regarding specific γδ T subsets in homeostatic and pathological conditions of human intestine are lacking. Therefore, by a multiparametric flow cytometry approach, we identified a novel human specific intestinal γδ IELs population constitutively expressing Natural Killer (NK) cell-specific cytotoxic receptors (NCRs), in association with several immune-regulating molecules including: CD56, CD8, CD28, NKG2C and NKG2D. Expression of NKp46 was restricted to Vδ1^{pos} (but not Vδ2^{pos}) γδ IELs and proved to be the dominant NCR compared to the lower level of NKp44 and almost undetectable expression of NKp30. The NKp46^{pos}/Vδ1^{pos} subset has gut-specific residency rather than systemic tissue distribution since no other analyzed human specimens, such as liver or lymph nodes showed expression of NCRs on γδ T cells. Moreover, we found no immune counterpart of the human NKp46^{pos}/γδ T cell subset in different anatomical sites of BALB/c or C57BL/6 mouse strains. NKp46^{pos} IELs reflects functionally higher anti-tumor activity compared to their NKp46^{neg}counterpart as evaluated by increased synthesis of both granzyme B (GRZ-B) and IFN-γ. The specific NKp46^{pos} phenotype is a feature of IL2/IL15-induced human thymic γδ T precursors that consecutively acquire anti-tumor activity. In addition, elevated occurrence of the intestinal NKp46^{pos}/Vδ1^{pos} T cell subset correlates with significantly lower tumor progression in patients with colorectal carcinoma (CRC). On the other hand, the tumor microenvironment negatively controls the incidence of both Vδ1 and NKp46^{pos} subset compared to the specific intestinal intraepithelial anatomical site. Collectively, this work identifies a novel human gut-specific differentiated NKp46^{pos} phenotype with

multifunctional immunoregulatory and cytotoxic anti-tumor activity, and with the potential to represent a prognostic marker of human CRC pathology.

1 Introduction

1.1 Overview of γδ T lymphocytes

Gamma-delta (γδ) T cell were discovered for the first time in the ‘80s as a different but specific subset of the great family of T lymphocytes population and are characterized by specific and unique properties (Vantourout & Hayday, 2013). In particular, γδ T lymphocytes have a distinctive T-cell receptor structure (TCR) expressed on their surface if compared to that expressed on conventional alfa-beta (αβ) T lymphocytes (Y Chien et al., 1987). In fact, in contrast to αβ T cells that have TCR composed of two glycoproteins defined as α- and β-chains, γδ T cells express a T cells receptor characterized by γ- and δ- chains (Y Chien et al., 1987). Despite the same thymic origin of αβ T lymphocytes, the frequencies of γδ T cells in human peripheral blood is very low compared to the frequencies of classical αβ T cells. γδ T cells represent only 1 to 5 % of total circulating T lymphocytes population (Yueh-hsiu Chien, Meyer, & Bonneville, 2014), on the contrary they are enriched in many organs such as skin, lung, cervix, uterus and intestine in which they correspond to 30-35 % of total lymphocytes. On the other hand, in mice γδ T cells are more abundant in peripheral tissues than the peripheral blood, indeed, they can reach up to 20-25 % within the digestive tract, and up to 40 % inside the skin (Yueh-hsiu Chien et al., 2014).

γδ T lymphocytes are not considered a homogeneous population, the V-D-J rarrangement during the maturation process can create different subpopulations of γδ T cells. In human γδ T cells can be distinguished in different subsets based on the

expression of δ chain. More specifically there are two main subsets, one that expresses Vδ2 chain and that is mainly coupled with Vγ9 and Vδ1 coupled with variable Vγ chains (Kabelitz, Marischen, Oberg, Holtmeier, & Wesch, 2005). Vδ1 T cells are the predominant subset found at mucosal districts sites such as the gastrointestinal tract, on the other hand Vδ2 T population is more abundant in peripheral blood (Dieter Kabelitz, Marischen, Oberg, Holtmeier, & Wesch, 2005) (Vantourout & Hayday, 2013). These two subset diversify not only for anatomical distribution but also for their effect functions. In particular Vδ1 act as a first line of defense against pathogens in the mucosal microenvironment providing a rapid cytotoxic response characterized by IFN-γ and GRZ-B secretion and appear to play a significant role in tissue homeostasis and repair (Silva-Santos, Serre, & Norell, 2015). In addition, Vδ1 T population drastically expand in the course of acute phase of HIV-1 (Human Immunodeficiency Virus) infection, and appear to be capable to strongly suppress HIV-1 replication activity (David Pauza, Poonia, Li, Cairo, & Chaudhry, 2015). On the other hand, Vγ9Vδ2 T cells play an early and essential role in sensing 'danger' by invading pathogens; furthermore, they can expand dramatically in peripheral blood, (up to 60% of total T cells) in many acute infections and in the presence of pathogens like *Mycobacterium tuberculosis* and *Plasmodium falciparum* (Lockhart, Green, & Flynn, 2006). In addition, Vγ9Vδ2 T subset has a potent and broad cytotoxic activity against human tumor cells (Silva-Santos et al., 2015). Recently a third important subset has been found with a different segment of δ chain defined as Vδ3 which represents a very small proportion in the peripheral blood (0.2% of circulating T cells), but seems to be slightly enriched in liver and in the blood of patients affected by chronic viral infections and some types of leukemia (Mangan et al., 2013). Despite the exact function of Vδ3 is not known, there are some studies suggesting an important role in anti-pathogen activity (Mangan et al., 2013) (Hunter, Willcox, Davey, & Kasatskaya, 2018).

Diversely from αβ T cells, γδ T lymphocytes do not preferentially require the peptide presentation mediated by the major histocompatibility complex (MHC) class I

and II, although it was demonstrated that some subsets can recognize MHC class Ib molecules (Vantourout & Hayday, 2013). Compared to the αβ-TCR, γδ-TCR can recognizes a small repertoire of antigens due to low set of possible combinations of γ and δ chains, in fact the estimated number of TCR γ and δ chains rearrangements is around 70-76 as compared to 2500-2950 for the α and β chains TCR (Yueh-hsiu Chien & Konigshofer, 2007).

1.1.1 Distribution and Development of γδ T lymphocytes

As their name suggests, γδ T cell develop largely in the thymus, where is generate their finally γδ T cell receptor (TCR) is generated via V-D-J recombination. Indeed, a common thymic progenitor can give rise to both αβ or γδ TCR, although this does not exclude the possibility of different subsets arising from qualitative discrete progenitors (McVay & Carding, 1996). Thymic selection represents a series of phases in which lymphocytes modify their cell surface markers in order to establish a phenotypic / functional immunological identity. γδ thymocytes development has been studied abundantly in mouse and it is commonly divided into 4 main steps: the first step, called double-negative 1 (DN1) is characterized by a lack of typically T cell markers like CD8 and CD4, but DN1 cells express high levels of CD44 glycoprotein. Subsequently, it begins the double-negative 2 step (DN2), characterized by the expression of CD44 and α chain of IL-2 receptor CD25. In DN2 phase there is the rearrangement of the β, γ and δ chains of the TCR which ends with the formation of definite TCR (McVay & Carding, 1996) (Triebel F, Faure F, Graziani M, Jitsukawa S, Lefranc MP, 1988). It has been shown that γδ-TCR formation is favored by high levels of the transcription factor SRY-box 13 (SOX13) (Vantourout & Hayday, 2013), furthermore unlike αβ T cells, γδ T cells undergo a functional pre-programming which might depends on early TCR stimulation. Otherwise it is clear that high levels of NOTCH1 expression supports the development of αβ TCR. If the rearrangement produces of αβ TCR follow the steps

DN3 and DN4 in which cells co-express CD4 and CD8 (double positive) before being selected positively as mature naive αβ T lymphocytes $CD4^{pos}$ or $CD8^{pos}$ (Vantourout & Hayday, 2013) (McVay & Carding, 1996) (Triebel F, Faure F, Graziani M, Jitsukawa S, Lefranc MP, 1988) (Muñoz-Ruiz, Sumaria, Pennington, & Silva-Santos, 2017) **(Fig. 1)**.

In mouse model was also observed that the first type of T cells is generated during the embryonic development and express the γ and δ in TCR structure (Ito et al., 1989). In particular, where identify "waves" of γδ T development that sequentially populate different tissues starting with skin at early embryonic age followed by tongue, reproductive tract and lastly intestinal IELs at peri and post-natal days (Asarnow et al., 1988). More specifically, the naive γδ T cells express Vγ5 chain and subsequently colonizes the epidermis and for this reason they are called dendritic epidermal γδ T cells (DETCs) (Asarnow et al., 1988). In the second moment γδ T cells are characterized by high expression of Vγ6 chain and colonizes all the reproductive tract (Asarnow et al., 1988). Specifically, DETCs plays an important role in the homeostasis of the epidermis, in fact they interact with keratinocytes and play an important role in epithelial repair after injury from the production of IGF-1 (insulin-like growth factor) and keratinocyte growth factor (Jameson & Havran, 2007) (Asarnow et al., 1988). Despite these studies little is known about the development of gamma delta cells in the mouse's intestine.

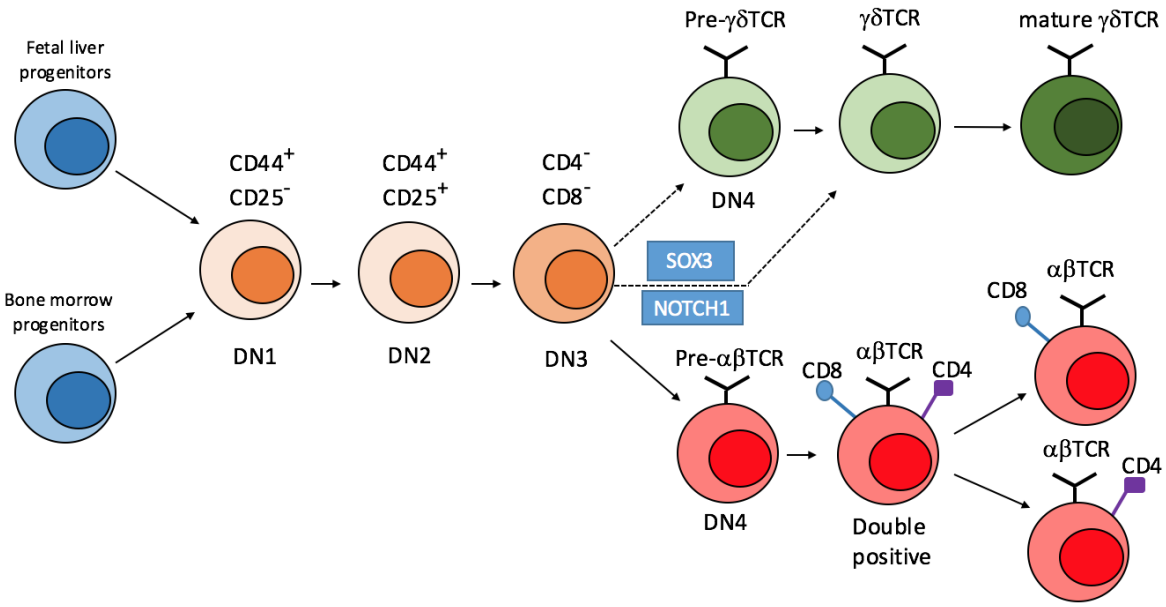


***Figure 1**: γδ T cells thymus-development.*

Schematic representation of γδ T lymphocytes development from fetal liver or bone marrow progenitors. The first step is called double-negative 1 (DN1) stage in which cells are characterized by $CD4^{neg}$, $CD8^{neg}$, $CD25^{neg}$, $CD4^{pos}$ immune-phenotype. Afterwards follow the phase double-negative 2 (DN2), characterized by rearrangement of the β–, γ– and δ– chains of the T cell receptor (TCR), in this stage cells become $CD4^{neg}$, $CD8^{neg}$, $CD25^{pos}$, $CD44^{pos}$. After DN2 stage there is the transient phase double negative 3 (DN3) in which cells are $CD4^{neg}$, $CD8^{neg}$, $CD25^{neg}$, $CD44^{pos}$. The transcriptional factor SOX13 plays an important role in this stage because support γδ TCR development. On the contrary high levels of NOTCH1 supports the development of αβ-TCR (not shown). In double negative stage 4 (DN4) appears the first TCR form called pre-TCR. Dashed lines indicate that γδ TCR can generate from progenitors with either a DN3 or a DN4 phenotype. γδ T lymphocytes can rapidly colonize the skin in mouse as a DETCs (dendritic epidermal T cells) while both in human and mouse these cells can become part of the intraepithelial lymphocytes (IELs). On the other hand, the development of αβ T lymphocytes continues with a phase called double positive in which cells express contemporaneously both CD8 and CD4 receptor, then follows a selection of $CD4^{pos}$T lymphocytes or $CD8^{pos}$ T lymphocytes that are released from thymus as conventional αβ T cells.

1.1.2 γδ T cells receptor ligands

Unlike for the αβ T lymphocytes, the ligands for γδ T cells are poorly identified, furthermore the low number of rearrangements of the γ and δ chains of the TCR suggests few and specific ligands for these cells. γδ T lymphocytes are not necessarily MCH restricted and a major part of the known ligands are self-molecules induced upon cell stress, injury, tumor transformation, virus and bacterial infections (Vantourout & Hayday, 2013). The extensive structural diversity of γδ-TCRs includes a series of antigens characterized by different origin, structure and properties (Y-h Chien & Bonneville, 2006).

One of the first molecules described for γδ TCR and specifically for Vγ9/Vδ2 subset were phosphoantigens such as (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) that is an intermediate of isoprenoid biosynthesis (Thedrez et al., 2007). This molecule is an essential metabolite of pathogenic bacteria including *Mycobacterium tuberculosis* that highly induces an increase in Vγ9/Vδ2 cells cytotoxic activity (Thedrez et al., 2007). Another phosphoantigen widely described as ligand for Vγ9/Vδ2 is isopentenylpyrophosphate (IPP) that is normally produced at low level by mammalian cells and at homeostatic concentrations (Thedrez et al., 2007) (Poupot & Fourniè, 2004). In particular, IPP is an intermediate of the mevalonate pathway which is used to make a large number of biomolecules. Normally the low concentrations of IPP does not influence the behavior of Vδ2Vγ9, although the process of tumor transformation can lead to the accumulation of IPP, thus making transformed cells susceptible to lysis by Vδ2Vγ9 T cells (Thedrez et al., 2007) (Poupot & Fourniè, 2004).

Other types of antigens capable to stimulate the γδ TCR are the stress-molecules MICA and MICB, firstly described as ligands for a NK cells receptor called NKG2D also expressed by γδ T cells (Bin Xu et al., 2011). In particular, it was shown that both MICA and MICB can stimulate specifically Vδ1 T cells, in a mode NKG2D-independent mode (Bin Xu et al., 2011) (O'Brien et al., 2007). Furthermore, the

expression of MICA and MICB can rapidly increase in tumour-trasformating cells as well as in damaged intestinal epithelial cells, and consequently can stimulate Vδ1 T cells cytotoxicity response (O'Brien et al., 2007) (Silva-Santos et al., 2015).

In the last 15 years many ligands for γδ T cells were discovered including: CD1c and CD1d molecules which mediates the recognition of lipid and glycolipid antigens and that are recognized specifically by Vδ1 T cells (Spada et al., 2000) , the mitochondrial F1-ATPase expressed on great part of tumor cells that promotes tumor recognition by Vδ2Vγ9 subset and the endothelial protein C receptor (EPCR) that increase during *Cytomegalovirus* infections and it is recognized specifically by V(γ)4V(δ)5 clone (Vermijlen et al., 2007) (Willcox et al., 2012) (Bruder et al., 2012) (Spada et al., 2000).

All together these data according to the prevalent concentration γδ T cells in mucosal surfaces, suggest that γδ T lymphocytes plays an important role in immune-surveillance as a first line of defense against pathogens and stress-related molecules in response to microbial and non-microbial tissue perturbation (A. C. Hayday, 2009). In addition to providing local tissue protection, this response can also provide an immediate source of cytokines, chemokines capable to regulate adaptive immunity response (A. C. Hayday, 2009).

1.1.3 γδ T cells functions

Although γδ T lymphocytes can perform many different functions **(Fig. 2)** that can depend on both tissue specialization and TCR chains restriction, their real contribution to the immune system is still under observation. γδ T cells are considered a primary source of soluble factor like cytokine and chemokines both in tissues and peripheral blood. Specifically it was demonstrated that both activated murine systemic and human peripheral blood γδ T cells are involved in the production of pro-

inflammatory cytokines like IFN-γ , tumor necrosis factor (TNF-α) and granzymes (Vantourout & Hayday, 2013) (Vermijlen et al., 2007). On the other hand, γδ T cells resident within the derma and the intestinal mucosa are able to release IL-17 cytokine, which plays an important role in bacterial and viral infections as well in the maintenance of gut homeostasis (Sutton, Mielke, & Mills, 2012). In particular IL-17 is pro-inflammatory cytokine that represent an important link between adaptive and innate immune system, in fact it is involved in the production of many other cytokines such as IL-6, GM-CSF, IL-1β, TGF-β, TNF-α and chemokines including IL-8, and MCP-1 (Chiricozzi et al., 2011) (Tyler et al., 2017) (Vantourout & Hayday, 2013).

During *Listeria moncytogenes* liver infection as well as in *Escherichia coli* intraperitoneal infection γδ T cells and specifically Vδ1 subset produce high quantity of IL-17, which is directly involved in neutrophil recruitment in order to favor the injury resolution (Hamada et al., 2008) (Sutton et al., 2012). Furthermore γδ T cells secreting IL-17 are involved in countering other bacterial and viral infections like enteritis caused by *Salmonella enterica*, *Mycobacterium tuberculosis* and *Respiratory syncytial virus* (RSV) (Lockhart et al., 2006) (Dodd, Riffault, Kodituwakku, Hayday, & Openshaw, 2009). Recently it was also demonstrated that an uncontrolled production of IL-17, γδ T cells-dependent, can promote tumor growth and expansion (Silva-Santos et al., 2015). Specifically it seems that Vγ4 γδ T cells strongly release IL-17 in tumor microenvironment that induces in tumor cells an upregulation of CXCL5 to enhance the infiltration of myeloid-derived suppressor cells (MDSC) in a CXCL5-CXCR2-dependent manner (Ma et al., 2014).

On the other hand, γδ T cells are also characterized by immuno-regulatory properties as in the case of mice DETCs that in addition to the production of pro-inflammatory cytokines can also express high levels of IL-13 that regulates B cells encouraging immunoglobulin E (IgE) secretion (Strid, Sobolev, Zafirova, Polic, & Hayday, 2011). Moreover it was demonstrated that activated γδ T cells can produce insulin growth factor 1 (IGF1) that regulates and influences stromal cells activity (Sharp,

Jameson, Cauvi, & Havran, 2005). Interestingly, in mice, γδ T cell isolated from spleen and liver and peripheral Vδ3 subsets are able to release IL-4 that is a key regulator of humoral and adaptive immunity by inducing B-cell and T-cell proliferation as well as differentiation of B cells into plasma cells (Jianqi et al., 2017). Furthermore, in a mouse model the specific skin subset of Vγ5Vδ1 is able to release kerotinocyte growth factor (KGF) which regulates cell regeneration capacity of epidermis after injury (Jianqi et al., 2017) (Vantourout & Hayday, 2013). This property was found also in mouse gut epithelium in which γδ T cells can have a protective and regenerative role that contributes to the maintenance of gut homeostasis and epithelial integrity. In fact, in a γδ deficient mouse model chemically-induced inflammatory bowel disease (IBD) using dextran sulfate sodium (DSS) the tissue repairing ability was compromised compared to wild type mouse (Nanno, Shiohara, Yamamoto, Kawakami, & Ishikawa, 2007).

γδ T cells are able also to interact with other immune cells such as B -lymphocyte, T-lymphocytes and dendritic cells (DC). For example, it is kwon that activated Vγ9Vδ2 T cells can produce CXCL13, also called B attracting chemokine 1 that control the organization and distribution of B cells within follicles of lymphoid tissues (Vermijlen et al., 2007) (Wen et al., 1996) (Poccia, 2007) (Vantourout & Hayday, 2013).

There is also a clear evidence for an impact of γδ T cells on DC maturation on both mice and humans. DCs in fact can respond to numerous molecular signals of infection, tumor and stress and migrate to lymph nodes in order to prime efferent adaptive responses. In this context both in human and mice tissue γδ T cells can collaborate with DC giving information about the status of tissue in order to regulate the immunogenic or tolerogenic response (Devilder et al., 2006).

γδ T cells can have also an antigen presenting cells function (APC) towards αβ T cells. In particular, circulating Vγ9Vδ2 can present antigen to $CD4^{pos}$T cells and cross-present antigen to $CD8^{pos}$ T cells (Brandes et al., 2009). Moreover, activated γδ T cells have the functions and characteristics of professional APCs cells comparable to those of DCs (Himoudi et al., 2012). In particular, Vγ9Vδ2 T cells express high levels of

CCR7 receptor that drives their migration to lymph node compartment in which they upregulate MHC class I/II and also the co-stimulatory molecule CD80/CD86 in order to complete the antigen presentation to T cells (Brandes, Willimann, & Moser, 2005). In the presence of antibody-opsonized target cells, γδ T lymphocytes can display both innate cytotoxic function and antigen-presenting capability (Himoudi et al., 2012) (Brandes et al., 2005).

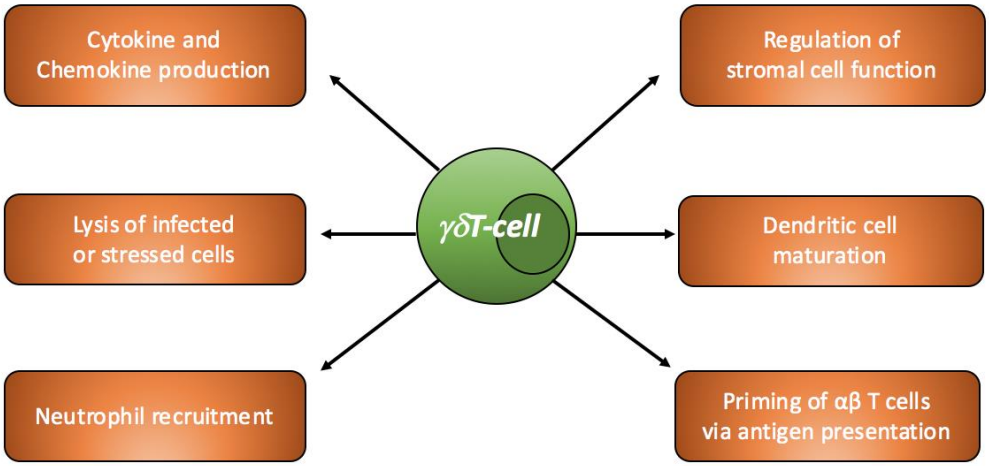


Figure 2:γδ T cells best functions

From literature the evidence that γδ T cells plays an essential role in defending the host against a broad range of infectious and sterile stresses is very high. In fact, γδ T cells are involved in many functions of immune response that can regulate both innate and adaptive immunity: 1) Production of a wide range of pro-inflammatory cytokines (IL-6 and TNF-α) and chemokines 2) Direct involvement in the destruction of bacterial or virus infected cells 3) Neutrophil recruitment via IL-17 production and resolution of injury 4) Production of growth factor like IGF1 for the regulation of neighboring stromal cells 5) Dendritic cell maturation 6) Antigen presenting cells (APC) to αβ T lymphocytes.

1.1.4 γδ T cells and their involvement in tumor immunity

It is kwon that γδ T cells can contribute to the immune response against malignancies. In the last years unexpected antitumor γδ T cell properties have been discovered **(Fig. 3)** demonstrated largely on the basis of their potent cytotoxicity and IFN-γ production (Silva-Santos et al., 2015) (Gao et al., 2003).

More specifically, IFN-γ can inhibit both tumor growth and angiogenesis thus reducing the survival of tumor cells (Y.-L. Wu et al., 2014). It was shown that γδ T cells have a high cytotoxicity against both solid and non-solid tumor such as neuroblastoma, melanoma, carcinoma epithelial-derived tumors and leukemia (Silva-Santos et al., 2015). Interestingly, γδ T antitumor response seems to be mediated by both TCR and classical Natural Killers cells receptors such as NKG2 family (NKG2A-C-D) and NCRs (Correia et al., 2011). In fact, in mice model DETCs response against carcinoma cells is mediated by both TCR and NKG2D engagement (Kong et al., 2009). Activation of NKG2D receptor is due to the recognition of induced-self proteins like RAET1/ULBP which are usually upregulated on the surface of stressed, malignant transformed, and infected cells (Kong et al., 2009) (Vantourout & Hayday, 2013). Furthermore, γδ T cells can also exert their antitumor activity through upregulation of Fas ligand (Fas-L) that induces apoptosis of target cells through interaction with TRAIL (Silva-Santos et al., 2015). Recently it was also demonstrated that activated γδ T cells can increase CD16 expression, a receptor that recognize segments of Fc structure of immunoglobulin G (Fcγ receptors) that enhance the antibody-dependent cellular direct-cytotoxicity against tumor targets (ADCC) (Zou et al., 2017) (Silva-Santos et al., 2015).

However, the specific subsets of γδ T cells (defined by their TCR repertoire and functional differences) may have different activity in anti-tumor immunity (D Kabelitz, Kalyan, Oberg, & Wesch, 2013). Indeed, many studies demonstrated that peripheral Vδ2 T cells have the capability to kill different types of tumors cells including various

epithelial carcinoma cancer cells, acute myeloid leukemia (AML) blasts, lymphoma cells as well as different types of cancer stem cells (Wrobel et al., 2007).

Vδ2 T cells are able to exert anti-tumor properties in both MHC-independent or dependent manner. Their cytotoxic antitumor potential can be activated following the TCR activation by recognition of tumor-associated phosphoantigens such as IPP or surface molecules overexpressed on tumor cells like human MutS homologue 2 (hMSH2) and, butyrophilin 3A1 (BTN3A1) (D Kabelitz et al., 2013). On the other hand, Vδ2 T cells engagement against tumor cells can be MHC-independent trough recognition of NKG2D related ligand such as MICA and UL16-binding proteins (ULBPs), both frequently expressed on tumor cells surface (Wrobel et al., 2007) (D Kabelitz et al., 2013). Interestingly, Vδ2 T cells can have tumor antigen-presenting function (TAPF) that results tumor antigen presentation to $CD8^{pos}$ cytotoxic T lymphocytes (D Kabelitz et al., 2013).

Similar to the Vδ2 subset, Vδ1 exert an anti-tumor action. In fact it was reported that activated peripheral Vδ1 population is able to kill acute lymphoblastic leukemia cells as well as primary myeloma cells (Meeh et al., 2006) (Correia et al., 2011) (Knight, Mackinnon, & Lowdell, 2012). Although ligands important for Vδ1 anti-tumor activity are largely unknown, however it has been shown that their response against tumor cells involves different molecular pathways such as: TCR-dependent pathways, signal delivered via activating receptors like NKG2D, natural cytotoxicity receptors NKp30 and DNAX accessory molecule-1 (DNAM-1) (Knight et al., 2012) (Correia et al., 2011). Interestingly, it has been observed that MICA molecule expressed on tumor cells can be directly recognized by TCR-Vδ1 complex, thus creating an "iperstimulation" consisting into high efficient antitumor activity mediated by increased levels of IFN-γ and Perforins (B. Xu et al., 2011) (D Kabelitz et al., 2013). On the other hand, a large type of tumor cells of hematological origin can also express high levels of ULBPs and in particular ULBP3 that can trigger cytotoxicity and/or cytokine production by Vδ1 subset (Poggi et al., 2004).

