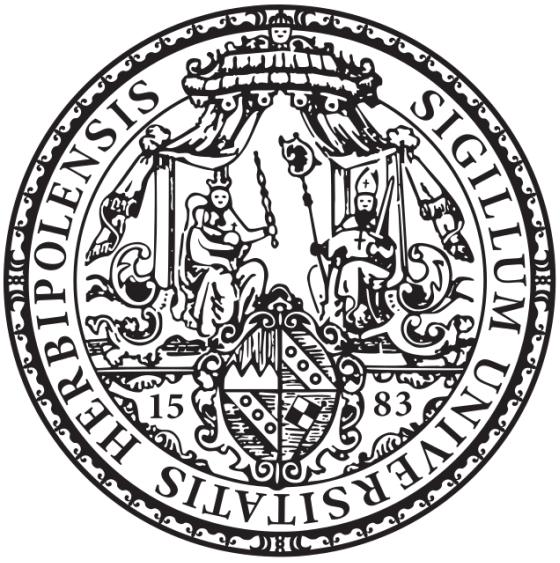


# BTN3A1 in the immune response of Vγ9Vδ2 T cells

# BTN3A1 in der Immunantwort der Vγ9Vδ2 T Zellen

Doctoral thesis for a doctoral degree at the Graduate School of Life Sciences,

Julius-Maximilians-Universität Würzburg,

Section of Infection and Immunity

Submitted by

Rubén Felipe Riaño Arias

From Necoclí, Antioquia, Colombia

Würzburg, 2016

Submitted on:

……..……..

Office stamp

Members of the *Promotionskomitee*:

Chairperson: Prof. Dr. Markus Engstler

Primary Supervisor: Prof. Dr. Thomas Herrmann

Supervisor (Second): Prof. Dr. Thomas Hünig

Supervisor (Third): Prof. Dr. med. Volker Kunzmann

Date of Public Defence: ……..……

Date of Receipt of Certificates: ……

Printed with the support of the German Academic Exchange Service.

Gedruckt mit Unterstützung des Deutschen Akademischen Austauschdienstes.

*Dedicado a Emelina, Edgar y Jorge Andrés.*
*Este triunfo es de ustedes.*

# ACKNOWLEDGEMENTS

I express my sincere gratitude to Prof. Herrmann for the opportunity to pursue my doctoral thesis under his supervision. Thanks for your advice and patience.

I am very thankful to Prof. Volker Kunzmann for his suggestions, ideas and support as a member of my PhD thesis committee.

I am extremely thankful to Prof. Thomas Hünig for his considerate support and for the great time during the Immunomodulation graduate program.

My special thanks to Prof. Michael Hudecek and Prof. Christine Krempl for their kind collaboration in some of my experiments.

It gives me immense pleasure to thank my labmates: Lisa, Mohindar, Daniel, and Alina… you made it easier and funnier for me.

I would also like to extend my appreciation to all the members (at all levels) of the Institute for Virology and Immunobiology, who helped me in different ways during my work.

I also would like to thank all my colleagues from the Immunomodulation graduate program. I had a good time with you in the seminar room and also during the retreats.

My sincere thanks to the Graduate School of Life Sciences for providing me the opportunity to live these learning experience. I am specially thankful to Gabriele Blum-Oehler for having always the door open to listen to me.

My special thanks to the German Academic Exchange Service (DAAD) for the funding of this transatlantic adventure. Here, I express my deepest sense of gratitude to Silke Hamacher for her huge help and concern.

My heartfelt thanks to Juancho, Cesitar, Eylin, Natalia, and Libia. No words are enough to express what you mean to me.

I am also extremely thankful to Paula and Adam for helping me with the writing of this thesis.

Finally, I thank my friends, in Germany and Colombia, for all their affection and love.

# SUMMARY

Human Vγ9Vδ2 T cells are the main γδ T cell subset in the circulation, accounting for up to 5% of the total peripheral blood lymphocyte population. They have been suggested to be important in response to tumors and infections. Their immune mechanisms encompass cell killing via cytotoxicity and secretion of pro-inflammatory cytokines such as IFNγ and tumor necrosis factor (TNF). The main stimulators of Vγ9Vδ2 T cells are isopentenyl pyrophosphate (IPP) and (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP), denominated phosphoantigens (PAg).

A major advance in the understanding of PAg detection and Vγ9Vδ2 T cell activation has been the identification of the butyrophlin 3A (BTN3A) proteins as key mediators in these processes. In humans, three isoforms constitute the BTN3A family: BTN3A1, BTN3A2, and BTN3A3; and their genes are localized on the short arm of chromosome 6. The role of BTN3A1 has been highlighted by BTN3A-specific monoclonal antibody 20.1 (mAb 20.1), which has an agonist effect and causes proliferation, expansion, and activation of primary human Vγ9Vδ2 T cells. On the other hand, BTN3A-specific monoclonal antibody 103.2 (mAb 103.2) is antagonistic, inhibiting the Vγ9Vδ2 T cell response. The actual mechanism underlying both PAg- and mAb 20.1-mediated activation is not completely elucidated, but the importance of BTN3A1 is clear.

The main objective of this dissertation was to characterize the role of BTN3A1 in the PAg-dependent and PAg-independent Vγ9Vδ2 T cell activation and to evaluate its contribution in the response to influeza A virus infected cells. This research work demonstrated, by using Vγ9Vδ2 TCR MOP-transduced murine cells (reporter cells), that human chromosome 6 (Chr6) is mandatory for PAg-induced stimulation, but not for stimulation with mAb 20.1. The reporter cells responded to mAb 20.1 in cultures with BTN3A1-transduced Chinese hamster ovary cells (CHO BTN3A1) as antigen

presenting cells. Nevertheless, for PAg-dependent activation the presence of Chr6 in CHO BTN3A1 was mandatory.

Although reporter cells expressing clonotypically different Vγ9Vδ2 TCRs showed similar PAg response, they clearly differed in the mAb 20.1 response. The reporter cell line transduced with Vγ9Vδ2 TCR D1C55 demonstrated essentially no response to mAb 20.1 compared to Vγ9Vδ2 TCR MOP cells. These findings were further supported by experiments performed with human PBMCs-derived Vγ9Vδ2 T cell clones. The results indicate heterogeneity in the PAg- and 20.1-dependent responses, in terms of CD25 and CD69 expression, among three different Vγ9Vδ2 T cells clones.

Co-cultures of reporter cells with Raji RT1B$^{l}$ and PAg plus mAb 20.1 or single chain antibody 20.1 (sc 20.1) revealed no additive or synergistic activating effects. In contrast, mAb 20.1 or sc 20.1 inhibited the PAg-mediated activation of the reporter cells.

The comparison of the relative contribution of the isoforms BTN3A2 and BTN3A3, in the activation of Vγ9Vδ2 T cells, was undertaken by overexpression of these isoforms in CHO cells. The results showed that BTN3A2 contributes to both PAg- and mAb-induced Vγ9Vδ2 T cell activation. On the contrary, BTN3A3 does not support PAg-mediated γδ T cell response.

Additionally, mutations in the proposed PAg- and mAb 20.1-binding sites of the extracellular BTN3A1 domain were generated by means of site-directed mutagenesis. These mutations revoked the mAb 20.1-induced Vγ9Vδ2 T cell activation, but not that induced by PAg.

Finally, co-cultures of Vγ9Vδ2 TCR MOP-transduced murine reporter cells with influenza A/PR/8/34-infected cells, or infection of PBMCs with this virus strain indicated that BTN3A1 might be dispensable for the Vγ9Vδ2 T cell response against influenza A.

The data of this research work points out that: i) in addition to BTN3A1, other Chr6-encoded genes are necessary for Vγ9Vδ2 T cell activation with PAg; ii) clonotypical (CDR3) differences influence the PAg- and mAb 20.1-mediated Vγ9Vδ2 T cell activation; iii) the PAg- and mAb 20.1-induced responses are not synergistic

and interfere with each other; iv) BTN3A2 and BTN3A3 isoforms differ in the ability to support PAg- or mAb 20.1-dependent Vγ9Vδ2 T cell activation; v) the importance of the intracellular B30.2 domain of BTN3A1, in the Vγ9Vδ2 T cell activation, might be higher than that of the extracellular domain; and vi) in spite of the importance of BTN3A1 in the activation of Vγ9Vδ2 T cells, it is possible that many molecules with redundant functions are involved in the elimination of influenza virus infection by these cells.

In summary, it is possible to hypothesize a model in which BTN3A1 detects prenyl pyrophosphates in the cytoplasm via its B30.2 domain and in association with another protein(s). The binding of PAg to this domain induces a multimerization of BTN3A1 or a conformational change of its extracellular domain (mimicked by mAb 20.1). These modifications might be recognized by the Vγ9Vδ2 TCR or by an associated T cell protein. In the case that the TCR directly recognizes BTN3A1, the intensity of the response will depend on the Vγ9Vδ2 TCR clonotype. Future research will allow to gain a better understanding of BTN3A1, its interaction with other proteins, its actual role in the activation of Vγ9Vδ2 T cells, and its importance in specific models of cancer or infection. This knowledge will be necessary to transform these cells into effective tools in the clinic.

# ZUSAMMENFASSUNG

Im Menschen stellen Vγ9Vδ2 T Zellen die größte Subpopulation an γδ T Zellen im Blut dar und machen bis zu 5% der Gesamtpopulation peripherer Blutlymphozyten aus. Sie spielen eine wichtige Rolle bei der Bekämpfung von Tumoren und Infektionen. Ihre Immunantwort umfasst cytotoxische Aktivität sowie Sekretion proinflammatorischer Zytokine wie IFNγ und dem Tumor Necrosis Faktor (TNF). Vγ9Vδ2 T Zellen werden am stärksten durch Isopentenylpyrophosphat (IPP) und (*E*)-4-hydroxy-3-methyl-but-2-enylpyrophosphat (HMBPP) stimuliert, welche als Phosphoantigene (PAg) bezeichnet werden.

Ein großer Schritt für das Verständnis der Phosphoantigenerkennung und Vγ9Vδ2 T Zellaktivierung war die Identifzierung der Schlüsselrolle, die Butyrophilin 3A (BTN3A) Proteinen in diesen Prozessen zukommt. Im Menschen existieren drei Isoformen von BTN3A (BTN3A1, BTN3A2 und BTN3A3), deren Gene auf dem kurzen Arm von Chromosom 6 lokalisiert sind. Die Rolle von BTN3A1 wurde durch den BTN3A spezifischen monoklonalen Antikörper 20.1 (mAk 20.1) besonders hervorgehoben, der eine agonistische Wirkung besitzt und Proliferation, Expansion, sowie Aktivierung primärer humaner Vγ9Vδ2 T Zellen hervorruft. Zudem existiert ein antagonistischer BTN3A spezifischer monoklonale Antikörper 103.2 (mAk 103.2), welcher Vγ9Vδ2 T Zellantworten inhibiert. Die der PAg- und mAk 20.1 vermittelten Aktivierung zugrunde liegenden Mechanismen wurden noch nicht vollständig aufgeklärt, die bedeutende Rolle von BTN3A1 in diesem Prozess ist jedoch eindeutig.

Das Ziel dieser Arbeit war es, die Rolle von BTN3A1 in der PAg abhängigen sowie unabhängigen Vγ9Vδ2 T Zellaktivierung zu charakterisieren und ihren Beitrag zu der Immunantwort gegen mit Influenza A Virus infizierte Zellen zu ermitteln. Durch die Nutzung Vγ9Vδ2 TCR MOP transduzierter muriner Zellen als Reporterzellen konnte gezeigt werden, dass das humane Chromosom 6 (Chr6) zwar für die PAg abhängige Stimulation, nicht jedoch für die Aktivierung durch mAk

20.1 zwingend notwendig ist. In Kultur mit BTN3A1 transduzierten “chinese hamster ovary” (CHO) Zellen antworteten die Reporterzellen auf mAk 20.1. Für eine PAg abhängige Aktivierung war jedoch zusätzlich die Anwesenheit des humanen Chr6 in CHO BTN3A1 Zellen Voraussetzung.

Obwohl Reporterzellen, die Vγ9Vδ2 TCRs verschiedener Klonotypen exprimierten, ähnliche PAg Antworten zeigten, unterschieden sie sich in der mAk 20.1 Antwort klar. Die mit Vγ9Vδ2 TCR D1C55 transduzierten Reporterzellen zeigten im Vergleich zu Vγ9Vδ2 TCR MOP Zellen nahezu keine mAk 20.1 abhängige Antwort. Diese Befunde wurden auch durch Experimente gestützt, die mit humanen, aus PBMCs gewonnenen Vγ9Vδ2 T Zellklonen durchgeführt wurden. Deren Resultate weisen, bezüglich der CD25 und CD69 Expression, auf eine heterogene PAg- und 20.1 abhängige Antwort der drei unterschiedlichen Vγ9Vδ2 T Zellklone hin.

Kokulturen von Reporterzellen mit Raji RT1B$^{l}$ und PAg plus mAk 20.1 oder dem Einzelkettenantikörper 20.1 (sc 20.1) zeigten keine additive oder synergistische aktivierende Wirkung, vielmehr wurde die PAg vermittelte Aktivierung der Reporterzellen durch mAk 20.1 oder sc 20.1 inhibiert.

Mittels Überexpression der beiden Isoformen BTN3A2 und BTN3A3 in CHO Zellen, wurde deren jeweiliger Beitrag zur Aktivierung von Vγ9Vδ2 T Zellen verglichen. Die Ergebnisse zeigten, dass BTN3A2 sowohl zu PAg als auch mAk induzierten Vγ9Vδ2 T Zellaktivierung beiträgt. BTN3A3 hingegen unterstützt die PAg vermittelte γδ T Zellaktivierung nicht.

Weiterhin wurden, mittels gerichteter Mutagenese, in den vorgeschlagenen PAg- und mAk 20.1 Bindungsstellen der extrazellulären BTN3A1 Domäne Mutationen generiert. Diese verhinderten die mAk 20.1-, jedoch nicht die PAg vermittelte Vγ9Vδ2 T Zellaktivierung.

Zuletzt zeigten Kokulturen von Vγ9Vδ2 TCR MOP transduzierten murinen Reporterzellen und Influenza A/PR/8/34 infizierten Zellen, sowie eine Infektion von PBMCs mit diesem Virusstamm, dass BTN3A1 für die Vγ9Vδ2 T Zellantwort gegen Influenza A entbehrlich sein könnte.

