The role of G-protein-coupled receptors in the biological activity of γδ T lymphocytes

Ph.D. thesis

In fulfillment of the requirements for the degree

"Doctor of Philosophy (Ph.D.)/Dr. rer. nat."

at

Friedrich Schiller University, Jena

Faculty of Biology and Pharmacy

Submitted to the Council of the Faculty of Biology and Pharmacy

of Friedrich Schiller University, Jena

by

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Jena, 2009

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Date of defense: 05-07-2010

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Abbreviations

αβ T cells	alpha-beta T cells
BSA	bovine serum albumin
Ca^{2+}	calcium
cAMP	cyclic adenosine-3',5'-monophosphate
CI	chemotactic index
Ctx.	cholera toxin
C5a	complement factor 5a
DAG	diacylglycerol
dimaprit	S-(3-dimethylaminopropyl)isopthiourea 2HCl (dimaprit-2HCl)
EGTA	ethyleneglycol-bis(beta-aminoethylether)-N,N'-tetraacetic acid
ERK	extracellular signal regulated kinase
FACS	fluorescence cytometry
FCS	fetal calf serum
FITC	fluoro-isothiocyanate
fMLP	N-formyl-Met-Leu-Phe
FPR	fMLP-receptor
FPRL1, FPRL2	fMLP receptor-like 1 and 2
γδ T cells	gamma-delta T cells
GCPR	G protein couple receptor
H1R, H2R, H3R, H4R	histamine receptor subyptes (1-4)
HTMT	6-[2-(-imidazolyl)ethylamino]-N-(4-trifluoromethylphenyl) heptanecarboxamide dimaltate
IL-	interleunkin-
IFN-	interferon
IP_3	inositol-1,4,5-trisphosphate
NK	cell natural killer cells
NFκB	nuclear factor 'kappa – light – chain –enhancer'
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PE	phycoerythrin
PIP_2	phosphatidylinositol (4,5)-bisphosphate
PIP_3	phosphatidylinositol (3,4,5)-trisphosphate
PI3K	phosphatidylinositol 3-kinase
Ptx.	pertussis toxin
Raji	human Burkitt's lymphoma cell line
RANTES	regulated on activation-normal T cell expressed and secreted
thioperamide	N-cyclohexyl-4-(1H-imidazol-4-yl)-1-piperidinecarbothioamide maleate salt (thioperamide)

Summary

The data collected in this work represent the first evidence that G-protein-coupled signalling exist in γδ T cells via histamine and fMLP receptors. The bigenic amine histamine and the bacterial peptide fMLP have been demonstrated to be novel chemoattractant factors for circulating human γδ T cells, which are critical members of the immunological tumor surveillance machinery. Here, we analyzed the influence of histamine on the interaction of human γδ T cells with tumor cells such as the A2058 human melanoma cell line, the human Burkitt's non-Hodgkins lymphoma cell line Raji, the T-lymphoblastic lymphoma cell line Jurkat, and the NK cell-sensitive erythroleukaemia line K562. We found that histamine inhibits the spontaneous cytolytic activity of γδ T cells in response to these cell lines. The downregulation of γδ T cell mediated cytotoxicity involves the histamine receptor subtype 2 (H_2R), the activation of G_s proteins and increased cAMP intracellular levels. On the other hand, histamine activates the common signalling pathways of chemotaxins such as G_i-protein-dependent actin reorganization, the increase of intracellular Ca^{2+} and the induction of migratory response in γδ T lymphocytes. Our data indicate that histamine contributes to the mechanism by which tumor cells escape immunological surveillance.

The bacterial-cell-wall-derived peptide N-formyl-Met-Leu-Phe (fMLP) is a well characterized chemotactic factor for phagocytes such as neutrophils, monocytes and dendritic cells. Here, we analyzed the influence of fMLP on isolated human peripheral blood γδ T cells. We found that fMLP induces intracellular calcium transients, actin reorganization, CD11b upregulation and the migration of γδ T cells. Pretreating γδ T cells with pertussis toxin inhibited all fMLP-stimulated cell responses, implicating the involvement of G_i proteins in the induced signalling cascade. The present data suggest that, in addition to phagocytes, N-formyl peptides also regulate the trafficking and activation of γδ T cells.

Zusammenfassung

Die Kommunikation zwischen Zellen ist eine essentielle biologische Funktion in multizellulären Organismen. Externe Signale können durch spezifische Rezeptoren der Zielzellen wahrgenommen werden und lösen Protein-Protein-Interaktionen, Protein-Phosphorylierungen und die Bildung sekundärer Botenstoffen aus und führen schließlich zu spezifischen, zellulären Reaktionen.

G-Protein gekoppelte Rezeptoren gehören der Superfamilie der sogennanten Serpentin Rezeptoren an, deren charakteristisches Merkmal sieben transmembranäre Helices sind. G-Proteine selbst sind aus drei Unterinheiten (α,β und γ) aufgebaut. Bindet ein Botstoff, beispielsweise Histamin oder fMLP an seinen Rezeptor, so wird durch eine Konformationswandlung die Bindungsstelle für G-Proteine freigelegt. Innerhalb des Immunsystems sind G-Proteine Regulatoren von zellulärer Reaktionen. Die G-Protein gekoppelte Rezeptoren sind an die Einrichtung von verschiedenen Zellfunktionen beteiligt. Beispielsweise induzieren Chemotaxine wie fMLP in neutrophilen Granulozyten G-Protein-abhängig die gerichtete Migration und die ROS-Produktion der Zellen. Mittels RT-PCR, Western blot Analyse und Flow Zytometrie wurden Histamin und fMLP Rezeptoren an der Oberfläche von aus dem peripheralen Blut isolierten humane $\gamma\delta$ T Zellen identifiziert. Histamin (2-(4-Imidazolyl)-ethylamin), das biogene Amin wird durch Abspaltung von Kohlendioxid aus der Aminosäure Histidin gebildet und insbesondere in Mastzellen, basophilen Granulozyten und Nervenzellen gespeichert. N-formyl-Met-Leu-Phe (fMLP) ist ein in den Bakterien vorkommender chemotaktische Faktor. Es wurde gezeigt, dass Histamin und fMLP als chemotaktische Faktoren für humane $\gamma\delta$ T Zellen funktionieren. Es wurde weiterhin demonstriert, dass die Migration der humanen $\gamma\delta$ T Zellen zu den beiden Faktoren eine G_i-Protein-abhängige Reaktion der Zellen ist. Wir haben nachgewiesen, dass es in humane $\gamma\delta$ T Zellen für Histamin drei unterschiedlichen Rezeptoren-Untereinheiten (H_1, H_2, H_4) existieren. Von denen der G_s-Protein gekoppelte H_2-Rezeptor für die Regulierung der Zytotoxizität Reaktion und der G_i-Protein gekoppelte H_4-Rezeptor für die chemotaktische Antwort der humane $\gamma\delta$ T Zellen auf Histamin verantwortlich ist.

1. Introduction

1.1. The immune system

The immune system is an organization of cells and molecules with specialized roles in defense against infection. There are two fundamentally different types of responses to invading microbes. Innate responses occur to the same extent however many times the infectious agent is encountered, whereas adaptive responses become more exact on repeated exposure to a given infection [1]. Innate and acquired responses usually work together to eliminate pathogens. The innate responses use neutrophils, monocytes, macrophages, basophils, mast cells, and eosinophils, natural killer cells, and γδ T cells. The molecular components of innate responses include complement, acute-phase proteins, and cytokines [1]. Acquired responses involve the proliferation of antigen-specific B and T cells. Specialized cells, called antigen presenting cells, present the antigen to lymphocytes and collaborate with them in the response to the antigen, to initiate the target-specific response. The response of the immune system to pathogens, immune complexes, and injured tissues depends in large part on the functional relationships between the different immune cells.

The accumulation of leucocytes at sites of inflammation and microbial infection in response to locally produced chemotactic factors is an important feature of the immune response [2]. The accumulation of selected populations of leukocytes is an orchestrated event that probably involves the coordinated expression of a variety of adhesion molecules, target-cell-specific chemokines, cytokines, growth factors and enzymes that contribute to the ensuing tissue remodelling, as defined by the degradation of existing and the deposition of new extracellular matrix proteins and proteoglycans, and angiogensis [3]. A large group of chemotactic factors such as all chemokines, histamine

and N-formyl peptides, e.g N-formyl-Met-Leu-Phe (fMLP) engage seven-transmembrane G-protein-coupled-receptors (GPCRs).

1.2.1. G-protein-coupled receptors (GPCRs)

With nearly 1000 members, G-protein-coupled receptors (GPCRs) constitute the largest group of cell-surface proteins that are involved in signal transduction. They account for more than 2% of the proteins that are encoded by the human genome [4]. GPCRs are often referred as serpentine receptors because they span the plasma membrane seven times with α-helical segments separated by alternating intracellular and extracellular loop regions. The N-terminus is located extracellularly, whereas the C-terminus is found intracellularly [5]. These receptors are activated by a diverse array of ligands, from peptide and non-peptide neurotransmitters, to chemokines and nucleotides [6.7]. Consistent with their ability to recognize many antigens, GPCRs participate in a wide variety of physiological functions. GPCRs are also involved in a number of human diseases: this involvement is reflected in the fact that they are targets directly or indirectly of 50-60% of all known therapeutic agents to date.

When receptors activated by agonists, the conformation of the transmembrane α-helices of GPCRs changes profoundly and previously masked G-protein-binding sites appear. This, in turn, promotes GDP-GTP exchange on the α-subunit, which results in their activation. Consequently, Gβγ- and GTP-bound Gα proteins stimulate effector molecules. The biochemical changes that are induced are highly dependent on the individual receptor-coupling specificity for each of the four families of mammalian G-protein subunits: α_s α_i α_q or $\alpha_{12/13}$ [8]. Receptors that are coupled to $G\alpha_s$ activate adenylyl cyclases, thereby increasing cyclic-AMP levels; whereas those that stimulate $G\alpha_i$-family members inhibit adenylyl cyclases and reduce cAMP levels as well as

activating phospholipases and phosphodiesterases [8]. Each G protein has a unique biological function. The G_s protein activates adenylyl cyclise, resulting in the accumulation of cyclic adenosine monophosphat (cAMP) in the cells. G_i proteins are highly homologous members of the family of heterotrimeric signal-transducing guanine nucleotide-binding proteins (G proteins) involved in coupling numerous receptors for extracellular signalling molecules to the inhibition of adenylyl cyclase, the stimulation of phospholipase C, or the opening of potassium channels [9, 10, 11]. The activation of phospholipase C results in the hydrolysis of phosphatidylinositol bisphosphate (PIP_2) and the generation of diacylglyceride (DAG), an activator of protein kinase C (PKC), and inositol(1,4,5)trisphosphate . [12]. The termination of GPCR signalling cascades after agonist activation is highly regulated and can occur through several mechanisms [8].

1.2.1.1. Toxins influencing G-protein coupled receptor pathways

There are two main subgroups of G proteins, the cholera-toxin-sensitive G proteins and the pertussis-toxin-sensitive ones. In the cholera-toxin-sensitive G proteins, the arginine residue present in the carboxy terminal of the α subunit of G_s will be ADP-ribosylated, whereas the pertussis toxin ADP ribosylates the cysteine residue in the carboxy terminal of the α subunit of G_i and G_o proteins while it inhibits their interaction with agonist-occupied receptors [13].

1.2.2. The role of adhesion molecules

Leukocytes must engage several sequential adhesion pathways to leave the circulation. Initially, tethers are formed by adhesion receptors that are specialized to engage rapidly and with high tensile strength. The most important initiators of adhesion are the three

selectins expressed on leukocytes L-selectin, endothelial cells (P- and E-selectin), and activated platelets (P-selectin) [14]. Selectin-mediated bonds are too impermanent to arrest cells at the vessel wall. As the blood flow exerts pressure, adhesion bonds dissociate at the cell´s upstream end and new bonds form downstream. This results in a rolling motion that is much slower than that of free-flowing cells. To stop rolling, cells must engage additional (secondary) receptor molecules, which belong to the integrin family. Integrins are obligate heterodimers containing two distinct chains, called the α- and β-subunits. They are expressed on the most of the cells and are responsible for anchoring the cells to the extracellular matrix. Only a limited number of integrins are expressed in leukocytes, e.g. the β_2-integrin CD11b/CD18 [15, 16, 17].

1.2. Chemotaxis of leukocytes and G-protein-coupled signalling

Chemotaxis is the ability of cells to detect and move towards the source of a chemoattractant signal. Such directional migration is essential for a variety of cellular processes, including cell movement during development, immune responses and wound healing, in addition to the metastasis of tumor cells. In response to chemoattractants, leukocytes rapidly polarize in the direction of the signal, forming a pseudopod on the side exposed to the highest chemoattractant concentration, and a uropod or a posterior domain on the opposite side of the cell. These structures become the leading and trailing edges of the migrating cell [18, 19]. The formation of the leading edge results from localized chemoattractant-induced F-actin assembly, whereas the cell´s side and posterior become enriched in assembled myosin II. The regulation of these cytoskeletal components during chemotaxis provides the driving and contracting forces required for cell motility **[20]**. Cells are able to detect and respond to chemoattractant concentrations that differ by as little as 2-5% between the front and the back of the cell. These

differences are then translated by the cell into a steep intracellular gradient of signalling components, leading to an asymmetric cellular response [21]. One of the first asymmetric responses to chemoattractant stimulation in leukocytes is the localized accumulation of phosphatidylinositol-3 kinases [PI(3,4,5)P_3], the product of class I phosphatidylinositol-3 kinases (PI3Ks), at the site of the new leading edge [21, 22]. PI3K, which is made up of multiple isoforms, comprises a regulatory p85 subunit and a catalytic p110 subunit, of which in αβT lymphocytes p110α and p110δ are expressed; both participate in the activity of the enzyme [23]. The three catalytic isoforms p110α, β, δ are classified as class IA PI3Ks; the only member of the class of IB PI3Ks is p110γ [24, 25]. The pharmacological inhibitors of PI3Ks are wortmannin [26] or LY294002 [27]. PI3K activation results in binding of p85 to adapters and in turn the enzyme to the plasma membrane and subsequently generates PI(3,4,5)P_3 and PI(3,4)P_2 by activating the catalytic subunit p110 [28]. This localized enrichment of [PI(3,4,5)P_3] at the leading-edge membrane of the cell recruits and activates proteins that preferentially bind to PI(3,4,5)P_3, including proteins that contain a plextrin-homology (PH) domain (e.g. protein kinase B [Akt/PKB], guanine nucleotide exchange factors [GEFs]) [29].

1.3. The role of γδ T cells

The immune system has maintained two different types of T cell receptors (TCRs) throughout evolution, αβ T cells and γδ T cells. γδ T cells form an independent population of lymphocytes expressing the γδ TCR genes [30, 31, 32] and representing a complex system of developmentally and functionally differentiated subsets [33]. γδ T cells are an ancient type of lymphocytes derived from haematopoetic stem cells; they share different characteristics with other immune cells such as the capacity to present antigens [34, 35] and the cytolitic effect [34, 36]. γδ T cells participate in the

polarization of Th_1- and Th_2- immune responses by releasing various cytokines and helping αβ T lymphocytes mature [37] and B cells [38]. γδ T cells, unlike αβ T lymphocytes, do not need MHC presentation by antigen presenting cells to recognize antigens. They are able to directly recognize natural phosphoantigens derived from plants [39], bacteria [40, 41, 42] protozoa [43], endogenous ligands from some tumors [47, 48, 49], and viruses [45, 46]. Early events in an immune response stimulate the production of cytokines; these direct the subsequent development of T cell subsets with discrete patterns of cytokine production. Cytokines secreted by human γδ T cells are able to activate both innate host defenses such as IFNγ, or IL-2 [34, 50, 48, 35, 49, 51] and humoral host defenses with IL-4 and IL-10 [50, 52, 53].

1.3.1. γδ T cell subtypes: Circulating and resident γδ T cells

Two main subsets of γδ T cells are distinguished on the basis of their location. Resident γδ T cells are found in the skin, uterine and epithelium, whereas the circulating/systemic γδ T cells are found in the peripheral blood or lymphoid tissues [54, 55]. The majority of circulating human γδ T cells express a TCR comprising the variable segments γ9 δ2 (Vγ9Vδ2 TCR). The functional characteristics of these cells are MHC-unrestricted cytolysis, production of Th1 type cytokines such as IFN-γ and TNF-α, and expansion in a variety of bacterial or parasite infections [56, 57, 58, 34]. In the peripheral blood of most healthy individuals, T cells expressing the Vδ2 gene paired with one particular Vγ chain (Vγ9) account for 50% to 90% of the γδ T cell population [34]. The major population of Vγ9Vδ2 T peripheral γδ T cells is strongly activated by a variety of bacteria and parasites [59, 36, 35]. Sequencing the rhesus monkey Vγ9Vδ2 TCR revealed a strong sequence homology to the human Vγ9Vδ2 TCR with important

preserved sequence motifs [60] macaque and human Vγ9Vδ2 TCR share several conserved motifs [61]. Intestinal intraepithelial γδ T cells frequently express the Vδ1 gene, which can associate with different Vγ elements [54].