Vδ1 T cells expanded from peripheral blood present high anti-tumor activity against solid tumors *in vitro*. Recently it was reported that fresh isolated human peripheral Vδ1 T cells show higher killing activity against human colon cancer cells compared to Vδ2 counterparts (D. Wu et al., 2015). Moreover, stimulation with PHA, IL-8 and IL-7 allows a specific expansion of peripheral Vδ1 subset, which show an enhanced anti-tumor cytotoxicity and unexpected ability to strongly suppress tumor growth (D. Wu et al., 2015). This ability of γδ T cells to counter tumor cells makes them an excellent candidate for onco-immunotherapy. Indeed, many experimental clinical trials have been performed to combat human tumors by using γδ T cells. In particular, Vδ2 T cells are easily expandable in vitro using IL-2 stimulation and therefore can be transferred into patients with tumors after being pre-activated with phospho-antigens or aminobiphosphonates (D Kabelitz et al., 2013). In this context, zoledronic acid is commonly used for the treatment of patients with osteoporosis and metastasis cancer patients, since it inhibits osteoclastic-mediated bone resorption. Furthermore, zoledronic acid interferes with mevalonate pathway leading to an accumulation of IPP and increasing susceptibility to γδ T cell mediate lysis (Wrobel et al., 2007) (Gober et al., 2003) (D Kabelitz et al., 2013). Interestingly, a complete remission of patient affected by metastatic renal cell carcinoma has been reported, after 6 monthly cycles of peripheral autologous γδ T injections, pre-activated and expanded *in vitro* with low dose of HMBPP and IL-2, combined with the infusion of zoledronate. This response was specifically associated with a strong increase production of INFγ by Vγ9Vδ2 T cells after the adoptive transfer (Kobayashi, Tanaka, Shimmura, Minato, & Tanabe, 2010).

In contrast to the antitumor activity γδ T cells may present a pro-tumor effect under specific conditions (D. Wu, Wu, Qiu, Wei, & Huang, 2017). It has been widely reported that infiltrated γδ T cells can promote the inflammatory process associated with tumor development as well as angiogenesis. In fact, γδ T cells are essential producers of IL-17 which strongly increase inflammation responses in tumor immunity and promotes angiogenesis (Wakita et al., 2010). In human colon rectal cancer IL-17^{pos} γδ

T cells can induce a severe immunosuppressive microenvironment associated with high production of tumor necrosis factor-α (TNF-α) and granulocytemacrophage colony-stimulating factor (GM-CSF) which are able to promote tumor growth. It follows that IL-17^{pos} γδ T cells infiltrating human colon cancer have a positive correlation with the tumor progression and with a worsening of clinical-pathological features (P. Wu et al., 2014). Also in breast cancer γδ T cells seem to favor tumor growth through the development of an immunosuppressive microenvironment by suppressing both naive and effector T cells and impairing DCs maturation and functions (Lo Presti, Dieli, & Meraviglia, 2014). It appears that γδ T cells infiltrating breast cancer are significant predictors of high recurrence and poor survival (Peng et al., 2007).

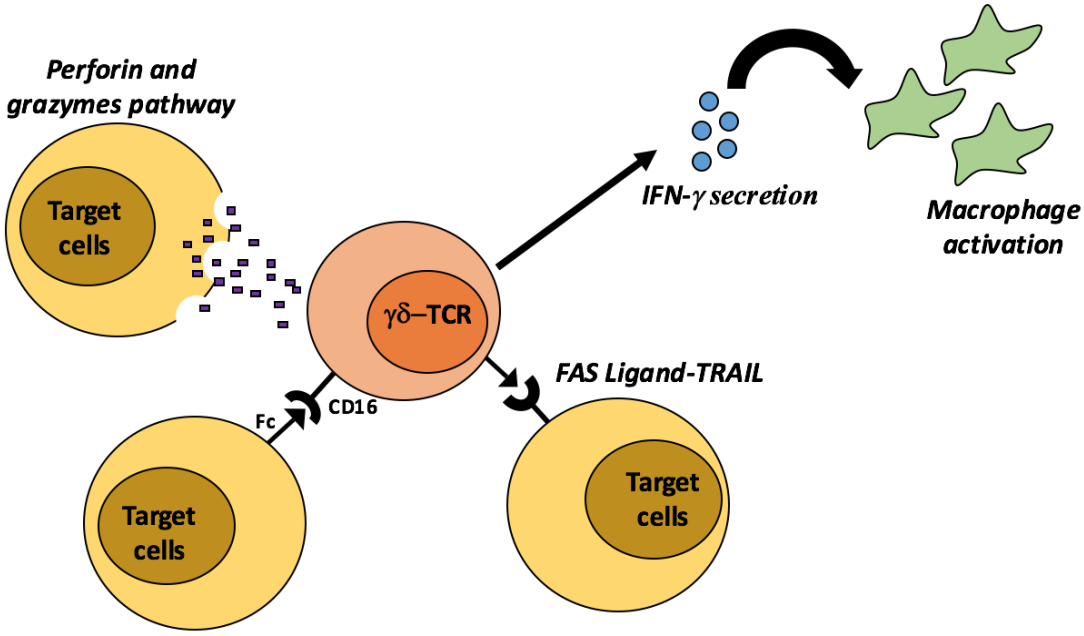


Figure 3: ***γδ-Antitumor properties***

γδ T cells show strong and different anti-tumor properties in fact they can release a lot a direct-destructing factors like granzymes and perforin that induce the apoptosis of tumor cells. Furthermore, activated γδ TCR increases CD16 expression and this leads them to mediated antibody-dependent cellular cytotoxicity. On the other hand, these cells can upregulate Fas-L and so induce an apoptosis TRAIL-dependent apoptosis. Finally, γδ T cells can also destroy indirectly tumor cells through regulation of macrophage recruitment via IFN-γ secretion.

1.2 Human Intestine and immune system

The human gut is a complex environment that in addition to play a key role in the digestive process it is also directly involved in the regulation of the immune system. The concept of intestinal immunity was born with Savage's observation of the human oral immunization against *Salmonella* that provides a strong defense against infections and bacterial growth (Savage, 1977).

Human gut is full of commensal bacteria which living in symbiosis with the host constitute a specific and great microenvironment called "microbioma". It is estimated that the human microbioma is composed of almost 10^{14} bacterial cells that are taxonomically heterogeneous (Savage, 1977). On the other hand, DNA analysis of microorganisms living in the human intestinal tract, realized using metagenomics technique with the HMP (human microbiome project), have identified over 3 million genes, 150 times those of the human species (Qin et al., 2010). Of the approximately 1000 species of microorganisms identified, every human being has at least 160 species, with many variations between one each individual (Qin et al., 2010).

The relationship between this series of microorganisms and the human gut can be considered an example of symbiosis, in fact human gut provides nutrients and a perfect microenvironment for bacterial survival, on the other side intestinal microflora participates to carbohydrate and vitamins synthesis reducing also intestinal permeability and increasing the epithelial defense mechanisms. Furthermore microbioma seems to directly regulate the intestinal immune system (Berg, Clemente, & Colombel, 2015). The immune system is controlled by many elements like immune organs, immune cells and soluble factors. The intestine mucosal immune system consists of three specific mucosal lymphoid elements which cooperate with each other: Peyer's patches, lamina propria and the epithelium.

1.2.1 Human intestinal tract: Anatomical overview

Human intestine is the tract between the pylorus and the anal orifice and it is the longest organ of the body with its 8 or more meters and represents the final part of the digestive process. It is composed of multi-layer walls and smooth muscles which regulate the absorption of nutrients as well as the formation and exit of the feces. From a macroscopically point of view human intestine is divided into “small intestine” in which occur the great part of the digestive process also favored by finger-like projections, known as *villi* and “large intestine” in which *villi* are absent and this favors the recovery of water and small nutritive molecules. The small intestine includes three specific segments: duodenum, jejunum and ileum. On the other hand, the large intestine is composed by caecum, colon and rectum in which the presence of a smooth surface favors the reabsorption of water, small nutrients as well as the defecation. More specifically at the level of the small intestine there is the absorption of 85% of the nutrients thanks to the presence of *villi* that increase the absorption surface. Each villus is coverd by extroflexions called microvilli or enterocytes which create a particular conformation that further increase the digestive and absorbing capacity of the intestine. Interestingly, enterocytes are cells that survive for a few days, as they grow older, these cells detach from the villus and pass into the intestinal lumen to be eliminated with the feces tand be replaced almost continually by younger cells.

This continuous regeneration is essential to maintain proper tissue homeostasis and a high digestive-absorbing efficiency of the intestine. All the intestine is furrowed by blood vessels and has many lymph glands that connect the organ with the blood and lymphatic circulatory system.

1.2.2 Gut-associated lymphoid tissue (GALT)

The gastrointestinal system represents a way of communication with the external environment and it is potentially in contact with a large number of pathogenic microorganisms (Robinson et al., 2004) (Cebra, Periwal, Lee, Lee, & Shroff, 1998). For this reason, a strong presence of the immune system in the mucous membranes is necessary to take under control these microorganisms. GALT represents the immune system associated with the gastro-digestive tract and it is an example of lymphoid tissue associated with the mucosa function (MALT) which is responsable of the protection from the attack by pathogens both in the primary and secondary immune-response (Habtezion, Nguyen, Hadeiba, & Butcher, 2016) (Jung, Hugot, & Barreau, 2010).

In particular, intestinal humoral immune system is based to release high levels of soluble IgA dimer which can be as a "shield" in intestinal lumen, preventing viral or bacterial infections, in fact it was demonstrated that germ-free mice have high number of IgA-secreting-plasmocytes (Ogra & Karzon, 1969) (Moreau & Corthier, 1988).
GALT can be subdivided into three principal compartments: Peyer's patches (PP), intraepithelial lymphocytes (IELs) and lamina propia lymphocytes (LPLs) (Cebra et al., 1998) **(Fig. 4).**

PPs patches are specific lymphoid follicles described in the intestinal epithelium and particularly abundant in jejunum and ileum. The number of Peyer's patches is high at age 15–25 and then declines continuously during aging. PPs patches are characterized by a follicle-associated epithelium (FAE) that differs from intestine-ephitelium because of presence of a lower number of *villi*, a very thin mucous layer and mostly for the permeability, in fact the follicle-associated epithelium is much less permeable for ions and other complex macromolecules (Markov, Falchuk, Kruglova, Radloff, & Amasheh, 2016). Peyer's patches are characterized by big aggregates of B lymphocytes with a smaller amount of T lymphocytes $CD4^{pos}/CD25^{pos}$ (10%) and $CD8^{pos}/CD25^{pos}$ (5%) (Jung et al., 2010). In the sub epithelial region Peyer's patches are enriched of DCs and

macrophages which meet the antigens thanks to the presence of microfold cells (M cells) which provide the uptake and transport of antigens from lumen. Interestingly both DCs and macrophages can also directly sample the lumen by extending dendrites through transcellular M cell-specific pores (Bonnardel et al., 2015) (Lelouard, Fallet, De Bovis, Méresse, & Gorvel, 2012).

PPs are important for the immune surveillance of the intestinal lumen and for facilitating the generation of the immune response within the mucosa. In fact, all pathogens that enter and accumulate in the gastrointestinal tract come into direct contact with the immune cells of PPs. Finally, the function of the PPs in the intestine can be compared to the role of the tonsils in the respiratory system, trapping foreign particles, with the aim of destroying them (Lelouard et al., 2012).

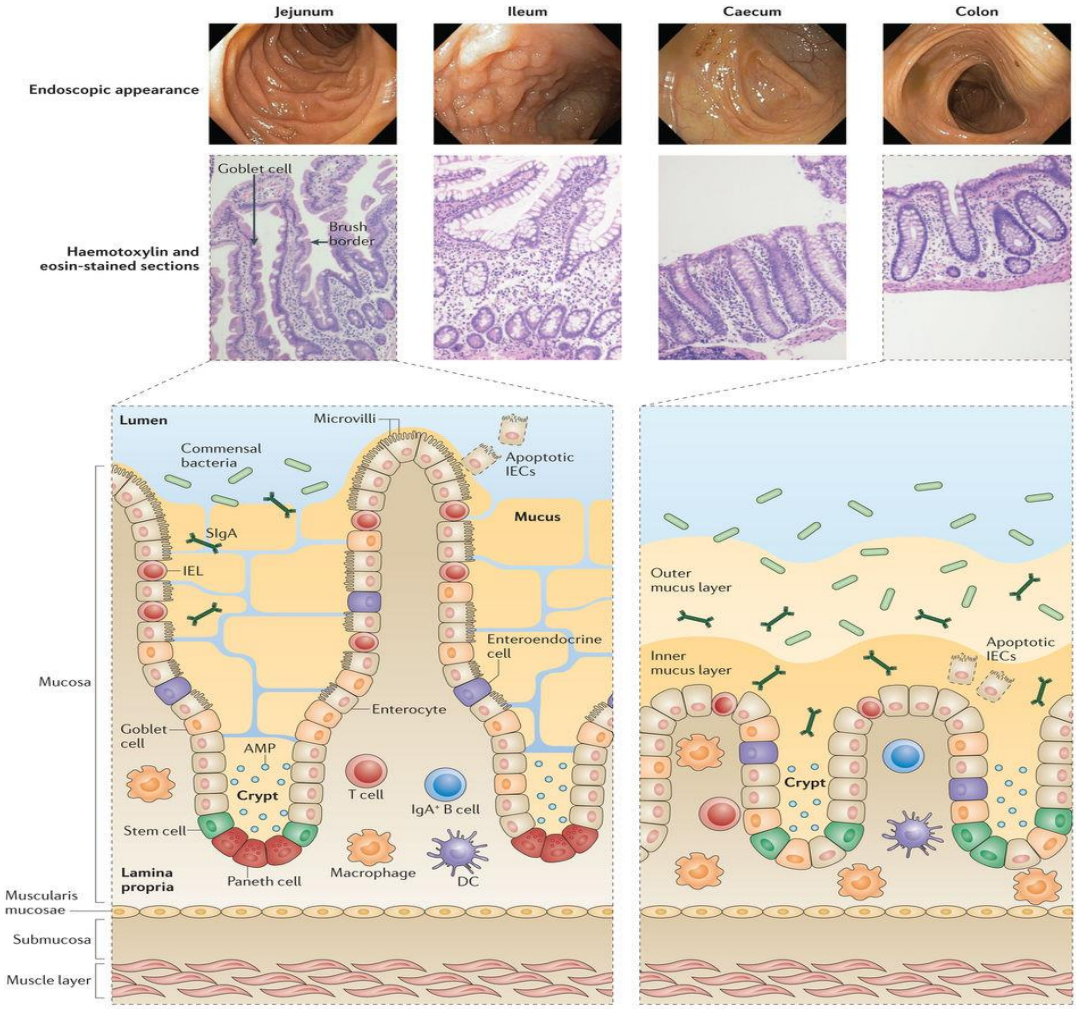
IELs are a heterogeneous group of lymphocytes present in the mammalian intestine and precisely between the basement membrane and the mucous layer. They are distributed through the intestine but they are more concentrated in the small intestine where they are the predominant subset of T lymphocytes (Deusch et al., 1991) (A. Hayday, Theodoridis, Ramsburg, & Shires, 2001). More specifically IELs T lymphocytes are considered a subpopulation of peripheral T lymphocytes from which they differ both phenotypically and functionally(A. Hayday et al., 2001). From a phenotypic point of view IELs normally do not express the memory T cells markers CD2, CD5, CD28, the lymphocyte function associated antigen 1 (LFA1) and thymus cell antigen 1 (Thy1) (Cheroutre, Lambolez, & Mucida, 2011) (Rocha, Vassalli, & Guy-Grand, 1994). Interestingly a great part of human and mice IELs show many phenotypic similarities with NK cells with CD16, CD122, DAP12 and lymphocyte antigen 49a (Ly49A) expression (Ohteki & MacDonald, 1993). Functionally IELs are important for the immune response, in fact the intestinal lumen is in contact with a large number of microorganisms including the external pathogens as well as the commensal cells that live in symbiosis with the intestinal microenvironment. For this reasons IELs lymphocytes have to perform both a protective and homeostatic functions (Vantourout

& Hayday, 2013) (A. Hayday et al., 2001)(Cheroutre et al., 2011). This double face of IELs is essential for intestinal balance that is based on the protective immunity against pathogens and the ability to preserve the integrity of the epithelial barrier by avoiding excessive immune responses. Among IELs T lymphocytes, two main populations can be distinguished according to the structure of the TCR, these are the αβ T cells (40-47% of total IELs lymphocytes) and the γδ T cells (20-30% of total IELs lymphocytes) (Sheridan & Lefrançois, 2010). Both αβ and γδ T cells can be further subdivided in two sub-populations on the basis of CD8 expression. Usually γδ T cells can be CD8αα^{pos} or CD8^{neg}, nevertheless recent studies have highlighted the presence of a new subgroup of human γδ T cells expressing CD8αβ with unique properties (Kadivar, Petersson, Svensson, & Marsal, 2016).(Sheridan & Lefrançois, 2010) (Gangadharan & Cheroutre, 2004). Interestingly γδ CD8αβ^{pos} are present in both blood and the human intestine with a significant enrichment in the latter. Furthermore, these cells are mostly Vδ1^{pos} and show cytotoxic potential in response to IL-2 stimulation by producing IFN-γ and TNF-α (Kadivar et al., 2016). Also IELs αβ T cells can be divided in two main subsets based on the composition of the CD8 coreceptor, particularly these cells are considered the progeny of conventional naïve CD8αβ T cells, but a large percentage expresses the CD8αα coreceptor and lacks specifically CD8β chain (Park, Moon, & Lee, 2016). On the contrary circulating T cells primarily express both CD8α and β chains (Gangadharan & Cheroutre, 2004) (Brandes et al., 2009). In both αβ and γδ IELs the role of the homodimer CD8αα is poorly understood but it was demonstrated that it can promote cytokines production without modulating lytic activity, proliferation, survival, or migration as well as prevent aberrant effector function (Cawthon, Lu, & Alexander-Miller, 2001).

Regarding IELs CD4^{pos} it is known that, in physiological conditions, they are low represented in IELs compartment if compared to systemic population and LPLs (Park et al., 2016).

Below the intestinal epithelium, there is a thin layer of connective tissue known as *lamina propria* which is rich of many and different cells such as DCs, fibroblasts, lymphocytes, macrophages, eosinophils and mast cells. Contrariwise the percentage of neutrophils in this compartment is very low, but their number can increase rapidly in condition of inflammation and/or infection (Fournier & Parkos, 2012). An important role in the intestinal immune response is played by LPLs that are a T cell population located specifically below the intestinal epithelium. Compared to IELs population that is mainly composed of T lymphocytes $CD8^{pos}$, LPLs are much more heterogeneous with a slight prevalence of $CD4^{pos}$ T cells characterized by effector or memory phenotype (Ogata & Itoh, 2016) (Park et al., 2016) .

In fact many studies in humans have confirmed that the $CD4^{pos}$ T cells in healthy *lamina propria* compartment express significant levels of $CD45RO^{pos}$, $CD62^{low}$, $CD69^{high}$, $CD25^{pos}$, Fas^{pos}, and $FasL^{pos}$ (Zeitz et al., 1991). However, another important marker of memory T cells, CD29, is not expressed at high levels in the LPLs, indicating that these cells differ from the classical memory T cells (Zeitz et al., 1991). On the other hand LPLs can express, especially in inflammatory conditions, gut homing $\alpha 4\beta 7$ integrin and CC-chemokine receptor type 9 (CCR9) (Papadakis et al., 2001) (T.Bergsbaken, 2015). Other studies in non-human primates infected by *C. trachomatis* have shown that LPLs in the presence of antigen do not proliferate, but are capable of supporting the immune response with immunoglobulin and cytokines (TGF-β, IFN-γ, IL-10) synthesis (Zeitz et al., 1991).LPLs T cells $CD8^{pos}$ are not present in large percentage in human intestine, despite these cells if activated are responsible of a strong cytotoxic response during pathogen infection (T.Bergsbaken, 2015)



<u>Figure 4</u>: Human intestinel anatomical and immunological organization

The human intestinal tract is composed by different regions, each of which is characterized by specific anatomical and physiological features. Starting from the top it is shown the diversity of the human intestinal epithelium in the Jejunum, Ileum, Caecum and Colon observed by both in endoscopy and immunohistochemistry technique. Below a schematic representation of the immunological organization of human intestine which includes the epithelium, the underlining lamina propria and the muscle layer of the muscularis mucosae. The epithelium and the lamina propria are two different immunological compartments with different lymphocytes populations. The mucosa is formed by extroflections called villi formed by enterocytes and on the bottom of which there are both stem cells and Paneth cells. Internally the mucosa is rich of immunological cells like DCs, neutrophils, macrophages and lymphocytes B and T called IELs. On the other hand, lamina propria is rich of lymphocytes called LPLs which include T and B cells with a slightly percentage of DCs and macrophages.

(Figure modified By Peterson & Artis, 2014)

1.2.3 γδ T cells and intestine

It is known that γδ T cells both in humans and mice, are more abundant in the intestine compared to peripheral blood. Here they play essential roles in barrier immune-surveillance and in tissue homeostasis and repair. Interestingly, intestinal IELs compartment (where γδ T cells are particularly enriched) is comparable between human and mouse in terms of microflora interaction and immunity properties. In both the species IELs are full of γδ and αβ T cells which are interspersed between epithelial intestinal cells just below the basal membrane (Nielsen, Witherden, & Havran, 2017). The fundamental contribution of γδ IELs to intestinal homeostasis is connect to their role in the regulation of intestinal permeability as well demonstrated by the decrease in intestinal junctional complexes observed in TCR δ -/- mice (Nielsen et al., 2017). Furthermore, this is related to an increased susceptibility to development of spontaneous colitis (Inagaki-Ohara et al., 2004). On the other hand, the protective role that γδ T cells have in the intestinal epithelium has been demonstrated using a mouse model of intestinal inflammatory disease induced chemically by the administration of dextran sodium sulfate (DSS). More specifically, TCR δ -/- mice were more susceptible to DSS dependent intestinal damage than Wilde Type, suggesting an important protective role of γδ T cells in intestinal epithelium (Nielsen et al., 2017). Another indication of the important role that γδ T cells play in intestine tissue homeostasis is their ability to influence the proliferation of intestinal epithelial cells. Intestinal γδ T cells produce high levels of keratinocyte growth factor type 1 (KGF-1), and in a TCRδ -/- mice it was shown a reduced proliferation of intestinal epithelial cells due to the lack of KGF-1 associated with γδ T cells deficiency (Komano et al., 1995). In addition in a large number of mouse models, it has been observed that γδ T cells can regulate inflammation. In fact, γδ T cells depletion in TCRδ -/- mice seems to be correlated with an increase in IFN-γ production in intestinal epithelium and consequent development of ulcerative colitis (Kühl et al., 2006) (Komano et al., 1995). Furthermore, the administration of γδ

IELs isolated from WT into TCRδ -/- mice determines an attenuation of colitis (Nielsen et al., 2017). Interestingly, Vδ1 T cells that represent the most widespread subset of γδ in the intestine are able to migrate specifically to the inflamed intestinal area and produce more IFN-γ than the static Vδ1(Nielsen et al., 2017).

It is also important to analyze the relationship between γδ T cells and intestinal microbiome. Several studies confirm that while the microbiome has a strong effect on the composition, behavior and number of αβ IELs, no significant interactions were observed for γδ IELs. In order to demonstrate this, it was observed how the development and the number of gut γδ IELs in mice germs-free dos not differ from those of WT mice, indicating that the microbiome has little or no effect on intestinal γδ IELs (Nielsen et al., 2017). Nevertheless it seems that only γδ IELs located in the small intestine are conditioned by the microbiome. In fact in a germ-free mouse model it was observed that the introduction of the microbiota collected from mice conventionally reared can drives a strong release of different antimicrobial peptides (AMPs), including that of the regenerated gamma 3 protein (RegIIIγ) (Ismail et al., 2011). RegIIIγ is the human equivalent of RegIIIα, both are type C lectins which are involved in bacterial killing specifically in Gram-positive elimination (Cash, Whitham, Behrendt, & Hooper, 2006) (Vaishnava et al., 2011). γδ IELs resident in small intestine can strongly regulate the production of RegIIIγ in the presence of gram-positive bacteria with consequent killing of the latter. Hence TCRδ -/- mice show difficulty in killing gram-positive intestinal bacteria due to the absence of production of sufficient levels of RegIIIγ (Vaishnava et al., 2011). This suggest that intestinal γδ IELs are the first rescuers essential to prevent or limit the penetration of the mucosa by intestinal bacteria.

Recently a family of Butyrophilin-like (Btnl) molecules which are part of the B7-family have been identified as an important regulator of γδ IELs behavior in both humans and mice intestine. Although the specific molecular mechanism remains unclear, it appears that the specific expression of Btln family molecules in the intestinal

epithelial tissue may contribute specifically to the cytotoxic activation of γδ IELs (Nielsen et al., 2017).

1.3 Natural Cytotoxicity Receptor (NCRs)

Natural cytotoxicity receptor (NCRs) were discovered in the 90s in human Natural Killer cells and they NKp46 (CD335), NKp44 (CD336), Nkp30 (CD337), (M Vitale et al., 1998) (Pende et al., 1999) (Vitale et al., 2001) (Pessino et al., 1998) (Sivori et al., 1997). NCRs belong to the immunoglobulin superfamily and regulate NK cells cytotoxic activity against cancer and pathogens-infected cells (Hudspeth, Silva-Santos, & Mavilio, 2013) triggering the lysis and secretion of different cytokines like IFN-γ and TNF-α (Yokoyama, Kim, & French, 2004). NKp46 and NKp44 are encoded respectively by *NCR1* and *NCR2* genes, on the other hand NKp30 is encoded by *NCR3* (Hudspeth, Silva-Santos, & Mavilio, 2013). More specifically *NCR2* and *NCR3* genes are located on chromosome 9 in MHC III locus, contrarily *NCR1* is located on chromosome 19 near the leukocyte regulatory complex (Hudspeth et al., 2013). Interestingly NCRs do not undergo somatic recombination in order to became active unlike the great part of T cell receptors. NKp46 and NKp30 are expressed constitutively on the surface of human NK from peripheral blood, on the contrary NKp44 receptor requires IL-2 or IL-15 stimulation (Pessino et al., 1998) (Sivori et al., 1997) (M Vitale et al., 1998). For a long time, NCRs were considered exclusive markers of NK cells, but recent studies have shown that NCRs can be expressed on the surface of other cells population such as T cells, NK-like cells and γδ T cells (Walzer, Jaeger, Chaix, & Vivier, 2007) (Hudspeth et al., 2013) (Von Lilienfeld-Toal et al., 2006) (Meresse et al., 2006) (Tang et al., 2008). Interestingly the functions of NCRs on different groups of T cells seem to mirror those of NCRs expressed on the surface of NK cells, controlling a large part of their behavior.

1.3.1 Natural cytotoxicity receptor structure

Although NCRs share similar functions, they have a profoundly different amino-acid sequence and molecular structure. These receptor are type I transmembrane proteins which belong to the Ig superfamily and are characterized by one/two extracellular immunoglobulin-like domain involved in the binding of specific ligands (Hudspeth et al., 2013). Furthermore each of these receptors contain a transmembrane domain formed by a short sequence of positive amino-acid that are able to interact with tyrosine-depending adaptor proteins start the signal transduction process (Kruse, Matta, Ugolini, & Vivier, 2014) (Hudspeth et al., 2013) **(Fig. 5)**.

NKp46 was the first NCR to be identified on NK cells surface, though later it was found in different T-cells subsets (αβ and γδ) as well as group 3 of ILCs (Hudspeth et al., 2012) (Meresse et al., 2006) (Spits et al., 2013). NKp46 is characterized by two extracellular C2-type Ig-like domains that are able to arrange at an angle of 85°(Ponassi et al., 2003). In addition this receptor contains a cytoplasmic tail that lacks classical signaling motifs, in return for this the adaptor protein CD3ζ and FcRγ can bind to NKp46 via their ITAM motifs that are able to recognize an arginine located in the NKp46 transmembrane structure (Ponassi et al., 2003) (Foster, Colonna, & Sun, 2003). NKp46 is also composed of a safe-peptide called D2-Ig domain (aa 136-155) that inhibits the uncontrolled binding to target cells as well the cytotoxic activity response (Jaron-Mendelson et al., 2012). The inhibitory capacity of D2 peptide is due to its ability to bind each other D2 domains precluding NKp46 homodimerization (Jaron-Mendelson et al., 2012). On the other hand NKp46 receptor shows high similarities to other protein of the Ig-like receptor family like ILT2 and glycoprotein VI (Jaron-Mendelson et al., 2012). These similarities suggest the involvement of the hinge region in the binding with specific peptides (Kruse et al., 2014).