Die Ergebnisse dieser Arbeit zeigen, dass i) zusätzlich zu BTN3A1, andere auf Chr6 befindliche Gene für die PAg abhängige Aktivierung von Vγ9Vδ2 T Zellen nötig sind; ii) klonotypische (CDR3) Unterschiede die PAg und mAk 20.1 vermittelte Vγ9Vδ2 T Zellaktivierung beeinflussen; iii) die PAg- and mAk 20.1 induzierten Antworten sich nicht verstärken, sondern beeinträchtigen; iv) sich die Isoformen BTN3A2 und BTN3A3 in der Fähigkeit, die PAg- oder mAk 20.1 abhängige Vγ9Vδ2 T Zellaktivierung zu unterstützen, unterscheiden; v) die intrazelluläre B30.2 Domäne von BTN3A1 eine größere Bedeutung für die Vγ9Vδ2 T Zellaktivierung haben könnte als die extrazelluläre; und dass vi) trotz der Bedeutung von BTN3A1 für die Aktivierung von Vγ9Vδ2 T Zellen, die Möglichkeit besteht, dass viele Moleküle mit redundanter Funktion bei der Eliminierung von Influenza Viren durch diese Zellen eine Rolle spielen.

Zusammenfassend lässt sich als Hypothese ein mögliches Modell aufstellen, in dem BTN3A1 in Assoziation mit einem oder mehreren zusätzlichen Proteinen zytoplasmatische Prenylpyrophosphate mittels der B30.2 Domäne detektiert. Die Bindung der PAg an diese Domäne würde dann eine Multimerisierung von BTN3A1 oder eine Konformationsänderung der extrazellulären Domäne (wie auch durch mAk 20.1 herbeigeführt) induzieren. Diese Modifizierungen könnten vom Vγ9Vδ2 TCR oder von einem assoziierten T Zellprotein erkannt werden. Für den Fall einer direkten Erkennung von BTN3A1 durch den TCR würde der Grad der T Zellantwort vom Vγ9Vδ2 TCR Klonotyp abhängen. Zukünftige Forschung wird ein besseres Verständnis von BTN3A1, dessen Proteininteraktionen, dessen Rolle in der Vγ9Vδ2 T Zellaktivierung, und dessen Bedeutung in spezifischen Krebs- oder Infektionsmodellen ermöglichen. Wissen, das benötigt wird, um diese Zellen effizient in klinischen Therapien einzusetzen.

# INDEX

# LIST OF FIGURES

# LIST OF TABLES

# 1. INTRODUCTION

## 1.1 The immune system

In the fight for survival, human beings and other eukaryotes have developed mechanisms to remain *immune* to the plethora of environmental challenges. The immune response is the organized reaction to macromolecules such as proteins, lipids, or polysaccharides, and small chemicals of self or foreign origin. The tissues, cells, and molecules in charge of the organization and management of the immune response constitute the immune system. According to the speed and specificity of the immune reactions, the immune system is conceptually divided into innate immunity and adaptive immunity. Innate immunity reacts immediately after stimulus and includes 1) physical, chemical, and microbiological barriers; 2) phagocytic cells (neutrophils, monocytes, and macrophages); 3) cells that release inflammatory mediators (eosinophils, basophils, and mast cells); 4) dendritic cells (DC); 5) natural killer (NK) cells and other innate lymphoid cells; along with 6) proteins such as the complement system or acute phase proteins [1-3].

Unlike innate mechanisms, adaptive immunity develops, temporally, after the immune challenge and becomes more vigorous, rapid, and refined after successive exposures to a particular stimulus. Lymphocytes are the key components of the adaptive immunity and the compounds recognized by them are called antigens. Adaptive immunity relies primarily on the use of antigen-specific receptors that are expressed by and drive the effector responses of T and B lymphocytes. Adaptive immunity recognizes and reacts to an enormous number of microbial and non-microbial antigens. This type of response bears some hallmarks: it is targeted to distinct antigens (specificity), covers a huge variety of them (diversity), increases after repeated stimulus by the same antigen (memory), generates a cell population from a single antigen-specific clone (clonal expansion), and adapts the effector

functions according to the stimulus (specialization) [1-3]. Both innate and adaptive immunity have in common the use of cytokines, a large group of secreted or membrane-bound proteins, with a wide variety of structures and functions such as induction of cell growth and proliferation, activation of effector functions, induction of apoptosis, and chemotaxis [1-3].

The innate and adaptive immune responses function in a cooperative fashion as components of an integrated system for host defense. The innate immunity stimulates and influences the adaptive immune response, and the latter enhances the mechanisms of the former. The complexity of the immune system is even higher. Even though its components are grouped into the innate or the adaptive immunity, some of them exert functions defined for members of the other type of immunity. The focus of this dissertation is a type of T lymphocyte, the $\gamma\delta$ T cells, which behave not only as cells of the adaptive immunity but also have many features of innate cells.

## 1.2 Antigen receptor of lymphocytes

The defense mechanisms of the innate immunity are phylogenetically older than those of the adaptive immunity. Pattern-recognition receptors such as Toll-like receptors (TLRs), Nod-like receptors (NLRs) and scavenger receptors —used by neutrophils, macrophages, and DC— are found even in plants and invertebrates [4]. These receptors recognize conserved pathogen-associated molecule patterns and are germ-line encoded proteins. In addition to the innate defense mechanisms, vertebrates have developed complex systems for lymphocyte antigen receptor diversification. Agnathans, the oldest vertebrate taxon, have lymphoid cells that assemble lymphocyte antigen receptor genes through the rearrangement of leucine-rich repeat-encoding modules [5]. These receptors are called variable lymphocyte receptors. On the other hand, adaptive immunity in jawed vertebrates (Gnathostomata) depends on B cell receptors (BCR) and T cell receptors (TCR), which are the main classes of antigen recognition receptors of lymphocytes and

belong to the immunoglobulin superfamily [6]. Proteins of this group contain globular structural motifs known as immunoglobulin (Ig) domains and are very important for the function of the immune system [7].

BCR and TCR molecules are generated by somatic recombination of variable (V), diversity (D), and joining (J) gene segments [8, 9]. Random recombination of these gene segments takes place in the progenitors of B and T cells in the primary lymphoid organs, under the orchestration of the products of recombination-activating gene 1 (RAG1) and RAG2 [6]. Since B lymphocytes are beyond the scope of this dissertation, the following content will focus on T lymphocytes and their TCR, placing special emphasis on the T cell subset of study.

A complete TCR is a heterodimer of two transmembrane polypeptide chains, $\alpha$ and $\beta$ or $\gamma$ and $\delta$, linked by a disulfide bond. Each chain consists of a variable domain and a constant domain. The first one is the amino-terminal portion of the TCR extracellular section and contains variable amino acid sequences that differ between distinct clones of lymphocytes. It holds the specificity for the antigen and the ability to bind to it. On the other hand, the constant domain, at the carboxy-terminal portion and proximal to the cell membrane, is not involved in antigen recognition [10]. The variable domain contains the complementarity-determining regions (CDR), also known as hypervariable regions, short segments that contain most of the variable residues that make contact with the antigen. The variable regions of each TCR chain contain three CDR, which means that an intact antigen receptor has a total of six CDR forming a complementary surface with the structure of the antigen [10].

In addition to serve in antigen recognition, a process that is dependent on major histocompatibility complex (MHC) molecules expressed on the cell surface of antigen-presenting cells (APC) or target cells [11, 12], TCR expression is pivotal in the development of T lymphocytes. They originate in the bone marrow and mature in the thymus; the environment of this organ provides the stimuli needed for their maturation from pro-T cells to the mature T cell stage. Through a series of organized steps, the selection of thymocytes (T cells in the thymus) for maturation is based on their ability to synthesize and express a functional TCR [13]. TCR

expression also allows to define the two main T cell lineages, αβ T cells and γδ T cells, and even T cell subpopulations regarding TCR repertoire; since T cell clones use differentially the V, D, and J gene segments to generate their TCR [14-16].

TCR molecules are associated with the CD3 proteins and ζ chain, capable of initiating intracellular biochemical pathways activated upon TCR engagement with their intracytoplasmic tails. The CD3 and ζ proteins are the same in all T lymphocytes independent of the TCR specificity. They are non-covalently associated with the TCR heterodimer and together with it form the TCR complex [17, 18]. Additionally, mature αβ T cells express either CD4 or CD8, which are correceptors expressed on the cell membrane and help to amplify the signaling pathways initiated after TCR-mediated activation [19]. As a result, T lymphocytes proliferate, differentiate into effector cells, acquire new functions, and protect themselves from or die by apoptosis.

T cells regulate cell function, mainly by cytokine production, or eliminate cells infected with intracellular microorganisms by means of cytotoxicity [16]. γδ T cells differ from αβ T cells in some aspects: they do not depend on restriction to MHC molecules to recognize antigens, most of them do not express the co-receptors CD4 or CD8, and their TCR repertoire is more limited [20, 21]. Functionally, γδ T cells share features with cells of the innate immune system; these include recognition of molecular patterns instead of specific peptidic antigens, rapid response to stimulation without clonal expansion [22-24], which confers on them the title of innate-like immune T cells.

## 1.3 Gamma delta TCR (γδ TCR)

In humans, the repertoire of V and J gene segments, used for TCR rearrangement by γδ T cells, is very restricted compared to the significant diversity of α and β gene segments. The gene segments used to encode the TCR γ chain map to chromosome 7 and are constituted by seven functional Vγ genes and five Vγ pseudogenes [25]. TCR δ and TCR α gene segments are closely linked and located

in a single locus mapped on to chromosome 14. The TCR δ gene family includes three true Vδ genes (Vδ1, Vδ2, and Vδ3), along with Vα genes that can be used in δ chain rearrangement; consequently considered Vδ genes: Vδ4 (Vα14), Vδ5 (Vα29), Vδ6 (Vα23), Vδ7 (Vα36), and Vδ8 (Vα38) [25]. The diversity of J gene segments, for both γ and δ chains, is also very low: five and four, respectively [25].

In contrast to the low number of V and J segments available for γδ TCR rearrangement, γδ T cells have the ability to use multiple D segment genes simultaneously during δ chain assembly, with the possibility to incorporate them in both the forward and reverse reading frames [25-27]. Additionally, random nucleotides can be added at the multiple junctional points of the V, D, and J gene segments, making the variability of the CDR3 in the TCR δ chain extraordinarily high [26, 27]. As a consequence, the potential TCR repertoire of γδ T cells is higher than that of αβ T cells [28, 29]. In spite of that, the actual repertoire of γδ TCR is rather limited, with invariant commonly occurring antigen receptors [30].

γδ TCR gene families have been highly conserved in vertebrates, but γδ T cells clearly differ between taxonomic groups in characteristics like TCR V genes usage, cell frequencies, tissue localization, phenotype, and function. Species such as human and mouse have around seven functional Vγ genes, whereas others like chicken can use more than 20 Vγ genes [20, 31]. Percentages of circulating γδ T cells in primates and rodents represent just 1-5% of the total circulating lymphocyte population, but in chickens they are very common and in ruminant species, they range from 5% to 70% of the peripheral blood lymphocytes [20]. Human γδ T cells are also localized in the intestinal epithelium, comprising around 40% of the intraepithelial lymphocytes (IELs) [32, 33]. Mice and rats bear in the skin a unique γδ T cell population designated as dendritic epidermal T cells [34]. Although γδ T cells of the majority of animal species do not express CD4 or CD8, up to 90% of rat γδ T cells are CD8 positive [35, 36]. Similarly, γδ T cells from cattle and avian species express this molecule [37, 38].

The diversity of TCR V genes, their special usage by γδ T cells in some tissues, and the patterns of combination in the different species define γδ T cell subsets with particular phenotypic, functional and homing features. In humans, the two

predominant subsets have been characterized as Vδ2$^{+}$ and Vδ2$^{-}$ γδ T cells. The former expresses a TCR that contains the V region pair Vγ9 and Vδ2 and are the main γδ T cell subpopulation in the circulation, accounting for up to 5% of the total peripheral blood lymphocyte population. They respond to a special type of compounds called phosphoantigens (PAg). The latter subset displays a TCR where the gene element Vδ1 is paired with various Vγ elements and is predominant in tissues such as gut, liver, or skin [22, 39].

## 1.4 Vγ9Vδ2 T cells in response to infection

Evidence of research during the last 20 years has highlighted the important participation of γδ T cells in the immunity to microorganisms. Research work from the early 1990s showed that γδ T cells respond to bacterial secreted proteins such as tetanus toxoid, staphylococcal enterotoxin, and listeriolysin [40-43]. These cells have been suggested to be important in response to several bacterial infections and γδ T cell deficient mice have a higher susceptibility to infections caused by different bacterial genera including *Mycobacterium, Escherichia, Listeria, Salmonella, Pseudomonas, Nocardia,* and *Klebsiella* [23]. *Mycobacterium tuberculosis* can specifically induce the expansion and activation of γδ T cells [44, 45]; which seems to be part of a protective response against the infection, as expansion and activation of effector Vγ9Vδ2 T cells allow to control bacillus replication or dissemination and to limit disease lesions in lungs in a macaque model of *M. tuberculosis* infection [46]. In SCID mice, adoptive transfer of human Vγ9Vδ2 T cells leads to successful control of infections caused by *Staphylococcus aureus, Escherichia coli* and *Morganella morganii* [47].

Vγ9Vδ2 T cells have also confirmed to have protective effects in acute and chronic viral infections. They have shown beneficial outcomes against influenza virus through direct killing of infected cells and antiviral cytokine production, controlling infection by human seasonal H1N1, pandemic H1N1, as well as avian H5N1 and H9N2 [48-50]. They can also induce CD4$^{+}$- and CD8$^{+}$-mediated

anti-influenza T cell response by processing and presenting virus-derived peptides [51]. Research evidence suggests that Vγ9Vδ2 T cells have an important contribution in HIV infection as well [52, 53]. γδ T cells, especially $V\delta1^+$ subpopulations, have also been involved in the control of infections caused by other viruses such as Epstein-Barr virus, human hepatitis virus C, and human cytomegalovirus [23, 54-56].