1.3.2. γδ T cell antigen recognition

γδ T cells have been shown to recognize classical MHC molecules (MHC I/II) as well as nonclassical MHC molecules, such as nonpolymorphic MHC-related CD1 protein and the closely related MHC class I b molecules T22 by TCR. MHC molecules are recognized as antigens, whereas the loaded peptides on the membrane do not play any role as ligands. In addition, γδ T cells can recognize non-MHC molecules, for examples, isopentenyl pyrophosphate, heat shock protein (Hsp), MICA/B, ULBP3, virus protein directly *via* γδ TCR or cooperatively through antigen specific receptor, such as NKG2D without antigen processing and presentation requirements [62, 63, 64, 65].

1.3.2.1. Bacterial antigens

Early reports on the reactivity of γδ T cells suggested that heat-shock proteins were the main stimulatory fraction of mycobacteria [66, 67]. However, later studies have shown that stimulation of γδ T cells by M. tuberculosis appears to occur mainly through the recognition of the phosphorylated nucleotides TUBag1-4 [68].

1.3.2.2. Amino-bisphosphonates

The antitumor effect of amino-bisphonates mainly results from their capacity to activate γδ T cells, as they have been found to induce Vγ9Vδ2 T cell proliferation in vitro [69] and in vivo [70] and to trigger the Vγ9Vδ2 TCR-mediated lysis of tumor lines [69].

Vγ9Vδ2 T cells are stimulated by cognate recognition of human cells pulsed with aminobisphosphates, e.g., pamidronate or zoledronate, probably because their pharmacological action leads them to accumulate of mevalonate metabolites [69, 71]. Amino-bisphosphonates induce Vγ9Vδ2 T cell proliferation in vitro and in vivo, and trigger the Vγ9Vδ2 TCR-mediated lysis of many tumor cell lines and reduce the survival of autologous myeloma cells [36].

1.3.2.3. Phosphoantigens

The most potent natural phosphoantigen is (E)-4hydroxy-3-methyl-but-enyl pyrophosphate, a metabolite of the nonmevalonate pathway of isoprenoidsynthesis, which is found in plants, bacteria and protozoa [43]. Less potent phosphoantigens are ubiquitous metabolites of isoprenoid synthesis, such as isopentenyl pyrophosphate (IPP) [72]. IPP-mediated γδ T cell activation does not require accessory cells as does the activation of the aminobisphosphonate pamidronate [73].

1.3.3. Receptors expressed on the surface of γδ T cells

1.3.3.1. TCR receptor

1.3.3.1.1. The classical TCR signalling pathway in T lymphocytes

The physiological ligand for T cell activation is a foreign peptide bound to a major histocompatibility complex (MHC)-encoded molecules presented on the surface of professional antigen presenting cells (APCs) such as dendritic cells (DC) [15]. Biochemical signals initiated by the TCR receptor make up α/β subunits and signal-transducing subunits ε, γ, δ, and ζ; these determine the specifity of the TCR receptor

activation and events initiated by other membrane proteins such as MHC receptors CD4/CD8; costimulators such as CD2, CD28 or integrins modulate the intracellular signaling thresholds required to initiate a T cell immune reaction [74]. The activation of tyrosine kinases initiates a cascade of signalling pathways in T cells resulting in the activation of small GTPases-signalling networks and the regulation of inositol phospholipid metabolism: this regulation is responsible for controlling intracellular calcium (Ca^{2+}) and the activity of diverse serin/threonine kinases, including members of the PKC family and phosphatidyl inositol-3 kinase (PI3K)-controlled serin kinases [29]. Unlike αβ T, γδ T cells are endowed with the capacity to interact with low-molecular-mass-phosphate-containing nonprocessed antigens, such as pyrophosphomonoesters [72] and alkylamines [39]. Recognition of these antigenic compounds is mediated by TCR but does not require presentation by conventional MHC molecules [75, 76].

1.3.3.1.2. NKG2D receptor

NKG2D belongs to a sub-family of C-type lectin-like receptors, which have lost the ability to bind both calcium and carbohydrate. In addition to TCR ligation, the activation of Vγ9Vδ2 T cells is modulated by a range of inhibitory and activating NK-cell receptors [77, 78].

NKG2D ligation is known to provide a powerful co-stimulus for the antigen-mediated activation of both $CD8^+$ and Vγ9Vδ2 and intraepithelial Vγ1Vδ1 T cells; however, the signalling pathways leading to the activation of these cell types are poorly understood. In Vγ9Vδ2 T cells, activation through NKG2D receptors happens independently of the antigen, as also happens in the NKG2D-mediated activation of NK cells [36]. Several lines of evidence show that both γδ T cell populations have antitumor reactivity [79, 48, 80], which they perform with their production of perforin [54, 50, 80] and granzymes

[34, 81]. Certain tumors and lymphomas activate γδ T cells via endogenous ligands in γδ TCR-dependent fashion [75, 48]; other ligands of tumors or transformated cells bind the NK cell receptor NKG2D on the surface of γδ T cells [79].

1.3.3.2. The role of γδ T cells as effector cells: the role of chemokines and cytokines

Early during the course of infection, cytokines produced by γδ T cells may help eliminate pathogens and contribute to effective cellular immunity against intracellular bacteria and parasites. Vδ2 T cells display a range of innate effector functions, including the rapid secretion of chemokines MIP-α, MIP-β, or RANTES [82, 83] and cytokines TNF-α and INF-γ [84, 85].

1.3.3.2.1. Response to chemokines

Chemokines or chemotactic cytokines are a family of small (7-15 kDa), inducible proteins, which can be classified into four families: C-X-C chemokines or α-chemokines, CC or β-chemokines C or γ-chemokines, and CX3C or δ-chemokines. Chemokines are implicated in multiple pathological processes, such as allergic disorders, autoimmune diseases and ischemia [12]. In addition to chemotaxis, chemokines have been shown to modulate a number of biological responses in leukocytes, including cellular activation, adhesion, enzyme release, degranulation, tumor cell lysis, and intracellular microbe death [86].

MCP-1/CCL2 [87], RANTES/CCL5, MIP-1α/CCL3 and MIP-1β/CCL4 were found to chemically attract αβ T lymphocytes and circulating γδ T cells, whereas neither αβ nor γδ showed response to IL-8/ CXCL8 or IFN-inducible protein 10 (IP-10/CXCL10) [88, 89]. The corresponding chemokine receptors - seven-transmembrane-spanning G

protein-coupled receptors - share structural features, and some of which are involved in intrathymic [90, 91] and memory T cell development, in orchestrating T and B cell interactions and in differentiating effector T cells [87, 92].

1.3.3.3. The role of γδ T cells as effector cells: the way of killing, cytolytic activity

γδ T cells not only inhibit the early stages of tumor development but also limit the progression of neoplastic cells; combined, these functtions suggest there are two or more different antitumor mechanisms in these cells**.** In summary, mouse studies have elucidated three different pathways by which γδ T cells may provide anticancer activities; direct killing of transformed cells, early IFN-γ production and critical immunoregulatory function. γδ T cell lines have been shown to efficiently kill extra- and intracellular *M. tuberculosis* bacilli through the release of the cytotoxic molecule granulysin, although granulysin kills both extra- and intracellular *M. tuberculosis*, eliminating the latter requires perforin [59]. Activated γδ T cells are shown to lyse target cells also by producting granzymes and perforin [36].

1.3.3.3.1. γδ T cells in the tumor microenvironment

The broad antigen-specific recognition of hematopoietic tumors by Vγ9Vδ2 T lymphocytes results in cytotoxic activity, inducing Th1 cytokine production and proliferation [77, 93]. Like NK cells, Vγ9Vδ2 T lymphocytes exert a cytotoxic activity that is controlled at the effector level by the expression of killer Ig-like receptors (KIR) [94] which interact with major histocompatibility complex class I (MHCI) molecules at the surface of the target [78, 95]. Tumor cell lines which are sensitive to this NK-like

cytolytic activity, such as the chronic myelogenous leukaemia cell line K562, often lack the ability to express MHC-class I molecules [96, 97, 98].

Some tumors and lymphomas, such as the B cell line Daudi, express endogenous ligands, which in turn activate γδ T cells in a Vγ9Vδ2 T cell-dependent fashion [75]. Moreover, both the percentage and the absolute number of circulating γδ T cells are known to be significantly reduced in patients with cutaneous primary melanoma, thus suggesting a specific deficit for this lymphocyte population. The reduction of γδ T cells correlated with the decrease of the Vδ2 T cell subset, which is involved in the reactivity toward microbial antigens and tumor cell antigens. The in vitro expansion of γδ T cells, particularly the Vδ2 T subset isolated from peripheral blood of patients with cutaneous primary melanoma, was significantly reduced; in summary, not only the proliferative capacity but the percentage of γδ T cells producing either TNF-α or IFN-γ is significantly reduced in melanoma patients [99].

1.3.3.3.1. γδ T cells in immunosuppression and cancer immunotherapy

Human γδ T cells are prime effector cell candidates for immune therapy against malignancy, since there is a growing evidence that Vγ9Vδ2 T cells have cytotoxic antitumor activitiy against a large range of tumor types [100, 101]. Activated Vγ9Vδ2 T cells provide alternative mechanisms for tumor-targeted recognition and killing, increasing the possibility that treatment with other modalities may enhance antitumor effects. Pre-treating tumor cells with low concentrations of chemotherapeutic agents may lead to their isoprenoid-mediated recognition and perforin following TCR-dependent rapid killing by Vγ9Vδ2 T cells [102].

1.4. Characterization of histamine and fMLP as chemoattractant factors

Since circulating γδ T cells have a pivotal role in host defense against invading pathogens, bacteria, and viruses as well as in the tumor microenvironment, and the existence of G-protein-coupled receptors on the surface of these cells has been not thoroughly examined, in this work we investigated the signal transduction pathways for histamine and fMLP in circulating γδ T cells. All the chemoattractants mentioned above are ligands of GPCRs and influence the immune response of leukocytes.

1.4.3. Histamine

Histamine (β-imidazolylethylamine) is an endogenous mediator involved in various responses of immune and non-immune cells; it is stored in the granules of tissue mast cells and blood basophlis and is released upon activation by IgE cross-linking [103]. The half-life of histamine in plasma is < 20 minutes, whereas the suppression of T-cells and phagocytes lasted ≥ 4 hours after subcutane histamine injection [104].

Beyond its physiological functions, histamine was described as an autocrine and paracrine/exogene growth factor for malignant melanomas and leukemic cells. Histamine and histidine decarboxylase (HDC) [105], the only enzyme that catalyzes histamine production, has been proven to be present in elevated concentrations in proliferating tissues, including tumor cells [106]. In the case of the myeloproliferative disease of chronic myeloid leukaemia (CML), this gene was the first known leukaemia-specific oncogene reported to be involved in the regulation of histamine production in neoplastic cells [107]. Malignant haematopoetic diseases characterized by hyperhistaminism in peripheral blood respond favorably to INF-α, an NK- and T-cell-activating cytokine [107, 108]. Histamine also interacts directly with H_2 receptors (H_2R) on T-cells to inhibit T-cell proliferation and cytokine production [109]; however, the

inhibition of T-cell function may not be expressed if histamine is combined with IL-2 [110, 111].

1.4.3.1. Histamine receptor subtypes

Histamine receptors are expressed on the surface of human monocyte-derived dendritic cells [112], eosinophils [113], mast cells [114], T cells, keratinocytes [115], and NK cells [116]. The effects of the numerous physiological activities of histamine are mediated by several histamine receptors. To date, four subtypes (H_1R, H_2R, H_3R and H_4R) of histamine receptors have been identified and cloned. All belong to the seven transmembrane domain G-protein- coupled receptor family, and coupled G proteins, and subsequently activated intracellular signals have been characterized [117]. In vitro studies made in human peripheral blood lymphocytes have demonstrate that histamine and the histamine receptor 2 (HR_2) agonists, impromidine and dimaprit, were effective in suppressing cell proliferation induced by low rather than high doses of PHA [118, 119, 120].

1.4.2. Formyl-methyl-leucyl-phosphate (fMLP)

N-formyl-methionine-leucine-phenylalanine (fMLP) is the prototype for N-formylated bacterial peptides and also a chemoattracting tripeptid. Its receptor in humans was pharmacologically defined as a high affinity binding site on the surface of neutrophils [121]. fMLP stimulates glucose uptake in CHO cells expressing chemoattractant-receptors which is due to the translocation of GLUT1 mediated by the Gα subunit through unknown pathways [122]. The fMLP-stimulated 2-DG uptake of human peripheral blood cells is inhibited by the PKC inhibitor calphostin C and tyrosine kinase inhibitor genistein [123]. In phagocytes, the chemoattractant fMLP activates the FPR to

stimulate diverse biological processes including chemotaxis, transcriptional activation, and actin reorganization [124].

1.4.2.1. The formyl peptide receptor (FPR)

First reports of the cloning and functional expression of human fMLP receptor, published in the early 1990s [125, 126], found the fMLPR to be constitutively active [127]. The formyl peptide receptor (FPR) genes of non human primates are 95-99% identical of their human ortholog [128]. Expression of the receptor has been described in phagocytic leukocytes, monocytes and neutrophils [129, 130], and is observed in immature dendritic cells, microglial platelets, spleen and bone marrow, non-haematopoetic cells and tissues such as hepatocytes, fibroblasts, astrocytes, neurons of autonomic nervous system, lung and lung carcinoma cells, thyroid, adrenals, heart, the tunica media of coronary arteries, uterus, ovary, placenta, kidney, stomach and colon [131]. The first ligand described for the FPR was the prototypical formylated peptide fMLP derived from *E. coli*, which binds and activate FPR with high affinity [131]. Because the formylation of peptides also occurs in mitochondria; the secondary release of formylated peptides secondary to cell death, might allow for the attraction of phagocytic leukocytes through FPR. Indeed disrupted mitochondria purified bovine mitochondrial N-formylatmethionyl-proteins, but not non-formylated mitochondrial proteins; the latter are chemoattractants for neutrophils in vitro [132]. The fMLP receptor is coupled to G_i proteins, as indicated by the total loss of cell response to their respective agonists upon exposure to pertussis toxin [121, 133, 134]. More specifically, the fMLP receptor is coupled to $G_{i\alpha1}$, $G_{i\alpha2}$ [135, 136, 137], $G_{i\alpha3}$ but coupling to G_o, G_z, $G_{\alpha16}$ [138] and in xenopus oocytes to G_q [139] has also been described [140, 141]. After ligation of the FPR, the heterodimeric G protein is activated, which dissociates into α

and βγ subunits; these subunits then activate the phospholipase C (PLC). The hydrolysis of phosphatidylinositol 4,5-bisphoshate (PIP_2) by PLC generates IP_3 [142], which releases intracellular calcium ($[Ca^{2+}]_i$) from endoplasmatic reticulum stores, and diacylglycerol (DAG); DAG, which activates protein kinase C (PKC) isofoms. The phosphoinositide 3-kinase (PI3K)-Akt/PKB pathway is also activated, in particular PI3Kγ, which is the isoform coupled to most chemoattractant receptors [143, 141].

Since fMLP such as IL-8 induce Ca^{2+} efflux and leukocyte chemotaxis, which are sensitive to PTX treatment, the fMLP receptor may use the same signal transduction pathways as the IL-8 receptors [144]. Co-transfection assays have been demonstrated that the fMLP receptor can specifically activate PLC β2 but not PLC β3 in a PTX-sensitive manner; thus, the interaction of the receptor with the G protein causes the release of the Gβγ subunits, which in turn specifically activate PLC β2 [138].

1.5. The aim of the study

The biogenic amine, histamine, has been described as playing a pathophysiological regulatory role in the cellular processes of various immune cells. This work analyzes the biological activity of histamine-influencing human peripheral blood γδ T lymphocytes, which are critical members of the immunological tumor surveillance. The bacterial cell-wall-derived peptide N-formyl-Met-Leu-Phe (fMLP) is a well characterized chemotaxin for phagocytes such as neutrophils, monocytes and dendritic cells. Both fMLP and histamine receptors belong to the family of G-protein-coupled seven-transmembrane receptors (GPCRs). Receptor ligand interactions are known to activate intracellular signal transduction pathways in neutrophils and monocytes, leading to directed motility, phagocytosis, superoxide anion generation, and degranulation responses culminating in death and elimination of invading microorganisms.

In this regard, the present study addresses the biological questions below:

1) What is the role of fMLP and histamine in the chemtaxis of human circulating γδ T lymphocytes?

2) What is the role of histamine in the cytolytic activity of human circulating γδ T lymphocytes?