In contrast to other NCRs NKp44 is not expressed on resting NK cells, but only on activated cells (Hudspeth et al., 2013) and despite this, it can be expressed on other

cells subset like T cells and DC (Fuchs, Cella, Kondo, & Colonna, 2005) (Vacca et al., 2008). Nkp44 is composed of only a single extracellular Ig domain V-type that is directly connected whit a 64-amino-acid stalk domain, a transmembrane domain and a small cytoplasmic tail formed by a sequence that represent the tyrosine dependent inhibitory motif ITIM (M Vitale et al., 1998) (Cantoni et al., 2003). Initially NKp44-ITAM domain was supposed to be no-functional, but later on it was clear its great importance in the negative regulation of NK cells activity and specifically in IFN-γ production (Pazina, Shemesh, Brusilovsky, Porgador, & Campbell, 2017). Furthermore the presence of positive lysine in the transmembrane domain allows this receptor to interact with the ITAM containing adaptor DNAX-activating protein 12 (DAP12) that activates the cytotoxic response (M Vitale et al., 1998) (Cantoni et al., 2003).

NKp30 is a protein of 30 kDa expressed constitutively like NKp46 in mature NK cells as well as other cells population such as cord blood T cells, γδ T cells and endometrial epithelial cells (Ponnampalam, Gargett, & Rogers, 2008) (Kruse et al., 2014) (Pende et al., 1999). NKp30 structure includes one short extracellular Ig domain, an intracellular domain rich of arginine and an intracellular domain without intrinsic signaling capacity (Li, Wang, & Mariuzza, 2011). The presence of the adaptor proteins CD3ζ and FcRγ favours the signal transduction trough the binding of arginine on the transmembrane domain. The NKp30 mRNA can be processed into six different splicing variants expressed in different cells subsets. Three of them (NKp30a/b/c) encode for an extracellular V-type domain, unlike the last three Nkp30 variants which encode for a domain C-type Ig (Pazina et al., 2017). Interestingly NKp30a/b/c variants are the most frequent and they can be expressed simultaneously on the same cell. In addition NKp30 a/b are important immunostimulatory isoforms, which can induce the release of Th1 cytokine like IFN-γ and TNF-α. On the contrary the isoform NKp30c is endowed with immunosuppressive activity due to IL-10 production (Delahaye et al., 2011).

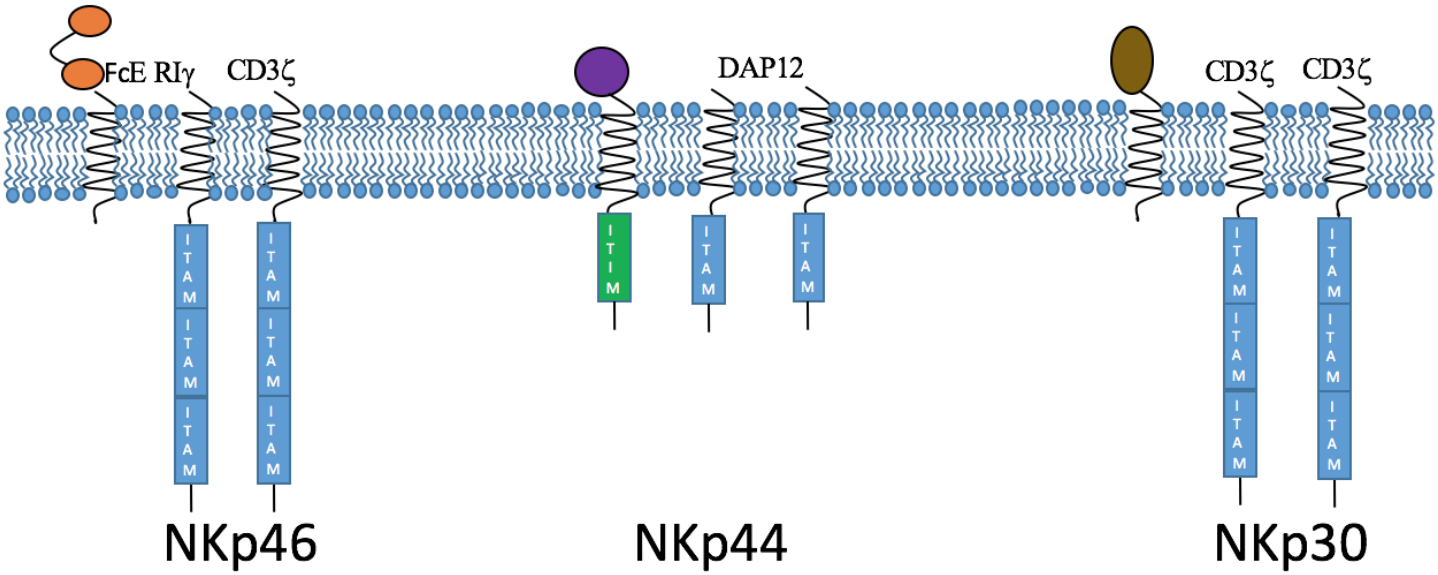


***Figure 5*: NCRs structure**

NCRs are characterized by type I transmembrane protein of the Ig superfamily and by one or two extracellular immunoglobulin-like domain involved in the binding of ligands. On the other side each of these receptors contain a transmembrane domain of positive amino-acids and an intracellular domain. To every NCR receptor are associated adaptor molecules CD3ζ, FcεRγ and Dap12 which are involved in signal transduction. NKp46 and 30 are also characterized by immunoreceptor tyrosine activating cytoplasmatic motif (ITAM) indicated in blue. On the contrary NKp44 is associated with the immunoreceptor tyrosine inhibition motif (ITIM) indicated in green.

1.3.2 NCRs Ligands

Although the structure, function and molecular mechanism of NCRs in immune cells are abundantly studied little is known about their ligands. In fact, the NCRs ectodomain is characterized by different binding sites that allow the interaction with structurally distinct molecules. Different type of molecules have been identified to interact with NCRs such as cell surface and intracellular proteins, as well as carbohydrate-molecules. (**Table 1**). Interestingly it was reported that the binding with many of these ligands has an activating effect on NCRs, despite many inhibitor molecules have been identified (Pazina et al., 2017).

1.3.2.1 Viral Ligands

Several viral-ligands have been identified for NCRs receptor. Early studies demonstrated that the viral hemagglutinin of influenza virus (HA) is able to interact with a specific site located on NKp46 ectodomain. This binding involves a cytotoxic response as well as an increase production of IFN-γ (Draghi et al., 2007). Interestingly was demonstrated that hemagglutinin of orthopox viruses, murine ectromelia and human vaccinia virus are able to interact to NKp30, NKp46 but not NKp44 receptor (Hudspeth et al., 2013) (Jarahian et al., 2011). Furthermore vaccinia infected cells seems to be less susceptible to NK cell cytotoxicity NCRs mediated compared to uninfected cells, this suggesting that HA of vaccinia virus can have an inhibitory effects on NCRs activity (Jarahian et al., 2011).

In addition it has been reported that NKp30 is able to interact specifically with pp65, an important tegument protein of human cytomegalovirus (HMCV). It was shown that HMCV infected cells are particularly resistant to NK cell mediated lysis, but this

resistance is lost in cells infected with HMCV pp65 deficient or traded with anti-Nkp30 antibodies. This may mean that pp65 protein can provide to HCMV a way to evade the immunity mediated by NK cells (Arnon et al., 2005).

NKp44 receptor is able to recognize the structural envelope protein domain III of the West Nile and Dengue flaviviruses in infected cells, this stimulates NK cells degranulation and IFN-γ production (Pazina et al., 2017). (Mandelboim et al., 2001)

Recently it was demonstrated that NCRs are able also to recognize both bacterial and parasites molecules, and consequently play an important role in the primarily immunological response against pathogens **(Table 1)**. More specifically it was shown that NKp30 and NKp46 in NK cells can bind directly Duffy binding like domain α (DBL-1α) of *Plasmodium falciparum* in order to induce lysis of malaria infected cells (Mavoungou, Held, Mewono, & Kremsner, 2007). In addition, interaction of Nkp44 with a BCG molecule of *Mycobacterium bovis bacillus* can increase the expression of CD56 on NK cells and cause the expansion of NK $CD56^{bright}$(Esin et al., 2008).

Receptor	Ligand	Source	Reference
NKp30	B7-H6	Tumor cells	(Brandt et al. 2009)
	BAG6	Stressed cells	(Pogge von Strandmann et al., 2007)
	pp65	HCMV	(Arnon et al. 2005)
	Pf/EMP1	*Plasmodium falciparum*	(Mavoungou et al. 2007)
	Viral HA	Poxvirus, Vaccinia Virus	(N. Bloushtain et al., 2004)
	Heparin and Heparan sulfate	Animal cells	(N. Bloushtain et al., 2004)
NKp44	PCNA	Tumor cells	(Korgun et al., 2006)
	Viral HA/HN	Influenza-virus, Sendai-virus	(Mandelboim et al., 2001)
	Sulfated proteoclycans	Animal cells	(Cantoni et al., 1999)
	Heparin and Heparan sulfate	Animal cells	(Horton & Mathew, 2015)
NKp46	Viral HA/HN	Influenza-virus, Sendai-virus	(Mandelboim et al., 2001)(Magri et al., 2011)
	Heparin and Heparan sulfate	Animal cells	(Sugahara & Kitagawa, 2002)
	Complement-factor P(Properdin)	Animal cells	(Kruse, Matta, Ugolini, & Vivier, 2014)
	Unknown	Stressed and tumor cells	(Kruse, Matta, Ugolini, & Vivier, 2014)

Table 1: Summary of the most important NCRs Ligands

1.3.2.2 NCRs tumor associated ligands

NCRs are also considered a key receptors in tumor cells recognitions and they are involved in NK anti-tumor activity. In fact many studies have demonstrated that NCRs are involved in the clearance of many tumors such as melanomas, leukemia and neuroblastoma (Hudspeth et al., 2013). Remarkably the cytolytic capacity of NCRs against tumor cells depends on their ability to recognize specific antigens. For example NCRs in NK cells are able to bind specific epitopes of heparan sulfate glycosaminoglycans that are overexpressed in tumor transforming cells (Jaron-Mendelson et al., 2012) (Sugahara & Kitagawa, 2002).

It was reported that NKp44 and at less extent NKp46 can interact with proliferating cell nuclear antigen (PCNA) which is an intranuclear molecule usually tightly associated with DNA and whose expression drastically increases on the surface of tumor transforming cells (Pazina et al., 2017). Interestingly the interaction between NKp44 and PCNA can inhibit cytotoxic functions of NK cells such as IFN-γ and TNF-α production. PCNA inhibition ability seems to be regulated specifically by the ITIM unit located on the cytoplasmic domain of NKp44 (Pazina et al., 2017). In particular, PCNA-NKp44 interaction induces a unique conformation of the receptor which creates an inhibitory signal for NK cells activity (Koch, Steinle, Watzl, & Mandelboim, 2013) (Pazina et al., 2017) (Esin et al., 2008). It was also show that NKp30 receptor is able to bind directly HLA-B associated transcript 3 (BAT3)/Bcl2 anthogene 6 BAG6 which is abundantly expressed in the cellular nucleus, but in stressed and tumor cells it can moves on the plasma membrane surface and subsequently it can be secreted via exosome vesicles (Brandt et al., 2009) (Arnon et al., 2005). BAT3/BAG6 is able to stimulate cytotoxic response and cytokines production on NK cells trough NKp30 stimulation signal (Koch et al., 2013). Finally B7-H7 expressed selectively on different tumor cell lines has been identified as a ligand for NKp30 that is able to trigger NK cells cytotoxicity against tumor cells (Brandt et al., 2009).

Aim of the project

In contrast to the peripheral blood γδ T cells, tissue resident γδ T cells have received less attention, and we are only beginning to understand how human organ-specialized γδ T compartments are functionally regulated and controlled. Only recently, a large analysis of 39 different types of human cancers, including colon rectal cancer (CRC) found intratumoral γδ T cells as the most favorable prognostic marker. While mice share some common innate-like functions of human γδ T cells, they differ significantly in terms of TCR specificity and tissue specialization. Human intestine is particularly enriched of γδ T cells, especially in the intraepithelial (IE) compartment, however, little is known about the origin, phenotype and functions of intestinal γδ T lymphocytes. Hence, there is a pressing need to comprehensively characterize human tissue-resident γδ T lymphocytes in order to understand the impact they may have on multiple diseases including cancer. More specifically we investigated if human intestine γδ T cells express NRCs in physiological condition, focusing on their function within intestine. Furthermore, we studied whether the intestinal microenvironment has an impact on the differentiation and effector functions of γδ T thymocytes precursor, cultivating them in conditions that recapitulated the intestinal microenvironment in both physiological and tumor conditions. To address this, we performed a translational study of human colonic γδ IELs in order to fully characterize phenotypic and functional properties of human intestinal γδ T cell in both physiological and pathological conditions such as CRC.

2. MATERIAL AND METHODS

2.1 Ethical statement

All human tissues have been obtained according to the Declaration of Helsinki. The collection of human samples for research purposes was ethically approved by the Institutional review Board (IRB) of Humanitas Research Hospital (HRH) for blood and gut specimens either free of diseases of affected by CRC or Inflammatory Bowel Diseases (IBDs) of patients undergoing surgical gut resection and for healthy skin of patients undergoing plastic surgical procedures (approval 2012/1021), for healthy lymph nodes of patients undergoing the removal of benign head-neck tumors (approval 2010/700), for healthy liver specimens of patients undergoing resection of CRC metastasis (approval 168/18). We also received the IRB approval from University of Palermo (UNIPA) for the collection of gut specimens affected by CRC of patients undergoing surgical gut resection (approval 13/2013) and from the IRB of TIGET/San Raffaele Institute for specimens of healthy thymus from pediatric patients undergoing cardiac surgery (approval TIGET07, TCTO-044). Each patient or the person having care and custody of the child signed a consent form, approved by the above IRB, that clarifies the donation of bio- specimens for this research project did not affect in any way the diagnosis, the therapies and the prognosis of the disease.

2.2 Human Tissue, cell isolation/culture and sample preparation

Human intestinal specimens were obtained from patients with colorectal cancer (CRC) and patients with inflammatory Crohn's disease undergoing gut resection. Specimens were provided by the Colon and Rectal Surgery Section of HRH, Rozzano, Italy and by

the UNIPA, Palermo, Italy. Healthy intestine tissues were collected from the distal area of the pathological tissue (≥10 cm) and macroscopically free from any disease. In order to obtain intestinal IELs, healthy mucosa was first dissected from the whole tissue and cut in small pieces, washed in Wash Buffer (WB) prepared with Hank's Balanced salt solution buffer without Ca2 and Mg2 (HBSS-/-; Lonza, Verviers, Belgium) supplemented with 1% penicillin/streptomycin/amphotericin (P/S/A; Invitrogen, Paisley, UK), treated with 2 mM of DL-Dithiothreitol (DTT) (Sigma Aldrich, Saint Louis, MO, USA) for 15 min at room temperature (RT), washed again and subsequently treated with 2 mM of Ethylene-diamine-tetra acetic acid (EDTA; Sigma Aldrich) for 30 min at 37°C/5% CO2. Cells suspension was filtered through 100 μm cell strainer (Thermo Fisher Scientific, Waltham, MA, USA) and cells were collected by centrifugation. The remaining tissue was used to isolate lamina propria lymphocytes (LPLs) by enzymatic digestion with 0.75 mg/mL of collagenase II (Sigma Aldrich) for 1 hour at 37°C/5% CO2. Obtained cells were filtered through 100 μm cell strainer and collected by centrifugation. Finally, both IELs and LPLs were isolated using 30/70% Percoll gradient (Sigma Aldrich). Pathological intestine specimens after were mechanically cut in small pieces and washed with WB, followed by enzymatic digestion with 1.5 μg/ml of collagenase IV (Life Technologies, Carlsbad, CA), 20μg/ml of hyaluronidase and 50 μg/ml of DNAase (both from Sigma Aldrich) for 2 hours at 37°C/5% CO2. Obtained cells were isolated by centrifugation and stained for flow cytometry analysis.

Human healthy thymus samples were obtained from children undergoing heart surgery for congenital heart diseases according to current clinical practice. Thymic tissue was cleaned from blood vessels and fat tissue and cut in small pieces. Thymocytes were recovered through mechanical smashing and kept in PBS (Corning; New York, NY, USA) with 1% of P/S, to preserve their viability or were frozen in fetal bovine serum (FBS; Lonza) with 10 % of DMSO (Lonza) and stored at -80°C. Thawed thymocytes were cultured in 96 well plate round bottom at a concentration of 10^6 cells /mL in X-

VIVO™ 15 serum-free hematopoietic cell medium supplemented with: 5% human AB serum, 1% P/S/A, 1% ultraglutamine, 1% Na pyruvate and 1% of non-essential amino acid solution (NEAA), all purchased from Lonza. Thymocytes cultured in vitro were stimulated with human recombinant IL-2 (200 U/mL, Miltenyi) or with IL-15, IL-7, IL-10, IL-12, IL-22, TGF-β, thymic stromal lymphopoietin (TSLP), all used at concentration 10 ng/mL and purchased from Peprotech (Rocky Hill, NJ, USA).

Human healthy liver tissues were obtained from patients who underwent hepatectomy for hepatic metastatic disease from CRC. Tissue specimen were collected from the distal area of the pathological tissue (≥ 4cm) and macroscopically free from any disease. Tissue dissociation was obtained by enzymatic digestion in gentleMACS™ Dissociator (Miltenyi) with 2 mg/mL of collagenase D (Roche Diagnostic; Indianapolis, IN,USA) for 45 minutes at 37°C/5% CO2. Cells then were filtered through 100 μm cell strainers, washed in HBSS-/- and lymphocytes were separated using 30/70 % Percoll gradient.

Human healthy skin was obtained from patients who undergo abdominoplasty or mastoplasty surgery procedure. Tissue were cut in small pieces and lymphocytes were recuperated by enzymatic digestion with 1.25 U/mL of Dispase II (Roche Diagnostic) initially for 16 hours at 4°C followed by 30 minutes at 37°C. Solution containing epidermal lymphocytes was filtered through 100 μm cell strainers, and cells were washed in HBSS-/- with 5mM EDTA. Remaining tissue was further digested with 1 mg/mL collagenase D and 1.25 U/mL of Dispase II 30 minutes at 37°C in order to recover dermis lymphocytes. Cells then were filtered through 100 μm cell strainers, washed in HBSS-/- with 5 mM EDTA and lymphocytes were separated using 30/70 % Percoll gradient.

Human healthy/non-reactive lymph nodes obtained from patients with Whartin's tumor or hyperplasia of salivary glands were mechanically smashed in HBSS-/- through 100 μm cell strainers and cells were collected by centrifugation.

Human peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of healthy volunteers (HRH, Rozzano, Italy) using Lympholyte® Cell Separation density

gradient solution (Cederlane laboratories, Burlington, Canada) according to the manufacturer's instruction.

Primary human colonic epithelial cells (HCoEpic, Cat. No.2950) purchased from ScienCell Research Laboratories (Sciencell Carlsbad, Carlsbad, CA, USA) were cultured on poly-L-lysine (Sigma Aldrich) following manufacturer's instructions in the specific colonic epithelial cell medium (CoEpiCM, Cat. No.2951) supplemented with colonic epithelial cell growth supplement (CoEpiCGS, Cat. No.2952) and 1% P/S/A.

Human leukemia cell line K562 (HRH Cells Bank) and colon cancer adenocarcinoma Caco2 (ATCC Cell, Middlesex, UK), mycoplasma free, were cultured in Iscove's Modified Dulbecco's medium (IMDM; Lonza) or Dulbecco's Modified Eagle's medium (DMEM; Lonza) respectively, both supplemented with 10% FBS, 1% P/S/A, 1% ultraglutamine. Additional supplements of 1% Na pyruvate and 1% NEAA were added to the Caco2 cell culture medium.

2.3 Mice

Balb-c and *C57BL/6* mice were purchased by Charles River (Wilmington, MA, USA) and maintained under pathogen-free conditions. Animal experiments adhered to the requirements of the European Commission Directive 86/609/EEC and to the Italian legislation (Decreto Legislativo 116; 27 January 1992). Experiments were approved by the Animal Care and Use Committee from Italian Ministry of Health (approval 158/2011).

Small intestine, colon and spleen specimens were dissected and collected at 4°C in RPMI 1640 (Lonza) medium supplemented with: 10% FBS, 1% P/S/A and 1% ultraglutamine. Cleaned from Peyers patches, small intestine and colon tissue were cut in small pieces and treated twice with 1 mM DTT solution (Sigma Aldrich) for 20 min at 37C°/5% CO_2. Cells were then filtered with 70 μm cell strainer and washed with WB.

Total lymphocytes were obtained using 30/70% of Percoll gradient (Sigma Aldrich). Spleen was mechanically smashed in a 100 μm cell strainer (Thermo Fisher Scientific), washed with the Wash buffer and filtered in 50 μm cell strainer to obtain single-cell suspension of splenocytes

2.4 Flow cytometry and antibodies

For multiparametric flow cytometry analysis a standard staining protocol for extracellular markers was used. Briefly, cells were first stained for live/dead discrimination by using Zombie Aqua™ fixable viability kit (BioLegend; San Diego, CA, USA) or 7-Aminoactinomycin D (7-ADD) (BD Pharmigen). Subsequently cells were washed with FACS WB (HBSS-/- with 2% of FBS), incubated with antibody (Ab) mix for 20 min in the dark at RT, washed again in and fixed in 1% paraformaldehyde (PFA; Santa Cruz Biotechnology, Dallas, TX, USA). Samples were acquired using LSRFortessa™ cell analyzer system or FACS Canto II (both from BD Bioscience; San Jose, CA, USA). FACS Aria III cell sorter (BD Bioscieces) was used for the specific cell subsets separation and in vitro assays, for sorting experiments cells were stained with 7-ADD to discriminate dead cells and the appropriate mix of Abs for 20 mins in the dark at RT. The optimal concentration of all Abs used in the study was defined performing a titration experiments. According to the guidelines for an accurate multicolor flow cytometry analysis fluorescence minus one (FMO) controls were used for flow cytometry analysis.

For phenotypic analysis, the following specific anti-human monoclonal antibodies (mAbs) were used. From BioLegend: CD3 (SK7; BV605), CD4 (RPAT4; BV570 or BV605), CD16 (3G8; AF700), CD69 (FN50; BV605), CD103 (BERACT9; PE), γδ TCR (B1; FITC), NKG2D (ID11; PECy7), NKp46 (9E2; BV421), CCR9 (L053E8; PerCPCy5.5); from BD Biosciences: CD8α (RPAT8; BV780), CD45 (H130; AF700), CD45 (2D; APC-Cy7), CD56 (B159; PE-CF594) , NKp30 (P3015; BV711); from

Miltenyi Biotec (Bergish Gladbach, Germany): CD45 (5B1; APCVio770), KIRs (REA293; APCVio770 and DX27; PerCPCy5.5), Vδ1 (REA173; PE), NKG2A (REA111; APC), CD3 (HIT3a; FITC), Vδ1 (REA 173; PE), NKp46 (9E2; PEVio770); from eBioscience: CD8β (5IDI8BEE; PECy7), CD69 (FN50; APC); from Beckman Coulter (Brea, CA,USA) Vδ2 (IMMU-389; FITC) and NKp44 (Z231; PECy5); from R&D system (Minneapolis, MN, USA) NKG2C (134591; PerCP).

Mouse γδ T lymphocytes were stained with live/dead discriminating marker 7-ADD and with the specific anti-mouse mix of mAbs: CD3 (17A2; FITC), NKp46 (29A1.4; AF700) (both from BD Pharmigen) and γδ TCR (eBioGL3; APC) (eBioscience).

Cell cytotoxic activity was assessed by anti-human CD107a (H4A3; PE,BD Biosciences). Intracellular staining was performed using the following antibody: anti-human Abs (BD Biosciences) for TNF-α (Mab11; PE), IFN-γ (B27; Bv711) and GZMB (GB11; AF647).

2.5 qPCR

Total RNA of sorted γδ T NKp46^{pos} and NKp46^{neg} IELs/thymocytes was extracted using RNasy micro-plus columns with RNase-free DNase treatment and RNA carrier (Qiagen Valencia, CA, USA) following the manufacturer's instructions. Extracted RNA was used to generate cDNA templates for qPCR using High-Capacity cDNA Reverse Transcription Kit with random primers and RNase inhibitor (Applied Biosystem, Foster City, CA, USA). 100 ng of total cDNA were pre-amplified for the specific genes expression using TaqMan®mRNA gene assays and TaqMan® PreAmp Master Mix (both from Applied Biosystem). QPCR reactions were performed by Real Time AB7900 (Applied Biosystem) with TaqMan® Universal PCR Master Mix and the specific TaqMan®mRNA gene assays (Applied Biosystem): NCR1 (HS00183683), IL10 (HS00961622), IL17A (HS00174383), TGFβ (HS00998133), IL22

(HS01574154), IFNγ (HS00989291), GZMB (HS01554355) and housekeeping S18 (HS01026310) and GAPDH (HS02758991).

PCR-based detection of mycoplasmal DNA was performed using BioMix™ (Bioline, London, UK) and GPO-1 (5'ACTCCTACGGGAGGCAGCAGT-3') and GPO-1 (5'ACTCCTACGGGAGGCAGCAGT-3') and MGSO (5'-TGCACCATCTGTCACTCTGTTAACCTC-3') primers from IDT (Coralville, IA, USA).

2.6 Cytotoxicity Assay

A flow cytometry-based lysis assay was performed in order to determine the cytotoxicity activity of human γδ NKp46^{pos} and γδ NKp46^{neg} T-cell isolated from both Thymus and IEL compartment against Human Leukemia cell line (K562) obtained from Humanitas Cells Bank, or against Colon Cancer Adenocarcinoma (Caco2, ATCC Cell, Middlesex, UK). Fresh IELs or thawed thymocytes stimulated with IL-2 (200 U/mL, Miltenyi) upon 6 days were cultured in 96 well plate round bottom at a concentration of 10^6 cells /mL in the presence or not of K562 (ratio of E:T = 1:1), and PE-conjugated anti-CD107a mAb was add in each well. After 4 hours of incubation at 37°C/ 5% CO_2 cells were collected, washed with HBSS-/- (Lonza), stained and acquired to LSR Fortessa™ for flow cytometry analysis. In all the experiments we used as a control of degranulation assay PBMC of heathy donors pre-stimulated for 1 hour with PMA (10 ng/mL) (Sigma Aldrich) and Ionomycin (10 mg/mL) (Sigma Aldrich).

2.7 Cytokine production assay

IFN-γ, TNF-α and Granzyme-β synthesis of γδ Nkp46$^{pos/neg}$ IELs was measured through flow cytometric-based intracellular staining assay, using as a stimulation factor the co-culture with target cells K562. Fresh IELs were plated in 96 well plate round bottom at a concentration of 10^6 cells /mL and measurements of cytokines were performed after 4 hours of incubation with or without K562 at 37 C°/ 5% of CO_2 (ratio of E:T = 1:1). Furthermore, Brefeldin A (Golgi Plug 1μg/mL, BD) was added in each well with the aim of blocking degranulation activity. IELs than were washed with HBSS-/- (Lonza) and an intracellular staining was performed using Fixation/ Permeabilization Solution Kit and Perm/Wash buffer, according to the manufacturer's instructions (BD Biosciences). Finally, samples were collected and acquired to LSR Fortessa.

2.8 Co-Culture Experiments

In order to observe how the intestinal microenvironment influences the expression of NKp46 receptor in γδ T cell defrost human thymocytes were cultured in 96 well plate round bottom at a concentration 10^6 cells /mL using in X-VIVO™ 15 Chemically Defined, Serum-free Hematopoietic Cell Medium (Lonza) enriched with: 5% human Serum (Lonza), 1% P/S/A (Lonza), 1% Ultraglutammine (Lonza), 1% Na pyruvate (Lonza) and 1% of Non-Essential Amino Acid Solution (NEEA, Lonza), IL-2 (200 U/mL, Miltenyi) for 3 days. Subsequently cells were stimulated with IL-15 (10 ng/mL, Peprotech) or TGF-β (10 ng/mL, Peprotech) or cultured with Caco2 (ATCC Cell) or Human Colonic Epithelial Cells HCoEpiC (Sciencell Carlsbad) at 37°C/ 5% CO_2 for 3 days upon of IL-2 (200U/mL, Miltenyi) stimulation, using for both the final

concentration of x 10^3 cells/well. At the end of the 6^{th} day of culture, cells were collected and stained for flow cytometry analysis.