In addition to bacterial and viral infections, γδ T cells participate in the immune response to other type of microorganisms such as *Candida albicans* or *Leishmania spp.* (mainly $V\delta1^+$ T cells)*;* and *Toxoplasma gondii* or *Plasmodium falciparum* (mainly $V\delta2^+$ T cells) [57-61]. An important mechanism of γδ T cell-mediated response to microorganisms is the killing of infected cells or pathogens via Fas-Fas ligand interaction as well as release of perforin, granzyme B, and granzyme M. Moreover, through secretion of pro-inflammatory cytokines such as interferon γ (IFNγ), interleukin 17 (IL-17) and tumor necrosis factor (TNF), different γδ T cell subpopulations contribute to control and eliminate microorganisms [62].

## 1.5 Vγ9Vδ2 T cells in response to tumor cells

Human γδ T cells take part in the anti-cancer immune response; they effectively kill tumor cells and produce protective cytokine responses against cancer [63-65]. In fact, Vγ9Vδ2 T cells have been employed in clinical trials for cancer therapy [66, 67]. Vγ9Vδ2 T cells are able to recognize and lyse malignant B cell lines of human origin, e.g., Daudi and RPMI 8226 [68, 69]. They have shown antitumor effects towards lymphoma, myeloma, prostate and breast cancer, among other types of cancer [64-67, 70].

It has been demonstrated that PAg positively affect the antibody-dependent cell-mediated cytotoxicity (ADCC) of Vγ9Vδ2 T cells [71]. The bioactivity of therapeutic anti-cancer monoclonal antibodies such as rituximab or alentuzumab is improved by the synthetic PAg, BrHPP. This improvement is due to an increased γδ T cell binding to tumor cells coated with these antibodies and to an enhanced

secretion of lytic granules from the γδ T cells, with the subsequent increment of ADCC [71]. Recent approaches have further supported the role of Vγ9Vδ2 T cells in the antitumor response. A reagent named [$(Her2)_2$ x Vγ9] tribody, a bispecific antibody that binds to Vγ9Vδ2 TCR-expressing cells and to Her2-positive pancreatic ductal adenocarcinoma cells, increases the Vγ9Vδ2 T cell-mediated cytotoxicity towards these cancer cells [72, 73]. Besides that, it has been shown that the combination of Vγ9Vδ2 T cells and an antibody specific for GD2, a ganglioside antigen expressed by neuroblastoma and Ewing's sarcoma, reduces tumor growth in a murine model of these two types of cancer [74].

Tumor cell detection is mainly undertaken by the γδ TCR and by NK cell receptors such as natural killer group 2, member D (NKG2D) [75, 76]. In regard to TCR recognition, one of the first findings indicated that ectopically expressed F1 ATPase in complex with apolipoprotein A1 has the ability to bind to a recombinant Vγ9Vδ2 TCR [77]. It has also been shown that the NKG2D ligand, UL16-binding protein 4 (ULBP4) can bind a soluble chimeric Vγ9Vδ2 TCR and activate Vγ9Vδ2 TCR-transfected $TCR^-$ Jurkat cells [78]. Nevertheless the actual importance of these ligands is controversial and these results still need to be independently confirmed by different research groups. NKG2D-mediated Vγ9Vδ2 T cell activation independent of the TCR has shown to trigger cytotoxicity and TNF production as well [79]. Efficient cytotoxic activity by this γδ T cell subset is also induced by over-expression of ULBP1, another NKG2D ligand, in hematologic tumors [80].

## 1.6 Vγ9Vδ2 T cells in interaction with other immune cell types

The complexity of the immune system limits the possibilities to set discrete boundaries between its components. Vγ9Vδ2 cells have a T cell nature, but they are also categorized as components of the innate immunity due to their capacity to respond quickly and free of MHC restriction to self and non-self molecules. Like the other immune players, Vγ9Vδ2 T cells not only have direct effects on their defined targets (microorganisms, stressed cells, and cancer cells), but they also influence

the immune response of other cells. *In vitro*, activated Vγ9Vδ2 T cells induce the upregulation of MHC class II and I molecules, CD80, CD86, CD83, CD25, CD40, and C-C chemokine receptor type 7 (CCR7) on immature DC [81-84]. This Vγ9Vδ2-mediated DC maturation reduces the ability of the latter cell lineage to take up soluble antigens [85] and allows them to produce IL-12 [81-84].

Moreover, Vγ9Vδ2 T cells influence the maturation of monocytes by inducing downregulation of CD14 as well as upregulation of CD40, CD86, HLA-DR, and markers of inflammatory DC such as CD83 and CD209, along with the production of IL-6, IFNγ, and TNF [86]. When exposed to Vγ9Vδ2 T cells, neutrophils attain an APC-like phenotype, with high expression of MHC class I and II molecules, CD40, CD54, CD64, and CD83, together with the capacity to take up soluble antigens and process them for presentation to $CD4^+$ and $CD8^+$ T cells [87].

Apart from influencing cells of myeloid lineage, Vγ9Vδ2 lymphocytes also provide B cell help, conditioning the humoral response. IL-21-primed Vγ9Vδ2 T cells assume characteristics of human follicular B helper T ($T_{FH}$) cells such as production of chemokine (C-X-C motif) ligand 13 (CXCL13), an important chemokine to recruit B cells to lymphoid organs and to organize the germinal centers [88]. *In vitro*-activated Vγ9Vδ2 T cells express CD40L, inducible T cell co-stimulator (ICOS), along with the production of IL-4 and IL-10 [89, 90], which are important factors for T cell-mediated B cell help. Vγ9Vδ2 T cells also promote the APC behavior of B cells by inducing them to increase the expression of CD40, CD86, and HLA-DR [91].

Strikingly, Vγ9Vδ2 T lymphocytes themselves can act as authentic professional APC, as defined by the effective processing of exogenous antigens via endocytosis or phagocytosis, and their capability to prime and induce differentiation of $CD4^+$ and $CD8^+$ T cells [51, 92-94]. All of this evidence points out the existence of an intricate network for immune surveillance, where γδ T cells are crucial participants that affect the transition from the innate to the adaptive response.

## 1.7 Target molecules of Vγ9Vδ2 T cells

γδ T cells recognize antigens that differ from the MHC-associated peptides presented to αβ T cells. The distinct γδ T cell subsets recognize defined groups of molecules such as MHC-ligands, non-peptide antigens, or phospholipids, among others [95]. Isopentenyl pyrophosphate (IPP) derived from *Mycobacterium smegmatis* was the first Vγ9Vδ2 T cell natural agonist identified. IPP and (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) —another Vγ9Vδ2 T cell activator, first identified in *E. coli* and mycobacteria— are well-known PAg, a family of natural compounds synthesized by many microorganisms and plants [96-99].

IPP and HMBPP share basically the same chemical structure, a diphosphate attached to an isoprene unit, but differ simply because HMBPP has an extra hydroxyl group and is around 30000 times more potent in activating Vγ9Vδ2 T cells than IPP [99, 100]. PAg are derived from metabolic pathways to generate isoprenoids, an ancient and diverse class of molecules that function as precursors of metabolites such as cholesterol, ergosterol, ubiquinones, and dolichols. These compounds play pivotal roles in cellular processes that include hormonal metabolism, growth regulation, intracellular signal transduction, vesicular transport, and photosynthesis, among others [101]. Two distinct and independent biosynthetic pathways serve to produce isoprenoids: the 1-deoxy-D-xylulose-5-phosphate (DOXP) pathway (also known as 2-C-methyl-D-erythritol 4 phosphate —MEP— pathway) and the mevalonate (MVA) pathway. Eukaryotic cells, fungi and archaebacteria use the MVA pathway, where IPP is generated after condensation of three molecules of acetyl-CoA. In contrast, most eubacteria, cyanobacteria, algae, plants and apicomplexan protozoa use the DOXP pathway. Here HMBPP functions as the immediate precursor of IPP, whose production initiates via the condensation of pyruvate and D-glyceraldehyde 3-phosphate [97, 102, 103].

When human peripheral blood mononuclear cells (PBMCs) are stimulated with HMBPP, the reactivity is restricted to the Vγ9Vδ2 lymphocyte subset, leading to

their expansion (in the presence of co-stimulation provided by IL-2), upregulation of cell surface activation markers, and secretion of proinflammatory cytokines [103-105]. Vγ9Vδ2 T cells stimulated with IPP produce considerable amounts of TNF; this response is attributed to a dysregulated metabolism and the consequent intracellular IPP accumulation from the MVA pathway [106, 107]. The best example of this scenario is depicted by the metabolic changes of tumor cells that translate to an unusual elevated production of IPP [107]. Moreover, infection with bacteria and viruses imposes a metabolic burden on target cells, which alters their MVA pathway, increasing their levels of IPP [108, 109].

Together with the direct stimulation of Vγ9Vδ2 lymphocytes with PAg, there are indirect ways to induce their activation. One of them is through the use of aminobisphosphonates (ABP), a group of drugs that includes zoledronate, pamidronate, alendronate, and others, used in the treatment of bone resorption diseases and some types of cancer [110-112]. ABP are pyrophosphate analogs with the ability to inhibit the function of the IPP-consuming enzyme farnesyl pyrophosphate synthase (FPPS), in the MVA pathway [113], thereby causing intracellular accumulation of IPP and sensitizing human cells to recognition by Vγ9Vδ2 T cells. In fact, cells treated with ABP become effective stimulators of primary Vγ9Vδ2 lymphocytes and of Vγ9Vδ2 TCR transductants, inducing proliferation, cell activation, cytokine production, and cytotoxicity [64, 106, 107, 114]. These effects are also achieved by the activation or over-expression of 3-hydroxy-3-methyl-glutaryl CoA (HMG-CoA) reductase, the upstream rate-controlling enzyme of IPP synthesis [107, 108]; whereas its inhibition, by mevastatin, decreases the production of IPP and thus, Vγ9Vδ2 T cell activation [107, 115]. Activation of Vγ9Vδ2 T lymphocytes via FPPS inhibition has also been demonstrated by alkylamines, a class of natural compounds with non-phosphate short alkyl chains and a terminal amino group, chemically distinct from ABP. Alkylamines are produced by both commensal and pathogenic bacteria as well as present in a variety of edible plants and products [115-117].

Altogether, Vγ9Vδ2 T cells react to (i) microbial HMBPP and (ii) intracellular IPP accumulation either as a product of stress conditions (infection and cell

transformation) or after FPPS inhibition. The precise mechanism of PAg recognition, however, remains poorly understood, but the importance of the Vγ9Vδ2 TCR in this scenario has long been highlighted [118-120]. Notably, it has been shown that Jurkat cells transfected with Vγ9Vδ2 TCR produce IL-2 in response to tumor cells and mycobacterial extracts [75, 114]. Moreover, classical events and signaling pathways triggered in T cells after TCR-mediated activation have also been reported in Vγ9Vδ2 T cell response mediated by PAg [121, 122]. It has been observed that cell-to-cell contact is required to activate Vγ9Vδ2 lymphocytes via PAg, and that only human APC can activate them [123-126]. This suggests that antigen-presenting molecules or surface-expressed co-stimulatory proteins are needed; and that they are ubiquitous, non-polymorphic, and species-specific. Though the knowledge accumulated about Vγ9Vδ2 T cells over the time after their discovery is substantial, it is still unclear how PAg are detected. Whether these compounds interact with some yet unknown surface molecule(s) that act as an antigen-presenting molecule, or whether they regulate the expression of determinants recognized by the Vγ9Vδ2 TCR are matters of intensive research.

## 1.8 Role of BTN3A in Vγ9Vδ2 T cell activation

A major advance in the understanding of PAg detection and Vγ9Vδ2 T cell activation came with the identification of the butyrophlin 3A (BTN3A) proteins (also known as CD277) as key mediators in these processes. Proteins from this family are type I membrane glycoproteins that participate in host homeostasis and have immunological functions. They are members of the Ig superfamily and share structural homology with B7 molecules [127-131]. In humans, three isoforms constitute the BTN3A family: BTN3A1, BTN3A2, and BTN3A3; and their genes are localized on the short arm of chromosome 6 (Chr6) [130, 132]. Their extracellular domains consist of an Ig variable-like amino-terminal domain and an Ig constant-like carboxy-terminal domain, with a high structural homology (>95%) between the three isoforms. Across the cell membrane, BTN3A1 and BTN3A3 have

an intracellular domain that belongs to the B30.2 family (PRY/SPRY), which has an additional extension of 70 amino acids in BTN3A3 and is not present in BTN3A2 [129, 133]. BTN3A are ubiquitous proteins in humans and primates with Vγ9Vδ2 T populations have also homologous proteins [129, 133, 134]. The reported co-evolution of TCR Vγ9, TCR Vδ2, and BTN3 genes in other eutherian placental mammals suggests a functional link between this γδ TCR and BTN3A proteins [135].

The initial and key evidence of the crucial role of BTN3A molecules in the Vγ9Vδ2 T cell response was the discovery that the mouse CD277-specific monoclonal antibody 20.1 (mAb 20.1) has an agonist effect and causes proliferation and expansion of primary human Vγ9Vδ2 T cells. Furthermore, it induced production of IFNγ, TNF, as wells as upregulation of CD69 and CD107 a/b [136]. The same study showed that the CD277-specific monoclonal antibody 103.2 (mAb 103.2) is antagonistic, inhibiting the Vγ9Vδ2 T cell response [136]. Since mAb 20.1 activates murine Vγ9Vδ2 TCR-transduced cells negative for BTN3A in co-culture with Raji cells [136], it is possible to infer that the mentioned antibodies do not bind to γδ T cells but to stimulatory or target cells. Crystal structure models have shown that mAb 20.1 binds to two separate BTN3A molecules and it is likely that this cross-linking results in their multimerization on the cell surface [134]. The antibody contacts BTN3A on the side, with the top of the BTN3A dimer available for binding to another receptor [134]. On the other hand, this model suggests a 1:1 binding stoichiometry of mAb 103.2 to the BTN3A dimer, a situation in which the antibody contacts the dimers on top, which might impair the plausible BTN3A binding to or sensing by the Vγ9Vδ2 TCR or another molecule [134].