2. Materials and methods

2.1. Materials

2.1.1. Biological Material

2.1.1.1. Cell lines

The human metastatic melanoma line A2058, from a brain metastasis in a 43-year-old man [145], the human Burkitt's non-Hodgkin lymphoma cell line Raji [146], the T-lymphoblastic lymphoma cell line Jurkat, established from the peripheral blood of a 14-year-old boy with acute lymphoblastic leukemia (ALL) at first relapse in 1976 [147] and the erythroleukaemia cell line K562 originating from patients with chronic myeloid leukaemia and blast crisis [148] were maintained at 37^{o}C in a 5% CO_2 incubator in RPMI 1640 supplemented with 10% fetal bovine serum, 10 U/ml penicillin, 10 U/ml streptomycin, and 1 mM L-glutamine [149].

2.1.1.2. Human peripheral blood γδ T lymphocytes

The use of human cells was approved by the Research Ethics Board of the Friedrich Schiller University of Jena. Human peripheral blood γδ T lymphocytes used in the work were isolated from the buffy coats of healthy volunteers produced by the Institute of Transfusion Medicine of Friedrich Schiller University of Jena.

2.1.2. Cell culture medium

Isolated human peripheral blood γδ T lymphocytes were cultured in RPMI medium supplemented with 10% fetal calf serum (FCS), 10 U/ml penicillin, 10 U/ml streptomycin, and 1 mM L-glutamine.

2.1.3. Reagents

Albumin from bovine serum (BSA)	Sigma-Aldrich, Taufkirchen
Cholera toxin (Ctx.)	Sigma-Aldrich, Taufkirchen
DNA Molecular Weight Marker	Roche Diagnostics, Mannheim
Dimethylsulfoxid (DMSO)	Sigma-Aldrich, Taufkirchen
FastStart Taq DNA Polymerase Kit	Roche Diagnostics, Mannheim
Fetal bovine serum, 10 U/ml	Promocell, Heidelberg
N-formyl-Met-Leu-Phe (fMLP)	Sigma-Aldrich, Taufkirchen,
Fura/2AM	MERK, Darmstadt
L-glutamine	Promocell, Heidelberg
Hank´s balanced salt solution (Hank´s BSS)	Promocell, Heidelberg
High Pure RNA Kit	Roche Diagnostics, Mannheim
Histamine	Sigma-Aldrich, Taufkirchen
HTMT dimaleate	TOCRIS (BIOZOL), Eiching
rHu interleukine-2 (Proleukine)	Chiron, Ratingen
imetit	TOCRIS (BIOZOL), Eiching
MicroScint-40 cocktail	PerkinElmer, Jügesheim
$Na_2{}^{51}CrO_4$ (100µCi)	Amersham, Freiburg
NBD-phallacidin	Invitrogen GmbH, Karlsruhe
Phosphate buffered saline (PBS)	Promocell, Heidelberg
Penicillin (10 U/ml)	Promocell, Heidelberg
Phaseolus vulgaris phytohemagglutinin (PHA)	Sigma-Aldrich, Taufkirchen
Pertusis toxin	Sigma-Aldrich, Taufkirchen
Primers	Invitrogen GmbH, Karlsruhe
RANTES	Promokine, Heidelberg

RPMI 1640 culture medium	Promocell, Heidelberg
SeaKem LE agarose	Cambrex, Tuafkirchen
Streptomycin, and 1 mM	Promocell, Heidelberg
Thioperamide malate salt (T123)	Sigma-Aldrich, Taufkirchen
Triton-X-100	Sigma-Aldrich, Taufkirchen
Wortmannin	Sigma-Aldrich, Taufkirchen

2.1.4. Antibodies

2.1.4.1. Flow cytometry analysis antibodies

anti-TCR γδ hapten-antibody	Miltenyi, Bergisch Gladbach
anti-hapten microbeads-FITC antibody	Miltenyi, Bergisch Gladbach
goat anti-mouse-FITC (sec. AB)	AL-Immunotools, Friesoythe
gouse anti-human cAMP antibody	Abcam, Cambridge
mouse anti-human CD88-FITC antibody	Acris Antibodies, Hiddenhausen
mouse anti-human fMLPR-PE antibody	BD Biosciences, Heidelberg
mouse anti-human Vγ9 TCR antibody	BD Biosciences, Heidelberg
mouse anti-human Vδ2 TCR antibody	BD Biosciences, Heidelberg
mouse anti-human-TCR-γ/δ-1 antibody	BD Biosciences, Heidelberg
mouse anti-human-TCR-α/β-1 antibody	BD Biosciences, Heidelberg

2.1.4.2. Western blot analysis antibodies

goat anti-human actin [(I-19:sc-1616)]	Santa Cruz Biotechnology, Heidelberg
goat anti-human H_1R (A20) antibody	Santa Cruz Biotechnology, Heidelberg
goat anti-human H_3R (P-15) antibody	Santa Cruz Biotechnology, Heidelberg

goat anti-human H_4R (IL-15) antibody	Santa Cruz Biotechnology, Heidelberg
rabbit anti-human H_2R (H-70) antibody	Santa Cruz Biotechnology, Heidelberg

2.1.5. Consumables

Cell culture flasks 75 cm^2	Greiner, Frickenhausen
Cell culture plates (12-, 24-, 96-well)	CellStar,
LS-Separation columns	Miltenyi, Bergisch Gladbach
Minifac tubes	Costar, Jena
Pipettes (5 ml, 10 ml, 25 ml)	Greiner, Frickenhausen
Pipette tips (10 µl, 100 µl and 1000 µl)	Greiner, Frickenhausen
Plastic tubes (500 µl, 1000 µl, 2000 µl)	Eppendorf, Hamburg
Syringes 60 ml Braun	Braun, Melsungen
Tubes (15ml, 50 ml)	Greiner, Frickenhausen

2.1.6. Buffers and solutions

Ammonium-chloride solution: 41.5 g NH_4Cl, 0.5 g $KHCO_3$, 0.179 g EDTA, 500 ml $H_2O_{(dest.)}$

PBS-BSA-EDTA solution: PBS pH 7.2, 0.5% BSA, 2 mM EDTA

EDTA solution: 0.5M, pH8.0

Ethidium bromide: 10 mg/ml

TBE buffer (5x), 1000 ml:

54 g Tris-base, 27.5 g boric acid, 20 ml 0.5 M EDTA (pH 8.0)

NET-G buffer (1x):

Tris-HCl pH 8.0 10 mM, NaCl 150 mM, EDTA 5 mM, Tween 20 0.05 % (v/v), Gelatine 0.05 % (v/v)

Running buffer (4x):

Tris-base, SDS 10% (v/v)

Stacking buffer (4x):

Tris-HCl pH 6.8 0.5 M, SDS 10 % (v/v)

Running gel (10%):

$H_2O_{(dest.)}$ 6.0 ml, Running buffer (4x) 3.6 ml, Acrylamide Stock 4.8 ml

Stacking gel (5%):

$H_2O_{(dest.)}$ 2.8 ml, Stacking buffer (4x)

1.2 ml, Acrylamide Stock 0.8 ml

Protein electrophoresis buffer (10x):

Tris-HCl pH 8.3 250 mM, Glycin 2 M, SDS 1 % (v/v)

Transfer buffer:

Tris 25 mM, Glycin 192 mM, Methanol 20 % (v/v)

Stripping buffer:

Tris-HCl pH 6.7 62.5 mM, 2-mercaptoethanol 100 mM, SDS 2% (v/v)

2.1.7. Equipment

Autoclave	Varioklav, Freiburg
Balance	Sartorius, Göttingen
Centrifuge 5804R	Eppendorf, Hamburg
Centrifuge RC-3B Sorvall	Heraeus, Langenselbold
Digital fluorescence microscope unit Atofluor	Zeiss, Jena
Electrophoresis gel chambers Gibco BRL	Life Technologies Inc., USA
Flow cytometer FACSCalibur	BD Biosciences, Heidelberg
Freezer -20°C	Siemens, Berlin

Freezer Hera -80°C	Heraeus, Langenselbold
Gamma counter TopcountTM	Packard Instruments,
Heating block HB-130	Unitek, Freiburg
Incubator CO_2	Sanyo, Sakata, Japan
Laminar-Air HB 2472 workbench	Heraeus, Langenselbold
Microplate Reader Emax	Molecular Devices, Freiburg
Microscope Telaval 3	Carl Zeiss, Jena
Pipettes Easypet	Eppendorf, Hamburg
Pipettes (20ml, 200ml, 1000ml)	Gilson, Hamburg
Power supply, PowerPac 300	Bio-Rad, USA
Thermomixer comfort	Eppendorf, Hamburg
Vortex	Bender&Hobein, Freiburg

2.2. Methods

2.2.1. Separation of blood leukocytes using the Ficoll-Paque gradient

A 50 ml buffy coat was diluted 1:1 with PBS (v/v). Carefully, 25 ml of the cell suspension was layered over 15 ml Ficoll-Paque PLUS solution in 50 ml conical tubes and centrifuged at 1800 rpm for 30 min at 20°C, without braking. Differential migration during centrifugation results in the formation of layers containing different cell types (Figure 2.2.1). The bottom layer contains aggregated erythrocytes. The layer above the erythrocyte layer contains mostly granulocytes. Because of their low density, the lymphocytes are found at the interface between the plasma and the Ficoll-Paque gradient with other slowly sedimenting particles (platelets and monocytes). The interphase cells (lymphocytes, monocytes and thrombocytes) were transferred by

Pasteur pipette to a new conical tube and subjected to short washing steps, with a balanced salt solution to remove platelets, Ficoll-Paque and plasma.

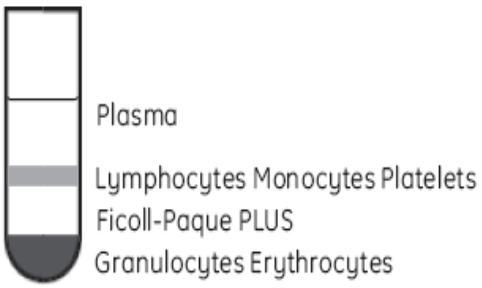


Figure 2.2.1. Differential migration during centrifugation results in the formation of layers containing different cell types.

2.2.1.1. Preparation of γδ T cells

The use of human cells was approved by the Research Ethics Board of the Friedrich Schiller University of Jena. Peripheral blood mononuclear cells (PBMCs) were isolated using the Ficoll separation protocol [33]. Briefly, a density gradient centrifugation of buffy coats was performed. The leukocyte-containing pellet was resuspended in phosphate-buffered saline (PBS) pH 7.2, supplemented with 0.5 % BSA and 2 mM EDTA, and the cells were labelled with an anti-TCR γδ hapten-antibody and anti-hapten micro-beads-FITC antibody. Labelled cells were separated with magnetic separation columns. Positive selected γδ T cells were cultured for 7-10 days in the presence of phaseolus vulgaris phytohemagglutinin (PHA) (2 μg/ml) for 3 days and IL-2 (100 IU/ml) until day 7 [80, 79, 99].

2.2.2. Flow cytometer

The flow cytometer enables a cell population to be acquired on the basis of its cell size and granularity. Additionally, surface molecules, or the purity or vitality of a cell population can also be examined by fluorescence. Purified cells and cells being cultured for 7-10 days were incubated with FITC-conjugated mouse anti-human-TCR-γ/δ-1 (1:200, BD Pharmingen) monoclonal antibodies for 30 min at 4°C. Isotype matched antibodies (IgG1 control, Immunotools) were used as negative control. Analysis was done with a FACSCalibur flow cytometer (BD Biosciences). All experiments were done with a purity of 96-99% of γδTCR positive T cell population. The cell viability was measured with the trypan blue exclusion test of cell viability and we accepted populations with a cell viability of min. 95%.

2.2.3. mRNA isolation, reverse transcription and polymerase chain reaction (RT-PCR) analysis

mRNA was isolated from $1x10^6$ human peripheral blood γδ T cells using a High Pure RNA isolation kit. A Fast Start Taq cDNA Polymerase Kit and a Fast Start Taq DNA Polymerase kit were used to obtain cDNA and PCR products. The primers were designed to recognize sequences specific for each target cDNA:

H_1R, (403 bp) : sense 5′-CATTCTGGGGGCCTGGTTTCTCT-3′ ;

antisense 5′-CTTGGGGGTTTGGGATGGTGACT-3′

H_2R, (497 bp): sense 5′- CCCGGCTCCGCAACCTGA-3′

antisense 5′-CTGATCCCGGGCGACCTTGA-3′

H_3R, (589 bp): sense 5′-CAGCTACGACCGCTTCTTGTC-3′

antisense 5′-GGACCCTTCTTTGAGTGAGC-3′

H_4R, (396 bp): sense 5′-GGTACATCCTTGCCATCACATCAT-3′

antisense 5′-ACTTGGCTAATCTCCTGGCTCTAA-3′

fMLPR (bp): sense 5′ TTC CGG ATG ACA CAC ACA GT

antisense 5′ TCC GAA CAA GTT GAT GTC CA

The phases of the PCR process were:

The initial phase: the denaturation of the DNA-double strand at 95ºC for 3 minutes

Main cycles: the amplification of the DNA with variable number of cycles (in three steps):

a. denaturation of the DNA-double strand (94ºC, 1 minute)

b. hybridization of the specific primers to the single-strand DNA (58 ºC, 60 ºC)

c. the Taq-polymerase chain reaction (amplification) (72 ºC, 1 minute)

The final phase: Taq-polymerase chain reaction (End-polymerization) (8 minutes)

2.2.4. Mobilization of Ca^{2+} transients

Intracellular free Ca^{2+} was measured in Fura-2-labeled γδ T cells using the digital fluorescence microscope unit Attofluor (Zeiss, Oberkochen, Germany). The purified circulating γδ T cells were resuspended in Hank´s 1% BSA solution and were loaded with 4 μM Fura-2/AM. The suspension was then incubated in dark, 37ºC for 30 minutes. After the incubation cells were washed two times and were resuspended in buffer and measured in dark. After the stimulation the fluorescence was measured and the ratio was calculated from the absorption between the wavelength 340 nm and 380 nm [150].

2.2.5. Filamentous (f) actin measurements

Probes of stimulated γδ T cells (10^6 /ml; 50ul/probe) were fixed in a 7.4 % formaldehyde buffer and mixed with the staining mixture containing 7.4 % formaldehyde, 0.33 μM NBD-phallacidin and 1 mg/ml lysophosphatidylcholine. The fluorescence intensity was measured by flow cytometry. The relative f-actin content was compared to unstimulated controls [151].

2.2.6. Migration assay

The chemotaxis of isolated human peripheral blood γδ T cells was performed in 48-well-microtechnic chambers from Neuro Probe (Gaithersburg, US). Wells in the bottom of the chamber were filled with 29 μl medium containing the indicated concentration of stimulus. Over this filled chamber, a polycarbonate membrane (thickness 10 μm, diameter of the pores 8 μm) and a gasket made of silicone was fixed. The device was screwed on the top of the gasket and the cells were added in 29 μl/well (resuspended in a concentration of $1x10^5$/ml) in the upper wells of the chamber. For the assay, the chamber was incubated for 240 minutes at 37°C. The non-migrating cells from the wells of the upper chamber were removed after the incubation period. The filter and gasket were then removed and the cells from the bottom chamber were collected, fixed in formaldehyde (3.7 %) and counted by flow cytometry. A chemotactic index (CI) was calculated as the ratio between stimulated and random migration.

2.2.7. In vitro cytotoxicity assay

Cytotoxicity was determined with a standard ^{51}Cr release assay. Target cells were labeled at 37°C for 1 h with 100 μCi $Na_2{}^{51}CrO_4$. Cells were washed and resuspended at

a cell density of 1×10^6 cells/ml in RPMI 1640 culture medium supplemented with 2 % fetal calf serum. Effector and target cells at different ratios (10:1; 5:1 and 2.5:1) were placed into individual wells of 96-well plates in a total volume of 200 µl at 37°C for 4 h. After incubation, 100 µl culture supernatant was collected from each well, mixed with a MicroScint-40 cocktail and analyzed with a gamma counter (TopcountTM, Packard Instruments). To obtain the value of total lysis, target cells were incubated with 2 % Triton-X. The percentage of specific lysis was calculated using the following formula:

$$\frac{\text{(experimental release - spontaneous release)}}{\text{(maximum release - spontaneous release)}} \times 100$$

2.2.8. Measurement of cAMP levels

γδ T cells (1 x 10^6 /ml) were fixed and made permeable before the intracellular staining was performed. The amount of intracellular cAMP in the γδ T cell preparation was determined by flow cytometry [152].

2.2.9. Western blot analysis

2.2.9.1. Lysis of the cells

Equal numbers of cells were washed and resuspended in PBS in 1.5 ml Eppendorf tubes and stimulated at 37°C. Supernatants were removed and cell pellets were transferred to ice and resuspended in an appropriate volume of lysis buffer containing 0.5 % of the detergent NP-40. Cell membranes were dissolved, while the cell cores and remaining cell compartments remained intact and were separated by centrifugation. The different protease inhibitors were added to the lysis buffer directly before use.