2.9 Migration Assay

With the purpose to characterize the CCR9-CCL25 dependents migration activity of human thymocytes precursor in gut microenvironment, a cell migration assay was performed. Defrost thymocytes were stimulated with IL-2 (200 U/mL, Milteny) upon 7 days in 96 well plate round bottom at a concentration of 2 x 10^6 cells /mL in enriched X-vivo medium (Lonza). Afterwards cells were sorted for γδ $Nkp46^{pos}$ and were plated in Transwell-24 units with 8.0 μm large pore (Corning, New York, NY, USA). Recombinant –soluble Human CCL25 (BioLegend) was used at 25 ng/mL and 50 ng/mL in 600 μL of enriched X-Vivo medium for the proliferation assay. On the other side sorted γδ $NKp46^{pos}$ thymocytes were resuspend in 100 μL of enriched X-Vivo Medium (Lonza) into the basket of Trans-Well and incubated for 6 hours at 37 C°/ 5% of CO_2. Subsequently cells were collected and counted for quantification.

2.10 Immunofluorescence assay

Freshly isolated tissue were fixed with 4% paraformaldehyde for 2 hours, washed and incubated overnight in 30% sucrose. Tissues were then embedded in OCT , frozen in a bath of isopentane cooled on dry ice, and cut in 8 μm-thick sections. Human NKp46 staining was then performed using polyclonal goat anti-human NKp46 (R&D Systems), polyclonal rabbit anti-human CD3 (Dako) and mouse anti-human TCRγδ (Coulter, Clone IMMU510) then followed by the following secondary antibodies raised in donkey

(Invitrogen): anti-rabbit Alexa488, anti-mouse Alexa594 and anti-goat Alexa647. Nuclei were counterstained with DAPI (Invitrogen) at concentration 1:50000. After staining, slides were dried, mounted with Prolong Gold (Invitrogen) and examined under Zeiss LSM 510 confocal microscope (Zeiss, Germany). Image processing was performed with Zeiss LSM and Adobe Photoshop software.

2.11 Hematoxylin and Eosin (H&E) staining

Formalin-fixed paraffin-embedded liver specimens, previously sectioned with a microtome HM310 Microm (GMI, MN, USA) at 2-4 μm thickness and mounted onto charged glass slides Superfrost^{+} (Thermo Fisher Scientific), were stained for H&E using a standard protocol. Briefly, after two changes of xylenes, rehydration in an ethanol/water gradient (100%, 90%, 70%), followed by washing in water, slides were stained with in Mayer's Hematoxylin solution (Dako, CA; USA) for 18 min, differentiated under running tap water for 10 min, then stained with 1% aqueous solution of Y Eosin (Dako) for 8 min, followed by three washes with 100% ethanol, and three washed with xylenes. Lastly H&E stained slides were mounted with Eukitt (Sigma Aldrich) and dry prior to imaging by Microscope BX51 (Olympus, Tokyo, Japan).

2.12 Statistics

Analysis was performed using GraphPad Prism version 7. The data were assessed by non-parametric approaches such as the unpaired *Mann-Whitney* test, or the *Wilcoxon* matched-pair test. The data are depicted as vertical bars corresponding to median value with scatter dots or ± standard deviation (SD) of the median. *P* values > 0.05 were considered not statistically significant (ns). Statistically significant *P* values were represented with GraphPad (GP) style and summarized with following number of

asterisks (*): *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$. Flow cytometry data *were analyzed by FlowJo software* version 9.4 and 9.6 (TreeStar; Ashland, OR, USA). Flow cytometry data analysis based on t-SNE algorithm were performed using FlowJo 9.6 software.

3. Results

3.1 Identification of a novel subset of NKp46^{pos} γδ T intestinal intraepithelial lymphocytes

Human healthy colon specimens obtained from patients who underwent surgical resection for colorectal cancer (CRC) were located at the distal part (≥ 10 cm in length) of the removed tumor core and were considered "tumor-free/healthy" after a conventional macroscopic and microscopic examination (hematoxylin-eosin staining) by pathologists **(Fig.6a-b)**. Gut specimens were then processed to obtain intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs). In order to do this gut tissues were processed with DTT/EDTA **(Fig.7a)** as described in the Methods section and γδ T cells were identified by the gating strategy shown in **(Fig.7b)**. We found a striking difference in the frequency of γδ T cells between IELs, LPLs and PBMCs. More specifically the frequency of γδ T cells appear to be significantly higher among IELs compared to LPLs and PBMC **(Fig. 7c)**. As expected, differently from circulating γδ T cells, both γδ IELs and LPLs presented high levels of the tissue-resident marker CD69 (Sathaliyawala et al., 2013)**(Fig. 7d)**. On the other hand, intraepithelial emplacement of γδ IELs was assessed by high expression of CD103 **(Fig. 7d)**, the main adhesion molecule involved in the specific retention of IELs in the epithelial layer (Cepek et al., 1994). Indeed, expression of CD103 (Cepek et al., 1994) in LPLs and blood γδ T cells was greatly lower or almost lacking **(Fig. 7d)**.

Using multiparametric flow cytometry we also observed that γδ IELs were characterized by a significant higher expression of CD56 and CD8 compared to γδ LPLs, while expressing low surface levels of CD4 **(Fig. 8a)**. These phenotypic features of γδ IELs were also associated with low amounts of inhibitory NK cell Receptors (iNKRs) (i.e. NKG2A and KIRs) and high expression of activating NKRs (aNKRs) (i.e. NKG2C and NKG2D), thus suggesting that γδ IELs are endowed with a

high cytolytic potential **(Fig. 8a)**. Since in our previously published data we observed Natural Cytotoxic Receptors (NCRs) expression upon *in vitro* activation with mitogenic stimuli in human circulating γδ T cells, we evaluated the expression of NCRs in gut resident γδ T lymphocytes (Correia et al., 2011). Interestingly, we identified a tissue specific subset of γδ T cells bearing NCRs that differ markedly from circulating γδ T cells of healthy individuals **(Fig. 8b)**. Expression of NKp46 has proved to be a dominant NCR in both IELs and LPLs populations with a significantly higher percentage in IELs compared to LPLs and PBMC **(Fig. 8b)**. Furthermore, the presence of NKp46^{pos} on γδ T cells in IEL compartment of human intestine was also confirmed by confocal microscopy analysis **(Fig. 8c)**. Levels of NKp44 receptor in γδ IELs were considerably less frequent, compared to that of NKp46; however, similar to what was observed for NKp46, the average of NKp44 expression was significantly higher in γδ IELs then in LPLs and in PBMC **(Fig. 8b)**. On the other hand, no significant differences were observed for NKp30 expression that resulted to have low and highly variable prevalence in all analyzed districts: IELs, LPLs and in PBMC **(Fig. 8b)**.

γδ IELs expressing CD8 have previously been characterized as a subset expressing CD8αα homodimers, which induces cell hypo-responsiveness/anergy (A. Hayday et al., 2001). Recently a novel CD8^{pos}αβ heterodimer γδTCR subset with a cytotoxic potential was found to be markedly enriched in human intestine and playing a key role in the homeostasis of GALT and in the pathogenesis of inflammatory bowel disease (Kadivar et al., 2016). Indeed, we observed that almost the 24.8% of the total γδ IELs presented CD8β chain on their surface **(Fig. 8d)**. Surprisingly, comparing flow cytometry analysis of the co-expression of both CD8α and CD8β chains in NKp46^{pos} γδ IELs we found that almost all cells present double staining for CD8α and CD8β, strongly indicating their CD8^{pos}αβ heterodimer phenotype **(Fig. 8d)**. This likely indicates that the presence of NKp46 identifies a specific cluster of CD8αβ/γδ IELs associated with immune effector-functions (i.e. cytotoxicity and cytokine productions)

rather than immune-tolerance.

Additional analysis based on t-distributed stochastic neighbor embedding (tSNE) on no-manual-gated CD3/CD45^{pos} lymphocytes and based on a 15-color panel (Live/dead-zombie, CD45, CD3, TCRγδ, CD69, CD103, CD56, CD8α and CD8β, CD4, NKG2A, NKG2C, NKG2D, NKp46, NKp44, NKp30) identified two main and non-overlapped clusters of NKp46^{neg} (C1) NKp46^{pos} (C2) γδ IELs distinct for NKp46 receptor expression, thus demonstrating existence of a novel intestinal NKp46^{pos} γδ IEL subset potentially involve in the gut mucosa immunosurveillance **(Fig. 9a)**. The relative heat map confirmed that C2 NKp46^{pos} cluster harbors distinctive and activating phenotype characterized by higher expression of NKp44 and NKp30, CD8β, CD56, NKG2C and NKG2D; while C1 comprised NKp46^{neg} γδ IELs by an expression of inhibitory KIRs **(Fig. 9b)**.

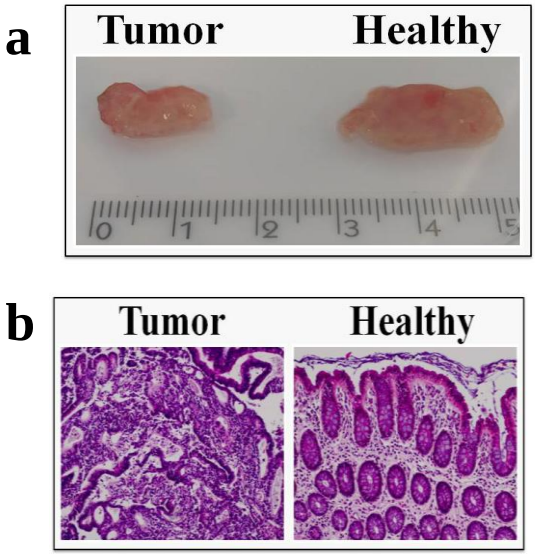


Figure 6*. Macroscopic and microscopic analysis of human colon specimen.* ***a****) Representative macroscopic example of specimens of human helthy colon (right) and in the presence Tumor (left) in patients with intestinal adenocarcinoma.* ***b****) Representative microscopic examples of a 10x hematoxylin-eosin staining showing specimens of intestine in healthy condition (right) and in the presence Tumor (left) of intestinal adenocarcinoma.*

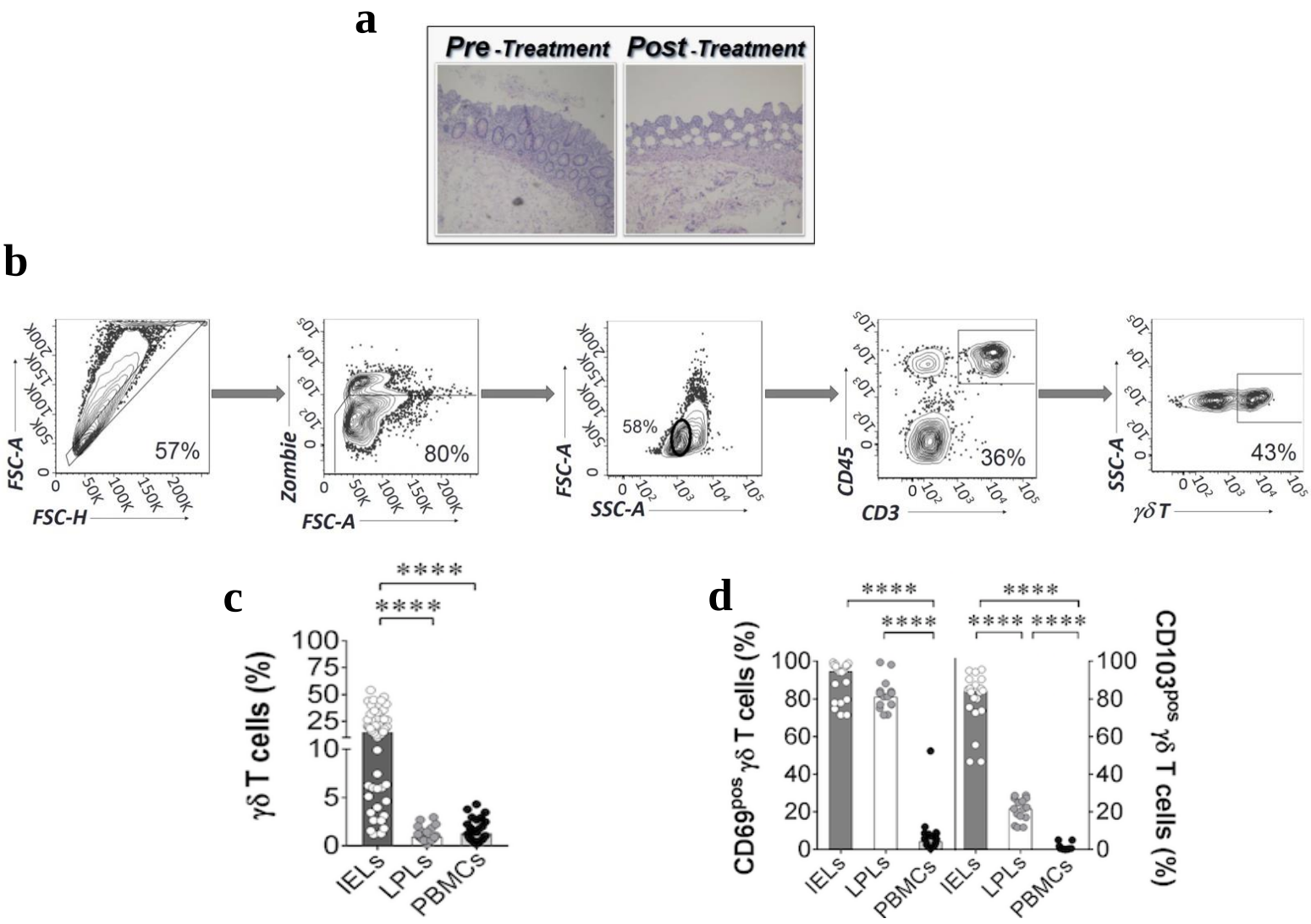


Figure 7: *Flow cytometry analysis of γδ T cells isolated from the crypts of healthy human intestine* ***a)*** *Representative example of a hematoxylin-eosin staining showing specimens of healthy human colon before (left) and after (right) the treatment with DL-Dithio-Threitolo (DDT) and Ethylene-Diamine-Tetra Acetic Acid (EDTA) to specifically remove the epithelial crypts****.*** *The left tissue was then enzymatically processed with collagenase II to obtain mononuclear cells from intestinal lamina propria.* ***b)*** *Representative example of flow cytometry dot plots showing the gating strategy used to identify viable $CD45^{pos}/CD3^{pos}$ γδ T lymphocyte both in intraepithelial (IEL) and lamina propria (LPL) compartments from specimens of human healthy colon;* ***c)*** *Summary statistical graph showing within the $CD45^{pos}/CD3^{pos}$ lymphocytes the percentages of γδ IELs (N=54 in white circles), γδ LPLs (N=20 in gray circles) from specimens of human healthy colon and γδ T peripheral blood mononuclear cells (PBMCs) (N=26 in black circles) of healthy donors;* ***d)*** *Summary statistical graph showing the expression (%) of CD69 (left) and CD103 (right) on γδ IELs (N=20 in white circles), γδ LPLs (N=15 in gray circles) from specimens of human healthy colon and on γδ T cell from PBMCs (N=20 in black circles) of healthy donors. P-value is represented as following: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, **** P ≤ 0.0001*

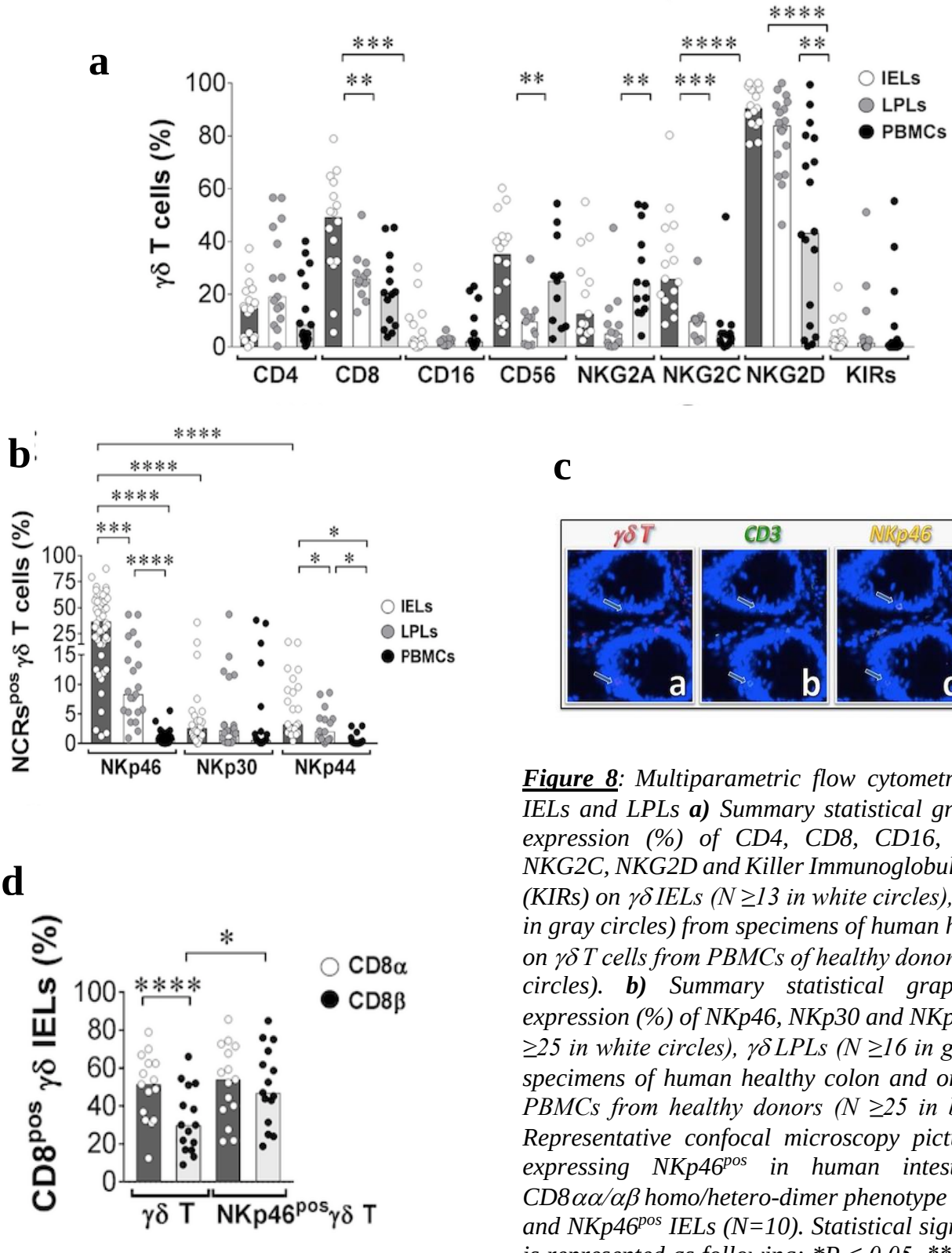


Figure 8: *Multiparametric flow cytometry analysis of γδ IELs and LPLs* ***a)*** *Summary statistical graph showing the expression (%) of CD4, CD8, CD16, CD56, NKG2A, NKG2C, NKG2D and Killer Immunoglobulin-like Receptors (KIRs) on γδ IELs (N ≥13 in white circles), γδ LPLs (N ≥10 in gray circles) from specimens of human healthy colon and on γδ T cells from PBMCs of healthy donors (N ≥13 in black circles).* ***b)*** *Summary statistical graph showing the expression (%) of NKp46, NKp30 and NKp44 on γδ IELs (N ≥25 in white circles), γδ LPLs (N ≥16 in gray circles) from specimens of human healthy colon and on γδ T cells from PBMCs from healthy donors (N ≥25 in black circles).* ***c)*** *Representative confocal microscopy picture of γδT cells expressing NKp46^{pos} in human intestinal tissue.* ***d)*** *CD8αα/αβ homo/hetero-dimer phenotype of total γδ T cells and NKp46^{pos} IELs (N=10). Statistical significance P-value is represented as following: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, **** P ≤ 0.0001.*

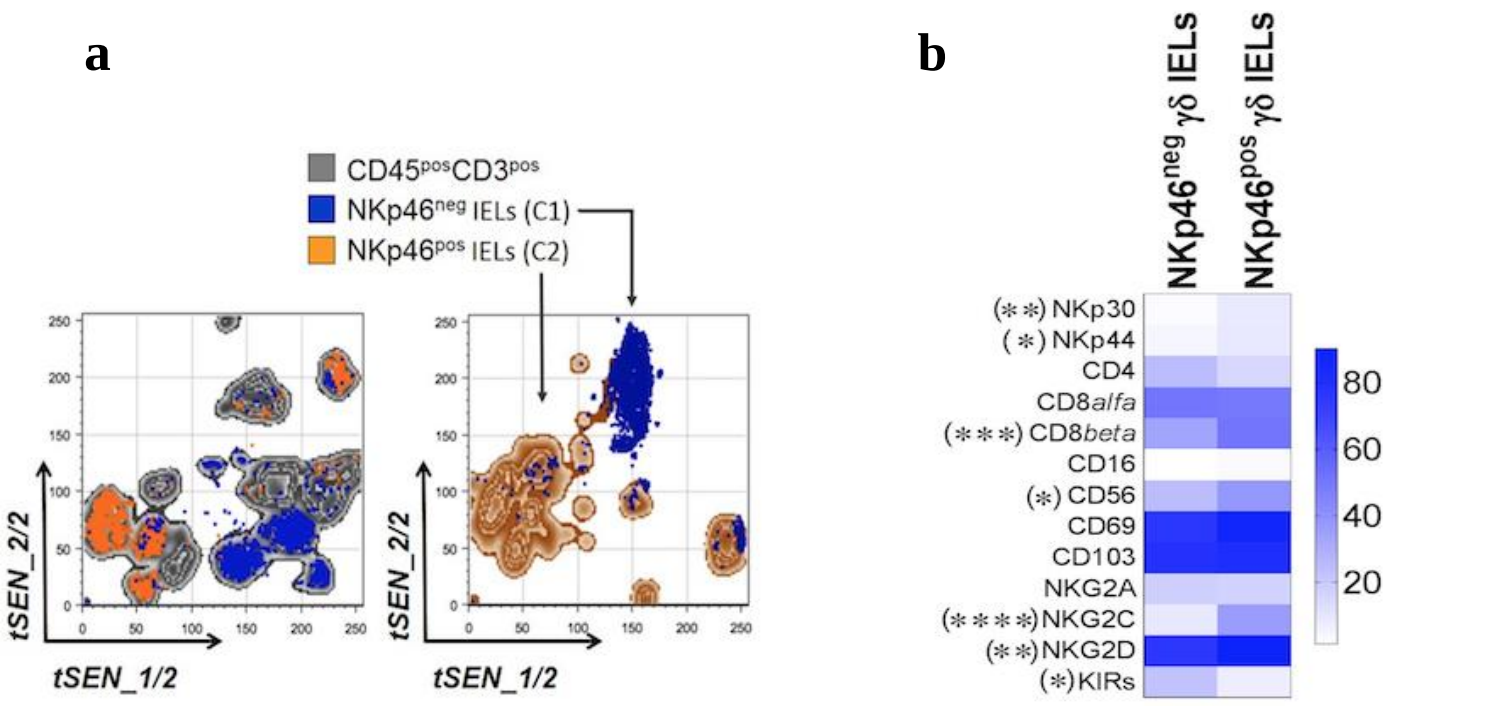


***Figure 9**: t-SNE analysis of γδ IELs isolated from human healthy colon. **a)** One representative specimen of human healthy colon showing the clustering of NKp46^{neg} (C1 in blue) and NKp46^{pos} (C2 in orange) γδ IELs within total CD45^{pos} /CD3^{pos} lymphocytes (gray). **b)** Heat-map showing the degree of expression of several surface markers on the NKp46^{neg} and NKp46^{pos} γδ IELs clusters defined as C1 and C2 (N=7). Statistical significance P-value is represented as following: *P≤0.05; **P≤0.01; ***P≤0.001; ****P≤0.0001.*

3.2 NKp46^{pos} γδ IELs are Vδ1 restricted and preferentially enriched in human intestine

Human γδ T cells are primarily identified by their Vδ chain usage and are classified in two main Vδ1 and Vδ2 subtypes (A. Hayday et al., 2001). Vδ1 characterize thymus and peripheral tissue, while Vδ2 constitute the majority of blood circulating γδ T cells (Vantourout & Hayday, 2013). To better characterize the novel subset of NKp46^{pos} γδ T cells, lymphocytes isolated from circulating blood, gut epithelium and lamina propria were stained whit antibodies specific for Vδ1 or Vδ2 TCR chain and identified by the gating strategy shown in **Fig. 10a**. As expected, blood circulating CD3^{pos} lymphocytes were mainly Vδ2 positive than Vδ1 and represented 74 % of total circulating γδ T cells (**Fig. 10b)**. On the contrary Vδ1^{pos} population was the dominate subset among IELs compared to Vδ2^{pos} and they reached respectively 54% and 32% of the relative distribution in total IELs (**Fig. 10b)**. On the other hand, no significant differences were observed in frequencies of Vδ1 and Vδ2 in LPLs compartment, and 30% of γδ LPLs neither express Vδ1 nor Vδ2 chain, (**Fig. 10b).** This suggests that the LPLs compartment in the gut might be, together with human liver, another preferential tissue where other subpopulations such as Vδ3^{pos} reside (D. Wu et al., 2017) (Chen, Zou, Teng, Zhang, & He, 2017).

Interestingly the expression of NCRs in IELs was much larger in the Vδ1 subset than in its Vδ2 counterparts **(Fig. 11a-b)**. In fact, there was a clear predominance of Vδ1 usage among NKp46^{pos} γδ IELs compared to the Vδ2 group **(Fig. 11b)**. Similarly, NKp44 receptor expression was significantly higher in Vδ1 then Vδ2 subsets. On the contrary both Vδ1 and Vδ2 IELs express very low levels of NKp30 **(Fig. 11b).**

Vδ1^{pos} IELs were characterized by a CD4^{neg}/CD8^{pos} phenotype with high surface levels of CD56 and NKG2D in the presence of low surface levels of inhibitor marker NKG2A and KIRs. These features characterize specifically Vδ1^{pos} IELs as their Vδ2^{pos}

counterparts express significantly lower levels of CD8 and CD56 together with significantly higher amounts of NKG2A **(Fig. 11c)**.

To compare the distribution of the specific NKp46^{pos}/Vδ1^{pos} subset in different human intestinal segments we analyzed IELs from the matched ileum and colon of healthy specimens obtained from patients with Crohn's disease. As similar percentage of Vδ1 and Vδ2 IELs in ileum and colon goes along with the comparable expression of NKp46 receptor in both compartments linked to the Vδ1 subset **(Fig. 12a-b)**. These results indicate that NKp46^{pos}/Vδ1^{pos} lymphocytes represent the largest and ubiquitous γδ T cell population within the epithelial compartment of both small and big intestine.

We also investigated the expression of NCR^{pos} γδ T cells in other human healthy tissues known to be enriched with high frequencies of γδ T cells such as skin, liver and lymph nodes (Nielsen et al., 2017) (Hudspeth et al., 2016). As expected, both human skin and liver compartments are enriched with the Vδ1 cell subset over the Vδ2 populations **(Fig. 12c)**. However, Vδ1^{pos} and Vδ2^{pos} subsets do not constitutively express NCRs in other peripheral tissue sites **(Fig. 12c)**. As expected in lymph nodes we did not find any prevalence in the frequency between Vδ1 and Vδ2 subsets; furthermore, cells purified from lymph nodes were negative for both NKp46 and NKp44 and express very low amount of NKp30 **(Fig. 12c)**.

Even though murine and human γδ T cells differ in several phenotypic features and tissue distribution, Vδ1 T cells have been shown to be more similar between the two species compared to their Vδ2 counterparts (Pang, Neves, Sumaria, & Pennington, 2012). In addition, NKp46, is the only NCR to be phylogenetically conserved in mice (A. Moretta, Bottino, Mingari, Biassoni, & Moretta, 2002).