The exact function of BTN3A molecules in the PAg-induced activation of Vγ9Vδ2 T cells remains controversial and currently two general models have been suggested to explain how PAg and BTN3A can work together to stimulate them. The first model describes BTN3A proteins as antigen-presenting molecules, whereby PAg bind to their extracellular domain and are directly recognized by the TCR [137]. The second model supports that the intracellular B30.2 domain acts as a PAg sensor with the ability to bind them, causing changes in the structural

conformation, distribution, or mobility of BTN3A and leading to γδ T cell stimulation [138]. Although these two models expose conflicting point of views regarding the mechanism of action of BTN3A, both emphasize the prominence of this family of proteins in the functioning of Vγ9Vδ2 T cells. In addition, after the identification of BTN3A as molecules able to communicate the presence of microorganisms or the metabolic state of a cell, through their interaction with PAg, it is possible to begin to suggest suitable experimental systems to explain this intriguing phenomenon.

## 1.9 Research objectives

Vγ9Vδ2 T cells remain an intriguing component of the immune system. Their functional location between innate and adaptive immunity makes them a very interesting cell subset. Additionally, their role in mechanisms of response to infection, cancer and their crosstalk with other immune cells render them a promising tool for therapies in these contexts. The knowledge gained on other immune players such as DC, B, and αβ T lymphocytes has permitted the modulation of the immune system and has led to medical contributions. Thus, understanding the signals that regulate Vγ9Vδ2 T cells and the molecular events that lead to APC or target cells to stimulate them will allow not only to gain more insight into γδ T cell biology, but will also help to develop new cell-based immunotherapies.

The current findings about BTN3A molecules support their role in the activation of Vγ9Vδ2 cells, but many questions remain open: Is there a requirement for additional accessory molecules, which together with BTN3A mediate complete γδ T cell responses? Does the diversity within CDR3γ and CDR3δ of Vγ9Vδ2 T cell clones translate into different profiles of response to PAg? What are the molecular features of the interaction between agonistic or antagonistic antibodies and BTN3A? What is the relative participation of the three BTN3A isoforms in the stimulation of these cells? How are BTN3A proteins involved in specific types of infections? Answers to these and further questions will allow, not only, to reach a deeper understanding of Vγ9Vδ2 T cell physiology, but will also help to understand their

participation in the immune response to microorganisms, tissue damage, and cancer. In addition, it will also provide the basis to develop effective strategies for their use in the clinic.

The main objective of this work was to characterize the role of BTN3A1 in the PAg-dependent and PAg-independent Vγ9Vδ2 T cell activation and to evaluate its contribution in the response to influenza virus-infected cells. This was accomplished through the following specific aims:

i. Test the ability of BTN3A1 alone to mediate Vγ9Vδ2 activation,
ii. Compare different Vγ9Vδ2 TCR clones in the PAg- and mAb 20.1-induced response,
iii. Assess the ability of mAb 20.1 to synergize with the PAg-elicited response,
iv. Compare the relative contribution of the three BTN3A isoforms in both PAg- and mAb 20.1-mediated Vγ9Vδ2 T cell activation,
v. Examine the effect of mutations in the BTN3A1 extracellular domain in the Vγ9Vδ2 response to PAg and mAb 20.1, and
vi. Evaluate the contribution of BTN3A1 in the Vγ9Vδ2 response to influenza A/PR/8/34 virus.

# 2. MATERIALS AND METHODS

## 2.1 Materials

### 2.1.1 Cell lines

| | |
|---|---|
| 293T | Transformed primary human embryonic kidney cells (ATCC CRL-1573). |
| 53/4 r/mCD28 Vγ9Vδ2 TCR MOP | 53/4 cells (a sister clone of the rat/mouse T-cell hybridoma 35/2), which express a rat TCR recognizing a peptide (amino acids 68 to 88) of guinea pig myelin basic protein ($gpMBP_{68-88}$) restricted to $RT1B^{l}$ and transduced for rat/mouse CD28 expression [139]. They are additionally transduced with the Vγ9Vδ2 TCR MOP. |
| 53/4 r/mCD28 Vγ9Vδ2 TCR D1C55 | 53/4 r/mCD28 cells transduced with the Vγ9Vδ2 TCR D1C55 [137]. |
| 53/4 r/mCD28 Vγ9Vδ2 TCR G115 | 53/4 r/mCD28 cells transduced with the Vγ9Vδ2 TCR G115, originated from the G9 γδ T cell clone [120]. |
| 53/4 r/mCD28 Vγ9Vδ2 TCR G115 cl5 | 53/4 r/mCD28 cells transduced with the Vγ9Vδ2 TCR G115 clone 5 (cl5) [140]. |

| | |
|---|---|
| 53/4 r/mCD28 γD1C55 δMOP | 53/4 r/mCD28 cells transduced with a γδ TCR containing the γ chain of Vγ9Vδ2 TCR D1C55 and the δ chain of Vγ9Vδ2 TCR MOP. |
| 53/4 r/mCD28 γMOP δD1C55 | 53/4 r/mCD28 cells transduced with a γδ TCR containing the γ chain of Vγ9Vδ2 TCR MOP and the δ chain of Vγ9Vδ2 TCR D1C55. |
| A375 BTN3A1 | Human malignant melanoma transfected with cDNA encoding BTN3A1, kindly provided by Gennaro De Libero (Experimental Immunology, Department of Biomedicine, University Hospital Basel, Basel, Switzerland). |
| A375 CD80 BTN3A1 | A375 BTN3A1 cells transduced with human CD80. |
| A549 | Human lung adenocarcinoma epithelial cell line, kindly provided by Christine Krempl (Institut for Virology and Immunobiology, Würzburg University, Würzburg, Germany). |
| CHO | Chinese hamster ovary cells. Referred as wild type (WT) cells in this work and transduced with human CD80. |
| CHO BTN3A1 | CHO cells transduced with human BTN3A1. |

| | |
|---|---|
| CHO BTN3A2 | CHO cells transduced with human BTN3A2. |
| CHO BTN3A3 | CHO cells transduced with human BTN3A3. |
| CHO BTN3A1 R65A | CHO cells transduced with a mutated human BTN3A1, in which arginine at position 65 is exchanged by alanine (R65A). |
| CHO BTN3A1 K37D | CHO cells transduced with a mutated human BTN3A1, in which lysine at position 37 is exchanged by aspartic acid (K37D). |
| CHO BTN3A1 K37D:Q100A | CHO cells transduced with a mutated human BTN3A1 containing the K37D mutation, together with the exchange of glutamine at position 100 for an alanine (K37D:Q100A). |
| CHO Chr6 | CHO cells monosomal for human Chr6 (GM11580, Human Genetic Cell Repository, Coriell Institute, Camden, New Jersey) and transduced with human CD80. |
| CHO Chr6 BTN3A1 | CHO Chr6 cells transduced with human BTN3A1. |
| CHO Chr6 BTN3A2 | CHO Chr6 cells transduced with human BTN3A2. |

| | |
|---|---|
| CHO Chr6 BTN3A3 | CHO Chr6 cells transduced with human BTN3A3. |
| CHO Chr6 BTN3A1 R65A | CHO Chr6 cells transduced with mutated human BTN3A1 R65A. |
| CHO Chr6 BTN3A1 K37D | CHO Chr6 cells transduced with mutated human BTN3A1 K37D. |
| CHO Chr6 BTN3A1 K37D:Q100A | CHO Chr6 cells transduced with mutated human BTN3A1 K37D:Q100A. |
| EBV-LCL | Epstein-Barr Virus-transformed B lymphoblastoid cell lines (EBV-LCL), kindly provided by Michael Hudecek, (Comprehensive Cancer Center Mainfranken, Würzburg University, Würzburg, Germany). |
| MDCK | Madin-Darby canine kidney cells, kindly provided by Peter Stäheli (Institute of Virology, Freiburg University, Freiburg, Germany). |
| RAJI RT1B$^{l}$ | Human Burkitt lymphoma (ATCC CCL-86) transduced with rat MHC class II molecule RT1B$^{l}$ [141]. |

### 2.1.2 Influenza virus strain

The influenza A/PR/8/34 (H1N1) virus strain was provided by Peter Stäheli (Institute of Virology, Freiburg University, Freiburg, Germany).

### 2.1.3 Reagents

| Product | Manufacturer |
|---|---|
| Agarose NEEO Ultra - Quality | Roth |
| Ampicillin | Gibco |
| Avicel® PH-101 | Fluka |
| *β*-Mercaptoethanol | Gibco |
| Biozym LE GP Agarose | Biozym |
| Boric acid | Roth |
| Bovine serum albumin (BSA) | Roth |
| Calcium chloride ($CaCl_2$) | Roth |
| Concanavalin A | Sigma |
| Cristal violet | Serva |
| Dimethyl sulfoxide (DMSO) | AppliChem |
| dNTP Set | Peqlab Biotechnologie |
| Ethanol | AppliChem |
| Ethylenediaminetetraacetic acid (EDTA) | AppliChem |
| Fetal calf serum (FCS) | Sigma |
| Formaldehyde solution 37% | Roth |
| GelRed | Reg Genaxxon |
| Gene Ruler 100bp and 1kb DNA Ladder | Thermo Scientific |
| Glucose (D(+)Glucose-monohydrate) | Roth |
| Glutamax (100x) | Gibco |
| Glycerin | AppliChem |
| 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) | Roth |

| | |
|---|---|
| Histidinol | Sigma |
| Histopaque® 1077 | Sigma |
| HMBPP | Sabine Amslinger, Regensburg University, Regensburg, Germany. |
| Human IL-2 | Novartis |
| Human serum (human male AB plasma) | Sigma |
| Isopropanol | AppliChem |
| L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin | Sigma |
| Loading dye | Thermo Scientific |
| Lysogeny broth (LB) medium | Gibco |
| Magnesium chloride ($MgCl_2$•$6H_2O$) | Ferak |
| Magnesium sulfate ($MgSO_4$) | AppliChem |
| Penicillin-Streptomycin (Pen-Strep) | Gibco |
| Phenol red | Merck |
| Polybren | Sigma-Aldrich |
| Potassium chloride (KCl) | AppliChem |
| Potassium dihydrogen phosphate ($KH_2PO_4$) | AppliChem |
| Sec-butylamine | Sigma |
| Super optimal broth (SOB) powder medium | AppliChem |
| Sodium azide ($NaN_3$) | Roth |
| Sodium butyrate ($Na(C_3H_7COO)$) | Sigma |
| Sodium carbonate ($Na_2CO_3$) | Merck |
| Sodium chloride (NaCl) | AppliChem |
| Sodium dihydrogen phosphate ($NaH_2PO_4$) | AppliChem |
| Sodium hydrogen carbonate ($NaHCO_3$) | Merck |
| Sodium hydrogen phosphate ($Na_2HPO_4$) | Roth |
| Sulfuric acid ($H_2SO_4$) | AppliChem |

| | |
|---|---|
| Triton X-100 | Sigma |
| Trypan blue | Sigma |
| Trypsin | BD Biosciences |
| Tween20 | Sigma |
| Zoledronate | Novartis |

### 2.1.4 Media, buffers and solutions

| | |
|---|---|
| Agarose plates | 25 g LB medium<br>15 g agarose<br>1 L $dH_2O$<br>autoclaved<br>ampicillin (final concentration 100 μg/mL) added under sterile conditions at 50°C<br>put in 10 cm Non-tissue culture Petri dishes split (20mL/dish) |
| Ampicillin | stock solution<br>(100 mg/mL)<br>100 mg ampicillin powder in 1 mL 70% ethanol |
| ATV | 80 g NaCl<br>4 g KCl<br>10 g D-(+)-glucose<br>5.8 g $NaHCO_3$<br>5 g trypsin<br>2 g EDTA<br>filled up to 10 L with $dH_2O$ |

| | |
|---|---|
| BSS I | 50 g glucose |
| | 3 g $KH_2PO_4$ |
| | 11.9 g $NaH_2PO_4$ |
| | 0.4 g phenol red |
| | filled up to 5 L with $dH_2O$ |
| | 110 mL of solution filled with $dH_2O$ to make 1 L |
| BSS II | 9.3 g $CaCl_2$ |
| | 20 g KCl |
| | 400 g NaCl |
| | 10 g $MgSO_4 \cdot 7H_2O$ |
| | 10 g $MgCl_2 \cdot 6H_2O$ |
| | filled up to 5 L with $dH_2O$ |
| | 110 mL of solution filled with $dH_2O$ to make 1 L |
| BSS/BSA | 0.2% BSA in BSS |
| Cell freezing solution | 50% FCS |
| | 40% RPMI 1640 |
| | 10% DMSO |
| Cristal violet solution | 1% Cristal violet diluted in 20% Ethanol |
| CTL-Medium | 500 mL RPMI 1640 |
| | 10% human serum |
| | 1% Glutamax |
| | 1% Pen-Strep |
| | 0.1% β-mercaptoethanol |

| | |
|---|---|
| DMEM−<br>(without HEPES) | Invitrogen |
| DMEM−<br>culture medium | 500 mL DMEM−<br>50 mL FCS (10% final concentration)<br>1.2 mL Pen-Strep solution |
| DMEM+<br>(with HEPES) | Invitrogen |
| DMEM+<br>culture medium | 500 mL DMEM+<br>50 mL FCS (10% final concentration)<br>1.2 mL Pen-Strep solution |
| EBV-LCL medium | 500 mL RPMI 1640<br>10% FCS<br>1% Glutamax<br>1% Pen-Strep |
| ELISA Blocking Reagent | PBS with 5% FCS |
| ELISA Coating Buffer | 0.84 g $NaHCO_3$<br>(0.1 M Bicarbonate buffer) 0.356 g $Na_2CO_3$<br>filled up to 100 mL with $dH_2O$<br>pH adjusted to 9.5 |
| ELISA Washing Buffer | 1 L PBS with 500 µL Tween20 |

| | |
|---|---|
| EMEM medium | (MDCK cell culture medium)<br>500 mL MEM Invitrogen<br>10% FCS<br>1% Pen-Strep |
| FACS-Buffer | 0.1% BSA + 0.05% $NaN_3$ in PBS |
| Formaldehyde solution | 4% formaldehyde in PBS |
| HBS 2x 50 mM | HEPES pH 7.1<br>10 mM KCl<br>12 mM glucose<br>280 mM NaCl<br>1.5 mM $Na_2HPO_4$<br>solution filtered in sterile conditions (45 μm) and pH adjusted to 7.1 |
| LB-Medium | 25 g LB medium (powder)<br>1 L $dH_2O$<br>autoclaved<br>ampicillin added before use (final concentration 100 μg/mL) |
| MDCK infection medium | 500 mL DMEM+<br>0.3% BSA |
| MDCK infection medium with TPCK-treated trypsin | 500 mL DMEM+<br>0.3% BSA<br>0.1 μg/mL TPCK-treated trypsin |

| | |
|---|---|
| PBS | 0.2 g KCl |
| | 0.2 g $KH_2PO_4$ |
| | 0.05 g $MgCl_2$ |
| | 8 g NaCl |
| | 1.15 g $Na_2HPO_4$ |
| | 0.167 g $CaCl_2 \cdot H_2O$ |
| | 0.1 g $MgCl_2 \cdot 6H_2O$ |
| | filled up to 10 L with $dH_2O$ |
| Plaque assay | 500 mL DMEM+ |
| (Avicel®) medium | 1.5% Avicel® |
| | 0.3% BSA |
| | 0.1 μg/mL TPCK-treated trypsin |
| Penicillin-streptomycin | 5 g penicillin |
| solution | 5 g streptomycin |
| | dissolved in 50 mL $dH_2O$ |
| RPMI 1640 | Invitrogen |
| RPMI 1640 | 500 mL RPMI 1640 |
| culture medium | 50 mL SC-supplement |
| | 25 mL FCS (10% final concentration) |

| | |
|---|---|
| SC-supplement | 50% heat-inactivated FCS<br>10% 100 mM sodium pyruvate<br>10% non-essential amino acids<br>10% Pen-strep (10 kU/mL)<br>5.84% glutamine solution (5%)<br>0.5% 50 mM β-mercaptoethanol |
| S.O.C.-medium | 25.5 g SOB powder medium dissolved in $dH_2O$<br>pH adjusted to 7.0<br>filled up to 980 mL with $dH_2O$<br>autoclaved<br>20 mL glucose solution (1 M) added |
| TBE 5x | 54 g Tris base<br>27.5 g boric acid<br>20 mL 0.5 M EDTA pH 8<br>filled up to 1 L with $dH_2O$<br>autoclaved |
| TE<br>(pH 8) | 10 mM Tris-HCl (pH 8)<br>1 mM EDTA (pH 8) |