Lysis buffer: 20 mM Tris-HCl pH 8.0; 137 mM NaCl; 2.7 mM KCl; 1 mM $MgCl_2$; 1 mM $CaCl_2$; 0.5% NP-40; 0.5 mg/ml BSA; 1 mM Na_3VO_4; 1 mM PMSF; 21 µg/ml Aprotinin; 12 µg/ml AEBSF; 5 µg/ml Leupeptin; 5 µg/ml Pepstatin A.

After 10 min on ice, the lysates were centrifuged (15 min, 14000 rpm, 4 °C) and the NP-40 soluble fraction containing membranes and cytosolic proteins were collected and transferred into 1.5 ml eppendorf tubes.

2.2.9.2. SDS-Polyacrylamide-Gel electrophoresis (SDS-PAGE)

After cells were lysed, the NP40-soluble fraction contained in the supernatant was removed and diluted with 5x Laemmli sample buffer [153] and heated to 95^{o}C for 5 minutes. Samples (20 µg protein/ lane) were electrophoresed in 10% SDS polyacrylamide gels (80 mA). Proteins separated by SDS-PAGE were transferred from the gel to PVDF membranes (HybondTM ECL^{TM}, Amersham Biosciences) by electrophoresis (Western blotting) 50V for 90 min. The primary antibody was diluted 1:2000 with NET-G buffer and incubated 12 hr at 4°C. After being washed with NET-G buffer, the membrane was incubated with the secondary antibody (1:10000 in NET-G) for 1 hour at room temperature. After being washed, the protein of interest was detected by enhanced chemical luminescence (ECL) utilizing the reaction of Luminol and H_2O_2. For this purpose, the membrane was incubated with the ECL (Perbio, Bonn, Germany) reagents for 1 min and the chemiluminescence signal was visualized with the Bio Imaging System MF-ChemiBIS 3.2 (Biostep, Germany).

To reprobe the membrane with different antibodies, the previously applied antibodies first had to be removed. For this purpose, the membrane was incubated with 100-200 ml stripping buffer for 45 min at 56°C. The membrane was then washed several times with

NET-G (1x), until no residual 2-mercaptoethanol could be detected. The blot was again blocked in NET-G (10x) for 1 h, and a new primary antibody applied as described.

2.2.10. Statistical analysis

Significant values ($p<0.05$) were determined using the nonparametric two-tailed Student's t-test.

3. Results

3.1. Histamine and γδ T cells: the involvement of pertussis-toxin- and cholera-toxin-sensitive functions of isolated human peripheral blood γδ T lymphocytes

3.1.1. γδ T cells expressed histamine receptors: H_1R, H_2R, and H_4R mRNAs

In order to characterize the biological activity of histamine in γδ T cells, the expression of different histamine receptor subtypes was analyzed. Using reverse transcriptase and polymerase chain reaction (RT-PCR) analysis, the expected products for the H_1R, H_2R and H_4R subtypes were detected in isolated human-peripheral-blood γδ T cells. In contrast, the H_3R was undetectable (Fig. 1). Moreover, H_1R, H_2R and H_4R expression in γδ T cells at the protein level has been shown by western blot analysis (Fig. 2).

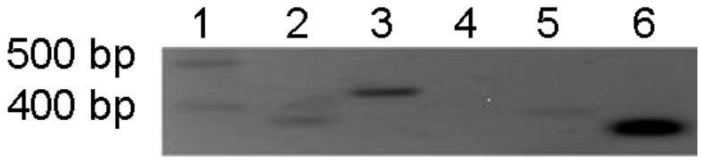


Fig. 1: **Expression of mRNA of H_1R, H_2R and H_4R in isolated human peripheral blood γδ T lymphocytes.** (A) γδ T cells were isolated from human peripheral blood and the expression of mRNA for histamine receptors was analyzed. Lane 1) DNA molecular weight marker XIV (100-1500bp); lane 2) H_1R (403 bp); lane 3) H_2R (497 bp); lane 4) H_3R (589 bp); lane 5) H_4R (396 bp); lane 6) β_2-microglobulin (259 bp). Experiments were repeated three times with identical results.

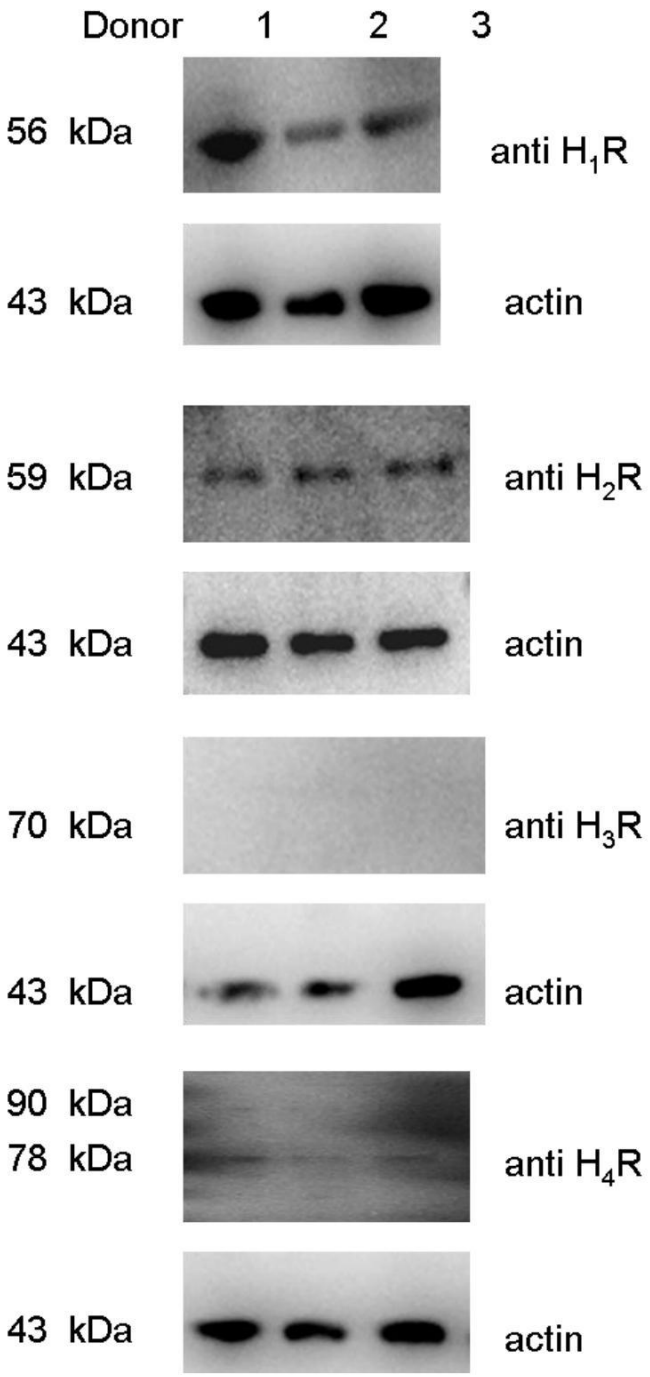
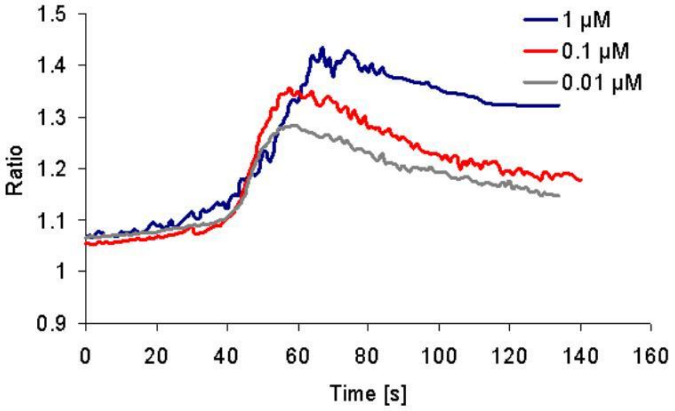


Fig. 2: **Expression of H_1R, H_2R and H_4R at a protein level in isolated human peripheral blood γδ T lymphocytes.** γδ T cells were isolated from human peripheral blood and the expression of different histamine receptor subtypes was analyzed by western blot analysis. Block 1) H_1R (56 kDa), actin (43 kDa); block 2) H_2R (59 kDa), actin (43 kDa); block 3) H_3R (70k Da), actin (43 kDa); block 4) H_4R (78/90 kDa), actin (43 kDa). Experiments were repeated three times with identical results.

3.1.2. Histamine induces actin polymerization, intracellular Ca^{2+} mobilization, and chemotaxis in γδ T cells through pertussis toxin-sensitive G_i proteins

Histamine induces Ca^{2+} transients in different types of leukocytes [154]. In accordance with the literature histamine also induced a rapid and concentration-dependent intracellular response in human γδ T cells isolated from peripheral blood (Fig. 3A). Ca^{2+} transients are mainly induced by mobilization from intracellular stores or by influx across the plasma membrane from the medium. In order to discriminate between these two mechanisms, experiments in the presence of ethyleneglycol-bis(beta-aminoethylether)-N,N'-tetraacetic acid (EGTA) were performed. Preincubating of γδ T cells with EGTA (4 mM) did not influence the histamine-initiated Ca^{2+} intracellular rise, which implicated the mobilization of Ca^{2+} from intracellular stores (data not shown). To investigate the involvement of G_i proteins in this response we took advantage of pertussis toxin. This toxin uncouples G_i proteins from serpentine receptors by ADP-ribosylation. Pretreating γδ T cells for 1 h with pertussis toxin (100 ng/ml) strongly inhibited the histamine-induced Ca^{2+} increase in γδ T cells, which in turn implicated the involvement of G_i proteins in this histamine response (Fig. 3B). To check the responsiveness of pertussis-toxin-treated cells, experiments with ionomycin were performed. Ca^{2+} transients induced by ionomycin were not influenced by pertussis toxin (data not shown).

A)



B)

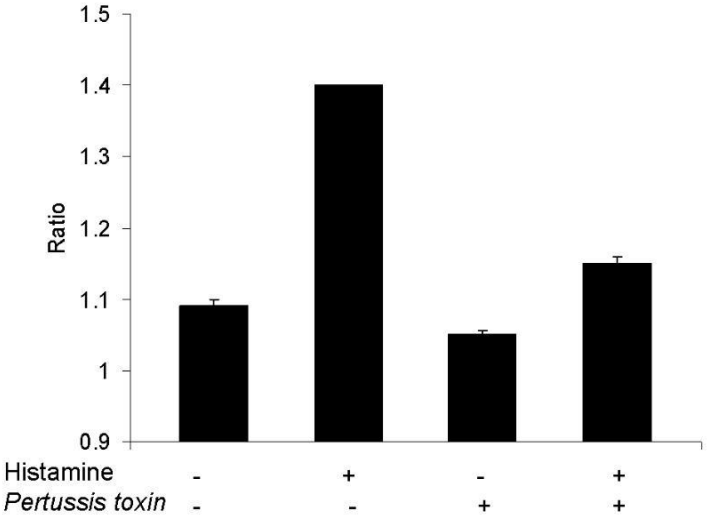
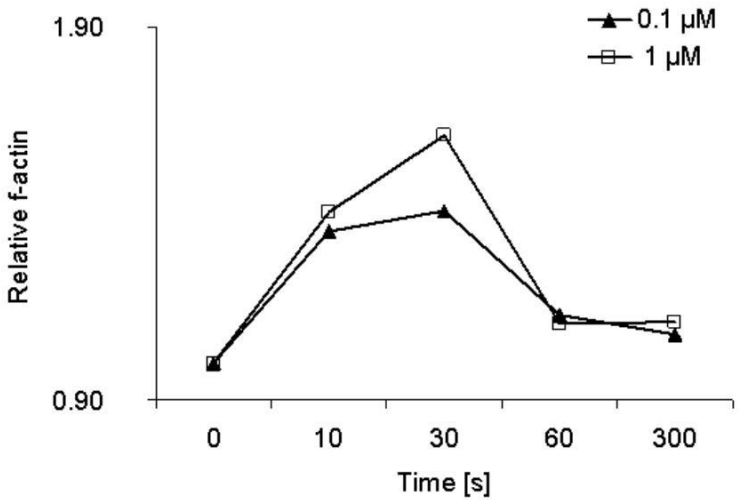


Fig. 3: Histamine induces calcium transients in isolated human peripheral γδ T lymphocytes. (A) Cells were loaded with Fura-2/AM and stimulated with 0.01 µM, 0.1 µM, and 1 µM histamine. Intracellular Ca^{2+} transients were followed by digital fluorescence microscopy and a ratio between 340 nm and 380 nm was calculated. Representative data from one experiment are shown. Experiments were repeated three times with identical results. (B) Cells were pre-incubated with or without pertussis toxin (100 ng/ml) for 90 min at 37°C and stimulated with 1 µM histamine. The ratio calculated after stimulation with and without histamine is shown. Data are means of three different experiments of three different donors ± SEM.

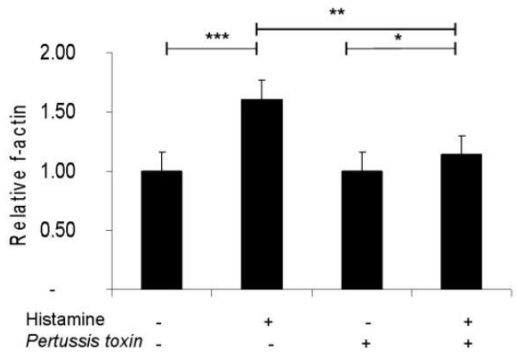
Next, actin reorganization was analyzed by flow cytometry. A rapid increase in f-actin content (circa 60%) was induced when γδ T cells were stimulated with histamine (Fig. 4A). The response was transient and reversible, with maximal values reaching within 30

seconds. To test the participation of G_i proteins in this response, γδ T cells were also pre-incubated with pertussis toxin before they were exposed to histamine (Fig. 4B). Pretreating γδ T cells with pertussis toxin almost completely abolished the effect of histamine on actin polymerization. In contrast pretreating them with cholera toxin did not result in a significant difference compared to control cells (Fig. 4C).

A)



B)



C)

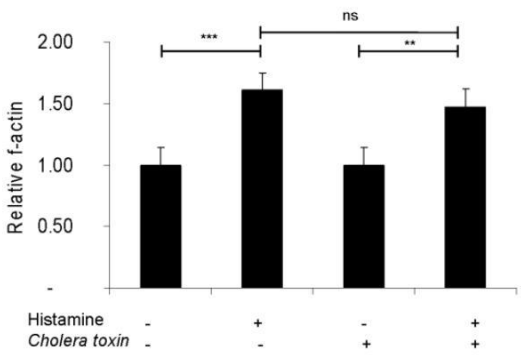
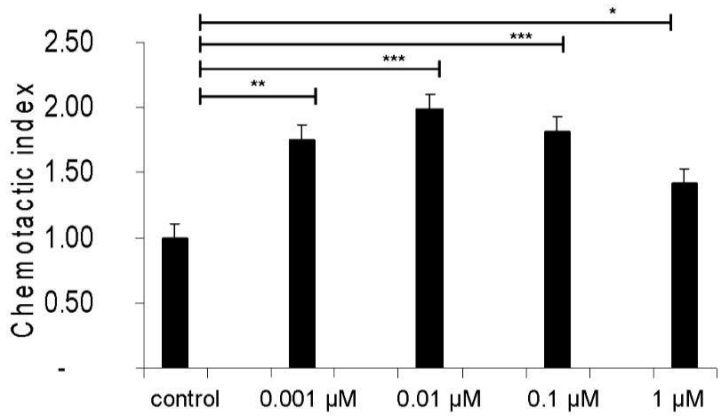


Fig. 4: Effects of histamine on actin polymerization in isolated human-peripheral-blood γδ T cells. (A) Cells were exposed to 0.1 μM - 1 μM histamine and f-actin content was measured by flow cytometry. (B) Cells were pre-incubated with or without pertussis toxin (100 ng/ml) for 90 min at 37°C and stimulated with 1 μM histamine for 30 seconds, and the increase in f-actin content was analyzed. (C) Cells were pre-incubated with or without cholera toxin (0.5 μg/ml) for 90 min at 37°C and stimulated with 1 μM histamine for 30 seconds and the f-actin content was analyzed. All data are means of three different experiments using three different donors ± SEM, (***: $p<0.0001$; **: $p>0.005$; *: $p>0.05$; ns: non-significant).

Intracellular Ca^{2+} transients and actin reorganization are prerequisites for cell migration. Therefore, human peripheral blood-isolated γδ T cells were exposed to different concentrations of histamine (0.01 μM - 1 μM), and migration across the porous-membrane was evaluated. Histamine induced a typical bell-shaped concentration dependent chemotactic response in γδ T lymphocytes (Fig. 5A). Maximal chemotactic responses were observed when cells were stimulated with 0.01 μM histamine. Moreover, histamine-stimulated migration was abolished by pretreating γδ T cells with pertussis toxin (100 ng/ml) (Fig. 5B).