To address the possibility that the specific NKp46^{pos} γδ T subset may have an immunological correspondent in mice we attempted to analyze diverse anatomical sites in BALB/c and C57BL/6 mouse models. Nevertheless, a small percentage of γδ T mice cells isolated from the mucosa of small intestine and colon as well as from spleen

presented NKp46 expression on their surface **(Table 2)**. This low recurrence was maintained independently from different immune Th1- and Th2-dominant response of mouse strains.

Overall, these data indicate that this newly disclosed subset of NKp46^{pos}/Vδ1^{pos} T cells that is preferentially expanded in the intestinal epithelium, is human specific and represents by far the largest IEL population in the gut.

In addition, there was no statistical significant correlation between the recurrences of total γδ IELs (**Fig 13a**) population or the specific NKp46^{pos}/Vδ1^{pos} subset (**Fig 13b)** and age of patients that resulted with the mean of age 64 and the range 22-92 years old. Similarly, male and female individuals have equivalent frequencies of both γδ IELs as well as a comparable expression level of NKp46^{pos} receptor on Vδ1 cells **(Fig 13c-d).**

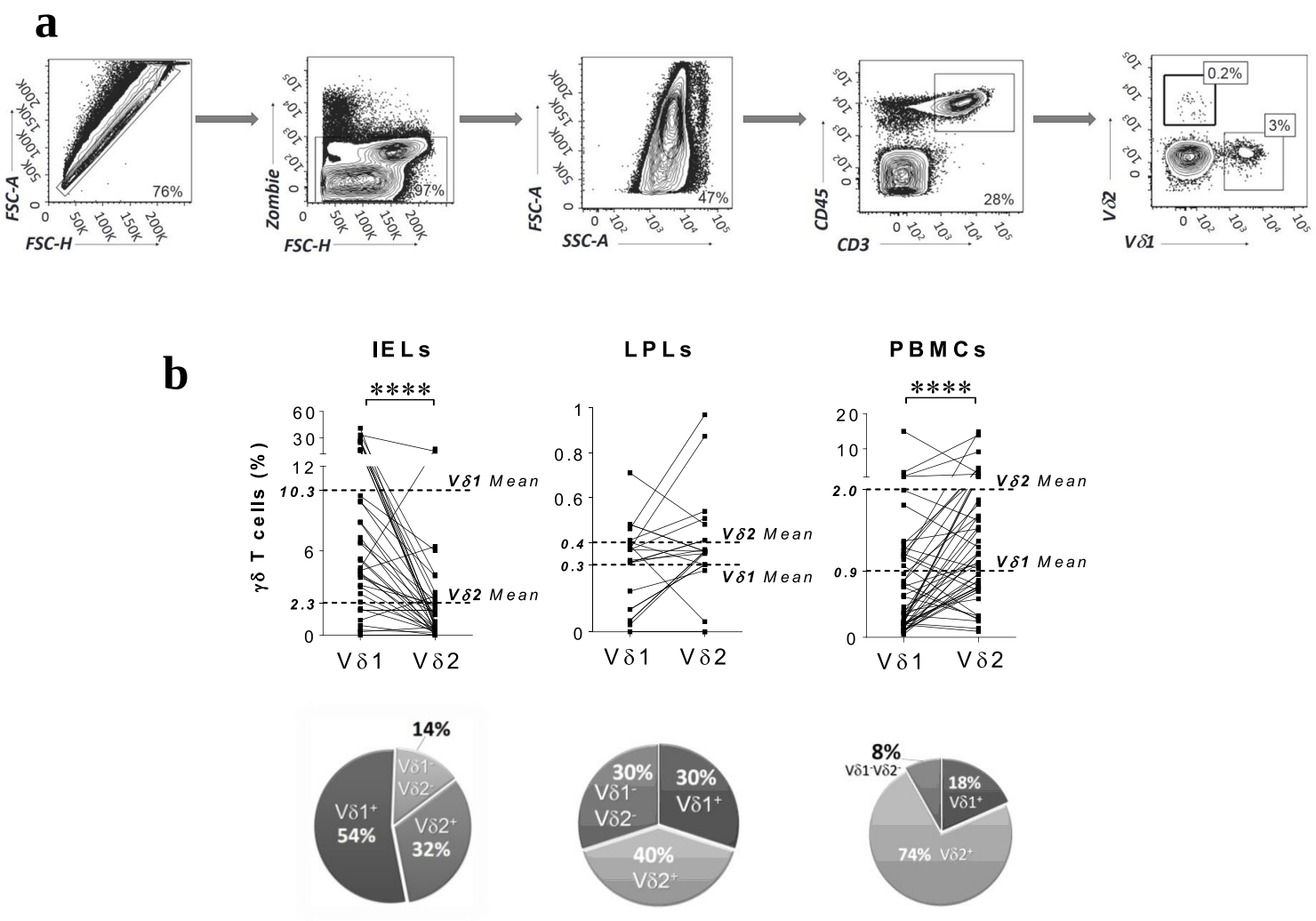


Figure 10: *Freshly isolated IELs, LPLs were stained as described in the Methods section and analyzed by flow cytometry in order to investigate the expression of Vδ1 or Vδ2 chains:* ***a)*** *Gating strategy of one representative plots experiment used to identify Vδ1 and Vδ2 subsets among total human intestinal IELs;* ***b)*** *Summary statistical graphs (upper line) showing within the entire CD45^{pos}/CD3^{pos} lymphocyte population the percentages of Vδ1 or Vδ2 IEL (N≥37) and LPL (N ≥22) subsets from specimens of human healthy colon and from PBMCs (N=49) of healthy donors. Pie charts (lower line) showing the percentages of Vδ1^{pos}, Vδ2^{pos} and Vδ1^{neg}/Vδ2^{neg} subsets within total γδ T cells of matched samples (N=10) of IELs and LPLs from specimens of human healthy colon and PBMCs of healthy donors. P-value is represented as following: **** P ≤ 0.0001*

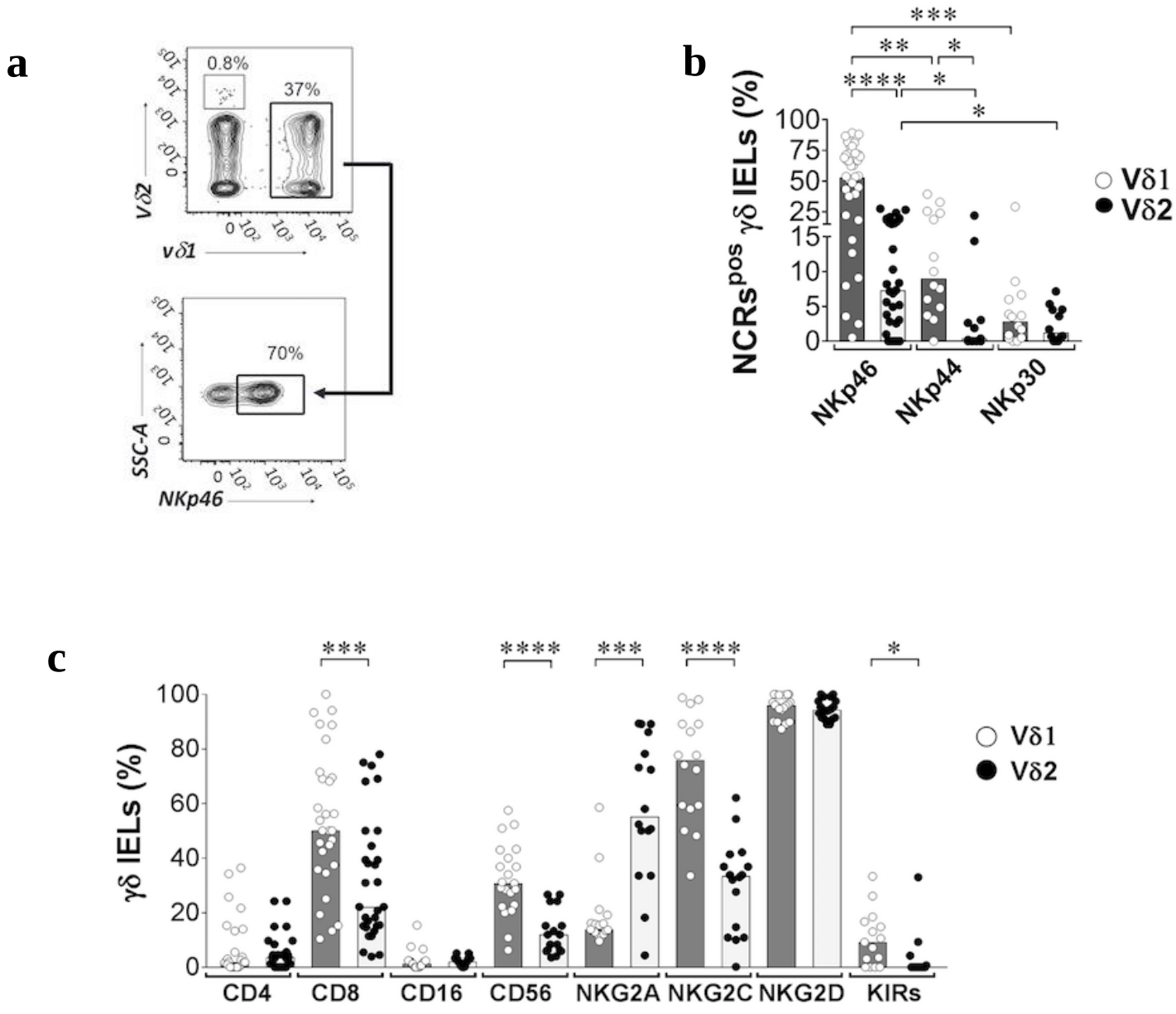


Figure 11: *Multiparametric flow cytometry analysis of human intestinal γδ IELs Vδ1 and Vδ2 subsets expressing NCRs.* ***a)*** *Representative example of contour plots showing the percentages of Vδ1 and Vδ2 T cell subset (upper panel) and of NKp46^{pos}/Vδ1^{pos} T cells (lower panel) within total purified γδ IELs from a specimen of human healthy colon.* ***b)*** *Summary statistical graph showing the frequencies of Vδ1 (white circles) and Vδ2 (black circles) IELs (N ≥20) from specimens of human healthy colon expressing NKp46, NKp30 and NKp44.* ***c)*** *Summary statistical graph showing expression of CD8, CD16, CD56, NKG2A, NKG2D and Killer Immunoglobulin-like Receptors (KIRs) on Vδ1 (white circles) and Vδ2 (Black circles) IELs (N ≥16) from specimens of human healthy colon. P-value is represented as following: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, **** P ≤ 0.0001.*

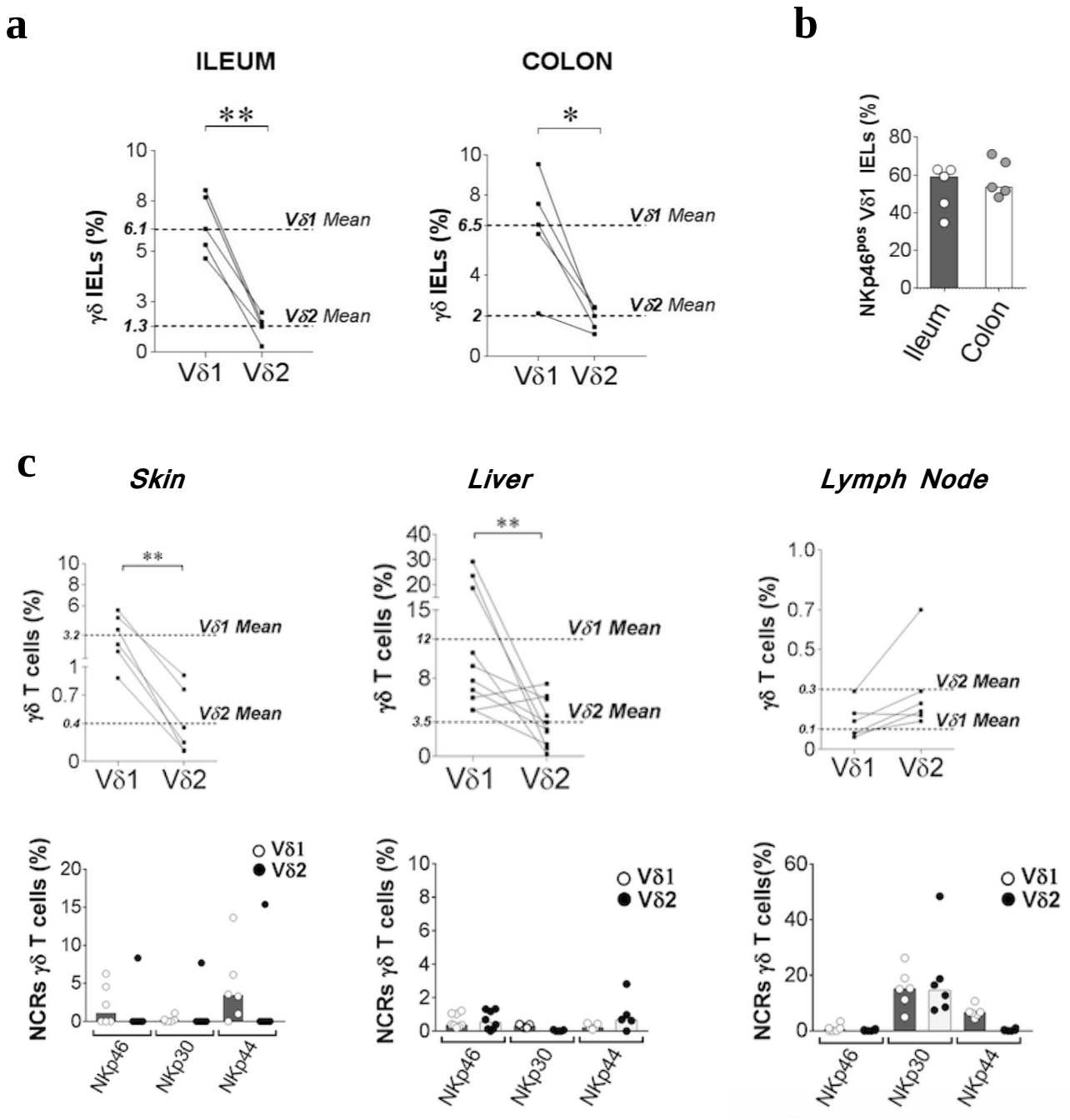


Figure 12: *Flow cytometry analysis of γδ T cells in different human tissues.* ***a)*** *Summary statistical graphs showing within the total CD45^{pos}/CD3^{pos} lymphocyte population the percentages of Vδ1 and Vδ2 IEL subsets from specimens of matched human healthy ileum (N=5) and colon (N=5).* ***b)*** *Summary statistical graph showing the frequencies of NKp46^{pos}/Vδ1^{pos} IELs from specimens of matched human healthy ileum (N=5 in white circles) and colon (N=5 in gray circles).* ***c)*** *Frequency of Vδ1 (N=6 withe circle) and Vδ2 (N=6 black circle) (upper graph) and NCR^{pos} Vδ1/Vδ2 (lower graph) subsets isolated from skin, liver and lymph node. P-value is represented as following: *P ≤ 0.05, **P ≤ 0.01.*

Table 2

Expression of NKp46 in mouse γδ T lymphocytes.

Sample / *Tissue*	Balb/c 5 weeks		C57BL/6 5 weeks	
	γδ T	NKp46^{pos} γδ T	γδ T	NKp46^{pos} γδ T
Colon (IELs)	**3.7 %** *(±0.28)*	**3.1 %** *(±0.28)*	**10.3 %** *(±0.66)*	**1.9 %** *(±0.16)*
Small Intestine (IELs)	**53.7 %** *(±1.41)*	**1.2 %** *(±0.58)*	**32.7 %** *(±0.77)*	**1.8 %** *(±0.99)*
Spleen (Splenocytes)	**0.7 %** *(±0.01)*	**4.1 %** *(±0.22)*	**0.9 %** *(±0.02)*	**2.9 %** *(±0.32)*

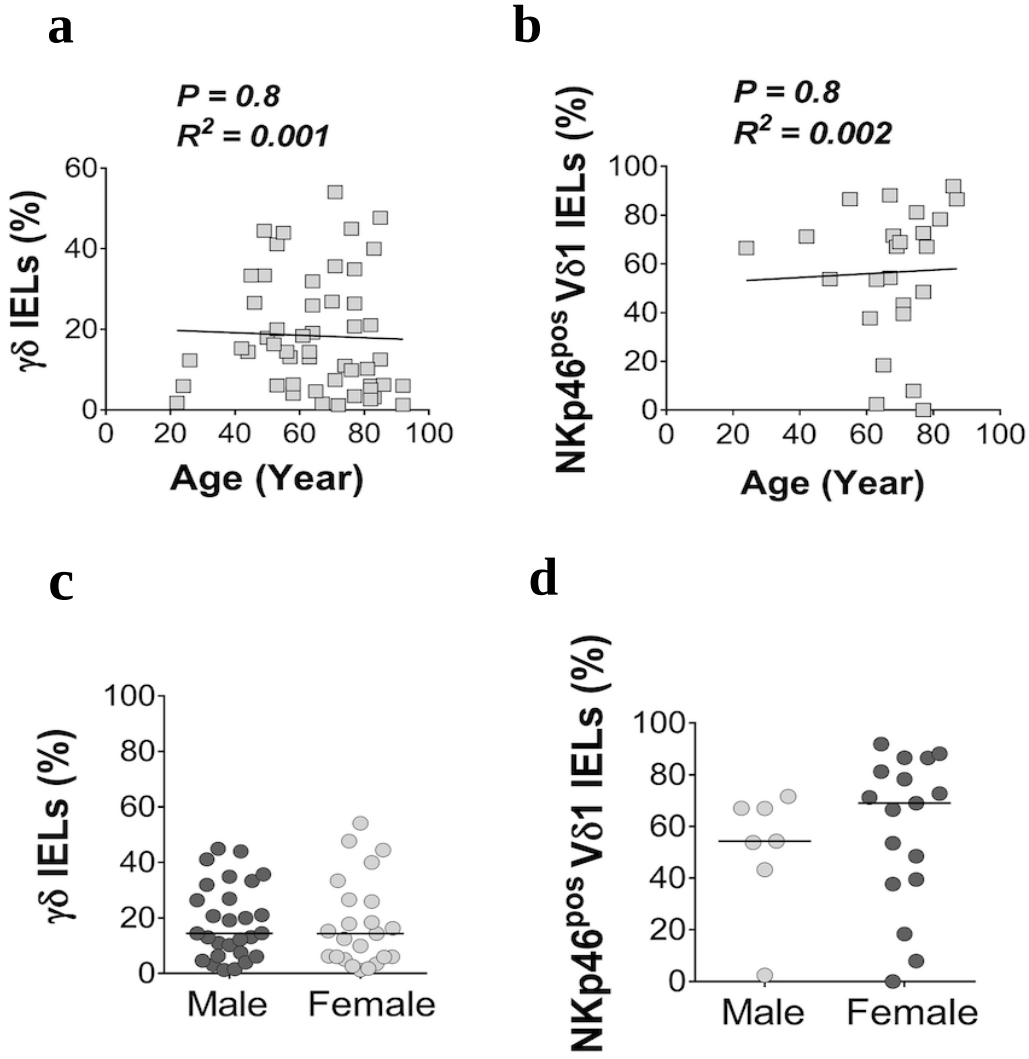


Figure 13: *Impact of sex and age on the frequency of γδ T cells and NKp46^{pos} Vδ1 cell subset within intraepithelial intestinal compartment.* ***(a-b)*** *Statistical analyses showing the correlation between the percentages of either γδ IELs (**a**) or NKp46^{pos} Vδ1* ***(b)*** *IELs specimens from human healthy colon and the age of donors enrolled in the study.* ***(c-d)*** *Summary statistical analyses showing the percentages of either γδ IELs* ***(c)*** *or NKp46^{pos} Vδ1* ***(d)*** *IELs from human healthy colon in male and female donors enrolled in the study*

3.3 Expression of NKp46 on γδ IELs is associated with higher degree of cytotoxicity and production of IFN-γ

Intestinal innate and adaptive immune cells are involved in important immune-regulatory functions to ensure both a correct homeostasis of GALT and a proper immune-surveillance against different pathogens and tumors. This includes the production of cytokines such as IL-10, IL-17, IL-22 and TGF-β that regulate the gut immune-microenvironment and the survival of intestinal epithelial cells (Bamias, Arseneau, & Cominelli, 2014). Hence, we tested the transcript levels of these cytokines on fresh FACS-sorted NKp46^{pos} and NKp46^{neg} γδ IELs compared to their γδ T cells circulating counterparts. Our results showed that both NKp46^{pos} and NKp46^{neg} γδ IELs express similarly high amounts of IL-22 mRNA compared to blood γδ T cells, thus highlighting their key roles in intraepithelial immune responses and homeostasis **(Fig.14 a)**. No differences were found for IL-10, IL-17 and TGFβ transcripts between NKp46^{pos} and NKp46^{neg} γδ IELs and circulating γδ T cells **(Fig.14 a)**.

We then analyzed their ability to kill human leukemia K562 cell line, a tumor cell target widely employed to test the cytotoxicity of γδ T cells (Gomes et al., 2010). The results showed that the intra-cellular levels of the cytolytic granule Granzyme B is constitutively higher in freshly purified NKp46^{pos} γδ IELs compared to the NKp46^{neg} counterparts **(Fig.14 b)**. Although incubation with K562 increases the amount of Granzyme B both in NKp46^{pos} and NKp46^{neg} γδ IELs, the overall levels of this cytotoxic granule is significantly higher in the first subset compared to the second one **(Fig.14 b)**. We then measured the degree of CD107a degranulation assay as a marker of cytolytic activity. Our results indicate that the frequencies of CD107a^{pos}/NKp46^{pos} γδ IELs are significantly higher compared to that CD107a^{pos}/NKp46^{neg} γδ IELs following the co-culture with K562 **(Fig.14 c)**. Indeed, low levels of CD107a expression characterize this latter subset of IELs even after the stimulation with the tumor cell target, thus indicating that the presence of NKp46 specifically marks cytolytic functions among γδ IELs

(Fig.14 c). Similar to results and statistic differences observed for CD107a we found higher IFN-γ production in NKp46^{pos} compared to NKp46^{neg} γδ IELs after incubation with K562 **(Fig.14 d)**

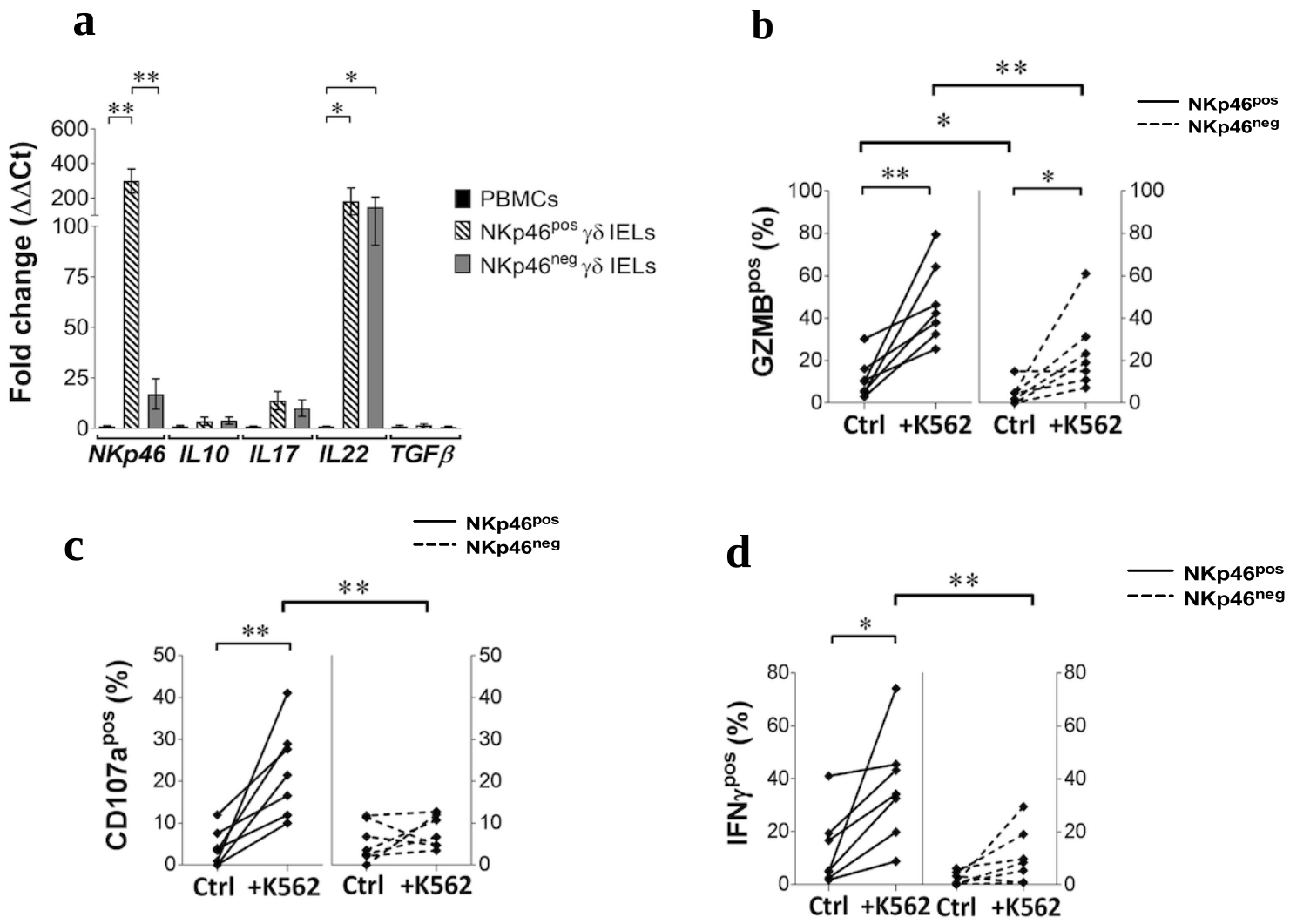


Figure 14: *Effector-functions of NKp46^{pos} γδ T IELs.* ***a)*** *Summary statistical graphs showing the transcript levels of NKp46, IL-10, IL-17, IL-22 and TGFβ in FACS-sorted NKp46^{pos} and NKp46^{neg} γδ IELs from specimens of human healthy colon and in FACS-sorted γδ T cell from PBMCs of healthy donors (N=6). Results are presented as fold changes ($2^{-\Delta\Delta CT}$) of target gene relative to PBMC samples and normalized to housekeeping gene GAPDH.* ***Freshly isolated IELs subsets were co-cultured without (Ctrl) or with K562 cells as described in the Methods section and analyzed by flow cytometry for:*** ***b)*** *Intracellular staining of GZMB, (N=7);* ***c)*** *Degranulation cell membrane marker CD107a (N=7).* ***d)*** *Intracellular staining of IFN-γ (N=7). Statistical significances were calculated with two-way, paired t-test. P-value is represented as following: *P ≤ 0.05, **P ≤ 0.01.*

3.4 Human γδ thymocytes resemble the phenotype and functions of NKp46^{pos} Vδ1 IELs following activation.

Maturation of γδ T cells occurs during thymus development that commits γδ T precursors to properly differentiate and migrate to peripheral tissues (Van de Walle et al., 2013). Recently it has been reported that acquisition of both cytotoxicity and ability to produce IFN-γ by γδ thymic precursors is not associated with the engagement of TCR, but requires activation with either IL-2 or IL-15 (Ribot, Ribeiro, Correia, Sousa, & Silva-Santos, 2014). In order to understand whether these two cytokines could induce the phenotype and functions similar to those of NKp46^{pos}/Vδ1^{pos} IELs at the level of γδ thymic precursors, we purified γδ thymocytes from healthy thymus collected from pediatric patients undergoing open cardiac surgical procedures. Stimulation of γδ thymocytes with either IL-2 or IL-15 induced a statistically significant increase of NKp46, while this was not the case for γδ thymocytes incubated with IL-7 **(Fig. 15a)**. Interestingly, the activation with IL-2 induced the preferential expansion of the NKp46^{pos}/Vδ1^{pos} cell subset, as we did not detect any significant increase of the Vδ2 cell compartment **(Fig. 15 b-c)**. IL-2 induces also on γδ thymocytes significant *de novo* expression of NKp44 and NKp30 receptors, although at less extent compared to that of NKp46 **(Fig. 15d)**. We did not detect any induced-expression of NCRs on αβ thymocytes or peripheral γδ T cells following the incubation with IL-2 **(Fig. 15d)**.