### 2.1.5 Vectors

| | |
|---|---|
| pEGZ | pIZ vector, which in addition to the zeocin resistance gene, includes a EGF gene (EGFP-Zeocin fusion gene). Kindly provided by Ingolf Berberich (Institute for Virology and Immunobiology, Würzburg University, Würzburg, Germany) [142]. |

| | |
|---|---|
| pEGZ BTN3A1 | pEGZ vector containing human BTN3A1 open reading frame. |
| pHIT 60 | codes for the *gag* and *pol* proteins of the Moloney murine leukemia virus (MoMLV). Kindly provided by Dirk Lindemann (Institute for Virology and Immunobiology, Würzburg University, Würzburg, Germany) [143]. |
| pczVSV-G | codes for the *env* protein of the Vesicular Stomatitis Virus. Kindly provided by D. Lindemann [144]. |
| SFG-GFP (S65T) CD80 | Retroviral expression vector, in which the originally encoded fragment of GFP gene, between the *NcoI/BamHI* restriction sites in SFG-GFP [145], was replaced by the a gene to code human CD80. |

### 2.1.6 Oligonucleotides

All oligonucleotides are listed in orientation 5' to 3'. All oligonucleotides were synthesized by Sigma Aldrich. Primers 1, 2, 3, and 4: created by Felipe Riaño. Primers 103, 104, 83, 84, EGN rev I, and EGN rev III: created by Lisa Starick. Primers 200 and 201: created by Mohindar Karunakaran (Table with primers on the next page).

**Table 1.** List of primers

| Name | Sequence |
|---|---|
| 1 BTN3A1 Mut 94 A fwd_1 | TGCACCGTATGCTGGGAGAACTT |
| 2 BTN3A1 Mut 94 A rev_2 | AAGTTCTCCCAGCATACGGTGCA |
| 3 BTN3A1 Mut 128 A fwd_3 | GTGTTATTTCGCAGATGGTGACT |
| 4 BTN3A1 Mut 128 A rev_4 | AGTCACCATCTGCGAAATAACAC |
| 103 BTN3A1 Mut. 66D fwd_103 | CATGGAGCTGGATTGGGTGAGTT |
| 104 BTN3A1 Mut. 66D rev_104 | AACTCACCCAATCCAGCTCCATG |
| 200 HsaBTN3A1EcoR1KozakFwd | CTATGAATTCGCCGCCACCATGAAAATGGC AAGTTTCCTGGC |
| 201 HsaBTN3A1RpBglIIRev | CTACAGATCTTCACGCTGGACAAATAGTCA GGG |
| 83 BTN3A1 Seq Mitte fwd_83 | ATGAAAAAGCCCTGGTGGAG |
| 84 BTN3A1 Seq Mitte rev_84 | CCCTTGGCACGCTGCACACT |
| EGN rev I | GCCCCATTCCAAGCGGCT |
| EGN rev III | AAAATAACATATAGACAAACGC |

### 2.1.7 Antibodies

**Table 2.** List of antibodies

| Antigen | Clone | Conjugation | Isotype | Manufacturer |
|---|---|---|---|---|
| Human BTN3A | 20.1 | ------ | mouse $IgG_1,\kappa$ | Daniel Olive's research group* [136] |
| Human BTN3A | 103.2 | ------ | mouse $IgG_{2a},\kappa$ | D.O. [136] |
| Human BTN3A | 20.1 | ------ | No isotype, single chain versions (sc Fvs) | Erin Adams' research group+[134] |
| Human BTN3A | 103.2 | ------ | scFv | E. A. [134] |
| Human CD3 | OKT3 | ------ | mouse $IgG_{2a},\kappa$ | Miltenyi |
| Human CD25 | M-A251 | PE | mouse $IgG_1,\kappa$ | Biolegend |
| Human CD69 | FN50 | PE | mouse $IgG_1,\kappa$ | BD Biosciences |
| Human CD80 | L307.4 | PE | mouse $IgG_1,\kappa$ | BD Biosciences |
| Human Vδ2 TCR chain | 4G6 | ------ | mouse $IgG_1,\kappa$ | Thomas Herrmann's research group [146] |
| Rat/mouse CD1d | 233 (WTH1) | ------ | mouse $IgG_{2a},\kappa$ | T. H. |
| Human CD1c | F10/21A3 | ------ | sc Fvs | E. A. |
| Unknown specificity | MOPC-31C | ------ | mouse $IgG_1,\kappa$ | BD Biosciences |
| Mouse IgG (H+L) | Polyclonal | FITC/PE | No isotype, donkey $F(ab')_2$ fragment | Jackson Immunoresearch laboratories |
| Mouse IgG | Polyclonal | ------ | ------ | Bethyl |

* (Institut Paoli-Calmettes, Marseille, France), + (University of Chicago, Chicago, USA). All antibodies were 1:10 diluted for use.

### 2.1.8 Kits

| Product | Manufacturer |
|---|---|
| BD OptEIA™ Mouse IL-2 ELISA Set | BD Biosciences |
| BD OptEIA™ TMB Substrate Reagent Set | BD Biosciences |
| BigDye® Term v3.1 CycleSeq Kit | Applied Biosystems |
| GeneJET™ Plasmid Miniprep Kit | Fermentas |
| JETQuick DNA Clean Up Spin Kit 50 | Genomed |
| JETstar Plasmid Purification Kit | Genomed |
| MiniElute™ Gel Extraction Kit | Quiagen |
| Phusion™ High-Fidelity DNA-Polymerase | Finnzymes |
| QIAquick Gel Extraction Kit | Qiagen |
| QIAquick PCR Purification Kit | Quiagen |
| Restriction enzymes (*BamHI, EcoRI, NcoI, MluI, PagI, PvuI, XhoI*) | Fermentas |
| T4 DNA Ligase | Fermentas |
| TCR$\gamma/\delta^+$ T Cell Isolation Kit (human) | Miltenyi Biotec |

### 2.1.9 Consumables

| Product | Manufacturer |
|---|---|
| 6-well plates (flat bottom) | Greiner |
| 12-well plates (flat bottom) | Greiner |
| 24-well plates (flat bottom) | Greiner |
| 48-well plates (flat bottom) | Greiner |
| 96-well plates (flat bottom) | Greiner |
| 96-well plates (round bottom) | Greiner |
| Centrifuge tubes (15 mL and 50 mL) | Greiner |
| Criovials (1.6 mL) | Sarstedt |
| Flow cytometry tubes (5 mL) | Sarstedt |
| Glass Pasteur pipettes with or without filter (1.5 mL) | Distributed by Laborbedarf Scheller |

| | |
|---|---|
| Microtubes (1.5 mL) | Eppendorf |
| Non-tissue culture dishes (Ø 10 cm) | Greiner |
| Pipette tips (10 µl, 200 µl, and 1000 µl) | Roth |
| Plastic pipettes (5 mL, 10 mL, and 25 mL) | Greiner |
| Polystyrene half-area 96-well EIA/RIA plates | Corning Incorporated |
| Single-use syringe (5 mL) | BD Biosciences |
| Sterile filters (0,25 µm and 0,45 µm) | Sartorius |
| Tips for Multipipette | Eppendorf |
| Tissue culture flasks (50 mL and 200 mL) | Greiner |
| Tissue culture dishes (Ø 6 cm) | Sarstedt |
| Tissue culture dishes (Ø 10 cm) | Greiner |

### 2.1.10 Computer programs and Internet addresses

| | |
|---|---|
| CellQuest 3.3 | BD Biosciences |
| Endnote X5 Thomson | Reuters |
| Excel 2011 | Microsoft |
| FlowJo 8.8.7 | Tree Star |
| PowerPoint 2011 | Microsoft |
| Prism 6 | GraphPad |
| Word 2011 | Microsoft |

Internet addresses

http://www.imgt.org/IMGT_vquest/share/textes/

http://www.ncbi.nlm.nih.gov/nuccore/NM_007048.5

http://www.ncbi.nlm.nih.gov/pubmed

## 2.2 Methods

### 2.2.1 Microbiology and molecular biology methods

#### *2.2.1.1 Site-directed mutagenesis of BTN3A1*

Mutations in the proposed PAg- and mAb 20.1-binding in the extracellular domain of human BTN3A1 (NCBI Reference Sequence: NM_007048.5) were introduced by polymerase chain reaction (PCR) using overlapping primers, which included the necessary minimal change in the nucleotide sequence for the point mutations. The PCR was carried out using the Phusion™ High Fidelity DNA polymerase (Finnzymes). Mutated BTN3A1 genes were cloned back into the EcoRI/BamHI cloning site of the vector pEGZ. To this end, the primers 200 and 201 were used for human BTN3A1 (see Table 1). All mutations were verified by sequencing of the insert (see 2.2.1.7).

PCR mixture:

| | |
|---|---|
| Template: | 1 μL (10 ng) |
| Primer Mix (10 μM each): | 2,5 μL |
| $dH_2O$: | 35 μL |
| Phusion™ DNA Polymerase: | 0,5 μL |
| Phusion™ buffer: | 10 μL |
| dNTPs: | 1 μL |

PCR program:

| | | | |
|---|---|---|---|
| Initial denaturation: | 30 sec | 98°C | |
| Denaturation: | 10 sec | 98°C | 25 cycles |
| Annealing: | 30 sec | 68°C | |
| Elongation: | 45 sec | 72°C | |
| Final elongation: | 10 min | 72°C | |
| Cooling: | ∞ | 4°C | |

Primers used to generate the mutations:

| Mutation | Mutation primer (See Table 1) |
|---|---|
| R65A | 1 + 2 |
| K37D | 103 + 104 |
| Q100A | 3 + 4 |

#### *2.2.1.2 Gel electrophoresis and DNA extraction from agarose gels*

For the analysis of DNA fragments by gel electrophoresis, 1% agarose gels were used. The DNA was transferred to the gel after mixture with running buffer (loading dye, Thermo Scientifc) and separated after applying a voltage of 120 V to the gel. The extraction of DNA fragments from agarose gels was done using the QIAquick Gel Extraction Kit (Qiagen) according to manufacturer's instructions.

#### *2.2.1.3 Cloning of mutated BTN3A1 into the pEGZ vector*

The mutated versions of the extracellular domain of human BTN3A1 were amplified using the primers 200 and 201 (see Table 1) and EGZ BTN3A1 as template. They were cloned into the EcoRI/BamHI restriction sites of the pEGZ vector. PCR preparation and PCR program see 2.2.1.1.

#### *2.2.1.4 Restriction digestion and ligation of insert and vector*

The restriction digestion to prepare a ligation was carried out for 2 h, at 37°C, using the appropriate restriction enzymes according to the manufacturer. Purification of insert and vector was performed by agarose gel separation after restriction digestion. Ligation of the purified insert and vector, in ratio of 3:1, was carried out using T4 DNA ligase (Fermentas) according to the manufacturer, at 4°C overnight.

### *2.2.1.5 Transformation of bacteria*

To transform competent bacteria, 1 μL of the ligation mixture was added to the bacteria (50 μL). This was followed by 10 min incubation on ice, 30 sec incubation at 42°C, and 2 min incubation on ice. Afterwards, 250 μL of S.O.C. medium were added to the bacteria. This bacterial culture was incubated for 1 h with shaking at 37°C. To select successfully transformed bacteria, the culture was streaked onto agar plates with the suitable antibiotic (for selection) and incubated overnight at 37°C.

### *2.2.1.6 DNA mini- and midi-preparation for isolation of plasmids*

To isolate the plasmids from successfully transformed bacterial clones, each selected growing clone was transferred, with a swab, from the colony on the selection plate to a glass tube containing 5 mL of LB medium with the corresponding antibiotic. The liquid culture was incubated with shaking at 37°C, overnight. The next day, 1.5 mL of dense bacterial culture were pelleted at 10000 rpm and room temperature (RT) for 2 min, and plasmids were isolated with a GeneJet$T^M$ Plasmid Miniprep Kit (Fermentas/Thermocientific) following the instructions of the manufacturer. The plasmids were analyzed by restriction digestion and sequencing. To obtain large amounts of plasmid, 50 mL LB medium with the corresponding antibiotic were inoculated with each selected clone, from the bacterial colony on the agar plate. The liquid culture was incubated with shaking at 37°C, overnight. The isolation of the plasmids was performed using the JetStar 2.0 MIDI kit (Genomed) according to the manufacturer's instructions.