A)



B)

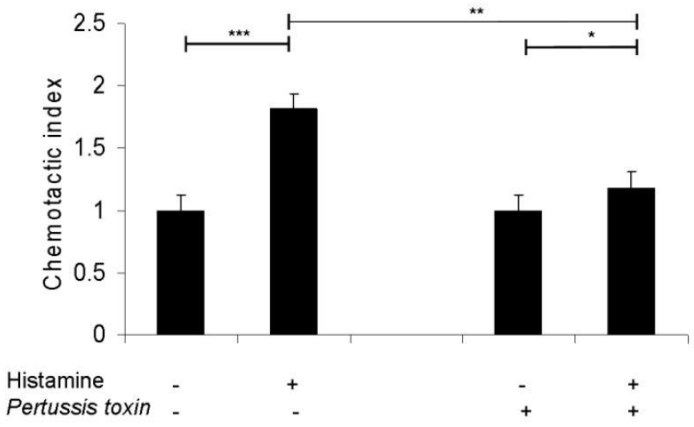
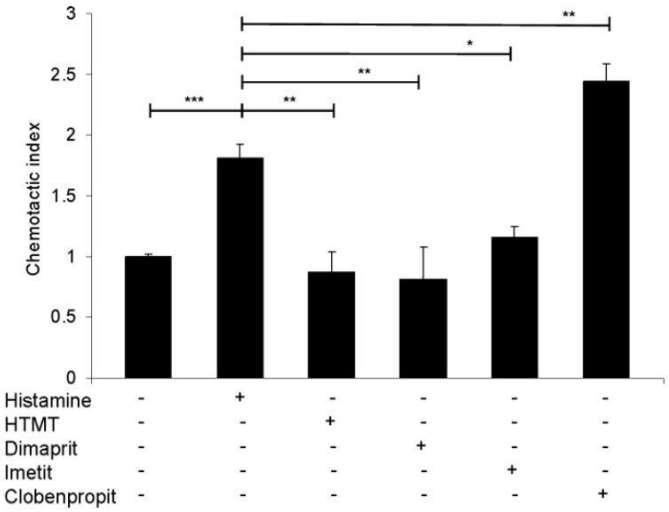


Fig. 5: Effects of histamine on chemotaxis in γδ T cells. Cells were exposed to different histamine concentrations (0.001 μM - 1 μM) in Boyden chambers. Migrated cells in the bottom wells of the Boyden chamber were fixed with formalin and counted by flow cytometry. (B) γδ T cells were pre-treated with pertussis toxin for 1h at 37°C, and migration in response to 0.1 μM histamine was analyzed. All data are means ±SEM, (***: $p<0.0001$; **: $p>0.005$; *: $p>0.05$).

To specify the subtype of histamine receptor involved in the histamine-induced chemotactic response in human peripheral blood γδ T cells, receptor-selective agonists and antagonists were used. The H_1R agonist 6-[2-(-imidazolyl)ethylamino]-N-(4-trifluoromethylphenyl) heptanecarboxamide dimaltate (HTMT), the H_2R agonist S-(3-dimethylaminopropyl) isopthiourea 2HCl (dimaprit-2HCl) and the H_3R agonist S-[2-(imidazol-4-yl)ethyl]isothiourea dihydrobromide (imetit) did not induce any significant change in chemotactic activity (Fig. 6A), whereas pretreating γδ T cells with the H_4R antagonist N-cyclohexyl-4-(1H-imidazol-4-yl)-1-piperidinecarbothioamide maleate salt (thioperamide) prevented histamine-induced migration (Fig. 6B), suggesting that in γδ T cells, migration in response to histamine occurs through H_4R.

A)



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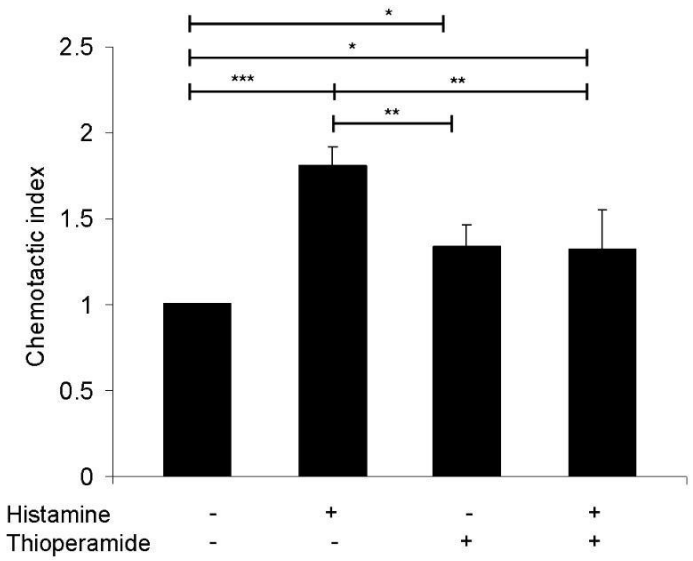


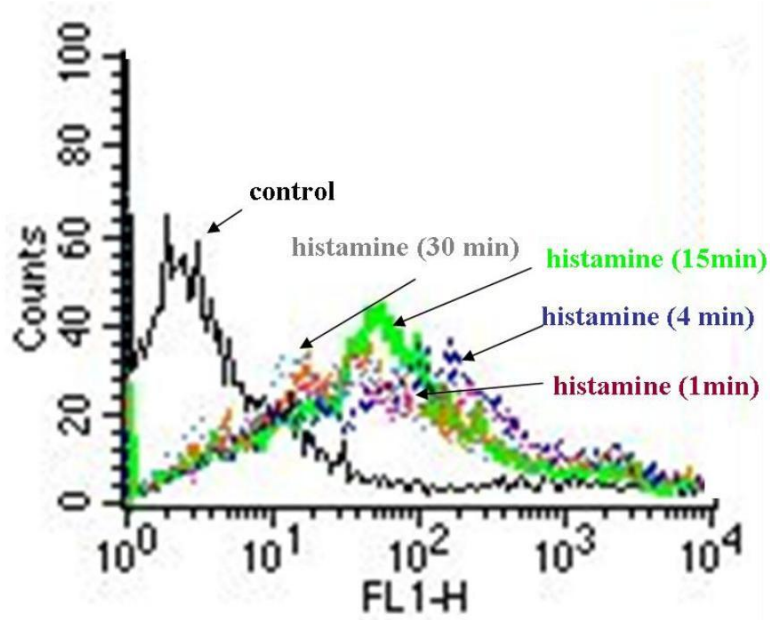
Fig. 6: Effects of histamine receptor agonists and antagonists on chemotaxis in γδ T cells. (A) γδ T cells isolated from healthy donors were exposed to histamine and isoform-selective agonists HTMT (H_1R), dimaprit (H_2R), and imetit (H_3R), and migration was analyzed. (B) γδ T cells were pre-treated with the H_4R antagonist thioperamide, and the migration assay was performed. All data are means ±SEM, (n=3); (***: $p<0.0001$; **: $p>0.005$; *: $p>0.05$).

3.1.3. Histamine-induced intracellular cAMP levels in γδ T cells

Histamine is known to affect the intracellular cAMP levels in human dendritic cells via H_2R and G_s proteins [112]. In order to characterize the functional expression of H_2R in

isolated human peripheral blood γδ T cells, intracellular cAMP levels after stimulation with histamine were determined by flow cytometry. A significant increase ($p<0.0001$) in cAMP levels, as reflected by increased in mean fluorescence intensity (MFI) was observed within 1 minute of histamine treatment (Fig. 7A). Moreover, cAMP levels reached a maximum after 4 minutes and remained high 30 minutes after histamine stimulation (Fig. 7B).

A)



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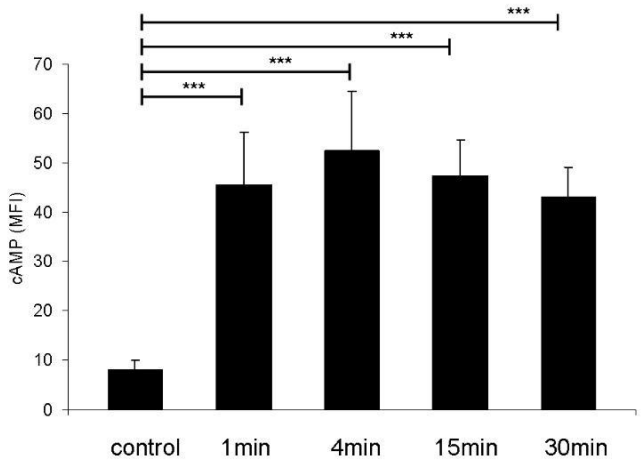


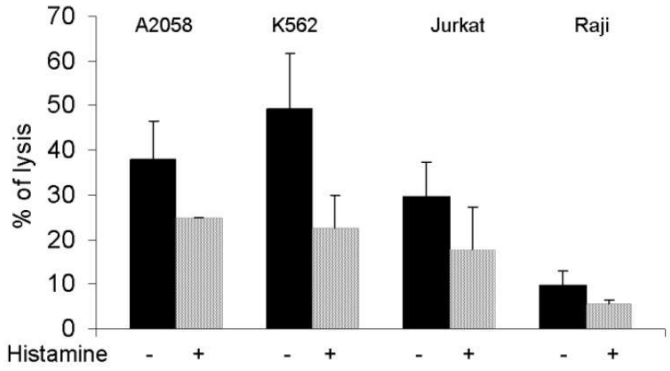
Fig. 7: Effect of histamine on intracellular cAMP levels in isolated human-peripheral-blood γδ T cells. (A) Distribution of fluorescence intensity in control cells and γδ T cells stimulated with 1 µM

histamine for 15 min is shown. Aliquots of cells were fixed and stained as described in Materials and Methods. Fluorescence intensity was measured by flow cytometry. Representative data from one experiment are shown, performed three times in triplicates. (B) Time course of cAMP levels after stimulation with 1 μM histamine. Experiments were repeated five times with γδ T cells isolated from different donors. Data are means ±SEM, (n=5); (***: $p<0.0001$).

3.1.4. Histamine affects the cytotoxic activity of γδ T cells in response to tumor cells

We have shown that the activation of G_s-protein-coupled receptors and the upregulation of cAMP lead to the downregulation of cytotoxic responses in NK cells [151]. In addition, γδ T cells are known to process cytolytic activity towards different human tumor cell lines such as the myeloid leukaemia cell line (K562), cutaneous malignant melanoma cells and the non-Hodgkins T cell line Jurkat [99, 155, 156]. To better characterize the cytolytic activity of γδ T cells, in vitro radioactive assays were performed using different cell lines. Target cells were labelled for 1 hour with chromium (100 μCi/10^6 cells) and co-cultured for 4 hours at 37°C with γδ T cells t o allow spontaneous cytotoxicity. As shown in (Fig. 8A), although γδ T cells displayed cytolitic activity in response all cell lines tested, their lytic capacity was greatest in response to K562 cells. Therefore, this cell line was chosen for further experiments to analyze the influence of histamine on on the cytolytic activity of γδ T cells. Histamine significantly reduced the cytolytic capacity of γδ T cells in response to K562 cells at all effector:target (E:T) ratios tested (Fig. 8B).

A)



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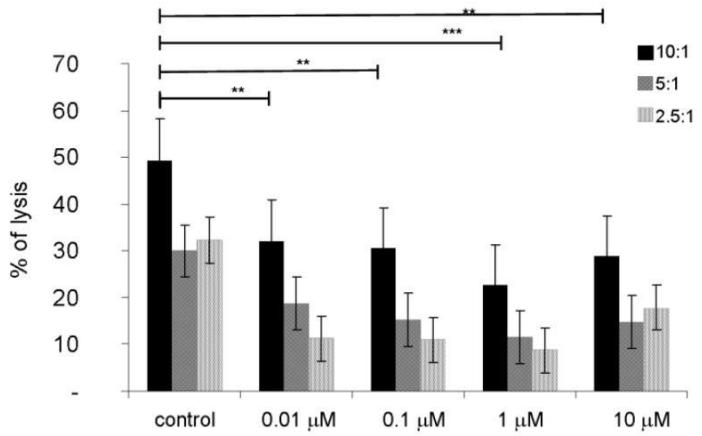
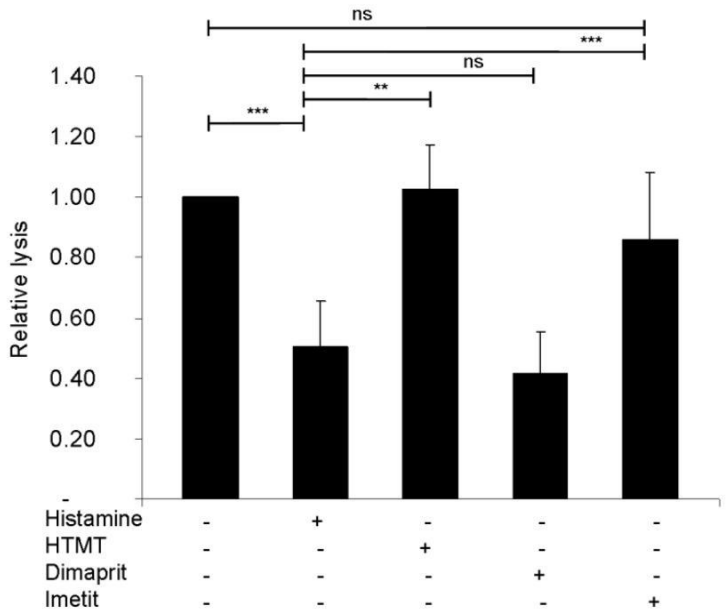


Fig. 8: Cytotoxic activity of γδT cells from healthy donors in response to tumor cell lines: (A) γδ T cells isolated from healthy donors were co-cultured with different tumor cell lines and their spontaneous cytolytic capacity was determined. (B) γδ T cells were stimulated with the indicated concentrations of histamine (0.01 µM - 10 µM) and cytotoxicity in response to the chronic myeloid tumor cell line K562 was analyzed. Cells were co-cultured in different effector: target (E:T) ratios as indicated. Data are means ±SEM, (n=3); (***: $p<0.0001$; **: $p>0.005$; *: $p>0.05$).

In order to specify which subtype of histamine receptor modulates cytotoxicity in γδ T cells, experiments with receptor-specific agonists and antagonists were performed. Isoform-specific H_1R- and H_3R-agonists did not affect the spontaneous lysis of K562 cells by γδ T cells, whereas the H_2R-agonist dimaprit significantly reduced the spontaneous lysis capacity of γδ T cells in response to K562 cells (Fig. 9A). Moreover, the H_4R antagonist did not prevent the histamine-induced effect on cytotoxicity while

the H_2R-antagonist prevented the histamine-induced effect (Fig. 9B). These experiments suggest that the modulatory effect of histamine on γδ T-cell-mediated cytotoxicity requires the activation of the H_2R isoform.

A)



B)

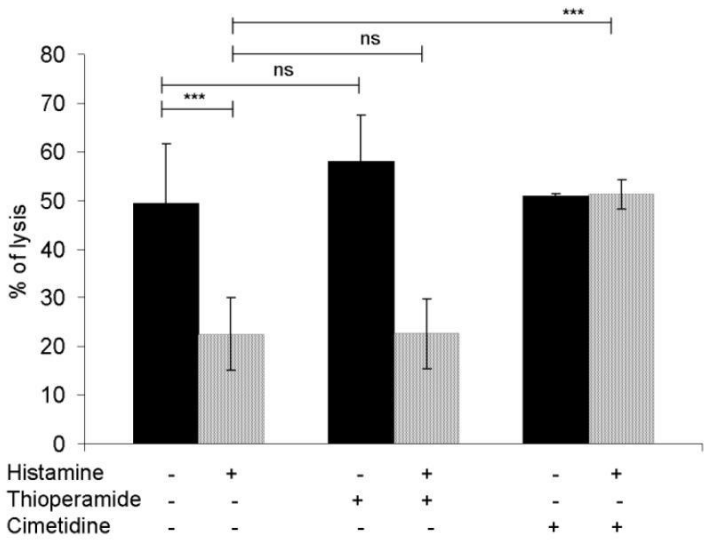


Fig. 9: Dimaprit inhibits the cytotoxic activity of γδ T cells. (A) γδ T cells were stimulated with 1 µM histamine, 10 µM H_1R-agonist HTMT, 10 µM H_2R-agonist dimaprit and 0.01 µM H_3R-agonist imetit and co-cultures with human myeloid cell line K562 were performed in order to analyze the level of cytolytic

activity. (B) γδT cells were stimulated with 1 µM histamine in the presence or absence of 10 µM H_4R-antagonist thioperamide, or the H_2R-antagonist, cimetidine and co-cultures with the human myeloid cell line K562 were performed in order to analyze cytolytic activity. Data are means ± SEM, (n=3); (***: $p<0.0001$; **: $p>0.005$; ns: non-significant).

We next determined the involvement of different G proteins on histamine-modulated cytotoxicity. Thus, γδ T cells were pretreated with G_i protein inhibitor pertussis toxin and the G_s activator cholera toxin, and their cytotoxic activity in response to K562 cells was examined (Fig. 10). Pertussis toxin did not significantly alter the effect of histamine on cytotoxicity. On the contrary, pretreating γδ T cells with cholera toxin alone inhibited cytotoxicity by more than 50% with respect to control cells untreated with histamine. This inhibitory effect of the cholera toxin was further enhanced by histamine.