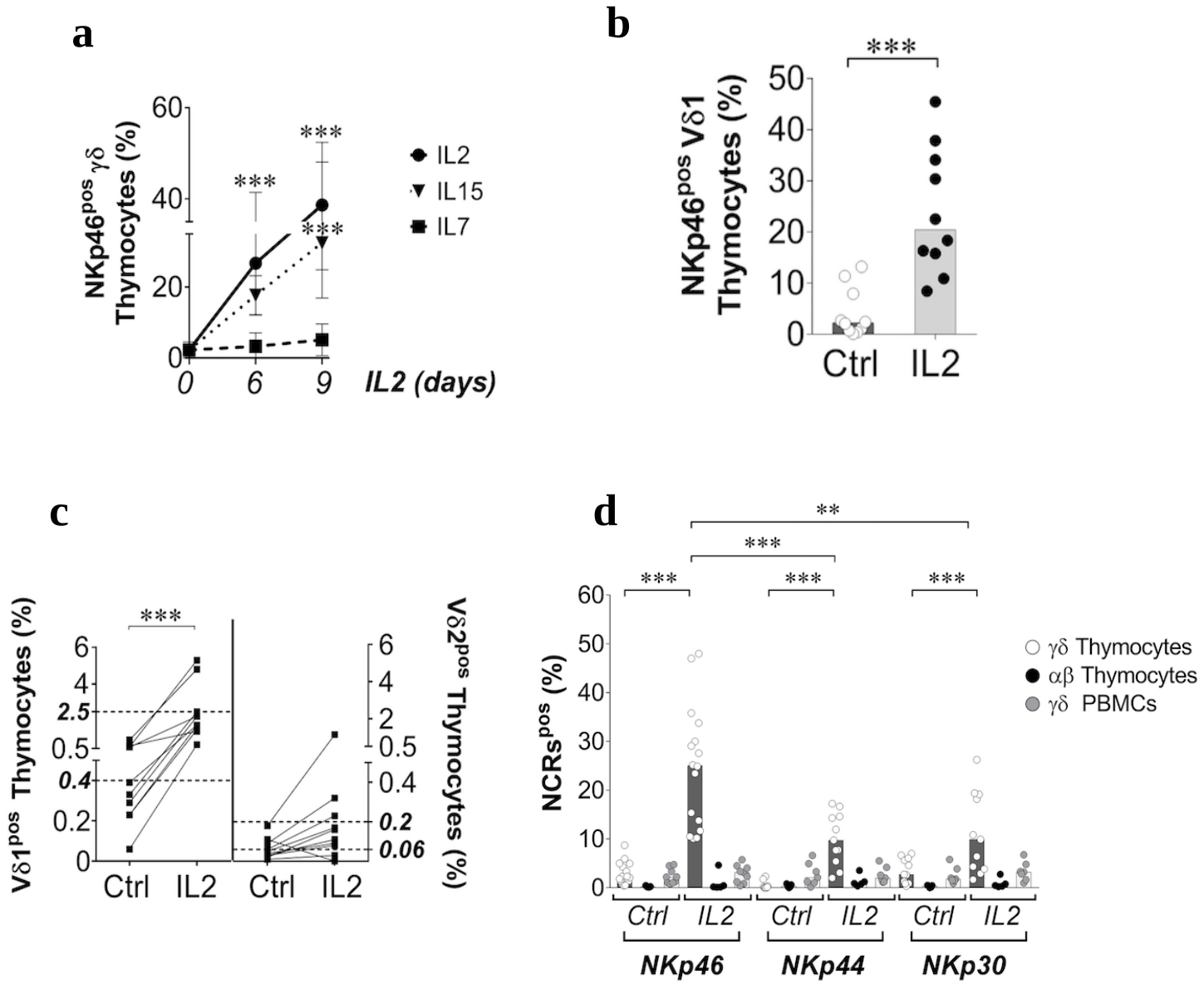


Figure 15: *Expansion of NKp46^{pos}/Vδ1^{pos} T cells following activation of thymocyte precursors.* ***a)*** *Summary statistical graphs showing the time course expression of NKp46 in γδ thymocyte precursors (N ≥5) cultured with IL-2 (200 U/mL) or IL-15 (10 ng/mL) or IL-7 (10 ng/mL).* ***b)*** *Summary statistical graphs showing the frequency of NKp46 expression in Vδ1 thymocytes cultured either in the absence (Ctrl) or in the presence of IL-2 for 6 days;* ***(c)*** *Summary statistical graphs showing the frequencies of Vδ1 (left graph) and Vδ2 (right graph) thymocytes cultured either in absence (Ctrl) or presence of IL-2 for 6 days (N=10).* ***(d)*** *Summary statistical graphs showing NCRs' expression in γδ and αβ thymocytes as well as in γδ T cells from PBMCs of healthy donor cultured either in absence (Ctrl) or in the presence of IL-2 for 6 days (N ≥5). P-value is represented as following: *P ≤ 0.01, **P ≤ 0.01 ***P ≤ 0.001.*

The phenotype of Vδ1^{pos} cells expanded from γδ thymocytes precursors after IL-2 activation resembles the one similar to Vδ1^{pos} IELs, freshly purified from human intestine **(Fig. 16a)**. Indeed, the incubation with IL-2 increases on proliferating Vδ1 thymocytes the expression of CD8, CD56 and NKG2D while decreases the surface levels of CD4 **(Fig. 16a)**. Only, NKG2A resulted differently expressed on Vδ1^{pos} cells generated from IL-2 activated thymic precursors compared to freshly purified intestinal Vδ1^{pos} IELs. Indeed, while this latter subset naturally expresses very low levels of this iNKR, the stimulation of thymic precursors with IL-2 induced a remarkably high *de novo* expression of NKG2A on Vδ1^{pos} T cells **(Fig. 16a).**

The IL-2 stimulation is also associated with an induced expression of CCR9, a chemokine-receptor kwon to be involved in the homing of γδ T lymphocytes to the gut mucosa **(Fig. 16a)** (Vantourout & Hayday, 2013). The CCR9 on γδ thymocytes, IL-2 activated and expressing NKp46, appeared functionally relevant as demonstrated by chemotactic response to its CCL25 ligand in a dose-dependent manner **(Fig. 16b)**. The acquisition of NKp46 phenotype following incubation with IL-2 on γδ thymocytes is coupled with higher transcription levels of GZMB **(Fig. 16c)** as well as with an increased degranulation capability (CD107a) against K562 and human tumor epithelial colorectal Caco2 cell line **(Fig. 16d)**. Altogether, these data show that IL-2 mediated differentiation of human Vδ1^{pos} T precursors results in the acquisition of the gut-like phenotype and an anti-tumor activity with a therapeutic potential for tissue *in situ* application.

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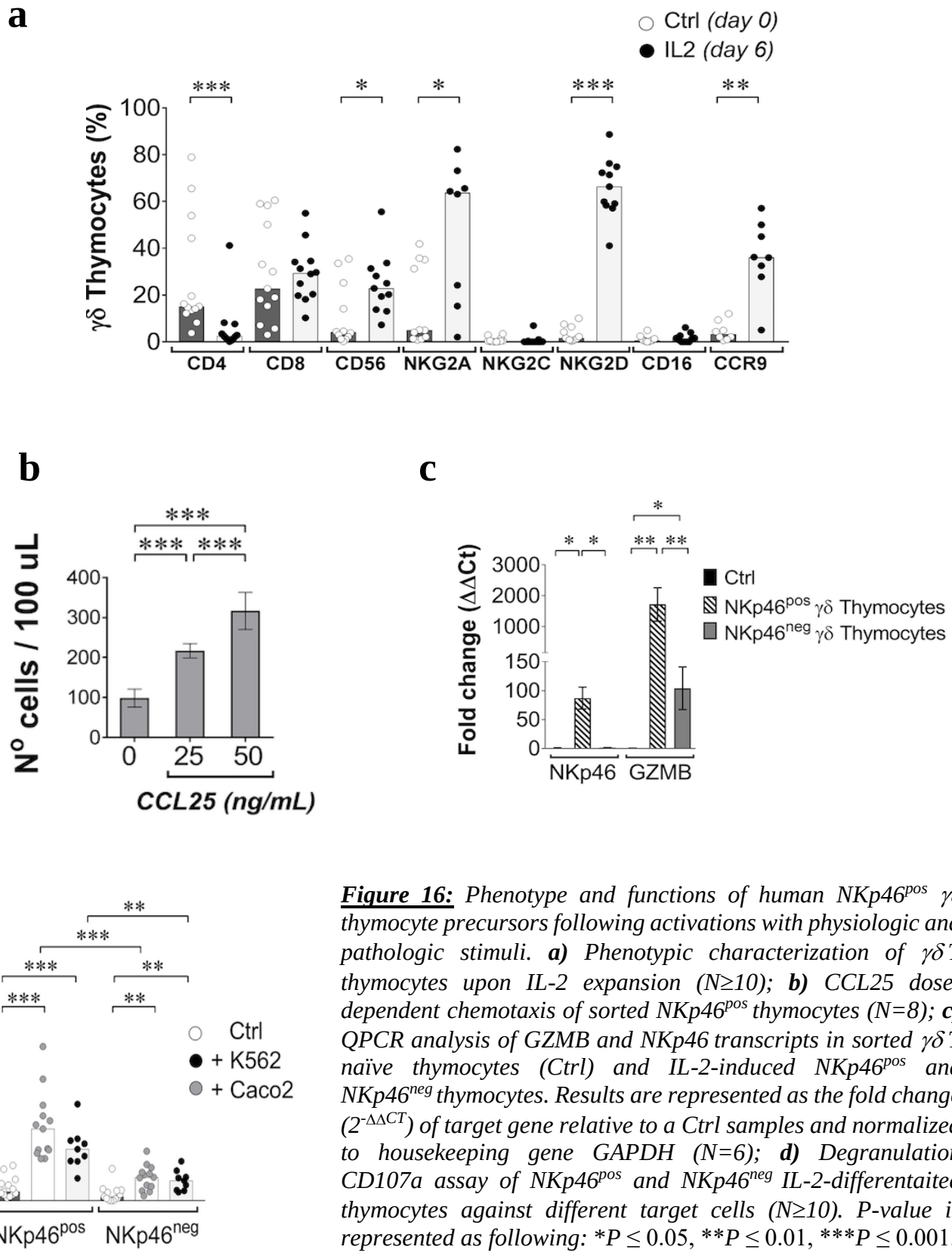


Figure 16: *Phenotype and functions of human NKp46^{pos} γδ thymocyte precursors following activations with physiologic and pathologic stimuli.* ***a)*** *Phenotypic characterization of γδ T thymocytes upon IL-2 expansion (N≥10);* ***b)*** *CCL25 dose-dependent chemotaxis of sorted NKp46^{pos} thymocytes (N=8);* ***c)*** *QPCR analysis of GZMB and NKp46 transcripts in sorted γδ T naïve thymocytes (Ctrl) and IL-2-induced NKp46^{pos} and NKp46^{neg} thymocytes. Results are represented as the fold change ($2^{-\Delta\Delta CT}$) of target gene relative to a Ctrl samples and normalized to housekeeping gene GAPDH (N=6);* ***d)*** *Degranulation CD107a assay of NKp46^{pos} and NKp46^{neg} IL-2-differentaited thymocytes against different target cells (N≥10). P-value is represented as following: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.*

3.5 Impact of intestinal microenvironment in the maturation of IL2-activated γδ thymocytes

The preferential enrichment of NKp46^{pos}/Vδ1^{pos} T cells in the intestinal epithelial compartment implicates their interaction with intestinal epithelial cells (IECs). Indeed, the survival and the retention of IELs depends on the ligand-activated transcription factor aryl hydrocarbon receptor (AHR) and IL-15 production by neighboring IECs (Qiu, Wang, Xiao, & Yang, 2015). We then incubated γδ thymocytes with IL-2 in the presence or absence of either primary Human Colonic Epithelial cells (HCoEpic) or IL-15 and we observed that both these elements have a synergistic effect in the increased expression of NKp46 on Vδ1^{pos} proliferating cells **(Fig. 17)**. In contrast, we did not observe any changes in NKp46 expression after co-cultured with thymic stromal lymphopoietin (TSLP), the soluble factor produced by IECs to induce T cell maturation at this tissue site (Ziegler & Liu, 2006) **(Fig. 17)**. We also assessed the possible roles of IL-10, IL-12 and IL-22 in modulating the surface levels of NKp46 on IL-2-differentatied γδ thymocytes. Again, these experiments did not reveal any impact of IL-10, IL-12 and IL-22 in modulating the expression of NKp46 on γδ thymocytes (data not shown). We also analyzed the contribution given by the transforming growth factor β (TGFβ) that emerged as a key architect of tumor microenvironment in CRCs with poor prognosis (Tauriello & Batlle, 2016). Interestingly, TGFβ exerts a significant inhibitory effect on the expression of NKp46 on IL-2 activated γδ thymocytes **(Fig. 17)**. Similar results were obtained when γδ IL-2 stimulated thymocytes were co-cultured with Caco2 cells, thus confirming that the intestinal tumor microenvironment is endowed with an escape mechanism impairing the cytolytic potential of NKp46^{pos}/Vδ1^{pos} cells.

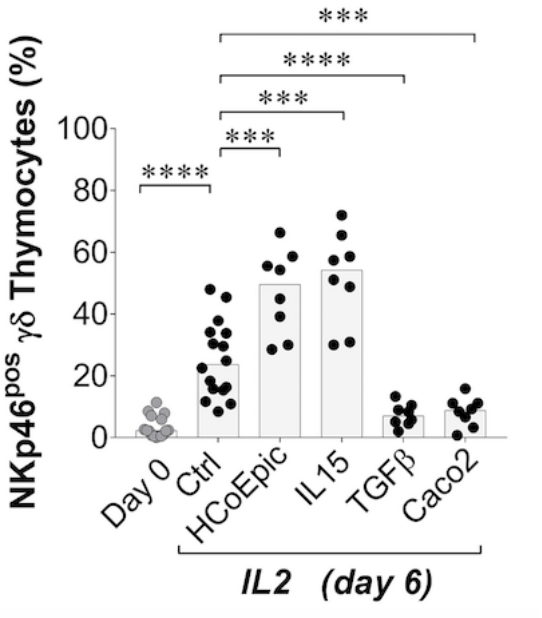


Figure 17: *Summary statistical graphs showing NKp46 expression on freshly purified thymocytes (Day 0) and on γδ thymocytes cultured with Il-2 for 6 days either in the absence (Ctrl) or in the presence of primary human colonic epithelial cells (HCoEpic) or IL-15 or Tumor Growth factor β (TGFβ) or Caco2 cell line (N ≥ 8). P-value is represented as following: ***P ≤ 0.001, **** P ≤* 0.0001.

3.6. Clinical outcome of NKp46^{pos}Vδ1^{pos} subset in human CRC

To comprehend the possible clinical outcome of the specific NKp46^{pos}Vδ1^{pos} subset in human gut disorders and in particular in CRC, we approached the patient-related data analysis. To investigate whether γδ T cells subsets in the "disease-free/healthy" tissue might represent prognostic marker in colon carcinoma we looked for a correlation between frequencies of different γδ T cells subsets and the TNM stage disease. Patients included in the analysis were restricted to either early (I/II) or late (III/IV) TNM stages. Furthermore, patients that required chemotherapy treatment before surgical resection of CRC were excluded from the analyses to avoid any bias on T lymphocytes recurrence. We first observed that the frequency of total γδ IELs in healthy fragments of colon from CRC patients was not predictive for the development of TNM stages disease **(Fig. 18a)** and no significant changes in both Vδ1 and Vδ2 subsets distribution were observed **(Fig. 18b)**.

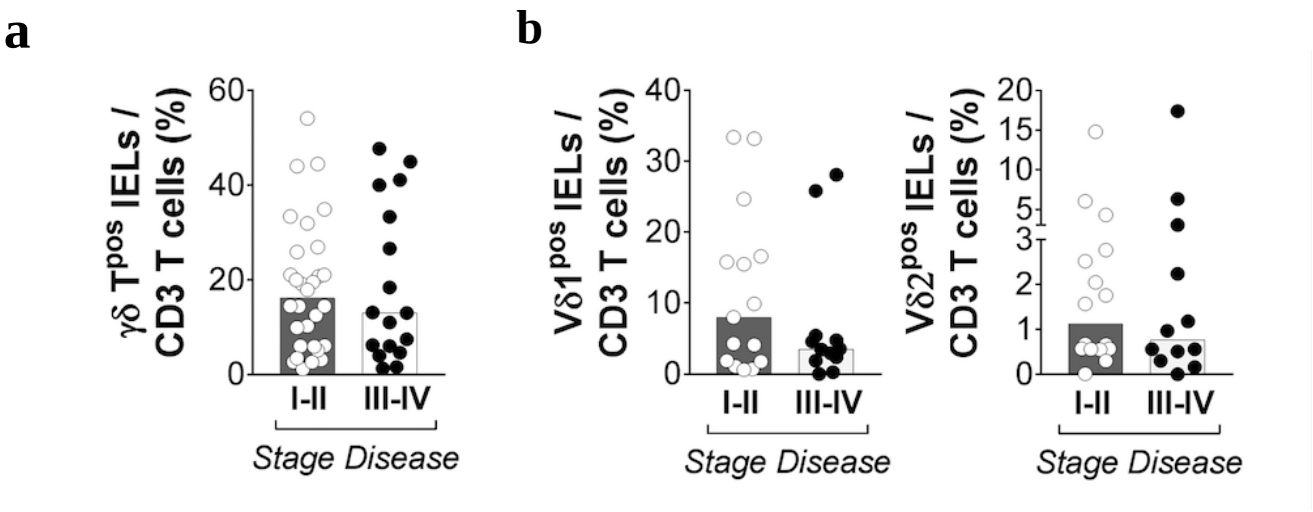
Interestingly, we found a trend of the inverse correlation between expression of NKp46 in γδ IELs and tumor progression. More specifically we observed that tumor-free gut specimens of CRC patients that developed higher stage III/IV contained a significantly lower frequency of NKp46^{pos}/Vδ1^{pos} IELs with average of (31.7% ±25.7), compared to those at stage I/II (72.5% ±12.4) **(Fig. 18c)**. This data indicates that higher occurrence of NKp46^{pos}/Vδ1^{pos} IELs may exert a beneficial effect on the very early stage of tumor progression. This positive influence was specific for the NKp46^{pos}/Vδ1^{pos} subset since no changes were observed in the much less represented cell subset of NKp46^{pos}/Vδ2^{pos} **(Fig. 18c)**.

We then evaluated the ability of NKp46^{pos}/Vδ1^{pos} subset to infiltrate CRC tumor mass. Since this adenocarcinoma originates directly from epithelial cells it lacks anatomical organization of normal gut mucosa in epithelial and lamina propria compartment. Therefore, in order to comprehend the valid occurrence of tumor-associated NKp46^{pos}/Vδ1^{pos} subset the analysis of cancer tissue was performed entirely

without separation into two distinct compartments of epithelium and lamina propria. The frequency of Vδ1 cells in tumor specimens was lower compared to the healthy IELs. **(Fig. 19a)**. Likewise, the average of tumor restricted NKp46^{pos}/Vδ1^{pos} was much lower compared to the abundance of this specific subset found in healthy IELs and was lower to the level observed in healthy LPLs **(Fig. 19b)**.

Recently it was observed that patients affected by some IBD such as ulcerative colitis (UC) are at higher risk of develop CRC (Janakiram & Rao, 2014). Therefore, we evaluated the frequency of Vδ1 cells and the specific NKp46^{pos}/Vδ1^{pos} subset in patients with UC. Similar to the tumor, patient affected by UC have decreased amount of both Vδ1**(Fig. 19a)** and NKp46^{pos}/Vδ1^{pos} cells **(Fig. 19b)**. Compared to healthy specimens and thus implicating pro-inflammatory conditions to promote the abolition of these cells.

Taken together, these data indicate that the presence of high frequencies of NKp46^{pos}/Vδ1^{pos} IELs in human intestines might be used as a positive prognostic marker to predict CRC progression. On the other side, both inflammatory and tumor microenvironments are associated with escape mechanisms inhibiting either the migration or the in situ-expansion of anti-tumor NKp46^{pos}/Vδ1^{pos} IELs.



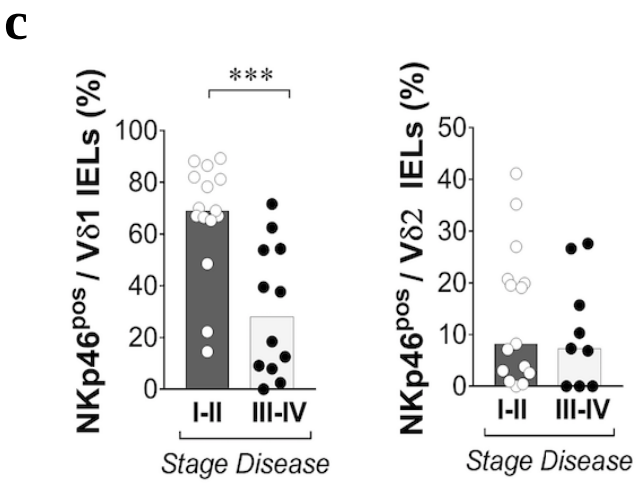


Figure18: *Clinical relevance of the NKp46^{pos}/Vδ1^{pos} subset in the pathogenesis of CRC.* ***a)*** *Statistical comparison of the incidence of total γδ IELs in healthy tissue of early (I-II) and late/metastatic (III-IV) stage disease of CRC patients (N ≥12).* ***b)*** *Frequencies of percentage of Vδ1 and Vδ2 subsets in healthy tissue of I/II and III/IV stage disease in CRC patients (N ≥12).* ***c)*** *Frequencies of percentage of NKp46^{pos}/Vδ1^{pos} and NKp46^{pos}/Vδ2^{pos} subset in healthy tissue of I/II and III/IV stage disease in CRC patients (N ≥ 15).P-value is represented as following: ***P ≤ 0.001.*

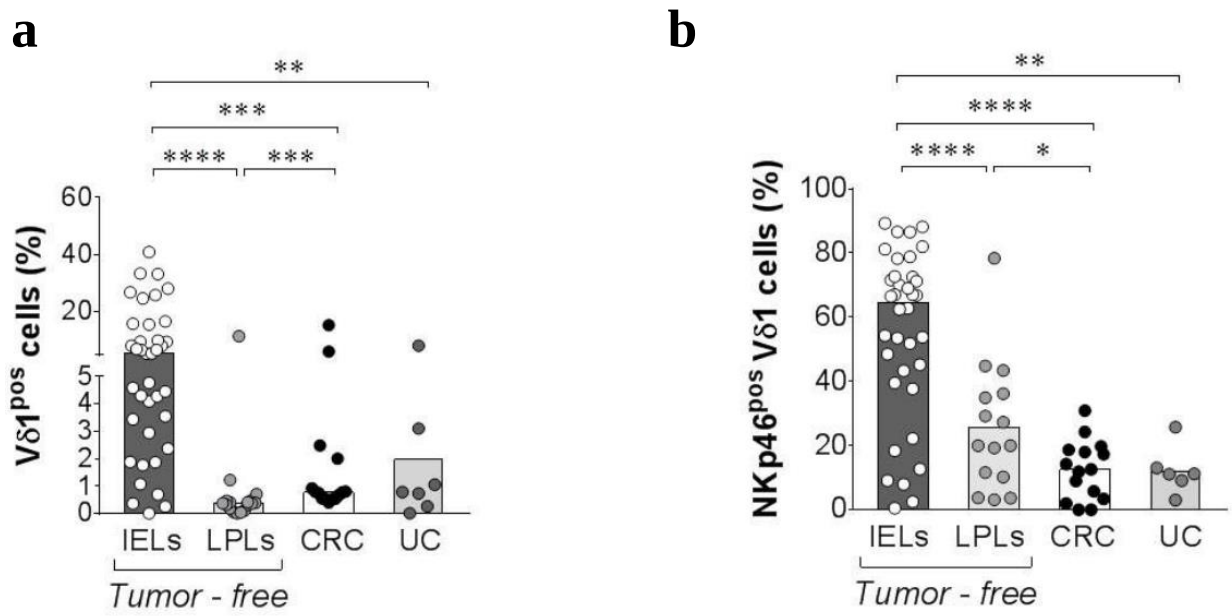


***Figure 19**: Summary statistical graphs showing the frequencies of total Vδ1^{pos} T cells (**a**) and of the NKp46^{pos}/Vδ1^{pos} T cell subset (**b**) in both epithelial (IELs) and lamina propria (LPLs) compartments of healthy/disease-free intestine (N ≥ 15) compared to those of infiltrating gut specimens of colon rectal cancer (CRC) (N=15) and ulcerative colitis (UC) patients (N=7). P-value is represented as following*: *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, **** $P \leq 0.0001$.

4. Discussion

NCRs were originally identified as germline encoded proteins constitutively and specifically expressed on NK cells (L. Moretta et al., 2002). NKp46 (Ncr1 in mice) seems particularly important for cancer surveillance, as several studies demonstrated that its deficiency leads to impaired eradication of tumors in experimental models (A. Glasner et al., 2012). Although NCRs are absent from circulating T cells, NKp46 and NKp44 were previously found on human intestinal IELs, in studies focused on pro-inflammatory αβ T cells in celiac disease (Meresse et al., 2006). The two NCRs were functional on αβ IELs, since they promoted IFN-γ secretion and degranulation. Interestingly, IL-15, which is overexpressed in celiac disease, was shown to endow NCR^{pos} IELs with lymphokine-activated killer functions, thus mediating tumor cell lysis in an NK-like, TCR-independent manner (Meresse et al., 2004). However, NCR^{pos} αβ IELs exclusively showed an effector phenotype in the highly restricted TCR repertoire which likely indicates previous TCR-mediated activation (Meresse et al., 2006). Altogether, these data suggested that chronic inflammation (as in celiac disease) promotes the differentiation of NK-like IELs.

By contrast, in this paper we identify a sizeable γδ IEL subset that constitutively expresses NCRs, especially NKp46, in healthy intestinal tissue. This is, to our knowledge, the largest "natural" (as opposed to induced) NCR^{pos} T cell subset yet identified. We thus believe the NCR^{pos} γδ IEL population is a gut-resident first line of defense against cellular transformation (or infection).

We previously found that the NCR^{pos} phenotype could be induced on pediatric γδ thymocytes upon stimulation with IL-15 or IL-2 (Ribot et al., 2014), which are the key determinants of the γδ T cell cytotoxicity program, in the absence of TCR stimulation (Ribot et al., 2014). This was consistent with a previous report where IL-15 or IL-2 (but not IL-7) were shown to induce NCR expression on T cells (both αβ and γδ) purified from cord blood, which accompanied the induction of cytotoxic functions (Tang et al.,

2008). On the other hand, this contrasts with our earlier observations with adult peripheral blood γδ T cells, where TCR activation, in the presence of IL-15 or IL-2, is indispensable to induced NCR expression (with NKp30, rather than NKp46, being the dominant family member) selectively on the Vδ1^{pos} subset of human γδ T cells (Correia et al., 2011). Importantly, we went on to show that NCR^{pos}/Vδ1^{pos} T cells hold great potential for adoptive cell therapy, as they displayed enhanced cytotoxicity against hematological tumors, both in vitro and in vivo (xenograft models) (Correia et al., 2011) (Almeida et al., 2016) Our current data highlight the enhanced cytotoxicity of NKp46^{pos}/Vδ1^{pos} T cells also against solid (epithelial-derived) tumors. In a complementary line of research, we also demonstrated that NCR^{pos}/Vδ1^{pos} T cells have potent anti-viral functions, both against CMV and HIV (Hudspeth et al., 2012). In both settings, NCRs (especially NKp30) were functionally relevant, as antibody cross-linking increased NCR^{pos}/Vδ1^{pos} T cell cytotoxicity, anti-tumor cytokine and anti-viral chemokine production (Correia et al., 2011) (Hudspeth et al., 2012)

The (patho)physiological relevance of our newly identified NKp46^{pos}/Vδ1^{pos} T cell subset is underscored by the clinical associations we found in CRC disease. In particular, we observed straight association between low frequencies of tumor surrounding NKp46^{pos}/Vδ1^{pos} cells and cancer progression suggesting an important role in the control of the development of high grade/metastatic disease. Interestingly, recent studies on NK cells observed that NKp46-mediated production of IFN-γ is able to control tumor progression in vivo, by inducing secretion of fibronectin (FN1) by tumors and thus affecting their structure and decreasing metastases (Ariella Glasner et al., 2018). Although T cells expressing Vδ1 are a dominant subset among all γδ T cells in CRC patients (Meraviglia et al., 2017) we detected significantly lower frequency of intratumoral Vδ1 compared to normal intraepithelial compartment. In addition, tumor-like conditions in the form of human colon cancer Caco2 cells and TGFβ which is overexpressed in CRC (Tauriello & Batlle, 2016) inhibit expansion of NKp46^{pos}/Vδ1^{pos} thymocytes. These data might partly explain how tumor local

environment protect the cancer cells from NKp46^{pos}/Vδ1-mediated immune elimination contributing to tumor progression.