### *2.2.1.7 DNA sequencing*

The DNA sequencing was performed using BigDye® v3.1 Term CycleSeq kit (Applied Biosystems) and was analyzed in an ABI 3100 sequencer (Applied Biosystems).

Mixture of sequencing PCR:

| | |
|---|---|
| Purified DNA: | 3 μL (750 ng) |
| Primer (10 μM): | 0.5 μL |
| 5x BigDye® Terminator 3.1 buffer: | 0.5 μL |
| BigDye® Terminator 3.1: | 1 μL |

PCR program for sequencing:

| | | | |
|---|---|---|---|
| Initial denaturation: | 30 sec | 96°C | |
| Denaturation: | 10 sec | 96°C | 25 cycles |
| Annealing: | 5 sec | 50°C | |
| Elongation: | 4 min | 60°C | |
| Final elongation: | 10 min | 60°C | |
| Cooling: | ∞ | 4°C | |

The sequencing was evaluated with the primers 83, 84, EGN rev I, and EGN rev II (see Table 1)

#### *2.2.1.8 Determination of the concentration of nucleic acids*

The spectrophotometric quantification of deoxyribonucleic acid (DNA) was performed using the NanoDrop 2000c (Thermo Scientific) according to the manufacturer's instructions.

### 2.2.2 Immunobiochemistry method

#### *2.2.2.1 Mouse IL-2 enzyme-linked immunosorbent assay (ELISA)*

The determination of mouse IL-2 in cell culture supernatant of stimulated cells was performed using the BD OptEIA™ Mouse IL-2 ELISA Set (BD Biosciences) and the BD OptEIA™ TMB Substrate Reagent Set (BD Biosciences) according to

manufacturer's instructions. The measurement of the developed ELISA plates was performed at 450 nm on a Vmax kinetic microplate reader (Molecular Devices).

### 2.2.3 Cell biology methods

#### *2.2.3.1 Routine cell culture*

Mammalian cells were cultured in a cell incubator at 37°C, 5% $CO_2$ and a $H_2O$ saturated atmosphere. Suspension and adherent cells were maintained with RPMI 1640 medium supplemented with 10% FCS (with the exception of 293T cells, which were cultivated in DMEM+). For routine maintenance, suspension cells were cultured in 12-well plates and adherent cells in 10 cm-tissue dishes. The cells were split in fresh culture medium every 3-4 days. For suspension cells, the split ratio was 1:3, whereas for adherent cells was 1:7-1:10. To split adherent cells, culture medium was discarded; 2 mL ATV were added to detach the cells. The cells were collected, pelleted, resuspended in 2 mL culture medium, and transferred to the tissue dishes in 5 mL fresh culture medium. Prior to every experiment, cells were counted with a Neubauer chamber under a light microscope and the percentage of viable cells was determined, by diluting the cells with trypan blue solution. Unstained cells were considered as alive and counted for the experiments.

#### *2.2.3.2 Freezing and thawing of cells*

To freeze a cell line $1x10^6$ to $5x10^6$ cells were pelleted, resuspended in 1 mL freezing medium, transferred to a pre-chilled 1.5 mL-cryovial, frozen at -80°C and after several weeks transferred to -140°C. To resume the culture of frozen cells, they were previously thawed at RT and washed twice with culture medium.

### *2.2.3.4 Retroviral transduction*

To express the gene of interest in mammalian cells, viral particle-mediated transduction was used. A three-plasmid expression system of retrovirus was used, infecting 293T cells to generate the viral vectors containing the gene of interest. Viral particles were released in the supernatant, which was later used to infect target cells [143]. This procedure was performed as follows:

**Day 1**: $1.5x10^6$ 293T cells were seeded in 5 mL DMEM+ medium in 6 cm-culture dishes. The dishes were gently shaken to achieve even spread of the cells, which was crucial for good transfection efficiency. The cells were incubated at 37°C, 5% $CO_2$ overnight.

**Day 2**: Culture medium of 293T cells was replaced with pre-warmed fresh DMEM medium without HEPES and incubated for 1 h to allow pH in the medium to reach equilibrium. Meanwhile, the transfection mix was prepared: 5 μg of each of the three vectors used (recombinant vector, pHIT60, and pVSV-G) were taken from the stock vial. 438 μL of sterile distillated water and 62 μL $CaCl_2$ 2M were added to the vectors. In a 15 mL-tube, 500 μL HBS 2X were pipetted. While vortexing, the 500 μL DNA-water-$CaCl_2$ mixture was added into the 15 mL-tube containing the HBS and incubated for 20 minutes at RT. Afterwards, the transfection mix was added dropwise all over the cell culture dish, which was gently shaken to distribute DNA evenly. The culture dish was incubated 6 h at 37°C, 5% $CO_2$ and the medium was replaced with 5 mL prewarmed fresh DMEM+ medium. The cells were incubated overnight in the same conditions.

**Day 3**: Culture medium was replaced with 5 mL prewarmed DMEM+ medium containing 10 mM sodium butyrate. Sodium butyrate activates the CMV promoter, inducing the expression of the genes of interest and those needed to generate the viral particles. The cells were incubated during 6 h at 37°C, 5% $CO_2$; afterwards the medium was replaced with 5 mL of prewarmed DMEM+. The cells were incubated in the same previous conditions.

**Day 4**: Supernatants from 293T cell culture were collected and filtered through 0.45 μm-filter, to keep the supernatants cell-free. $1x10^5$ target cells were taken into a 15 mL-tube and suplemented with 3 mL of viral supernatant containing 4 μg/mL

polybrene. The cells were centrifuged at 2200 rpm, 37°, for 3 h. Immediately after centrifugation, the supernatant was discarded and the cell pellet was resuspended in 3 mL of fresh culture medium and incubated in 6 cm-dishes (if they were adherent cells) or in 6-well plates (if they grew in suspension) at 37°C, 5% $CO_2$.

#### *2.2.3.5 Identification of transduced cells*

After transduction with the gene of interest, the cells were cultured with the suitable culture medium and the appropriate antibiotic (e.g. histidinol for CHO cells containing Chr6) for 3-4 days. Afterwards, the cells were analyzed by flow cytometry (see 2.2.3.9) for the expression of fluorescent proteins like EGZ or stained with specific antibodies against the protein of interest. Once the cells were found to be positive for the transduced gene, they were sorted using a FACS Aria III machine to obtain a complete positive population. The cells were the maintained in culture using the routine cell culture methods.

#### *2.2.3.6 Stimulation of 53/4 r/m CD28 Vγ9Vδ2-transduced reporter cells*

The ability of Vγ9Vδ2 TCR transductants to respond to PAg was tested by co-cultures, in 96-well plates, with either Raji RT1B$^{l}$ cells or adherent cells such as CHO cells and A375 melanoma cells. Adherent cells were seeded the previous day. The ratio APC:responder cells was 1:1 with suspension APC and 1:5 with adherent APC. The cells were stimulated with different concentrations of HMBPP, zoledronate, sec-butylamine, agonistic 20.1 antibody, or antagonistic 103.2 antibody. The plates were incubated during 20 h at 37°C, 5% $CO_2$. The IL-2 produced in response to the stimuli was determined by ELISA (see 2.2.2.1).

#### *2.2.3.7 Isolation of PBMCs*

PBMCs were isolated from heparinized fresh blood obtained from healthy donors under informed consent. Separation of PBMCs was done using Histopaque® 1077.

15 mL of Histopaque® 1077 were added into a 50 mL-conical tube and 30 mL of whole blood were transferred to the tube. The sample was centrifuged at 1180x g for 15 min without brakes applied. After centrifugation, the PBMCs fraction (concentrated as an interphase between the plasma and the separation medium) was gently aspirated with a 5 mL-pipette and transferred to a new 50 mL-tube. The cells were washed twice with 50 mL ice-cold BSS/BSA at 461x g, 4°C, for 5 min. Finally, the PBMCs were resuspended in 5 mL RPMI 10% FCS.

#### *2.2.3.8 Stimulation of primary Vγ9Vδ2 T cells with zoledronate-pulsed CHO cells*

To evaluate the ability of Vγ9Vδ2 T cells from PBMCs to respond to PAg, $1x10^5$ CHO cells were seeded in 48-well plates and incubated overnight, at 37°C, 5% $CO_2$. The cells were then treated with either 1 μM or 10 μM zoledronate and further incubated for 20 h. Afterwards, they were washed twice with pre-warmed PBS containing 10% FCS and co-cultured with $2x10^5$ freshly isolated PBMCs during 20 h. Non-adherent cells were collected and stained with anti-Vδ2 (clone 4G6), washed and stained with FITC-labeled $F(ab')_2$ fragment donkey anti-mouse IgG. To measure CD69 expression (T cell activation marker), cells were stained with anti-CD69 PE. The percentage of CD69 on $V\delta2^+$ cells or $V\delta2^-$ negative cells was assessed by flow cytometry (See 2.2.3.9).

#### *2.2.3.9 Flow cytometric analysis of cells*

Flow cytometry was performed on a FACSCalibur (BD Biosciences) using the software CellQuest 3.3 (BD Biosciences) to measure the samples. The samples were analyzed with FlowJo 8.8.7 (Tree Star). $2x10^5$ (CHO cells or other cells lines) to $5x10^5$ (primary cells) cells were used per staining condition. Staining was carried out in FACS buffer and samples were washed with an excess of buffer between incubations. The incubation with the antibodies was performed at 4°C. Two protocols for staining were used. In the simplest of them cells were counted, washed with FACS buffer, centrifuged at 1600 rpm for 5 min. The specific

antibodies (directly conjugated to a flourochrome such as FITC or PE) were added to the cells and incubated for 30 min. Afterwards they were washed with FACS buffer and samples were measured. In the second flow cytometry protocol, the cells were first incubated for 30 min with an uncojugated mouse anti-human antibody. After washing and centrifuging the cells, they were further incubated for 30 min with a fluorocrome-conjugated $F(ab')_2$ fragment donkey anti-mouse IgG. The cells were then washed, centrifuged and incubated for 15 min with an excess of mouse IgG serum antibodies. Finally, the cells were incubated for 30 min with antibodies directly conjugated to fluorochromes and samples were measured.

#### *2.2.3.10 Vγ9Vδ2 T cell cloning by single cell dilution*

To generate Vγ9Vδ2 T cell clones PBMCs were isolated (see 2.2.3.7), Vγ9Vδ2 were negatively selected by magnetic activated cell sorting (MACS) using the human TCRγ/$\delta^+$ T Cell Isolation kit (Miltenyi Biotec), and single cell dilution cultures were performed. The cells were cultured in 96-well plates in CTL medium for two weeks in presence of 30ng/mL anti-CD3 (clone OKT-3), 100 nM HMBPP, and 50 U/mL IL-2 along with irradiated EBV-LCL and irradiated PBMCs as feeder cells (see 2.2.3.11). Three clones were selected based on their purity as Vγ9Vδ2 T cell population (designated as clone A, clone B and clone C). They were further expanded, for two weeks, in the same culture conditions mentioned before.

#### *2.2.3.11 Irradiation of feeder cells*

EBV-LCL and PBMCs functioning as feeder cells in the Vγ9Vδ2 T cell cloning experiments were irradiated in a Faxitron Cabinet X-ray System 43855F CP160 Option (Faxitron X-ray LLC). EBV-LCL were irradiated to 80 Gy for 16.6 min, and PBMCs to 30 Gy for 6 min.

### *2.2.3.12 Stimulation of PBMCs-generated Vγ9Vδ2 T cells clones*

To measure the response of Vγ9Vδ2 T cell clones from PBMCs to HMBPP and mAb 20.1; $1x10^5$ Vγ9Vδ2 T cell clones were co-cultured with $1x10^5$ Raji $RT1B^l$ in presence of 200 nM HMBPP, 2 μg/mL mAb 20.1, 0.2 μg/mL mAb 20.1 or 0.02 μg/mL mAb 20.1. The cultures were incubated for 20 h at 37°C, 5% $CO_2$. The cells were collected and cell activation of the Vγ9Vδ2 T cell clones, in terms of CD25 upregulation, was measured by flow cytometry (see 2.2.3.9).

### *2.2.3.13 Replication of influenza A/PR/8/34 in MDCK cells*

In order to generate cell culture supernatants with influenza A/PR/8/34 virus, $5x10^6$ MDCK cells were seeded in T25 culture flasks, using 5 mL MDCK culture medium, the day previous to infection with the virus, and incubated overnight at 37°C, 5% $CO_2$. 80%-90% was considered as the ideal percentage of confluence of the monolayer. Before infection, MDCK culture medium was replaced, after washing the cells three times with PBS, by 1 mL MDCK infection medium without TPCK-treated trypsin. A virus dilution was prepared by taking 3 μL of the virus stock, and adding them into 97 μL of MDCK infection medium without TPCK-treated trypsin. 3 μL of the virus dilution were added to 1 mL MDCK infection medium without TPCK-treated trypsin. Afterwards, MDCK cells were inoculated with the viruses and incubated for 1 h, at 37°C, 5%$CO_2$, with gentle shake of the flasks every 20 min. Next, the medium was replaced with 5 mL MDCK infection medium with 0.1 μg/mL TPCK-treated trypsin. The cells were incubated at 37°C, 5%$CO_2$. The culture supernatant (containing the viruses produced by the infected cells) were harvested at days 2 and 4 after infection, aliquoted, and kept at -80°C.

### *2.2.3.14 Influenza A/PR/8/34 plaque assay*

To determine the virus titers in the culture supernatant of MDCK infected cells, $1x10^6$ MDCK cells (pro well) were seeded in 24-well plates using 1 mL MDCK

medium culture, and incubated overnight at 37°C, 5% $CO_2$. The cells were seeded in triplicates for the following virus dilutions: $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$, and $10^{-6}$. The culture medium was discarded, the cells were washed with PBS at RT, and 1 mL MDCK culture medium was added to them. Meanwhile, virus dilutions were prepared from the culture supernatants previously harvested (from 2.2.3.13) as follows: 25 μL of virus supernatant were pipetted to 225 μL MDCK infection medium, this dilution constituted the $10^{-1}$ dilution. Next, 25 μL of the $10^{-1}$ dilution were added to 225 μL MDCK infection medium, to obtain the $10^{-2}$ dilution. This process was further repeated until obtaining the $10^{-6}$ dilution. The culture medium was then removed from the MDCK cells, which were inoculated with 150 μL of each virus dilution. They were incubated 1 h, 37°C, 5%$CO_2$, with gentle shakes every 20 min.