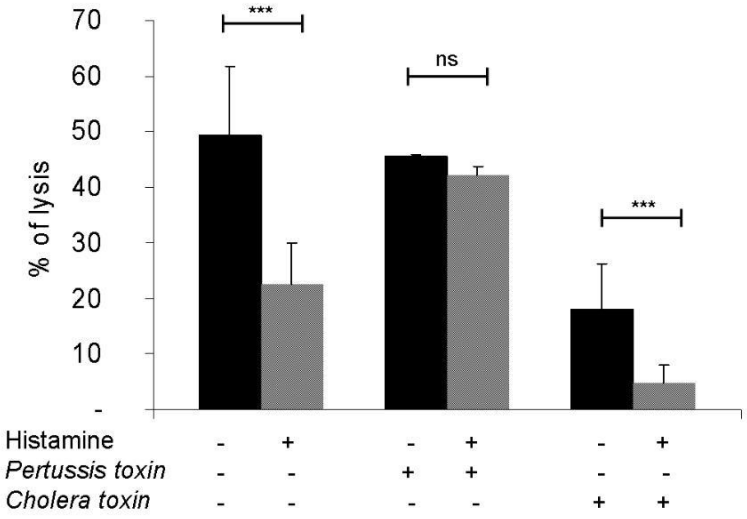
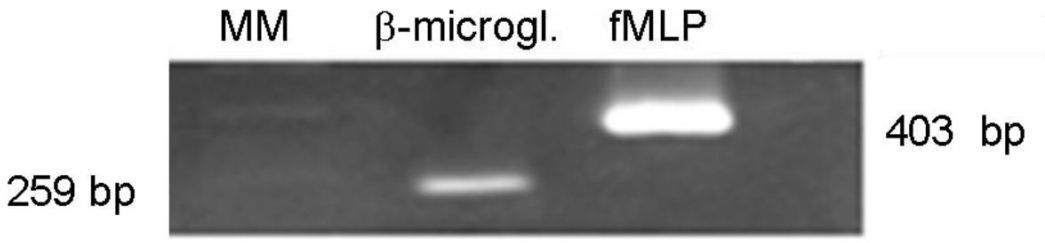


Fig. 10: Cytotoxic activity of γδ T cells in resonse to tumor cell line K562 is dependent on G_s proteins γδ T cells were pre-incubated with or without cholera toxin (0.5 µg/ml) or pertussis toxin (100 ng/ml) for 1h at 37°C. Thereafter, γδ T cells were stimulated or not with histamine and co-cultured with K562 cells. Data are means ± SEM, (n=3); (***: $p<0.0001$; ns: non-significant).

3.2. fMLP and γδ T cells: involvement of pertussis-toxin-sensitive functions of isolated human-peripheral-blood γδ T lymphocytes

The expression of the mRNA of the fMLP receptor was analyzed using reverse transcriptase and polymerase chain reaction (RT-PCR). The expected products for the fMLP receptor were detected in isolated human-peripheral-blood γδ T cells by gel electrophoresis (Fig. 11A). No PCR-product could be obtained by omitting reverse transcription (data not shown). In addition, the expression of fMLP proteins on the surface was analyzed. The fMLP expressions on γδ T cells are shown by flow cytometry (Fig. 11B+C). Neutrophils were used as control cells (Fig. 11D+E).

A)



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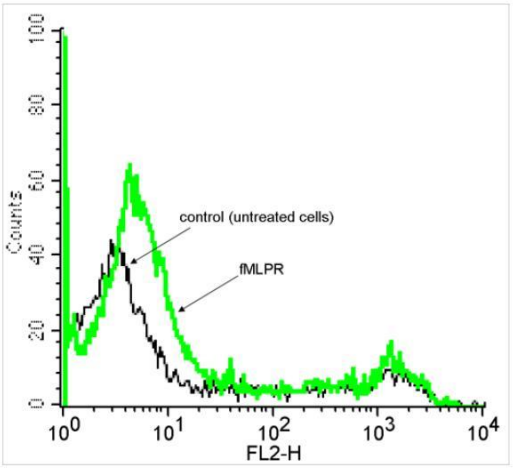
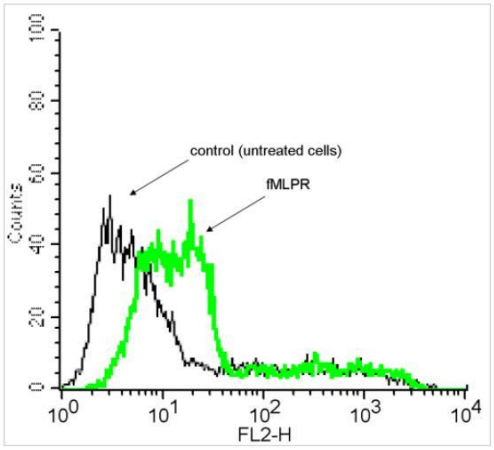
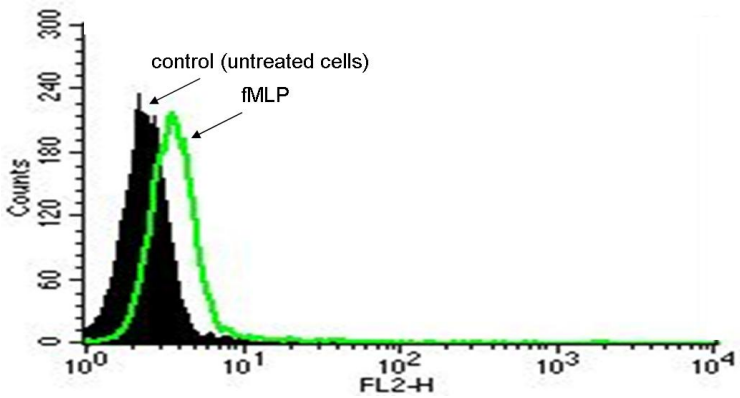


C)



D)



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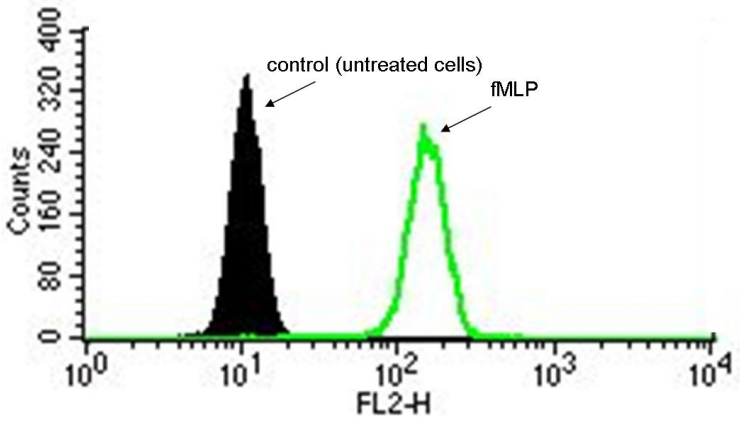


Fig. 11: **Expression of fMLP mRNA by RT-PCR and surface receptors detected by flow cytometry staining in isolated human peripheral blood γδT lymphocytes.** (A) γδ T lymphocytes isolated from human peripheral blood express the mRNA for fMLP receptors. (B;C;D;E) γδ T lymphocytes express the cell surface receptors for fMLPR. γδ T lymphocytes were isolated and cultured as detailed in Materials and Methods. Cells were incubated for 30 min at 4°C with PE-conjugated anti-fMLPR mAb (B: γδ T lymphocytes extracellular staining; C: γδ T lymphocytes intracellular staining D: neutrophils extracellular staining; E: neutrophils intracellular staining). Fluorescence intensity of 10 000 cells/sample was analyzed by flow cytometry. Abscissa: fluorescence intensity FL-2H, PE; FL-1H, FITC; ordinate: number of cells.

fMLP is able to induce Ca^{2+} transients in neutrophils [157]. Here, the influence of fMLP on Ca^{2+} in γδ T cells was measured by digital fluorescence microscopy after loading the cells with Fura-2. fMLP induced a rapid and concentration-dependent intracellular response in human γδ T cells isolated from peripheral blood (Fig. 12A). Ca^{2+} transients

are mainly created by their mobilization from intracellular stores or by their influx across the plasma membrane from the medium. In order to distinguish between these two mechanisms, experiments in the presence of EGTA in the medium were performed. The preincubation of γδ T cells with EGTA (4 mM) did not influence the fMLP-initiated intracellular Ca^{2+} rise, implicating the mobilization of Ca^{2+} from intracellular stores (data not shown). To investigate the involvement of G_i proteins in this response, we took advantage of pertussis toxin. This toxin uncouples G_i proteins from serpentine receptors by ADP-ribosylation. Pretreating γδ T cells for 1h with pertussis toxin (100 ng/ml) inhibited the fMLP-induced Ca^{2+} increase in γδ T cells (Fig. 12B). To check the responsiveness of pertussis-toxin-treated cells, experiments with ionomycin were performed. Ca^{2+} transients induced by ionomycin were not influenced by pertussis toxin (data not shown).

A) B)

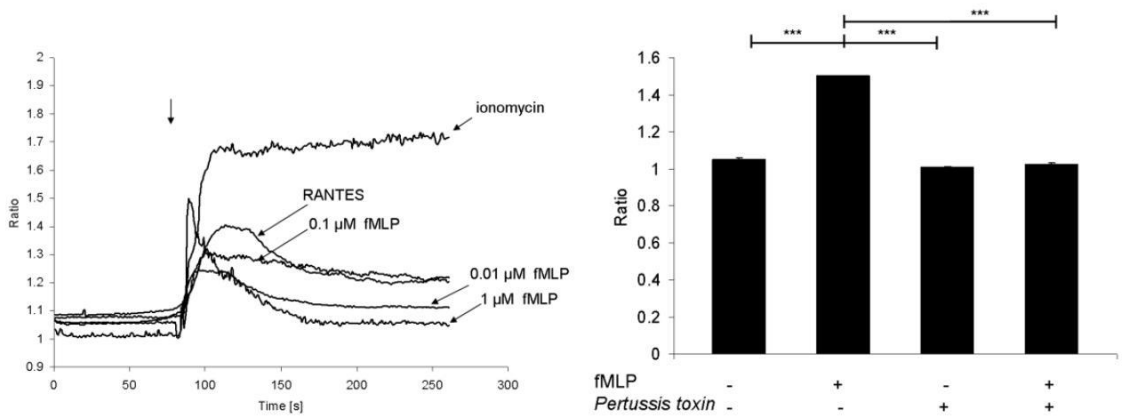
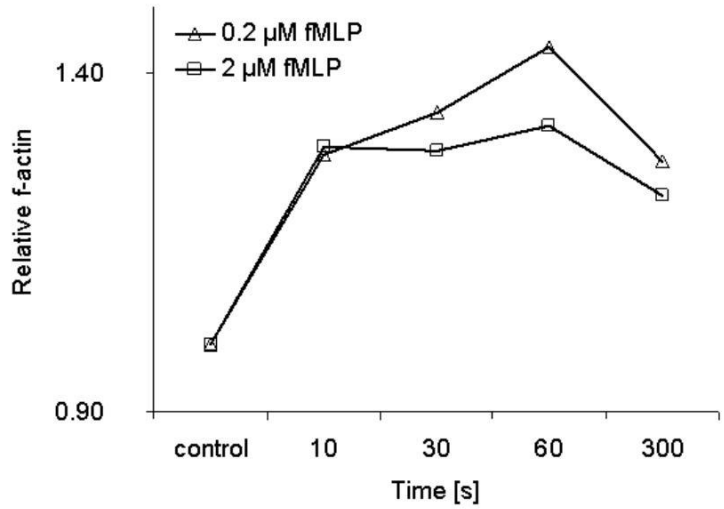


Fig. 12: fMLP activation induces intracellular Ca^{2+} transients in human peripheral γδT lymphocytes. (A) Cells were loaded with Fura-2/AM and stimulated with different concentrations of fMLP (0.01 µM -1 µM). Intracellular Ca^{2+} transients were followed in fura2-labelled human peripheral blood γδ T lymphocytes. fMLP induces a rapid and concentration-dependent increase of intracellular calcium Ca^{2+}. Ionomycin and RANTES were used as positive controls. Changes in fluorescence intensity were followed spectrofluorometrically after stimulation of the cells with fMLP, and the ratio between 340

nm and 380 nm was calculated. (B) Cells were preincubated with pertussis toxin (100 ng/ml) for 90 min at 37°C and stimulated with fMLP (1 μM). Representative data from three experiments using cells isolated from different donors are shown.

The reorganization of f-actin is a prerequisite for the migration of different types of leukocytes [158, 159]. Next, the influence of fMLP on the f-actin content in human peripheral blood γδ T cells was analyzed by flow cytometry. Rapid f-actin formation was induced when γδ T cells were stimulated with fMLP (Fig. 13A). The increase in f-actin content upon fMLP stimulation was transient, but extended for at least 5 min. Pretreating γδ T cells with pertussis toxin completely abolished the effect of fMLP on f-actin polymerization (Fig. 13B).

A)



B)

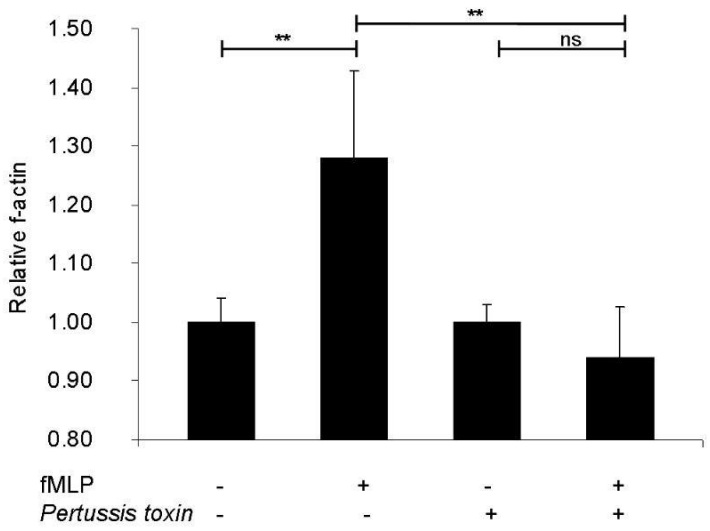
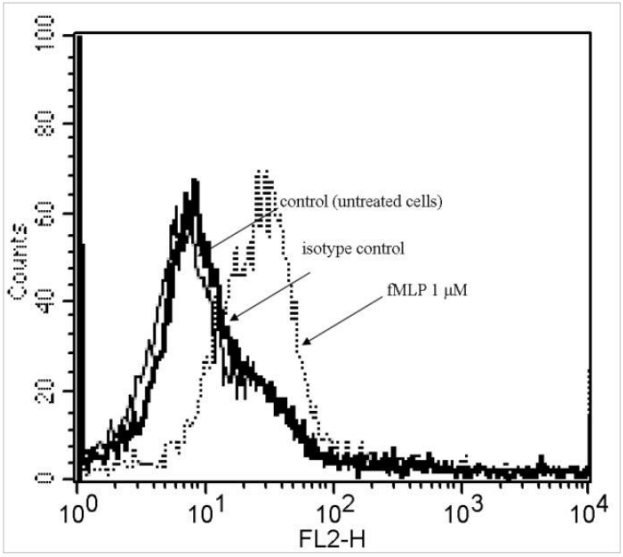


Fig. 13: Effects of fMLP on actin polymerization in isolated human-peripheral-blood γδT cells. (A) fMLP induced f-actin polymerization in γδT cells. (B) Cells were pre-incubated without or with pertussis toxin (100ng/ml) for 90 min at 37°C and stimulated (10s) with a concentration of 0.2 μM fMLP. The relative f-actin content after 0s, 10s, 30s, and 300s was measured by flow cytometry. A significant increase in f-actin polymerization was observed 30s after fMLP stimulation compared to the untreated control. Data are means ± SEM, (n=3); (**: $p<0.0001$; ns: non-significant).

CD11b is known to be the key β_2-integrin involved in cell adhesion and leukocyte chemotaxis [160]. Thus we measured the influence of fMLP on CD11b expression by flow cytometry. fMLP induced a concentration-dependent upregulation of CD11b on the surface of human γδ T lymphocytes (Fig. 14A-B).