NKp46 has been shown to be involved in the killing of various cancer cells (Halfteck et al., 2009) (Sivori et al., 2000), however no cellular ligand(s) has been identified so far. NCR^{pos} Vδ1^{pos} T cells may recognize tumor cells based on their surface expression of ligands such as heparan sulfate proteoglycans, previously shown to bind both NKp46 and NKp30 (Bloushtain et al., 2004). Interestingly, a recent report in NK cells showed that over-expression of human NKp46 in clusters at the cell membrane associates with cytoskeletal rearrangement and lytic granules polarization to the immune synapse (Hadad et al., 2015). On the other hand, the NKp46^{pos} γδ IELs phenotype is also characterized by increased expression of NKG2C, an activating receptor that binds the non-classical HLA-E molecule which expression is a part of the stress response of IECs (Perera et al., 2007). Interestingly**,** increased expression of HLA-E was established as a good prognostic indicator in CRC (Benevolo et al., 2011) and as such was proposed, in concert with the levels of infiltrating leukocytes expressing activating or inhibitory forms of NKG2-receptors, as useful parameter for prognosis (Versluis et al., 2017). Interestingly, the less represented Vδ2 IEL subset displayed significantly higher expression of the inhibitory NKG2A receptor that share the cognate ligand (HLA-E) with activating NKG2C molecule. Therefore, while NKp46 expression marks enhanced cytotoxicity among γδ IELs, other receptors must also contribute to the distinctive functional properties of this new IEL subset.

IL-2/ IL-15-mediated expression of NKp46, without TCR-restriction, found in cord blood-derived γδ T cells (Tang et al., 2008) and in pediatric Vδ1 thymocytes, in contrast to what we observed in adult Vδ1 blood T lymphocytes suggest that the ontogeny of the NKp46^{pos}/Vδ1^{pos} phenotype might depend on the distinctive features of pre and post-natal Vδ1 thymocytes as well as require gut-dependent experience. The origin and development of IELs are still the subject of debate, however, murine model shows that γδ thymocytes follow waves that sequentially populate different tissues

starting with skin at early embryonic age followed by tongue, reproductive tract and intestinal IELs at peri and post-natal days, whereas postnatal thymocytes give rise systemic γδ T cells. Although IL-2-activated γδ thymocytes share phenotypic and functions with the gut-resident γδ T cells, they differ for expression of some immunoregulatory markers such as NKG2A and NKG2C thus indicating additional gut-controlled differentiation**.** In the gut, there are multiple cellular sources of IL-15 including intestinal epithelial cells (IECs), dendritic cells (DCs) and IELs (Qiu et al., 2015). IECs are able to upregulate IL-15 secretion upon activation of TLR4 and NOD2 pathways thus suggesting that the microbiota contribute to the constitutive intestinal expression of IL-15 during steady state conditions (Jiang et al., 2013). On the other hand, IL-2 secreted by the neighboring αβ IELs, the main source of IL-2 in the gut mucosa, supports the growth of γδ IELs (Chu, Chen, Wu, Kuo, & Liao, 1999). In fact, it is worth pointing out that the epithelium site is the preferential anatomical location for NKp46^{pos}/γδ T cells compared to the lamina propria. All these data suggest that the development of the specific NKp46^{pos}/Vδ1^{pos} phenotype depends on gut environmental factors.

In summary, the finding that human colon tumors coexist with an NKp46-expressing γδ T population with enhanced anti-tumor potential, capable to modify cancer progression, represents a prospective avenue for development of novel immunotherapies. We thus believe that the "Delta One T cell" protocol, which upregulates NCR expression on Vδ1 T cells while expanding them to large numbers for adoptive transfer (Almeida et al., 2016), may be a promising strategy for colon cancer treatment

REFERENCES

Almeida, A. R., Correia, D. V., Fernandes-Platzgummer, A., Da Silva, C. L., Da Silva, M. G., Anjos, D. R., & Silva-Santos, B. (2016). Delta one T cells for immunotherapy of chronic lymphocytic leukemia: Clinical-grade expansion/differentiation and preclinical proof of concept. *Clinical Cancer Research*, *22*(23), 5795–5804. https://doi.org/10.1158/1078-0432.CCR-16-0597

Arnon, T. I., Achdout, H., Levi, O., Markel, G., Saleh, N., Katz, G., … Mandelboim, O. (2005). Inhibition of the NKp30 activating receptor by pp65 of human cytomegalovirus. *Nature Immunology*, *6*(5), 515–523. https://doi.org/10.1038/ni1190

Asarnow, D. M., Kuziel, W. A., Bonyhad, M., Tigelaar, R. E., Tucker, P. W., & Allison, J. P. (1988). Limited diversity of γδ antigen receptor genes of thy-1$^+$ dendritic epidermal cells. *Cell*, *55*(5), 837–847. https://doi.org/10.1016/0092-8674(88)90139-0

Bamias, G., Arseneau, K. O., & Cominelli, F. (2014). Cytokines and Mucosal Immunity. *Current Opinion in Gastroenterology*, *30*(6), 547–552. https://doi.org/10.1097/MOG.0000000000000118

Benevolo, M., Mottolese, M., Tremante, E., Rollo, F., Diodoro, M. G., Ercolani, C., … Giacomini, P. (2011). High expression of HLA-E in colorectal carcinoma is associated with a favorable prognosis. *Journal of Translational Medicine*, *9*, 184. https://doi.org/10.1186/1479-5876-9-184

Berg, D., Clemente, J. C., & Colombel, J. F. (2015). Can inflammatory bowel disease be permanently treated with short-term interventions on the microbiome? *Expert Review of Gastroenterology and Hepatology*, *9*(6), 781–795. https://doi.org/10.1586/17474124.2015.1013031

Bloushtain, N., Qimron, U., Bar-Ilan, A., Hershkovitz, O., Gazit, R., Fima, E., … Porgador, A. (2004). Membrane-Associated Heparan Sulfate Proteoglycans Are Involved in the Recognition of Cellular Targets by NKp30 and NKp46. *The Journal of Immunology*, *173*(4), 2392–2401. https://doi.org/10.4049/jimmunol.173.4.2392

Bonnardel, J., Da Silva, C., Henri, S., Tamoutounour, S., Chasson, L., Montañana-Sanchis, F., … Lelouard, H. (2015). Innate and Adaptive Immune Functions of Peyer's Patch Monocyte-Derived Cells. *Cell Reports*, *11*(5), 770–784. https://doi.org/10.1016/j.celrep.2015.03.067

Brandes, M., Willimann, K., Bioley, G., Lévy, N., Eberl, M., Luo, M., … Moser, B. (2009). Cross-presenting human γδ T cells induce robust CD8+ αβ T cell responses. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(7), 2307–12. https://doi.org/10.1073/pnas.0810059106

Brandes, M., Willimann, K., & Moser, B. (2005). Professional Antigen-Presentation Function by Human γδ T Cells. *Science*, *309*(5732), 264 LP-268. Retrieved from http://science.sciencemag.org/content/309/5732/264.abstract

Brandt, C. S., Baratin, M., Yi, E. C., Kennedy, J., Gao, Z., Fox, B., … Levin, S. D. (2009). The B7 family member B7-H6 is a tumor cell ligand for the activating natural killer cell receptor NKp30 in humans. *The Journal of Experimental Medicine*, *206*(7), 1495–1503. https://doi.org/10.1084/jem.20090681

Bruder, J., Siewert, K., Obermeier, B., Malotka, J., Scheinert, P., Kellermann, J., … Dornmair, K. (2012). Target specificity of an autoreactive pathogenic human gd-T cell receptor in myositis. *Journal of Biological Chemistry*, *287*(25), 20986–20995. https://doi.org/10.1074/jbc.M112.356709

Cantoni, C., Bottino, C., Vitale, M., Pessino, A., Augugliaro, R., Malaspina, A., … Biassoni, R. (1999). NKp44, A Triggering Receptor Involved in Tumor Cell Lysis by Activated Human Natural Killer Cells, Is a Novel Member of the Immunoglobulin Superfamily . *The Journal of*

Experimental Medicine, *189*(5), 787–796. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2192947/

Cantoni, C., Ponassi, M., Biassoni, R., Conte, R., Spallarossa, A., Moretta, A., … Bordo, D. (2003). The Three-Dimensional Structure of the Human NK Cell Receptor NKp44, a Triggering Partner in Natural Cytotoxicity. *Structure*, *11*(6), 725–734. https://doi.org/https://doi.org/10.1016/S0969-2126(03)00095-9

Cash, H. L., Whitham, C. V, Behrendt, C. L., & Hooper, L. V. (2006). Symbiotic Bacteria Direct Expression of an Intestinal Bactericidal Lectin. *Science (New York, N.Y.)*, *313*(5790), 1126–1130. https://doi.org/10.1126/science.1127119

Cawthon, A. G., Lu, H., & Alexander-Miller, M. A. (2001). Peptide Requirement for CTL Activation Reflects the Sensitivity to CD3 Engagement: Correlation with CD8 Versus CD8 Expression. *The Journal of Immunology*, *167*(5), 2577–2584. https://doi.org/10.4049/jimmunol.167.5.2577

Cebra, J. J., Periwal, S. B., Lee, G., Lee, F., & Shroff, K. E. (1998). Development and maintenance of the gut-associated lymphoid tissue (GALT): the roles of enteric bacteria and viruses. *Developmental Immunology*, *6*(1–2), 13–8. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2276005&tool=pmcentrez&rendertype=abstract

Cepek, K. L., Shaw, S. K., Parker, C. M., Russell, G. J., Morrow, J. S., Rimm, D. L., & Brenner, M. B. (1994). Adhesion between epithelial cells and T lymphocytes mediated by E-cadherin and the αEβ7 integrin. *Nature*, *372*, 190. Retrieved from http://dx.doi.org/10.1038/372190a0

Chen, H., Zou, M., Teng, D., Zhang, J., & He, W. (2017). Characterization of the diversity of T cell receptor γδ complementary determinant region 3 in human peripheral blood by Immune Repertoire Sequencing. *Journal of Immunological Methods*, *443*, 9–17. https://doi.org/https://doi.org/10.1016/j.jim.2017.01.009

Cheroutre, H., Lambolez, F., & Mucida, D. (2011). The light and dark sides of intestinal intraepithelial lymphocytes. *Nature Reviews. Immunology*, *11*(7), 445–456. https://doi.org/10.1038/nri3007

Chien, Y., & Bonneville, M. (2006). Gamma delta T cell receptors. *Cellular and Molecular Life Sciences : CMLS*, *63*(18), 2089–2094. https://doi.org/10.1007/s00018-006-6020-z

Chien, Y., Iwashima, M., Wettstein, D. A., Kaplan, K. B., Elliott, J. F., Born, W., & Davis, M. . (1987). T-cell receptor delta gene rearrangements in early thymocytes. *Nature*, *330*(24), 401–404. https://doi.org/10.1038/330722a0

Chien, Y., & Konigshofer, Y. (2007). Antigen recognition by gammadelta T cells. *Immunological Reviews*, *215*, 46–58. https://doi.org/10.1111/j.1600-065X.2006.00470.x

Chien, Y., Meyer, C., & Bonneville, M. (2014). gd T Cells: First Line of Defense and Beyond. *Annual Review of Immunology*, *32*(1), 121–155. https://doi.org/10.1146/annurev-immunol-032713-120216

Chiricozzi, A., Guttman-Yassky, E., Suárez-Farĩas, M., Nograles, K. E., Tian, S., Cardinale, I., … Krueger, J. G. (2011). Integrative responses to IL-17 and TNF-α in human keratinocytes account for key inflammatory pathogenic circuits in psoriasis. *Journal of Investigative Dermatology*, *131*(3), 677–687. https://doi.org/10.1038/jid.2010.340

Chu, C.-L., Chen, S.-S., Wu, T.-S., Kuo, S.-C., & Liao, N.-S. (1999). Differential Effects of IL-2 and IL-15 on the Death and Survival of Activated TCRgdt; Intestinal Intraepithelial Lymphocytes. *The Journal of Immunology*, *162*(4), 1896 LP-1903. Retrieved from http://www.jimmunol.org/content/162/4/1896.abstract

Correia, D. V, Fogli, M., Hudspeth, K., Gomes, M., Mavilio, D., Silva-santos, B., & Gomes da silva, M. (2011). Differentiation of human peripheral blood Vd1+ T cells expressing the natural cytotoxicity receptor NKp30 for recognition of lymphoid leukemia cells. *Blood*, *118*(4), 992–

1001. https://doi.org/10.1182/blood-2011-02-339135.
David Pauza, C., Poonia, B., Li, H., Cairo, C., & Chaudhry, S. (2015). γδ T cells in HIV disease: Past, present, and future. *Frontiers in Immunology*, *6*(JAN), 1–11. https://doi.org/10.3389/fimmu.2014.00687
Delahaye, N. F., Rusakiewicz, S., Martins, I., Ménard, C., Roux, S., Lyonnet, L., … Zitvogel, L. (2011). Alternatively spliced NKp30 isoforms affect the prognosis of gastrointestinal stromal tumors. *Nature Medicine*, *17*(6), 700–707. https://doi.org/10.1038/nm.2366
Deusch, K., Lüling, F., Reich, K., Classen, M., Wagner, H., & Pfeffer, K. (1991). A major fraction of human intraepithelial lymphocytes simultaneously expresses the γ/δ T cell receptor, the CD8 accessory molecule and preferentially uses the Vδ1 gene segment. *European Journal of Immunology*, *21*(4), 1053–1059. https://doi.org/10.1002/eji.1830210429
Devilder, M.-C., Maillet, S., Bouyge-Moreau, I., Donnadieu, E., Bonneville, M., & Scotet, E. (2006). Potentiation of Antigen-Stimulated Vγ9Vδ2 T Cell Cytokine Production by Immature Dendritic Cells (DC) and Reciprocal Effect on DC Maturation. *The Journal of Immunology*, *176*(3), 1386 LP-1393. Retrieved from http://www.jimmunol.org/content/176/3/1386.abstract
Dodd, J., Riffault, S., Kodituwakku, J. S., Hayday, A. C., & Openshaw, P. J. M. (2009). Pulmonary V gamma 4+ gamma delta T cells have proinflammatory and antiviral effects in viral lung disease. *Journal of Immunology (Baltimore, Md. : 1950)*, *182*(2), 1174–81. https://doi.org/182/2/1174 [pii]
Draghi, M., Pashine, A., Sanjanwala, B., Gendzekhadze, K., Cantoni, C., Cosman, D., … Parham, P. (2007). NKp46 and NKG2D Recognition of Infected Dendritic Cells Is Necessary for NK Cell Activation in the Human Response to Influenza Infection. *The Journal of Immunology*, *178*(5), 2688–2698. https://doi.org/10.4049/jimmunol.178.5.2688
Esin, S., Batoni, G., Counoupas, C., Stringaro, A., Brancatisano, F. L., Colone, M., … Campa, M. (2008). Direct binding of human NK cell natural cytotoxicity receptor NKp44 to the surfaces of mycobacteria and other bacteria. *Infection and Immunity*, *76*(4), 1719–1727. https://doi.org/10.1128/IAI.00870-07
Foster, C. E., Colonna, M., & Sun, P. D. (2003). Crystal structure of the human natural killer (NK) cell activating receptor NKp46 reveals structural relationship to other leukocyte receptor complex immunoreceptors. *The Journal of Biological Chemistry*, *278*(46), 46081–6. https://doi.org/10.1074/jbc.M308491200
Fournier, B. M., & Parkos, C. A. (2012). The role of neutrophils during intestinal inflammation. *Mucosal Immunology*, *5*(4), 354–366. https://doi.org/10.1038/mi.2012.24
Fuchs, A., Cella, M., Kondo, T., & Colonna, M. (2005). Paradoxic inhibition of human natural interferon-producing cells by the activating receptor NKp44. *Blood*, *106*(6), 2076–2082. https://doi.org/10.1182/blood-2004-12-4802
Gangadharan, D., & Cheroutre, H. (2004). The CD8 isoform CD8αα is not a functional homologue of the TCR co-receptor CD8αβ. *Current Opinion in Immunology*, *16*(3), 264–270. https://doi.org/10.1016/j.coi.2004.03.015
Gao, Y., Yang, W., Pan, M., Scully, E., Girardi, M., Augenlicht, L. H., … Yin, Z. (2003). γδ T Cells Provide an Early Source of Interferon γ in Tumor Immunity. *The Journal of Experimental Medicine*, *198*(3), 433–442. https://doi.org/10.1084/jem.20030584
Glasner, A., Ghadially, H., Gur, C., Stanietsky, N., Tsukerman, P., Enk, J., & Mandelboim, O. (2012). Recognition and Prevention of Tumor Metastasis by the NK Receptor NKp46/NCR1. *The Journal of Immunology*, *188*(6), 2509–2515. https://doi.org/10.4049/jimmunol.1102461
Glasner, A., Levi, A., Enk, J., Isaacson, B., Viukov, S., Orlanski, S., … Mandelboim, O. (2018). NKp46 Receptor-Mediated Interferon-g; Production by Natural Killer Cells Increases Fibronectin 1 to Alter Tumor Architecture and Control Metastasis. *Immunity*, *48*(1), 107–119.e4. https://doi.org/10.1016/j.immuni.2017.12.007

Gober, H.-J., Kistowska, M., Angman, L., Jenö, P., Mori, L., & De Libero, G. (2003). Human T Cell Receptor γδ Cells Recognize Endogenous Mevalonate Metabolites in Tumor Cells. *The Journal of Experimental Medicine*, *197*(2), 163–168. https://doi.org/10.1084/jem.20021500

Gomes, A. Q., Correia, D. V., Grosso, A. R., Lança, T., Ferreira, C., Lacerda, J. F., … Silva-Santos, B. (2010). Identification of a panel of ten cell surface protein antigens associated with immunotargeting of leukemias and lymphomas by peripheral blood γδ t cells. *Haematologica*, *95*(8), 1397–1404. https://doi.org/10.3324/haematol.2009.020602

Habtezion, A., Nguyen, L. P., Hadeiba, H., & Butcher, E. C. (2016). Leukocyte Trafficking to the Small Intestine and Colon. *Gastroenterology*, *150*(2), 340–354. https://doi.org/10.1053/j.gastro.2015.10.046

Hadad, U., Thauland, T. J., Martinez, O. M., Butte, M. J., Porgador, A., & Krams, S. M. (2015). NKp46 clusters at the immune synapse and regulates NK cell polarization. *Frontiers in Immunology*, *6*(SEP), 1–11. https://doi.org/10.3389/fimmu.2015.00495

Halfteck, G. G., Elboim, M., Gur, C., Achdout, H., Ghadially, H., & Mandelboim, O. (2009). Enhanced In Vivo Growth of Lymphoma Tumors in the Absence of the NK-Activating Receptor NKp46/NCR1. *The Journal of Immunology*, *182*(4), 2221 LP-2230. Retrieved from http://www.jimmunol.org/content/182/4/2221.abstract

Hamada, S., Umemura, M., Shiono, T., Tanaka, K., Yahagi, A., Begum, M. D., … Matsuzaki, G. (2008). IL-17A Produced by γδ T Cells Plays a Critical Role in Innate Immunity against Listeria monocytogenes Infection in the Liver. *Journal of Immunology (Baltimore, Md. : 1950)*, *181*(5), 3456–3463. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2859669/

Hayday, A. C. (2009). γδ T Cells and the Lymphoid Stress-Surveillance Response. *Immunity*, *31*(2), 184–196. https://doi.org/10.1016/j.immuni.2009.08.006

Hayday, A., Theodoridis, E., Ramsburg, E., & Shires, J. (2001). Intraepithelial lymphocytes: Exploring the Third Way in immunology. *Nature Immunology*, *2*(11), 997–1003. https://doi.org/10.1038/ni1101-997

Himoudi, N., Morgenstern, D. A., Yan, M., Vernay, B., Saraiva, L., Wu, Y., … Anderson, J. (2012). Human γδ T Lymphocytes Are Licensed for Professional Antigen Presentation by Interaction with Opsonized Target Cells. *The Journal of Immunology*, *188*(4), 1708 LP-1716. Retrieved from http://www.jimmunol.org/content/188/4/1708.abstract

Horton, N. C., & Mathew, P. A. (2015). NKp44 and Natural Cytotoxicity Receptors as Damage-Associated Molecular Pattern Recognition Receptors. *Frontiers in Immunology*, *6*, 31. https://doi.org/10.3389/fimmu.2015.00031

Hudspeth, K., Donadon, M., Cimino, M., Pontarini, E., Tentorio, P., Preti, M., … Mavilio, D. (2016). Human liver-resident CD56(bright)/CD16(neg) NK cells are retained within hepatic sinusoids via the engagement of CCR5 and CXCR6 pathways. *Journal of Autoimmunity*, *66*, 40–50. https://doi.org/10.1016/j.jaut.2015.08.011

Hudspeth, K., Fogli, M., Correia, D. V, Mikulak, J., Roberto, A., Della, S., … Mavilio, D. (2012). Engagement of NKp30 on V δ 1 T-cells induces the production of CCL3 , CCL4 and CCL5 and suppresses HIV-1 replication Engagement of NKp30 on Vδ1 T-cells induces the production of CCL3 , CCL4 and CCL5 and suppresses HIV-1 replication. *Online*, *119*(17), 4013–4016. https://doi.org/10.1182/blood-2011-11-390153

Hudspeth, K., Silva-Santos, B., & Mavilio, D. (2013). Natural cytotoxicity receptors: Broader expression patterns and functions in innate and adaptive immune cells. *Frontiers in Immunology*, *4*(MAR), 1–15. https://doi.org/10.3389/fimmu.2013.00069

Hunter, S., Willcox, C., Davey, M., & Kasatskaya, S. (2018). Human liver infiltrating γδ T cells are composed of clonally expanded circulating and tissue-resident populations. *Journal of Hepatology*. https://doi.org/10.1016/j.jhep.2018.05.007

Inagaki-Ohara, K., Chinen, T., Matsuzaki, G., Sasaki, A., Sakamoto, Y., Hiromatsu, K., …

Yoshimura, A. (2004). Mucosal T Cells Bearing TCRγδ Play a Protective Role in Intestinal Inflammation. *The Journal of Immunology*, *173*(2), 1390 LP-1398. Retrieved from http://www.jimmunol.org/content/173/2/1390.abstract

Ismail, A. S., Severson, K. M., Vaishnava, S., Behrendt, C. L., Yu, X., Benjamin, J. L., … Hooper, L. V. (2011). γδ intraepithelial lymphocytes are essential mediators of host–microbial homeostasis at the intestinal mucosal surface. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(21), 8743–8748. https://doi.org/10.1073/pnas.1019574108

Ito, K., Bonneville, M., Takagaki, Y., Nakanishi, N., Kanagawa, O., Krecko, E. G., & Tonegawa, S. (1989). Different gamma delta T-cell receptors are expressed on thymocytes at different stages of development. *Proceedings of the National Academy of Sciences of the United States of America*.