Afterwards, the inocula were removed and the cells were overlayed with 1 mL 1.5% Avicel® medium with 0.1 μg/mL TPCK-treated trypsin. The cell cultures were incubated during 3 days at 37°C, 5% $CO_2$. Subsequently, the Avicel® medium was removed and the cells were fixed with 1 mL 4% formaldehyde (in PBS) for 15 min at RT. After that, the formaldehyde was discarded, the cells were treated with 1 mL 1% crystal violet (in 20% ethanol), and incubated 15 min at RT on shaking plate. Finally, the plates were rinsed with water and the virus titer, as plaque-forming units per milliliter (PFU/mL), were determined. To obtain these values, the plaques on the plates were counted and averaged out; the result was multiplied by the dilution where the plaques were counted; and this product was divided by the inoculum volume (0.15 mL). The final result was the virus titer in PFU/mL.

#### *2.2.3.15 Stimulation of Vγ9Vδ2 TCR transductants with influenza-infected cells*

To evaluate the capacity of murine cells expressing the Vγ9Vδ2 TCR to respond to influenza A virus, $1x10^4$ A549 lung epithelial carcinoma cells were infected with influenza A/PR/8/34 at different multiplicities of infection (MOI) —2, 4, 6, 8, and 10— and co-cultured with Vγ9Vδ2 TCR MOP cells during 20 h. The levels of IL-2 produced by the responder cells were determined by ELISA (see 2.2.2.1).

### *2.2.3.16 Stimulation of primary Vγ9Vδ2 T cells with influenza A virus*

The ability of Vγ9Vδ2 T cells to respond to influenza A virus and the role of BTN3A1 in this mechanism were measured by the infection of freshly isolated PBMCs, during 10 h, with influenza A/PR/8/34 virus at a MOI of 5 and treatment with 200 ng/mL mAb 103.2. Upregulation of CD69 and CD25 was chosen as a marker for Vγ9Vδ2 T cell activation and was determined by flow cytometry (see 2.2.3.9).

### *2.2.3.17 Statistical analysis of data*

The statistical analysis was performed using Prism 6.0d (GraphPad).

# 3. RESULTS

## 3.1 BTN3A1 and other genes on human chromosome 6 are necessary for PAg-induced activation

BTN3A1 has been identified as a key mediator for PAg-induced Vγ9Vδ2 T cell activation and its gene locus has been mapped on to human chromosome 6 (Chr6). The contribution of other Chr6-encoded genes in such process, however, it is not yet established. Taking advantage that the proteins of the BTN3A family are present in primates and other placental mammals, but not in rodents, rodent cells were used to evaluate their relative contributions in Vγ9Vδ2 T cell activation. To test whether BTN3A1 alone has the ability to mediate PAg activation by rendering murine cells into PAg-presenting cells; Chinese hamster ovary cells (CHO) and CHO cells monosomal for human Chr6 (CHO Chr6) were retrovirally transduced with BTN3A1 and with human CD80 (huCD80). These cells were cocultured with 53/4 rat/mouse T hybridoma cells overexpressing a rat/mouse CD28 construct and transduced with the Vγ9Vδ2 TCR MOP (responder cells).

The genes to code the γ chain and the δ chain are connected by a 2A sequence and contained in the same vector—. Interaction between CD28 on the responder cells and CD80 on the CHO cell lines is mandatory to obtain PAg-induced response (Figure 1). The αβ TCR of the responder cells is specific for the peptide 68-88 of the guinea pig myelin basic protein (gpMBP). This antigen receptor is restricted to rat MHC class II molecule $RT1B^{l}$. The co-expression of both Vγ9Vδ2 TCR and αβ TCR facilitates testing the specificity of PAg-mediated response. The cell cultures were stimulated with grading doses of HMBPP, zoledronate, sec-butylamine (a type of alkylamine), and mAb 20.1 during 20 h. IL-2 production was the criterion to determine activation of the responder cell and was measured by a commercial ELISA kit.

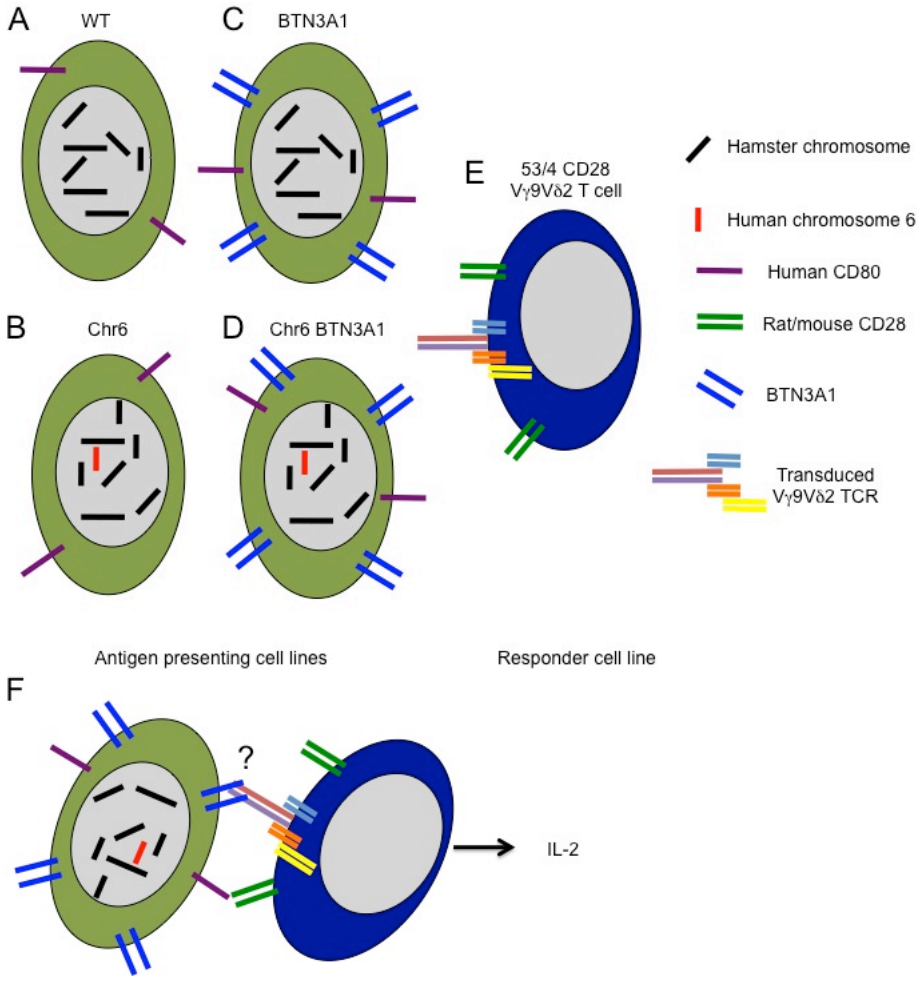


**Figure 1. Experimental system of Vγ9Vδ2 T cell activation.** This approach takes advantage of the lack of BTN3A molecules and Vγ9Vδ2 TCR in rodent species, and therefore their inability to respond to PAg to evaluate the relative contribution of those molecules in this context. The antigen presentation was undertaken by Chinese hamster ovary (CHO) cells (WT) and CHO cells containing human chromosome 6 (Chr6). Some of them were retrovirally-transduced with human BTN3A1 (BTN3A1 and Chr6 BTN3A1). As responder cells, mouse/rat T cell hybridomas transduced with the Vγ9Vδ2 TCR MOP were employed. The proper interaction of APC and responder cells is dependent on the binding of CD80 (expressed, at the same levels, by the former cells) and CD28 (expressed by the latter cell line). The readout for the activation of the responder cells was IL-2 production.

CHO (used as control cells, and designated as wild type — WT — cells) and CHO BTN3A1 cells (designated as BTN3A1 cells) did not induce any response neither to HMBPP nor to zoledronate. However, the presence of Chr6 in CHO Chr6 cells (designated as Chr6 cells) led to a significant higher response to both types of PAg (Figures 2A, 2B, 2D, and 2E). The response to these two stimulants was

significantly increased with overexpression of BTN3A1 in CHO Chr6 BTN3A1 cells (designated as Chr6 BTN3A1 cells), inducing higher levels of IL-2 compared to Chr6 cells (Figures 2C and 2F). HMBPP induced a much higher response compared to zoledronate as shown by the difference in the levels of IL-2 induced by these stimulators (Figures 2C and 2F).

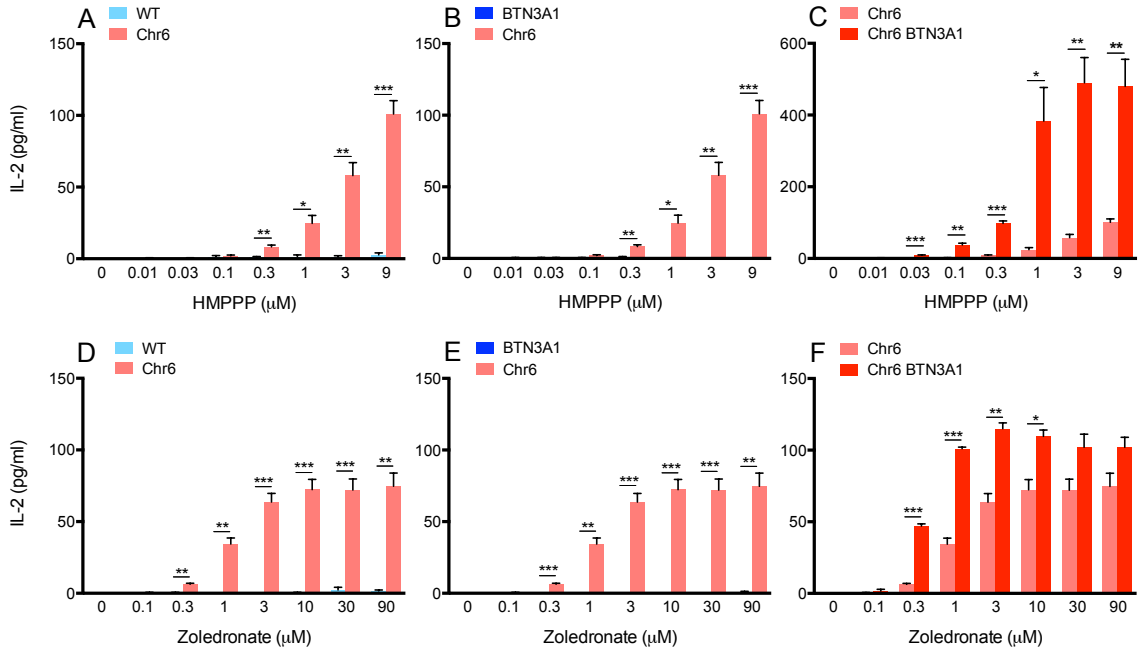


**Figure 2. PAg-mediated Vγ9Vδ2 T cell activation depends on the presence of both BTN3A1 and Chr6 in the APC.** (A-C) 1x10$^4$ CHO (WT, BTN3A1, Chr6, and Chr6 BTN3A1) cells were seeded in 96-well plates and incubated overnight. They were then stimulated with grading doses of HMBPP in the presence of 5x10$^4$ 53/4 r/m CD28 Vγ9Vδ2 TCR MOP (responder) cells for 20 h. The concentration of IL-2 produced by the responder cells was determined by an ELISA commercial kit (D-F). Cultures were performed as previously described but using the indicated concentrations of zoledronate as stimulus. (A-F) All samples were cultured in duplicates. Data are shown as mean ± SEM of three independent experiments. *$p$<0.05, **$p$<0.01, and ***$p$<0.001 (Unpaired Student's $t$-test).

Although BTN3A1 cells did not show any response to PAg, they induced significantly higher production of IL-2 when stimulated with mAb 20.1. compared to both Chr6 cells and Chr6 BTN3A1 cells (Figures 3A and 3B). Even if the response mediated by Chr6 cells appeared to be low, it was significantly higher when

compared to WT cells (Figure 3C) and, agreeing with the results obtained with PAg, it was considerably increased with overexpression of BTN3A1 (Figure 3D). This response to mAb 20.1 was specific, since Chr6 BTN3A1 did not respond to the isotype control of this antibody (Table 3).

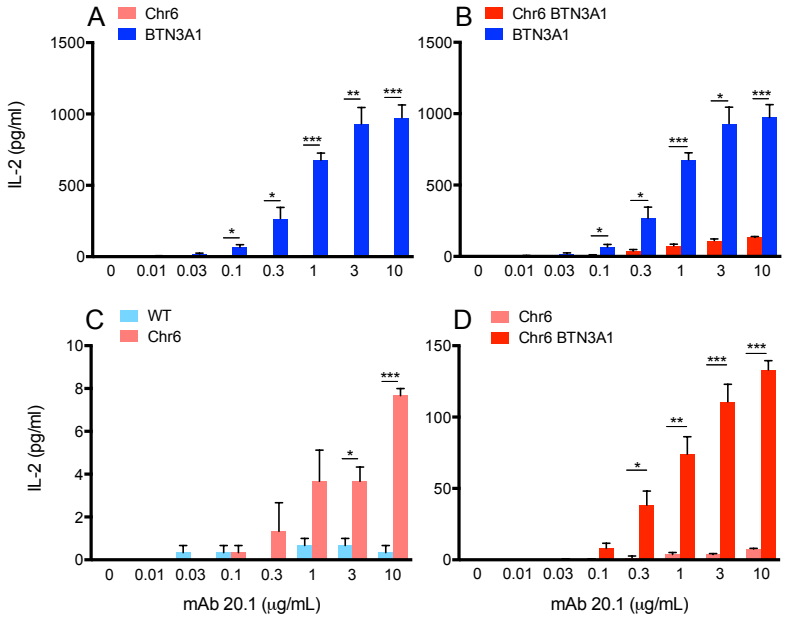


**Figure 3. mAb 20.1-evoked Vγ9Vδ2 TCR activation relies only on BTN3A1 cell surface expression and is independent of the presence of Chr6 in the APC.** (A-D) $1x10^4$ CHO cells were seeded in 96-well plates and incubated overnight. After this, they were stimulated with different concentrations of mAb 20.1 in co-culture with $5x10^4$ responder cells during 20 h. The amount of IL-2 produced by these cells was determined by an ELISA commercial kit. All samples were cultured in duplicates. Data are shown as mean ± SEM of three independent experiments. **p*<0.05, ***p*<0.01, and ****p*<0.001 (Unpaired Student's *t*-test).