A)



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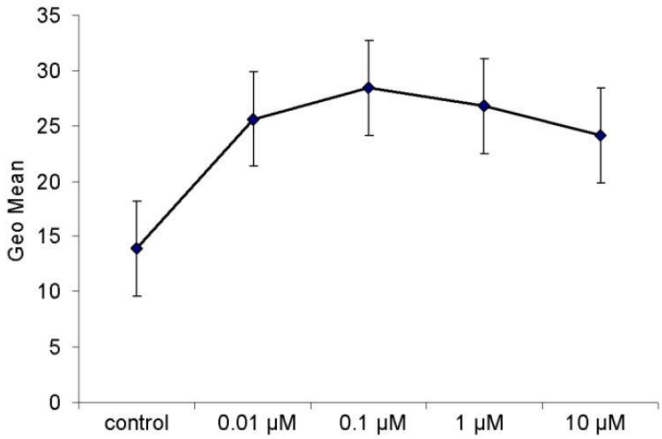
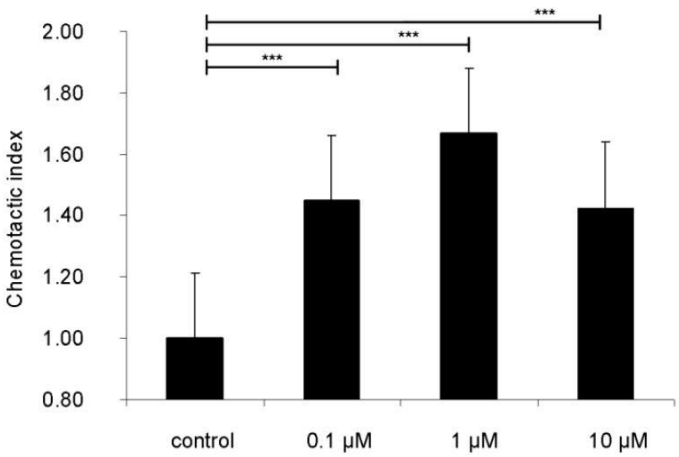


Fig. 14: fMLP-induced CD11b expression in γδT cells. γδ T cells were stimulated with the indicated concentrations of fMLP and then incubated for 30 minutes at 37°C. The reaction was stopped by diluting the sample to 100 times the original concentration with ice-cold buffer. Samples were incubated for 40 minutes on ice with PE-conjugated anti-CD11b mAbs and then analyzed by flow cytometry. Representative histograms of stimulated (0.01 µM - 10 µM fMLP) (A-B) and unstimulated anti-CD11b-stained γδ T cells are shown. Representative data from three experiments using cells isolated from different donors are shown.

Intracellular Ca^{2+} transients, actin reorganization and CD11b expression are prerequisites for cell migration in various types of leukocytes. Therefore, isolated human-peripheral-blood γδ T cells were exposed to different concentrations of fMLP

(0.01 µM - 1 µM), and migration across a porous membrane was evaluated. Both fMLP induced a typical bell-shaped concentration-dependent chemotactic response in γδ T lymphocytes (Fig. 15A). Again, fMLP-stimulated migration was abolished by pretreating γδ T cells with pertussis toxin (Fig. 15B).

A)



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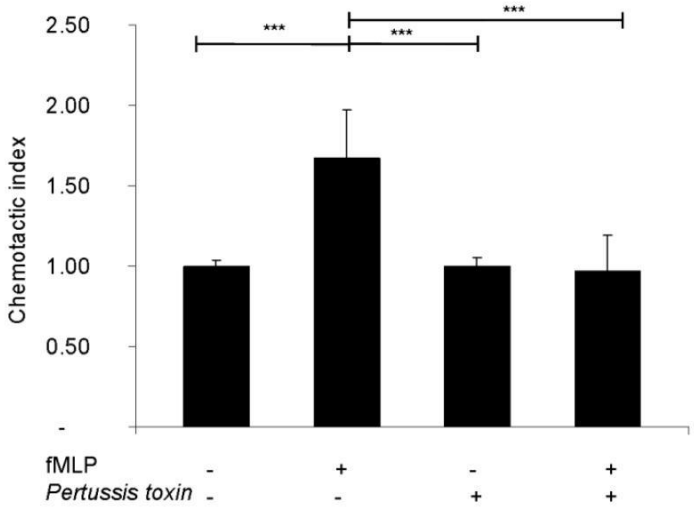

Fig. 15: fMLP-induced chemotaxis of circulating γδT cells. (A) Cells were exposed to different fMLP concentrations (0.1 µM-10 µM) in Boyden chambers. Migrated cells collected from the bottom wells were fixed with formalin and counted by flow cytometry. (B) γδ T cells were pre-treated with the G_i protein inhibitor pertussis toxin. fMLP-induced migration of isolated human-peripheral-blood γδ T lymphocytes is significantly inhibited by pertussis toxin. Data are means ±SEM, (***: $p<0.0001$; **: $p>0.005$; ns: non-significant).

4. Discussion

4.1. γδ T cells

γδ T cells are thought to be major effector cells in a number of infectious diseases, e.g. tuberculosis [66, 67], brucellosis [161], listeriosis [162], leishmaniasis [163], malaria [164], and toxoplasmosis [165]. The recruitment and activation of γδ T lymphocytes to sites of inflammation are caused by various agents and natural phosphoantigens derived from plants [39], bacteria [40, 41, 166], protozoa [43], endogenous ligands [32, 48, 35, 49, 167] and viruses [167, 168]. Early events in an immune response involving γδ T cells stimulate the production of cytokines that direct innate host defenses and the subsequent development of T cell subsets with discrete patterns of cytokine production. γδ T cells are a population of lymphocytes expressing functional γδ T cell receptor genes [30]. This subtype of T cells, presumably an ancient type of lymphocyte, is derived from haematopoietic stem cells that share certain characteristics with other immune cells, e.g. the capacity for antigen presentation, immune modulatory properties and cytolytic activity [34, 80]. Two main subsets of γδ T cells are distinguished according to their location. Resident γδ T cells are found in skin, uterine and epithelial tissues, whereas circulating/systemic γδ T cells can be isolated from peripheral blood or lymphoid tissues [54, 55]. In contrast to αβ T lymphocytes, γδ T cells do not need antigens presenting on classical MHC molecules for recognition [55]. Instead, they recognize antigens bound to CD1 molecules. [45, 169]. Several lines of evidence implicate γδ T cells in primary host defenses as well as in tumor surveillance. They are also known to attack bacterial and virus-bearing cells as well as transformed cells [79]. This cytotoxic activity is mediated by the production and release of perforin and granzymes [79, 34]. The antitumoral activity of γδ T cells is either mediated via

endogenous ligands in γδ TCR-dependent fashion or depends on the interaction of the cells with NK cell receptor NKG2D [75, 79].

Different subpopulations of immune cells can infiltrate tumor lesions, although there is usually a limited number of T lymphocytes detectable even in those neoplasias (such as melanoma) in which the lymphocyte infiltrate appears to correlate with disease course [169]. The limited number of T lymphocytes in the microenvironment of tumors suggests that mechanisms acting in down-modulating antitumor immune responses are likely to be at work [170]. Two lymphocyte subsets T cells and NK cells are frequently found within malignant tumors and in the surrounding inflammatory tissue. Fractions of these intra- or peritumoral lymphocytes are frequently impaired compared to T and NK cells in peripheral blood or in adjacent non-malignant tissue, a phenomenon referred to as tumor-induced immunosuppression [171, 172]. Tumor and tumor-associated cells actively and directly inhibit immune responses by secreting of immunosuppressive cytokines such as interleukin (IL)-10 [173] and transforming growth factor (TGF)-β [173, 174], as well as by skewing $CD4^+$ T cell responses into T_H2 rather than T_H1-dominated immunoreactions [175, 176].

MCP-1/CCL2 [87], RANTES/CCL5, MIP-1α/CCL3 and MIP-1β/CCL4 were found to be chemically attractive for αβ T lymphocytes and circulating γδ T cells, whereas neither αβ nor γδ responded to IL-8/ CXCL8 or IFN-inducible protein 10 (IP-10/CXCL10) [89]. The corresponding chemokine receptors are seven-transmembrane-spanning G-protein-coupled receptors. They share structural features and some of them are involved in intrathymic [90], and memory T cell development, others in the orchestration of T and B cell interactions and the differentiation of effector T cells [87].

4.2. G protein-coupled receptor pathways in circulating γδ T cells: histamine and fMLP receptor pathways

Our data suggest that γδ T cells´ responses to the stimulation of histamine or fMLP are dependent on G protein-coupled receptor pathways. The heterotrimeric G proteins are composed of three subunits: α, β and γ. About twenty α subunits have been reported, belonging to four families. Two distinct G protein subtypes are sensitive to bacterial toxins: cholera toxin ADP-ribosylates the arginine residue present in the carboxy terminal of the α subunit of G_s, whereas pertussis toxin ADP-ribosylates the cystein residue in the carboxy terminal of the α subunit of G_i or G_o proteins [177]. Each G protein has a unique biological function. The G_s protein activates adenylyl cyclise, resulting in the accumulation of cyclic adenosine monophosphat (cAMP) in the cells. G_i proteins are highly homologous members of the family of heterotrimeric signal-transducing guanine nucleotide-binding proteins (G proteins) and by coupling numerous receptors for extracellular signalling molecules to inhibit adenylyl cyclase, stimulate phospholipase C, or open potassium channels [9,10]. The activation of phospholipase C results in the hydrolysis of phosphatidylinositol bisphosphate (PIP_2) and the generation of diacylglyceride (DAG), an activator of protein kinase C (PKC), and inositol(1,4,5)trisphosphate [12].

4.2.1. Histamine affects γδ T cell effector functions: the involvement of pertussis-toxin- and cholera-toxin-sensitive functions of isolated human peripheral blood γδ T lymphocytes

Since its discovery in 1910, histamine has been regarded as one of the most important mediators in allergy and inflammation and is known to be involved in smooth muscle-stimulating, vasodepressor action and is involved in reactions during anaphylaxis [178].

Although histamine is located in most body tissues, it is highly concentrated in the lungs, skin and gastrointestinal tract [179], where it has been shown to regulate both the secretion of gastric acid in the stomach and the functions of several neurological transmitters [180, 181]. In the central nervous system, histamine is involved in regulating drinking, body temperature, and blood pressure and in perceiving pain [182, 183]. Histamine has also been described as an autocrine/paracrine or exogenous growth factor for cancer cells, e.g. malignant melanomas and leukaemia cells. In the case of chronic myeloid leukaemia, the secretion of histamine is the consequence of a leukaemia-specific oncogene [107].

Histamine is known to regulate humoral and cellular immunity by controlling the production of pro-inflammatory cytokines, the expression of adhesion molecules and the migration of inflammatory cells such as eosinophils, dendritic cells, NK cells and αβ T cells [184, 185]. Histamine and histidine decarboxylase (HDC) [105], the only enzyme that catalyzes histamine production, has been proven to be present in elevated concentrations in proliferating tissues including tumor cells [157]. In the case of the myeloproliferative disease chronic myeloid leukaemia (CML), this gene was the first known leukaemia-specific oncogene reported to be involved in the regulation of histamine production in neoplastic cells [107]. By mediating the immune response histamine also interacts directly with H_2 receptors (H_2R) on T cells to inhibit T cell proliferation and cytokine production [109]. H_2R antagonists increase the likelihood that patients with gastric and colorectal cancer will survive [186, 187]. Histamine mediates the dose-dependent inhibition of human IL-12 and stimulates IL-10 via H_2R receptors [188]; moreover, it inhibits the IFN- γ production of T_H1 cells but has no effect on IL-4 production from T_H2 clones [189]. Furthermore, histamine directly enhances the production of human IgE from B cells [190], while it inhibits T cell cytotoxicity.

Available data suggest that histamine induces a T_H2 shift at the level of monocytes [191].

It has been previously shown that γδ T cells exposed to histamine do not expand [39]; however, our data show that γδ T cells express three distinct histamine receptor subtypes and functionally respond to two of them. To better understand the role of histamine in the cross-talk between immune cells and tumor cells, we performed cell studies and co-culture experiments with γδ T cells. Here, we demonstrated that histamine stimulates actin polymerization and Ca^{2+} transients in a concentration-dependent manner and migration in a typical bell-shaped concentration curve. Moreover, we showed that the increase in intracellular Ca^{2+} is due to mobilization from intracellular stores, inasmuch as it is insensitive to the chelation of extracellular Ca^{2+}. A long-lasting migration response requires continuous interaction between migration-inducing ligands and the cell surface receptors that can be activated via actin polymerization to induce the "cell motor". Therefore, a gradient of ligands, continuously available cell surface receptors and a very sensitive signal transduction mechanism are necessary to transmit the external signal to the internal cellular movement machinery. Thus, a low concentration of chemotactic ligands can activate and direct the cell over a long period of time. At high ligand concentrations, the receptors at the cell surface are very quickly occupied and consequently desensitized as well as internalized via endocytosis. In this case, the chemotactic index is low because ligands find no form of receptors at the cell surface that can be activated and are not able to induce movement until either novel transcriptionally regulated receptors are synthesized or the internalized receptors are recycled [192].

Histamine is able to act by binding to different G protein-coupled receptors. In order to demonstrate the participation of G_i proteins in these stimulated cell responses, experiments with pertussis toxin were performed. This toxin selectively uncouples G_i

proteins from the intracellular sites of receptors by ADP-ribosylation. Pretreating γδ T cells with pertussis toxin blocked histamine-induced actin polymerization, Ca^{2+} transients and migration in γδ T cells; these consequences implicate the involvement of G_i proteins in these cell responses. Principally, histamine is able to act by binding to different receptor subtypes, H_1R, H_2R, H_3R, and H_4R.

In the present work, reverse transcription-polymerase chain reactions revealed the mRNA expression of H_1R, H_2R and H_4R in γδ T cells. No mRNA expression for H_3R was detected. Moreover, H_1R, H_2R and H_4R expression at the protein level has been shown by Western blot analysis. The involvement of the different receptors in these cell responses was dissected using isoform-specific receptor agonists and antagonists. Our experiments revealed that histamine regulates actin polymerization, Ca^{2+} transients and chemotaxis via H_4R, but provided no evidence for the involvement of H_1R, H_2R and H_3R in these cell responses. This receptor-isoform specific cell regulation is consistent with reports in eosinophils, mast cells and NK cells [185, 114]. Therefore, one can assume that H_4R in γδ T cells activates pertussis-toxin-sensitive heterotrimeric G_i proteins, which in turn dissociate into the guanosine triphosphate-α subunit and free βγ dimers. The latter activates phospholipase $C\beta_2$ [193]. This enzyme cleaves phosphatidylinositol (4,5)-bisphosphate into diacylglycerol and the inositol trisphosphate, which mobilizes Ca^{2+} from intracellular stores [194]. In leukocytes, G_i proteins regulate the reorganization of the actin cytoskeleton independently of activated phospholipase C [195]. These G_i-protein-coupled signalling pathways are essential components of the migration response in different subtypes of leukocytes [196].

Unlike classical chemotaxin receptors, such as chemokine receptors or the complement C5a receptor, the coupling of different types of histamine receptors is pleiotropic,

including the interactions of H_2R with G_s proteins with the consequent activation of adenylyl cyclase.

Our cell studies combining histamine and isoform-specific receptor agonists or antagonists showed enhanced cAMP levels and H_2R activation in γδ T cells. In different subtypes of leukocytes, e.g. NK cells and $CD8^+$ T cells, the cytotoxicity response by cAMP has been reported to be inhibited [197]. Our data show that the spontaneous cytolytic activity of isolated human peripheral blood γδ T cells is prevented by histamine. Neither HTMT nor imetit nor thioperamide altered the spontaneous cytolytic capacity of γδ T cells, but it was inhibited by dimaprit, suggesting that H_2R may be involved in the inhibitory effect of histamine on the cytotoxicity of γδ T cells in human peripheral blood. Moreover, it has been shown that cholera toxin impairs cytotoxicity in αβ T lymphocytes and NK cells [198].

As the literature suggests [198], we found that the G_s-protein activator cholera toxin inhibited the spontaneous cytotoxicity of γδ T cells, enhancing cAMP levels. Infiltration by lymphocytes, macrophages, mast cells, and neutrophils is a hallmark of inflammatory, defense and tissue repair reactions, which are often present in tumors. Various types of tumor-infiltrating macrophages and lymphocytes are considered to be potential effectors of anti-tumor immunity and may interfere with tumor expression [199]. In this work, we showed that histamine, which is present in the inflammatory and neoplastic microenvironment, induces the migration of isolated human peripheral blood γδ T cells; in contrast, the spontaneous cytolytic effect of γδ T cells is prevented by histamine [108, 200]. Neither HTMT nor imetit nor thioperamide altered the spontaneous cytolytic effect of γδ T cells, but it was inhibited by dimaprit, suggesting that H_2R may be involved in the inhibitory effect of histamine on human peripheral

blood γδ T cell-mediated cytotoxicity. Our data suggest that histamine contributes to the ability of tumor cells to escape the immunological surveillance.