Jameson, J., & Havran, W. L. (2007). Skin gd T-cell functions in homeostasis and wound healing. *Immunological Reviews*. https://doi.org/10.1111/j.1600-065X.2006.00483.x

Janakiram, N. B., & Rao, C. V. (2014). The Role of Inflammation in Colon Cancer. In B. B. Aggarwal, B. Sung, & S. C. Gupta (Eds.), *Inflammation and Cancer* (pp. 25–52). Basel: Springer Basel. https://doi.org/10.1007/978-3-0348-0837-8_2

Jarahian, M., Fiedler, M., Cohnen, A., Djandji, D., Hämmerling, G. J., Gati, C., … Momburg, F. (2011). Modulation of nkp30- and nkp46-mediated natural killer cell responses by poxviral hemagglutinin. *PLoS Pathogens*, *7*(8). https://doi.org/10.1371/journal.ppat.1002195

Jaron-Mendelson, M., Yossef, R., Appel, M. Y., Zilka, A., Hadad, U., Afergan, F., … Porgador, A. (2012). Dimerization of NKp46 Receptor Is Essential for NKp46-Mediated Lysis: Characterization of the Dimerization Site by Epitope Mapping. *The Journal of Immunology*, *188*(12), 6165–6174. https://doi.org/10.4049/jimmunol.1102496

Jiang, W., Wang, X., Zeng, B., Liu, L., Tardivel, A., Wei, H., … Zhou, R. (2013). Recognition of gut microbiota by NOD2 is essential for the homeostasis of intestinal intraepithelial lymphocytes. *The Journal of Experimental Medicine*, *210*(11), 2465–2476. https://doi.org/10.1084/jem.20122490

Jianqi, W., Lei, X., Xu, H., Haiyan, H., Feifei, Q., Song, B., … Beixing, L. (2017). Role of γδ T cells in exacerbated airway inflammation during reinfection of neonatally primed mice in adulthood. *Journal of Medical Virology*, *89*(12), 2108–2115. https://doi.org/10.1002/jmv.24914

Jung, C., Hugot, J.-P., & Barreau, F. (2010). Peyer's Patches: The Immune Sensors of the Intestine. *International Journal of Inflammation*, *2010*, 1–12. https://doi.org/10.4061/2010/823710

Kabelitz, D., Kalyan, S., Oberg, H. H., & Wesch, D. (2013). Human Vdelta2 versus non-Vdelta2 gammadelta T cells in antitumor immunity. *Oncoimmunology*, *2*(3), e23304. https://doi.org/10.4161/onci.23304

Kabelitz, D., Marischen, L., Oberg, H. H., Holtmeier, W., & Wesch, D. (2005). Epithelial defence by gamma-delta T cells. *International Archives of Allergy and Immunology*, *137*(1), 73–81. https://doi.org/10.1159/000085107

Kadivar, M., Petersson, J., Svensson, L., & Marsal, J. (2016). CD8αβ $^{+}$ γδ T Cells: A Novel T Cell Subset with a Potential Role in Inflammatory Bowel Disease. *The Journal of Immunology*, *197*(12), 4584–4592. https://doi.org/10.4049/jimmunol.1601146

Knight, A., Mackinnon, S., & Lowdell, M. W. (2012). Human Vdelta1 gamma-delta T cells exert potent specific cytotoxicity against primary multiple myeloma cells. *Cytotherapy*, *14*(9), 1110–1118. https://doi.org/https://doi.org/10.3109/14653249.2012.700766

Kobayashi, H., Tanaka, Y., Shimmura, H., Minato, N., & Tanabe, K. (2010). Complete remission of lung metastasis following adoptive immunotherapy using activated autologous gammadelta T-cells in a patient with renal cell carcinoma. *Anticancer Research*, *30*(2), 575–579. https://doi.org/30/2/575 [pii]

Koch, J., Steinle, A., Watzl, C., & Mandelboim, O. (2013). Activating natural cytotoxicity receptors

of natural killer cells in cancer and infection. *Trends in Immunology*, *34*(4), 182–191. https://doi.org/10.1016/j.it.2013.01.003

Komano, H., Fujiura, Y., Kawaguchi, M., Matsumoto, S., Hashimoto, Y., Obana, S., … Itohara, S. (1995). Homeostatic regulation of intestinal epithelia by intraepithelial gamma delta T cells. *Proceedings of the National Academy of Sciences of the United States of America*, *92*(13), 6147–6151. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC41659/

Kong, Y., Cao, W., Xi, X., Ma, C., Cui, L., & He, W. (2009). The NKG2D ligand ULBP4 binds to TCRγ9/δ2 and induces cytotoxicity to tumor cells through both TCRγδ and NKG2D. *Blood*, *114*(2), 310 LP-317. Retrieved from http://www.bloodjournal.org/content/114/2/310.abstract

Kruse, P. H., Matta, J., Ugolini, S., & Vivier, E. (2014). Natural cytotoxicity receptors and their ligands. *Immunology and Cell Biology*, *92*(3), 221–229. https://doi.org/10.1038/icb.2013.98

Kühl, A. A., Pawlowski, N. N., Grollich, K., Loddenkemper, C., Zeitz, M., & Hoffmann, J. C. (2006). Aggravation of intestinal inflammation by depletion/deficiency of γδ T cells in different types of IBD animal models. *Journal of Leukocyte Biology*, *81*(1), 168–175. https://doi.org/10.1189/jlb.1105696

Lelouard, H., Fallet, M., De Bovis, B., Méresse, S., & Gorvel, J. (2012). Peyer's patch dendritic cells sample antigens by extending dendrites through M cell-specific transcellular pores. *Gastroenterology*, *142*(3), 592–601. https://doi.org/10.1053/j.gastro.2011.11.039

Li, Y., Wang, Q., & Mariuzza, R. A. (2011). Structure of the human activating natural cytotoxicity receptor NKp30 bound to its tumor cell ligand B7-H6. *The Journal of Experimental Medicine*, *208*(4), 703–714. https://doi.org/10.1084/jem.20102548

Lo Presti, E., Dieli, F., & Meraviglia, S. (2014). Tumor-Infiltrating γδ T Lymphocytes: Pathogenic Role, Clinical Significance, and Differential Programing in the Tumor Microenvironment. *Frontiers in Immunology*, *5*, 607. https://doi.org/10.3389/fimmu.2014.00607

Lockhart, E., Green, A. M., & Flynn, J. L. (2006). IL-17 Production Is Dominated by T Cells rather than CD4 T Cells during Mycobacterium tuberculosis Infection. *The Journal of Immunology*, *177*(7), 4662–4669. https://doi.org/10.4049/jimmunol.177.7.4662

Ma, S., Cheng, Q., Cai, Y., Gong, H., Wu, Y., Yu, X., … Liu, H. (2014). IL-17A produced by ???? T cells promotes tumor growth in hepatocellular carcinoma. *Cancer Research*, *74*(7), 1969–1982. https://doi.org/10.1158/0008-5472.CAN-13-2534

Magri, G., Muntasell, A., Romo, N., Sáez-Borderías, A., Pende, D., Geraghty, D. E., … López-Botet, M. (2011). NKp46 and DNAM-1 NK-cell receptors drive the response to human cytomegalovirus-infected myeloid dendritic cells overcoming viral immune evasion strategies. *Blood*, *117*(3), 848 LP-856. Retrieved from http://www.bloodjournal.org/content/117/3/848.abstract

Mandelboim, O., Lieberman, N., Lev, M., Paul, L., Arnon, T. I., Bushkin, Y., … Porgador, A. (2001). Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. *Nature*, *409*, 1055. Retrieved from http://dx.doi.org/10.1038/35059110

Mangan, B. A., Dunne, M. R., O'Reilly, V. P., Dunne, P. J., Exley, M. A., O'Shea, D., … Doherty, D. G. (2013). CD1d restriction and Th1/Th2/Th17 cytokine secretion by human Vδ3 T cells. *Journal of Immunology (Baltimore, Md. : 1950)*, *191*(1), 30–34. https://doi.org/10.4049/jimmunol.1300121

Markov, A. G., Falchuk, E. L., Kruglova, N. M., Radloff, J., & Amasheh, S. (2016). Claudin expression in follicle-associated epithelium of rat Peyer's patches defines a major restriction of the paracellular pathway. *Acta Physiologica*, *216*(1), 112–119. https://doi.org/10.1111/apha.12559

Mavoungou, E., Held, J., Mewono, L., & Kremsner, P. G. (2007). A Duffy binding-like domain is involved in the NKp30-mediated recognition of Plasmodium falciparum-parasitized erythrocytes by natural killer cells. *The Journal of Infectious Diseases*, *195*(10), 1521–1531.

https://doi.org/10.1086/515579
McVay, L. D., & Carding, S. R. (1996). Extrathymic Origin of Human γδ T Cells during Fetal Development. *Journal of Immunology*, *157*(7), 2873–2882. Retrieved from https://www.scopus.com/inward/record.uri?eid=2-s2.0-0030265684&partnerID=40&md5=f5bcd5020702c788dd8c6c5b6e1ddc9d
Meeh, P. F., King, M., O'Brien, R. L., Muga, S., Buckhalts, P., Neuberg, R., & S., L. L. (2006). Characterization of the gd T cell response to acute leukemia. *Cancer Immunology, Immunotherapy*, *55*(9), 1072–1080. https://doi.org/10.1007/s00262-005-0094-6
Meraviglia, S., Lo Presti, E., Tosolini, M., La Mendola, C., Orlando, V., Todaro, M., … Dieli, F. (2017). Distinctive features of tumor-infiltrating γδ T lymphocytes in human colorectal cancer. *OncoImmunology*, *6*(10), e1347742. https://doi.org/10.1080/2162402X.2017.1347742
Meresse, B., Chen, Z., Ciszewski, C., Tretiakova, M., Bhagat, G., Krausz, T. N., … Jabri, B. (2004). Coordinated induction by IL15 of a TCR-independent NKG2D signaling pathway converts CTL into lymphokine-activated killer cells in celiac disease. *Immunity*, *21*(3), 357–366. https://doi.org/10.1016/j.immuni.2004.06.020
Meresse, B., Curran, S. A., Ciszewski, C., Orbelyan, G., Setty, M., Bhagat, G., … Jabri, B. (2006). Reprogramming of CTLs into natural killer–like cells in celiac disease. *The Journal of Experimental Medicine*, *203*(5), 1343–1355. https://doi.org/10.1084/jem.20060028
Moreau, M. C., & Corthier, G. (1988). Effect of the gastrointestinal microflora on induction and maintenance of oral tolerance of ovalbumin in C3H/HeJ mice. *Infection and Immunity*, *56*(10), 2766–2768.
Moretta, A., Bottino, C., Mingari, M. C., Biassoni, R., & Moretta, L. (2002). What is a natural killer cell? *Nature Immunology*, *3*, 6. Retrieved from http://dx.doi.org/10.1038/ni0102-6
Moretta, L., Bottino, C., Pende, D., Mingari, M. C., Biassoni, R., & Moretta, a. (2002). Human natural killer cells: their origin, receptors and function. *Eur J Immunol*, *32*(5), 1205–1211. https://doi.org/10.1002/1521-4141(200205)32:5{<}1205::AID-IMMU1205{>}3.0.CO;2-Y
Muñoz-Ruiz, M., Sumaria, N., Pennington, D. J., & Silva-Santos, B. (2017). Thymic Determinants of gamma-delta T Cell Differentiation. *Trends in Immunology*, *38*(5), 336–344. https://doi.org/10.1016/j.it.2017.01.007
Nanno, M., Shiohara, T., Yamamoto, H., Kawakami, K., & Ishikawa, H. (2007). γδ T cells: Firefighters or fire boosters in the front lines of inflammatory responses. *Immunological Reviews*, *215*(1), 103–113. https://doi.org/10.1111/j.1600-065X.2006.00474.x
Nielsen, M. M., Witherden, D. A., & Havran, W. L. (2017). γδ T cells in homeostasis and host defence of epithelial barrier tissues. *Nature Reviews. Immunology*, *17*(12), 733–745. https://doi.org/10.1038/nri.2017.101
O'Brien, R. L., Roark, C. L., Jin, N., Kemal Aydintug, M., French, J. D., Chain, J. L., … Born, W. K. (2007). γδ T-cell receptors: Functional correlations. *Immunological Reviews*, *215*(1), 77–88. https://doi.org/10.1111/j.1600-065X.2006.00477.x
Ogata, M., & Itoh, T. (2016). Gamma/delta intraepithelial lymphocytes in the mouse small intestine. *Anatomical Science International*, *91*(4), 301–312. https://doi.org/10.1007/s12565-016-0341-2
Ogra, P. L., & Karzon, D. T. (1969). Poliovirus Antibody Response in Serum and Nasal Secretions Following Intranasal Inoculation with Inactivated Poliovaccine. *J Immunol*, *102*(1), 15–23. Retrieved from http://www.jimmunol.org/content/102/1%5Cnhttp://www.jimmunol.org/%5Cnhttp://www.jimmunol.org/cgi/content/abstract/102/1/15
Ohteki, T., & MacDonald, H. R. (1993). Expression of the CD28 costimulatory molecule on subsets of murine intestinal intraepithelial lymphocytes correlates with lineage and responsiveness. *European Journal of Immunology*, *23*(6), 1251–1255. https://doi.org/10.1002/eji.1830230609
Pang, D. J., Neves, J. F., Sumaria, N., & Pennington, D. J. (2012). Understanding the complexity of

γδ T-cell subsets in mouse and human. *Immunology*, *136*(3), 283–290. https://doi.org/10.1111/j.1365-2567.2012.03582.x

Papadakis, K. A., Prehn, J., Moreno, S. T., Cheng, L., Kouroumalis, E. A., Deem, R., … Targan, S. R. (2001). CCR9–Positive lymphocytes and thymus-expressed chemokine distinguish small bowel from colonic Crohn's disease. *Gastroenterology*, *121*(2), 246–254. https://doi.org/https://doi.org/10.1053/gast.2001.27154

Park, Y., Moon, S.-J., & Lee, S.-W. (2016). Lineage re-commitment of CD4CD8αα intraepithelial lymphocytes in the gut. *BMB Reports*, *49*(1), 11–17. https://doi.org/10.5483/BMBRep.2016.49.1.242

Pazina, T., Shemesh, A., Brusilovsky, M., Porgador, A., & Campbell, K. S. (2017). Regulation of the functions of natural cytotoxicity receptors by interactions with diverse ligands and alterations in splice variant expression. *Frontiers in Immunology*, *8*(MAR). https://doi.org/10.3389/fimmu.2017.00369

Pende, D., Parolini, S., Pessino, A., Sivori, S., Augugliaro, R., Morelli, L., … Moretta, A. (1999). Identification and Molecular Characterization of Nkp30, a Novel Triggering Receptor Involved in Natural Cytotoxicity Mediated by Human Natural Killer Cells. *The Journal of Experimental Medicine*, *190*(10), 1505–1516. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2195691/

Peng, G., Wang, H. Y., Peng, W., Kiniwa, Y., Seo, K. H., & Wang, R.-F. (2007). Tumor-Infiltrating γδ T Cells Suppress T and Dendritic Cell Function via Mechanisms Controlled by a Unique Toll-like Receptor Signaling Pathway. *Immunity*, *27*(2), 334–348. https://doi.org/https://doi.org/10.1016/j.immuni.2007.05.020

Perera, L., Shao, L., Patel, A., Evans, K., Meresse, B., Blumberg, R., … Mayer, L. (2007). Expression of nonclassical class I molecules by intestinal epithelial cells. *Inflammatory Bowel Diseases*, *13*(3), 298–307. Retrieved from http://dx.doi.org/10.1002/ibd.20026

Pessino, A., Sivori, S., Bottino, C., Malaspina, A., Morelli, L., Moretta, L., … Moretta, A. (1998). Molecular cloning of NKp46: a novel member of the immunoglobulin superfamily involved in triggering of natural cytotoxicity. *The Journal of Experimental Medicine*, *188*(5), 953–960. https://doi.org/10.1084/jem.188.5.953

Peterson, L. W., & Artis, D. (2014). Intestinal epithelial cells: regulators of barrier function and immune homeostasis. *Nature Reviews Immunology*, *14*, 141. Retrieved from http://dx.doi.org/10.1038/nri3608

Poccia, A. M. and F. (2007). gamma-delta T Cells and Dendritic Cells: Close Partners and Biological Adjuvants for New Therapies. *Current Molecular Medicine*. https://doi.org/http://dx.doi.org/10.2174/156652407782564345

Poggi, A., Venturino, C., Catellani, S., Clavio, M., Miglino, M., Gobbi, M., … Zocchi, M. R. (2004). Vδ1 T Lymphocytes from B-CLL Patients Recognize ULBP3 Expressed on Leukemic B Cells and Up-Regulated gdTCR by Retinoic Acid. *Cancer Research*, *64*(24), 9172 LP-9179. Retrieved from http://cancerres.aacrjournals.org/content/64/24/9172.abstract

Ponassi, M., Cantoni, C., Biassoni, R., Conte, R., Spallarossa, A., Pesce, A., … Bordo, D. (2003). Structure of the human NK cell triggering receptor NKp46 ectodomain. *Biochemical and Biophysical Research Communications*, *309*(2), 317–323. https://doi.org/10.1016/j.bbrc.2003.08.007

Ponnampalam, A. P., Gargett, C. E., & Rogers, P. A. W. (2008). Identification and hormonal regulation of a novel form of NKp30 in human endometrial epithelium. *European Journal of Immunology*, *38*(1), 216–226. https://doi.org/10.1002/eji.200636429

Poupot, M., & Fournié, J. J. (2004). Non-peptide antigens activating human Vγ9/Vδ2 T lymphocytes. *Immunology Letters*, *95*(2), 129–138. https://doi.org/10.1016/j.imlet.2004.06.013

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, S., Manichanh, C., … Yang, H. (2010). A human

gut microbial gene catalog established by metagenomic sequencing. *Nature*, *464*(7285), 59–65. https://doi.org/10.1038/nature08821.A

Qiu, Y., Wang, W., Xiao, W., & Yang, H. (2015). Role of the intestinal cytokine microenvironment in shaping the intraepithelial lymphocyte repertoire. *Journal of Leukocyte Biology*, *97*(5), 849–857. https://doi.org/10.1189/jlb.3RU1014-465R

Ribot, J. C., Ribeiro, S. T., Correia, D. V., Sousa, A. E., & Silva-Santos, B. (2014). Human Thymocytes Are Functionally Immature and Differentiate into Cytotoxic Type 1 Effector T Cells upon IL-2/IL-15 Signaling. *The Journal of Immunology*, *192*(5), 2237–2243. https://doi.org/10.4049/jimmunol.1303119

Robinson, M. E., George, S. Z., Dannecker, E. A., Jump, R. L., Hirsh, A. T., Gagnon, C. M., & Brown, J. L. (2004). Sex differences in pain anchors revisited: Further investigation of "most intense" and common pain events. *European Journal of Pain*, *8*(4), 299–305. https://doi.org/10.1016/j.ejpain.2003.10.003

Rocha, B., Vassalli, P., & Guy-Grand, D. (1994). Thymic and extrathymic origins of gut intraepithelial lymphocyte populations in mice. *The Journal of Experimental Medicine*, *180*(2), 681–6. https://doi.org/10.1084/jem.180.2.681

Sathaliyawala, T., Kubota, M., Yudanin, N., Turner, D., Camp, P., Thome, J. J. C., … Farber, D. L. (2013). Distribution and compartmentalization of human circulating and tissue-resident memory T cell subsets. *Immunity*, *38*(1), 187–197. https://doi.org/10.1016/j.immuni.2012.09.020

Savage, D. C. (1977). Microbial Ecology of the Gastrointestinal Tract. *Annual Review of Microbiology*, *31*(1), 107–33. https://doi.org/10.1002/9780470281840

Sharp, L. L., Jameson, J. M., Cauvi, G., & Havran, W. L. (2005). Dendritic epidermal T cells regulate skin homeostasis through local production of insulin-like growth factor 1. *Nature Immunology*, *6*(1), 73–79. https://doi.org/10.1038/ni1152

Sheridan, B. S., & Lefrançois, L. (2010). Intraepithelial Lymphocytes: To Serve and Protect. *Current Gastroenterology Reports*, *12*(6), 513–521. https://doi.org/10.1007/s11894-010-0148-6

Silva-Santos, B., Serre, K., & Norell, H. (2015). γδ T cells in cancer. *Nature Reviews Immunology*, *15*(11), 683–691. https://doi.org/10.1038/nri3904

Sivori, S., Parolini, S., Marcenaro, E., Castriconi, R., Pende, D., Millo, R., & Moretta, A. (2000). Involvement of natural cytotoxicity receptors in human natural killer cell-mediated lysis of neuroblastoma and glioblastoma cell lines. *Journal of Neuroimmunology*, *107*(2), 220–225. https://doi.org/10.1016/S0165-5728(00)00221-6

Sivori, S., Vitale, M., Morelli, L., Sanseverino, L., Augugliaro, R., Bottino, C., … Moretta, A. (1997). p46, a Novel Natural Killer Cell–specific Surface Molecule That Mediates Cell Activation . *The Journal of Experimental Medicine*, *186*(7), 1129–1136. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2211712/

Spada, F. M., Grant, E. P., Peters, P. J., Sugita, M., Melián, A., Leslie, D. S., … Brenner, M. B. (2000). Self-recognition of CD1 by gamma/delta T cells: implications for innate immunity. *The Journal of Experimental Medicine*, *191*(6), 937–48. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2193122&tool=pmcentrez&rendertype=abstract

Spits, H., Artis, D., Colonna, M., Diefenbach, A., Di Santo, J. P., Eberl, G., … Vivier, E. (2013). Innate lymphoid cells-a proposal for uniform nomenclature. *Nature Reviews Immunology*, *13*(2), 145–149. https://doi.org/10.1038/nri3365

Strid, J., Sobolev, O., Zafirova, B., Polic, B., & Hayday, A. (2011). The intraepithelial T cell response to NKG2D-ligands links lymphoid stress surveillance to atopy. *Science*, *334*(6060), 1293–1297. https://doi.org/10.1126/science.1211250

Sugahara, K., & Kitagawa, H. (2002). Heparin and heparan sulfate biosynthesis. *IUBMB Life*, *54*(4), 163–175. https://doi.org/10.1080/15216540214928

Sutton, C. E., Mielke, L. A., & Mills, K. H. G. (2012). IL-17-producing γδ T cells and innate lymphoid cells. *European Journal of Immunology*, *42*(9), 2221–2231. https://doi.org/10.1002/eji.201242569

T.Bergsbaken, M. B. (2015). Proinflammatory microenvironments within the intestine regulate differentiation of tissue-resident CD8 T cells responding to infection, *16*(4), 406–414. https://doi.org/10.1038/ni.3108.Proinflammatory

Tang, Q., Grzywacz, B., Wang, H., Kataria, N., Cao, Q., Wagner, J. E., … Verneris, M. R. (2008). Umbilical Cord Blood T cells Express Multiple Natural Cytotoxicity Receptors After IL-15 Stimulation, but Only NKp30 Is Functional. *Journal of Immunology (Baltimore, Md. : 1950)*, *181*(7), 4507–4515. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2614673/

Tauriello, D. V. F., & Batlle, E. (2016). Targeting the Microenvironment in Advanced Colorectal Cancer. *Trends in Cancer*, *2*(9), 495–504. https://doi.org/10.1016/j.trecan.2016.08.001

Thedrez, A., Sabourin, C., Gertner, J., Devilder, M. C., Allain-Maillet, S., Fournié, J. J., … Bonneville, M. (2007). Self/non-self discrimination by human γδ T cells: Simple solutions for a complex issue? *Immunological Reviews*, *215*(1), 123–135. https://doi.org/10.1111/j.1600-065X.2006.00468.x

Triebel F, Faure F, Graziani M, Jitsukawa S, Lefranc MP, H. T. (1988). A unique V-J-C-rearranged gene encodes a gamma protein expressed on the majority of CD3+ T cell receptor-alpha/beta-circulating lymphocytes. *The Journal of Experimental Medicine*, *167*(2), 694–699. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2188842/

Tyler, C. J., McCarthy, N. E., Lindsay, J. O., Stagg, A. J., Moser, B., & Eberl, M. (2017). Antigen-Presenting Human γδ T Cells Promote Intestinal CD4 $^{+}$ T Cell Expression of IL-22 and Mucosal Release of Calprotectin. *The Journal of Immunology*, 1700003. https://doi.org/10.4049/jimmunol.1700003

Vacca, P., Cantoni, C., Prato, C., Fulcheri, E., Moretta, A., Moretta, L., & Mingari, M. C. (2008). Regulatory role of NKp44, NKp46, DNAM-1 and NKG2D receptors in the interaction between NK cells and trophoblast cells. Evidence for divergent functional profiles of decidual versus peripheral NK cells. *International Immunology*, *20*(11), 1395–1405. https://doi.org/10.1093/intimm/dxn105

Vaishnava, S., Yamamoto, M., Severson, K. M., Ruhn, K. A., Yu, X., Koren, O., … Hooper, L. V. (2011). The antibacterial lectin RegIIIγ promotes the spatial segregation of microbiota and host in the intestine. *Science (New York, N.Y.)*, *334*(6053), 255–258. https://doi.org/10.1126/science.1209791

Van de Walle, I., Waegemans, E., De Medts, J., De Smet, G., De Smedt, M., Snauwaert, S., … Taghon, T. (2013). Specific Notch receptor–ligand interactions control human TCR-αβ/γδ development by inducing differential Notch signal strength. *The Journal of Experimental Medicine*, *210*(4), 683–697. https://doi.org/10.1084/jem.20121798

Vantourout, P., & Hayday, A. (2013). Six-of-the-best: unique contributions of γδ T cells to immunology. *Nature Reviews Immunology*, *13*(2), 88–100. https://doi.org/10.1038/nri3384

Vermijlen, D., Ellis, P., Langford, C., Klein, A., Engel, R., Willimann, K., … Eberl, M. (2007). Distinct Cytokine-Driven Responses of Activated Blood T Cells: Insights into Unconventional T Cell Pleiotropy. *The Journal of Immunology*, *178*(7), 4304–4314. https://doi.org/10.4049/jimmunol.178.7.4304

Versluis, M. A. C., Marchal, S., Plat, A., de Bock, G. H., van Hall, T., de Bruyn, M., … Nijman, H. W. (2017). The prognostic benefit of tumour-infiltrating Natural Killer cells in endometrial cancer is dependent on concurrent overexpression of Human Leucocyte Antigen-E in the tumour microenvironment. *European Journal of Cancer*, *86*, 285–295. https://doi.org/10.1016/j.ejca.2017.09.008

Vitale, M., Bottino, C., Sivori, S., Sanseverino, L., Castriconi, R., Marcenaro, E., … Moretta, A.

(1998). NKp44, a novel triggering surface molecule specifically expressed by activated natural killer cells, is involved in non-major histocompatibility complex-restricted tumor cell lysis. *The Journal of Experimental Medicine*, *187*(12), 2065–72. https://doi.org/10.1084/jem.187.12.2065

Vitale, M., Falco, M., Castriconi, R., Parolini, S., Zambello, R., Semenzato, G., … Moretta, A. (2001). Identification of NKp80, a novel triggering molecule expressed by human NK cells. *European Journal of Immunology*, *31*(1), 233–242. https://doi.org/10.1002/1521-4141(200101)31:1<233::AID-IMMU233>3.0.CO;2-4

Von Lilienfeld-Toal, M., Nattermann, J., Feldmann, G., Sievers, E., Frank, S., Strehl, J., & Schmidt-Wolf, I. G. H. (2006). Activated γδ T cells express the natural cytotoxicity receptor natural killer p44 and show cytotoxic activity against myeloma cells. *Clinical and Experimental Immunology*, *144*(3), 528–533. https://doi.org/10.1111/j.1365-2249.2006.03078.x

Wakita, D., Sumida, K., Iwakura, Y., Nishikawa, H., Ohkuri, T., Chamoto, K., … Nishimura, T. (2010). Tumor-infiltrating IL-17-producing γδ T cells support the progression of tumor by promoting angiogenesis. *European Journal of Immunology*, *40*(7), 1927–1937. https://doi.org/10.1002/eji.200940157

Walzer, T., Jaeger, S., Chaix, J., & Vivier, E. (2007). Natural killer cells: from CD3-NKp46+ to post-genomics meta-analyses. *Current Opinion in Immunology*, *19*(3), 365–372. https://doi.org/10.1016/j.coi.2007.04.004

Wen, L., Pao, W., Wong, F. S., Peng, Q., Craft, J., Zheng, B., … Hayday, A. C. (1996). Germinal center formation, immunoglobulin class switching, and autoantibody production driven by "non alpha/beta" T cells. *The Journal of Experimental Medicine*, *183*(5), 2271–82. https://doi.org/10.1084/jem.183.5.2271

Willcox, C. R., Pitard, V., Netzer, S., Couzi, L., Salim, M., Silberzahn, T., … Déchanet-Merville, J. (2012). Cytomegalovirus and tumor stress surveillance by binding of a human γδ T cell antigen receptor to endothelial protein C receptor. *Nature Immunology*, *13*(9), 872–879. https://doi.org/10.1038/ni.2394

Wrobel, P., Shojaei, H., Schittek, B., Gieseler, F., Wollenberg, B., Kalthoff, H., … Wesch, D. (2007). Lysis of a broad range of epithelial tumour cells by human gdT cells: Involvement of NKG2D ligands and T-cell receptor- versus NKG2D-dependent recognition. *Scandinavian Journal of Immunology*, *66*(2–3), 320–328. https://doi.org/10.1111/j.1365-3083.2007.01963.x

Wu, D., Wu, P., Qiu, F., Wei, Q., & Huang, J. (2017). Human γδT-cell subsets and their involvement in tumor immunity. *Cellular & Molecular Immunology*, *14*(3), 245–253. https://doi.org/10.1038/cmi.2016.55

Wu, D., Wu, P., Wu, X., Ye, J., Wang, Z., Zhao, S., … Xu, J. (2015). Ex vivo expanded human circulating V d 1 gd T cells exhibit favorable therapeutic potential for colon cancer, (March), 1–13.

Wu, P., Wu, D., Ni, C., Ye, J., Chen, W., Hu, G., … Huang, J. (2014). γδT17 Cells Promote the Accumulation and Expansion of Myeloid-Derived Suppressor Cells in Human Colorectal Cancer. *Immunity*, *40*(5), 785–800. https://doi.org/10.1016/j.immuni.2014.03.013

Wu, Y.-L., Ding, Y.-P., Tanaka, Y., Shen, L.-W., Wei, C.-H., Minato, N., & Zhang, W. (2014). γδ T Cells and Their Potential for Immunotherapy. *International Journal of Biological Sciences*, *10*(2), 119–135. https://doi.org/10.7150/ijbs.7823

Xu, B., Pizarro, J. C., Holmes, M. A., McBeth, C., Groh, V., Spies, T., & Strong, R. K. (2011). Crystal structure of a T-cell receptor specific for the human MHC class I homolog MICA. *Proceedings of the National Academy of Sciences*, *108*(6), 2414–2419. https://doi.org/10.1073/pnas.1015433108

Xu, B., Pizarro, J. C., Holmes, M. A., McBeth, C., Groh, V., Spies, T., & Strong, R. K. (2011). Crystal structure of a γδ T-cell receptor specific for the human MHC class I homolog MICA. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(6),

2414–2419. https://doi.org/10.1073/pnas.1015433108
Yokoyama, W. M., Kim, S., & French, A. R. (2004). The Dynamic Life of Natural Killer Cells. *Annual Review of Immunology*, *22*(1), 405–429. https://doi.org/10.1146/annurev.immunol.22.012703.104711
Zeitz, M., Schieferdecker, H. L., Ullrich, R., Jahn, H. U., James, S. P., & Riecken, E. O. (1991). Phenotype and function of lamina propria T lymphocytes. *Immunologic Research*, *10*(3), 199. https://doi.org/10.1007/BF02919693
Ziegler, S. F., & Liu, Y.-J. (2006). Thymic stromal lymphopoietin in normal and pathogenic T cell development and function. *Nature Immunology*, *7*, 709. Retrieved from http://dx.doi.org/10.1038/ni1360
Zou, C., Zhao, P., Xiao, Z., Han, X., Fu, F., & Fu, L. (2017). γδ T cells in cancer immunotherapy. *Oncotarget*, *8*(5), 8900–8909. https://doi.org/10.18632/oncotarget.13051