4.2.2. fMLP affects effector functions of γδ T cells: the involvement of pertussis-toxin-sensitive functions of isolated human peripheral blood γδ T lymphocytes

Gram-negative bacteria-derived and synthetic N-formyl peptides play key chemotactic roles for phagocytic cells. N-formyl-Met-Leu-Phe (fMLP) is the prototype for N-formylated peptides. Its receptor in humans has been pharmacologically defined as a high affinity binding site on the surface of phagocytes such as neutrophils [121]. Natural fMLP was subsequently purified and identified in supernatants of gram negative bacteria. Nevertheless, over the past five years, data from several groups have indicated that fMLP receptors might act in a more complex manner. A large number of non-formylated peptide ligands for the fMLP receptor, including derivates of mitochondrial proteins, have been identified. Moreover, physiological ligands for the fMLP receptor also presumably derive also from viral envelope proteins and the fibriolytic cascade [133]. However, it is currently thought that the main effector cells activated by fMLP represent phagocytes such as neutrophils, dendritic cells and monocytes. Recently, the reduced resistance of fMLP receptor knockout mice to infection by Listeria monocytogenes has been reported [201]. Since there is good evidence that γδ T cells are major effector cells in listeriosis, the direct effect of fMLP on cell adhesion and migration was analyzed in these cells.

fMLP exerts its biological functions through three different receptor subtypes: fMLP-receptor (FPR), and fMLP receptor-like 1 and 2 (FPRL1, FPRL2). Human FPR was first defined biochemically as a high affinity binding site for the prototype fMLP on the surface of neutrophils. Two additional human genes, designated FPRL1 and FPRL2

(FPR-like), were subsequently isolated. [202, 203, 204]. It is generally accepted that FPRL1 shares signal transduction features with FPR. Both receptors are sensitive to pertussis toxin and possess a high degree of amino acid identity in the cytoplasmic signalling domains [205].

Here, we demonstrated the expression of the fMLP receptor (FPR) surface protein on γδ T cells. Moreover, we showed that fMLP stimulates Ca^{2+} transients in γδ T cells. The increase in intracellular Ca^{2+} is due to mobilization from intracellular stores, inasmuch as it is insensitive to extracellular chelation by EGTA. Pretreating γδ T cells with pertussis toxin inhibited both fMLP-induced Ca^{2+} transients. This toxin selectively uncouples G_i proteins from the intracellular sites of receptors by ADP ribosylation. These data suggest that fMLP in γδ T cells induces similar or identical signal pathways as in phagocytes. Binding fMLP to their receptors might induce GDP/GTP exchange in the $G\alpha_i$ subunit of heterotrimeric G proteins. The activated heterotrimeric G protein complex (GTPform) is unstable and dissociates into $G\alpha_i$ subunits and free $\beta\gamma$ dimers, which activate phospholipase $C\beta_2$ [194]. This enzyme cleaves phosphatidylinositol (4,5)-bisphosphate into diacylglycerol and the inositol trisphosphate, which in turn mobilizes Ca^{2+} from intracellular stores [195].

In leukocytes, G_i proteins regulate the reorganization of the actin cytoskeleton independently of activated phospholipase C [196]. These G_i-protein-coupled signalling pathways are essential components of the migratory response in different subtypes of leukocytes [197]. To determine whether fMLP-treated cells can initiate the signalling required for actin reorganization γδ T cells were activated with different concentrations of fMLP (0.1 μM - 10 μM) labelled with NBD and analyzed by flow cytometry. Rolling of the cells is mediated by the selectin family of adhesion molecules and their counter-structural carbonhydrate ligands, such as sialyl Lewis X, on leukocytes and sulphated

polysaccharides, such as fucoidan, on endothelial cells. The more stable adhesion, sticking, to the vessel wall is mediated by CD11/CD18 leukocyte adhesion molecules (β_2 integrins) on leukocytes and intercellular adhesion molecules (ICAMs) on endothelial cells. After attaching firmly, leukocytes start to migrate across the endothelium via intercellular junctions into the subendothelial space. Finally, leukocytes become attached to the inflammatory sites a process known as chemotaxis through the production of exogenous and endogenous chemoattractant mediators. Exogenous chemotaxins include bacterial oligopeptides of the fMLP type, lectins, denatured proteins, some lipids and lipopolysaccharides. Endogenous chemotaxins are produced by the host organism and are humoral (complement fragment C5a and C3a, fibrinopeptides, kallikrein and plasminogen activators) or cellular (from different cells: leukotrine B_4, platelet activated factor, chemotactic cytokines) [206, 207].

We demonstrated that fMLP induces actin polymerization and CD11b upregulation in a concentration dependent manner and migration in a typical bell-shaped concentration curve. The requirements of these cell responses were discussed above already in the chapter for histamine.

4.3. Conlcusion

The data collected in this work represent the first evidence that G-protein-coupled signalling exist in γδ T cells via histamine and fMLP receptors. The bigenic amine histamine and the bacterial peptide fMLP have been demonstrated to be novel chemoattractant factors for circulating human γδ T cells, which are critical members of the immunological tumor surveillance machinery. Here, we analyzed the influence of histamine on the interaction of human γδ T cells with tumor cells such as the A2058 human melanoma cell line, the human Burkitt's non-Hodgkins lymphoma cell line Raji, the T-lymphoblastic lymphoma cell line Jurkat, and the NK cell-sensitive erythroleukaemia line K562. We found that histamine inhibits the spontaneous cytolytic activity of γδ T cells in response to these cell lines. The downregulation of γδ T cell mediated cytotoxicity involves the histamine receptor subtype 2 (H_2R), the activation of G_s proteins and increased cAMP intracellular levels. On the other hand, histamine activates the common signalling pathways of chemotaxins such as G_i-protein-dependent actin reorganization, the increase of intracellular Ca^{2+} and the induction of migratory response in γδ T lymphocytes. Our data indicate that histamine contributes to the mechanism by which tumor cells escape immunological surveillance.

The bacterial-cell-wall-derived peptide N-formyl-Met-Leu-Phe (fMLP) is a well characterized chemotactic factor for phagocytes such as neutrophils, monocytes and dendritic cells. Here, we analyzed the influence of fMLP on isolated human peripheral blood γδ T cells. We found that fMLP induces intracellular calcium transients, actin reorganization, CD11b upregulation and the migration of γδ T cells. Pretreating γδ T cells with pertussis toxin inhibited all fMLP-stimulated cell responses, implicating the involvement of G_i proteins in the induced signalling cascade. The present data suggest that,

in addition to phagocytes, N-formyl peptides also regulate the trafficking and activation of γδ T cells.

4.4. Outlook

Our data suggest that the responses of circulating human γδ T cells to histamine are mediated by two different G-protein-coupled pathways. Data suggest that the downstream pathways of cAMP is involved in the responses of γδ T cells to histamine stimulation. Further analysis of the involvement of phospholipase C (PLC), protein kinas B (Akt) and protein kinase A is required.

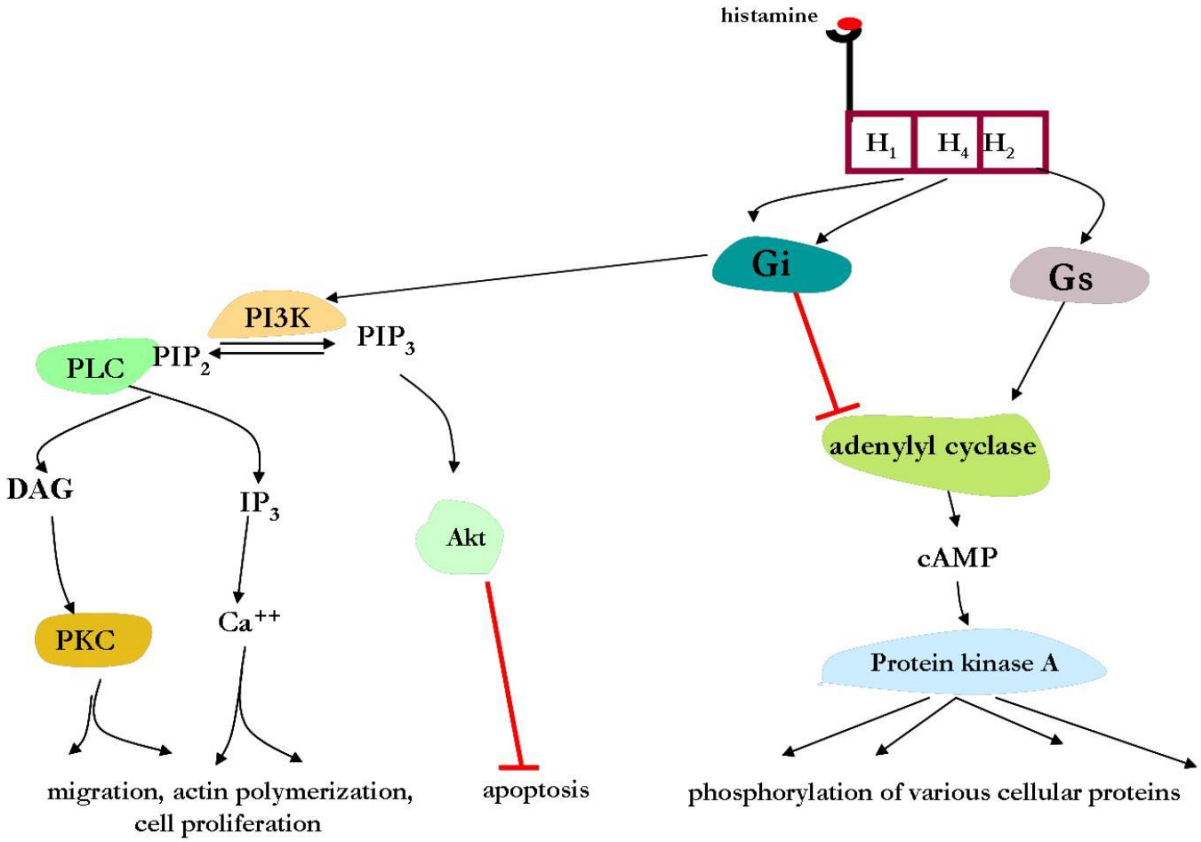

Figure 4.4.1. The possible G-protein-coupled receptor signalling in γδ T cells via the histamine receptor subtypes H_1R, H_2R, H_4R. Histamine may recruit the γδ T cells to the site of tumors and inhibit their cytolytic functions. Once there, γδ T cells secrete different chemokines, e.g. RANTES, that may provide tumor development. (Abbreviations: **PI3K**: phosphatidylinositol 3-kinase; **PIP_2**: phosphatidylinositol 4,5-bisphosphate; **PIP_3:** phosphatidylinositol (3,4,5)-trisphosphate**; PLC:** phospholipase C; **DAG:** diacylglycerol ; **PKC**: protein kinase C ; **IP_3**: inositol-1,4,5-trisphosphate; **Ca^{++}**: calcium; **Akt**: proteinkinase B; **cAMP**: cyclic adenosine-3',5'-monophosphate; H_1R, H_2R and H_4R: histamine receptor subtypes 1,2 and 4; G_i: G_i protein; G_s: G_s protein).

Furthermore, in the case of the fMLP receptor pathway in circulating human γδ T cells, further studies analyzing the different subtypes of the fMLP receptor family are required to describe the exact receptor pathway involved in the responses of γδ T cells to fMLP.

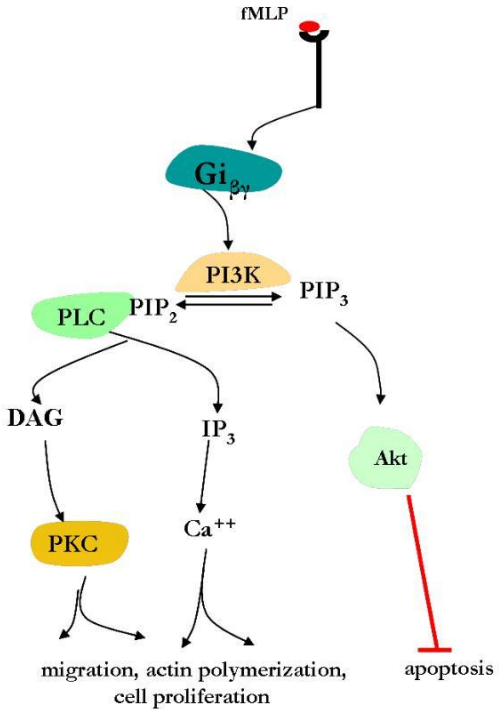

Figure 4.4.2. The possible G-protein-coupled signalling pathway in γδ T cells via the fMLP receptor. (Abbreviations: **PI3K**: phosphatidylinositol 3-kinase; **PIP_2**: phosphatidylinositol 4,5-bisphosphate; **PIP_3:** phosphatidylinositol (3,4,5)-trisphosphate**; PLC:** phospholipase C; **DAG:** diacylglycerol ; **PKC**: protein kinase C ; **IP_3** : inositol-1,4,5-trisphosphate; **Ca^{++}** : calcium; **Akt**: proteinkinase B; **fMLP**: N-formyl-Met-Leu-Phe).

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6. Acknowledgments

I would like to express my gratitude to all those who made it possible for me to complete this thesis.

I want to thank the Hans Knöll Institute the International Leibnitz Research School for giving me the fellowship and all the support and help, especially Prof. Axel Brakhage and Prof. Peter Zipfel and Dr. Dorit Schmidt.

I have furthermore to thank to Prof. Reinhard Weztker and Prof. Johannes Norgauer who encouraged me to go ahead with my thesis, for all their support and all our discussions which helped me in all the time of research for and writing of this thesis.

My former colleagues from the Department of Dermatology at the Friedrich Schiller University of Jena who supported me in my research work. I want to thank them for all their help, support, interest and valuable hints. I am especially obliged to Dr. Mariana Lagadari, Dr. Katja Lehmann, Luciana Berod, Suzana Cubillos and Susann Piehler.

Emily Wheeler looked closely at the final version of the thesis for English style and grammar, correcting both and offering suggestions for improvement.

Especially, I would like to give my special thanks to my family, whose patient love enabled me to complete this work.

7. Selbständigkeitserklärung / Declaration

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Hilfsmittel, Literatur und persönlichen Mitteilungen angefertigt habe.

Ich versichere, dass ich diese Arbeit noch an keiner anderen Hochschule eingereicht habe, um ein Promotionsverfahren eröffnen zu lassen. Die Hilfe eines Promotionsberaters wurde von mir nicht in Anspruch genommen. Dritte haben von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit der vorgelegten Dissertation stehen. Die Promotionsordnung der Biologisch-Pharmazeutischen Fakultät ist mir bekannt.

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Jena, 6. Dezember 2009

8. Curriculum Vitae

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2003-2005	Undergraduate Research, Master thesis, Institute of Physiology /Medical School/, University of Pécs, Pécs, Hungary Supervisors: Prof. Dr. med. Lénárd László and Prof. Dr. Gábriel Róbert (Master thesis: **The action of angiotensin II on water intake and salt appetite in the central nervous system, focused on the zona incerta**)

Brief Chronology of Employment:

2005-2006	Predoctoral Fellow, Cancer Research Institute USA–Guest Researcher Fellowship Mikrobiologiskt och Tumörbiologiskt Centrum (MTC), Karolinska Institute, Stockholm, Sweden Supervisors: Prof. Dr. med. Eva Klein and Prof. Dr. med. Georg Klein
2006-2009	PhD student, International-Leibnitz-Research-School-Fellowship Department of Experimental Dermatology, Friedrich Schiller University of Jena, Jena, Germany Supervisors: Prof. Dr. med. Johannes Norgauer and Prof. Reinhard Wetzker (Phd thesis: **The role of G-protein-coupled receptors in the biological activity of γδ T lymphocytes**)
2009-date	Post doctoral Fellow, Laboratory of Pediatric Gastroenterology Erasmus University Medical Center, Rotterdam, The Netherlands

Technical skills:

Cell culture, protein chemistry (western blotting, immunoprecipitation), nucleic acid purification, RT-PCR, gel electrophoresis, flow cytometry analysis, chemotaxis-assays, ELISA assays, ^{51}Cr radioactive assay, fluorescence microscopy, animal handling (rats), processing stereotaxical implantation surgery of canules for microinjections into the CNS.

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3. Bagi É., K. Tóth, **K. Truta-Feles**, L. Lénárd: Angiotensinergic regulation of salt hunger in the zona incerta of rat brain. *Acta Physiologica Hungarica*, 92(3-4): 237, 2005.

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6. Lagadari M, **Truta-Feles K**, Lehmann K, Berod L, Ziemer M, Idzko M, Barz D, Kamradt T, Maghazachi AA, Norgauer J.: Lysophosphatidic acid inhibits the cytotoxic activity of NK cells: involvement of G_s protein-mediated signaling. *Int. Immunol.* 2009 Jun;21(6):667-77. Epub 2009 May 21.

7. **K. Truta-Feles**, M. Lagadari, K. Lehmann, L. Berod, S. Cubillos, S. Piehler, Y. Herouy, D. Barz, T. Kamradt, A.A. Maghazachi, and J. Norgauer: A novel role for histamine as a modulator of γδ T cell migration and cytotoxicity: Involvement of G_i and G_s protein-coupled signalling pathways. (in press)

8. **K. Truta-Feles**, K. Lehmann, M. Lagadari, L. Berod, S. Cubillos, S. Piehler, D. Barz, A.A. Maghazachi, T. Kamradt, and J. Norgauer: A novel role for fMLP as a modulator of human γδ T cell migration: Involvement of G_i protein-coupled signalling pathways. (submitted)

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