**Table 3.** IL-2 levels in 20 h-cultures of Vγ9Vδ2 TCR MOP murine cells stimulated with mAb 20.1 and its isotype control.

| Stimulus | Cell line | | | | | |
|---|---|---|---|---|---|---|
| mAb 20.1 (mg/mL) | Chr6 BTN3A1 | | | Raji RT1B$^{l}$ | | |
| 10 | 146 | 127 | 126 | 45 | 22 | 17 |
| 3 | 134 | 92 | 106 | 42 | 22 | 17 |
| 1 | 98 | 58 | 66 | 36 | 19 | 20 |
| 0,3 | 58 | 27 | 30 | 38 | 13 | 15 |
| 0,1 | 15 | 6 | 3 | 33 | 15 | 14 |
| 0,03 | 0 | 1 | 0 | 21 | 12 | 12 |
| 0,01 | 0 | 0 | 0 | 10 | 5 | 4 |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Isotype control (mg/mL) | | | | | | |
| 10 | 0 | 0 | 0 | 0 | 1 | 0 |
| 3 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0,3 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0,1 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0,03 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0,01 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 |

CHO Chr6 BTN3A1 cells and Raji RT1B$^{l}$ cells were used as APC. Data are pg/mL.

To understand the unexpected results of mAb 20.1-stimulation with CHO cell lines, they were stained with mAb 103.2— which binds with higher affinity to BTN3A1 than mAb 20.1— to measure BTN3A1 levels on the cell surface by means of flow cytometry (Figure 4). Consequently, with the results of the mAb 20.1 stimulation assays, the levels of BTN3A1 were higher on BTN3A1 cells (Figure 4 and 5). Chr6 BTN3A1 cells also showed expression of this molecule but at lower levels compared to BTN3A1 cells (Figure 4 and 5). Chr6 cells did not show any expression of BTN3A1, which subsequently explains the poor ability of these cells to induce IL-2 when stimulated with mAb 20.1 (Figure 4, 5, 3A, and 3D). Nevertheless, this also poses the question about the BTN3A1-specificity of the previous results obtained with PAg. To solve this problem, an inhibition assay was performed to test the capacity of mAb 103.2 to abolish PAg-mediated Vγ9Vδ2 T cell activation. Chr6 cells were treated with either 3 μM HMBPP or 10 μM zoledronate and grading

doses of mAb 103.2 or its respective isotype control (233 WTH1). Raji RT1B$^{l}$ cells in presence of 3 μM HMBPP were used as control cells. The Chr6 cell-mediated responses to both HMBPP and zoledronate were significantly abrogated by mAb 103.2 (Figures 6A and 6B). In the two cases the percentages of maximum activation (IL-2 concentration at 0 ng/mL of antibody) dropped to levels below 7%. The response mediated by Raji RT1B$^{l}$ cells was also statistically decreased (Figure 6C). These results exclude the possibility that the findings obtained with PAg stimulation are not BTN3A1-specific and make clear the functionality of BTN3A1 in Chr6 cells in spite of its imperceptible levels.

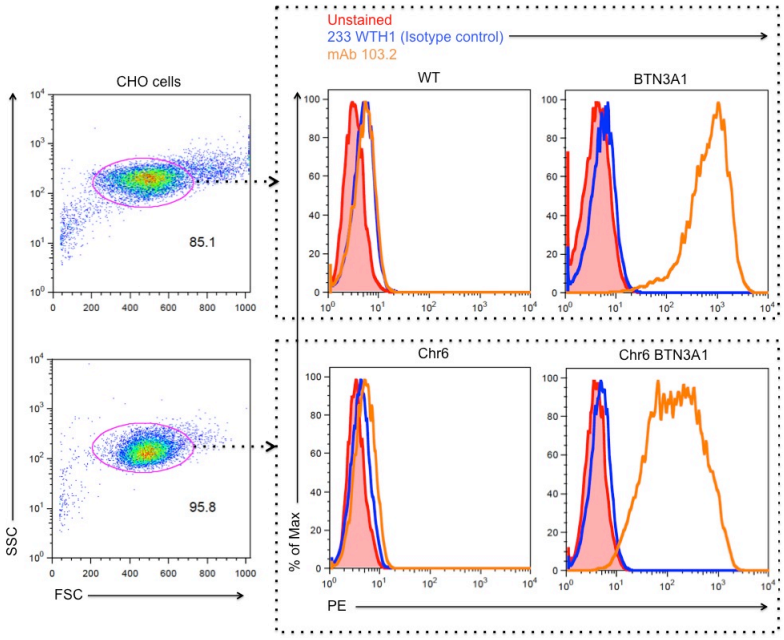


**Figure 4. Extracellular BTN3A1 staining, on CHO cells, is specific.** 2x10$^{5}$ CHO cells of the different types were stained with mAb 103.2 or 233 WTH1 (isotype control), washed and stained with PE-labeled F(ab')$_2$ fragment donkey anti-mouse IgG. MFI of staining was determined by flow cytometry. From SSC-FSC dot plots (where CHO cells were gated), histograms depicting their staining with the specific antibody and its isotype control were generated.

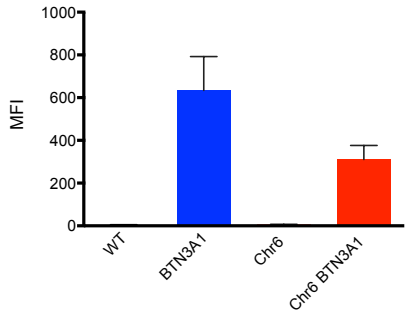


**Figure 5. Cell membrane staining of BTN3A1, by mAb 103.2, differs between CHO cells lines.** $2x10^5$ CHO cells of the different types were stained with mAb 103.2, washed and stained with PE-labeled $F(ab')_2$ fragment donkey anti-mouse IgG. The MFI was determined by flow cytometry. Data are shown as mean ± SEM of three independent experiments.

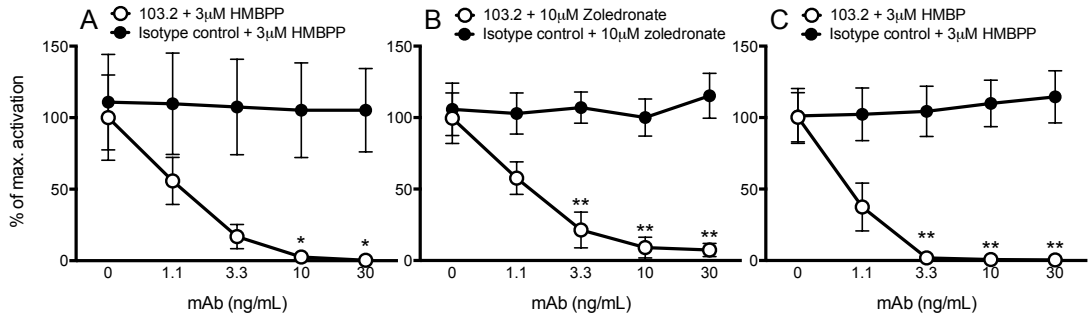


**Figure 6. mAb 103.2 abrogates PAg-induced response.** (A) $1x10^4$ CHO Chr6 cells were seeded in 96-well plates and incubated overnight. They were stimulated with either mAb 103.2 or its isotype control (233 WTH1) in presence of 3 μM HMBPP in co-culture with $5x10^4$ responder cells for 20 h. The amount of IL-2 was measured by ELISA. (B) Cultures were performed as previously described, but using 10 μM zoledronate as stimulus. (C) Cultures were done as in „A“, but using Raji $RT1B^l$ cells as APC. All samples were cultured in duplicates. Data are shown as mean ± SEM of three independent experiments and normalized to percentage of maximum activation, where 100% is the concentration of IL-2 produced in response to 3 μM HMBPP and 10 μM zoledronate in absence (0 ng/mL) of mAb 103.2: 89 pg/mL in “A”, 81 pg/mL in “B”, and 145 pg/mL in “C”. *$p$<0.05 and **$p$<0.01 (Multiple Student’s $t$-tests).

Chr6 cells and Chr6 BTN3A1 cells lacked the ability to induce activation of the responder cells when stimulated with sec-butylamine (Figure 7), which may require additional cellular compounds not present in murine cells or present in human chromosomes other than Chr6.

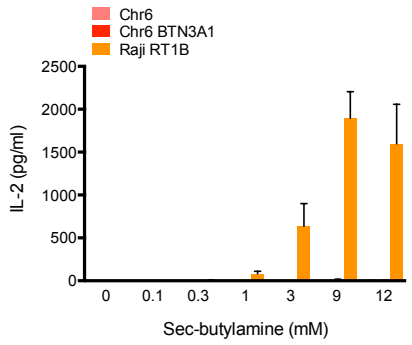


**Figure 7. CHO cells do not induce response to sec-butylamine.** $1x10^4$ Chr6 and Chr6 BTN3A1 CHO cells (seeded from previous day) and $5x10^4$ Raji RT1B cells were stimulated with different concentrations of sec-butylamine and co-incubated 20 h with responder cells. Levels of IL-2 were evaluated by ELISA. All samples were cultured in duplicates. Data are depicted as mean ± SEM of three independent experiments.

In order to expand upon the previous results, the ability of BTN3A1 to convert CHO cell lines into APC for freshly isolated human Vγ9Vδ2 T cells was tested. To this aim, WT cells, BTN3A1 cells, Chr6 cells, and Chr6 BTN3A1 cells were pulsed with either 1 μM or 10 μM zoledronate for 20 h. After washing the cells with PBS to eliminate residual zoledronate, they were further co-cultured with primary human PBMCs for another 20 h. The non-adherent fraction was obtained from the cultures and then stained with an anti-Vδ2 TCR chain antibody. In order to determine the percentage of activated Vγ9Vδ2 T cells, an anti-CD69 antibody was used for staining and measurement by flow cytometry. From a side scatter-forward scatter (SSC-FSC) dot plot, where the lymphocyte population was selected, a second dot plot was generated to obtain the Vδ2 subset (equivalent to Vγ9Vδ2 T cells). Finally, histograms showing CD69 expression on either $V\delta2^+$ cells were generated (Figure 8).

A concentration of 1 μM zoledronate induced a lower proportion of activated Vγ9Vδ2 T cells than 10 μM zoledronate (Figure 8 and 9). According to the results obtained with the murine transductant cellls, the presence of Chr6 was mandatory to obtain Vγ9Vδ2 activation, since neither WT cells nor BTN3A1 cells could lead to CD69 expression on the $V\delta2^+$ subset (Figures 8, 9A, and 9B). The percentage of

$V\delta2^+CD69^+$ cells yielded after co-culture with Chr6 cells was slightly increased when Chr6 BTN3A1 cells were used as stimulator cells (Figures 8 and 9C). Taken together, these experiments provide proof that in spite of the importance of BTN3A1 to mediate $V\gamma9V\delta2$ T cell response, other genes on Chr6 are required for PAg-elicited activation.

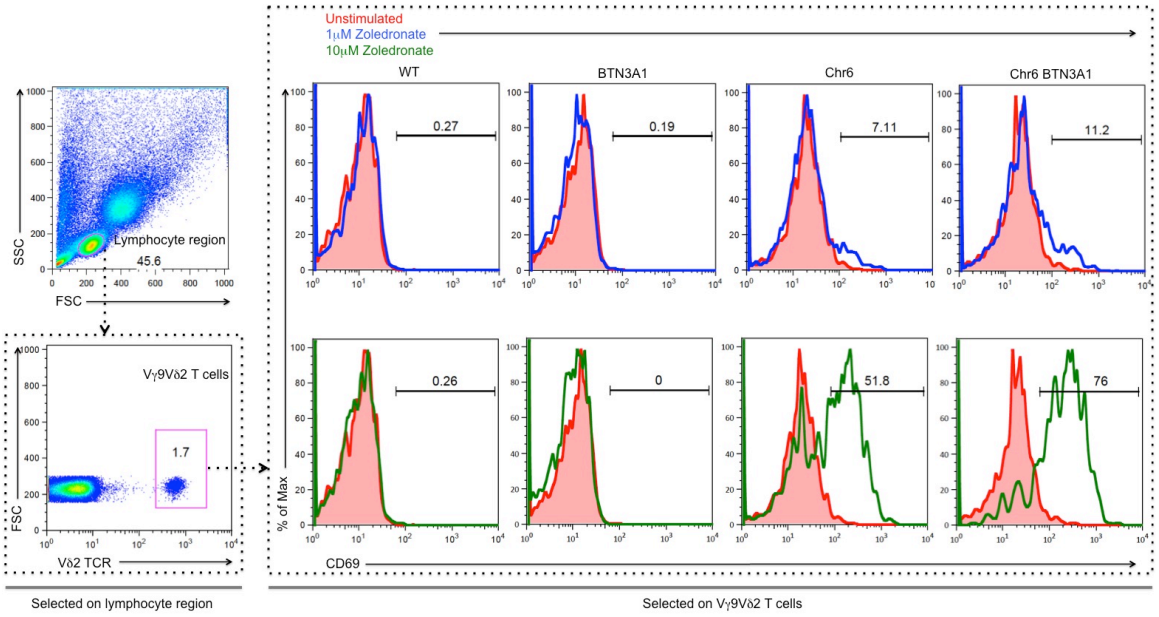


**Figure 8. Strategy to determine the percentage of Vγ9Vδ2 T cells expressing the activation marker CD69.** $1x10^5$ CHO cells were seeded overnight in 48-well plates. The cells were then treated with either 1 μM or 10 μM zoledronate and further incubated for 20 h. After that, they were washed twice with pre-warmed PBS containing 10% FCS and co-cultured with $2x10^5$ freshly isolated PBMCs during 20 h. Non-adherent cells were collected and stained with anti-Vδ2 (clone 4G6), washed and stained with FITC-labeled $F(ab')_2$ fragment donkey anti-mouse IgG. To measure CD69 expression, cells were stained with anti-CD69 PE. The percentage of CD69 on $V\delta2^+$ cells or $V\delta2^-$ negative cells was assessed by flow cytometry. Analyses were done on the lymphocyte region, from where the $V\gamma9V\delta2$ T cells were gated to generate histograms with the frequencies of $CD69^+$ cells for both evaluated zoledronate concentrations.

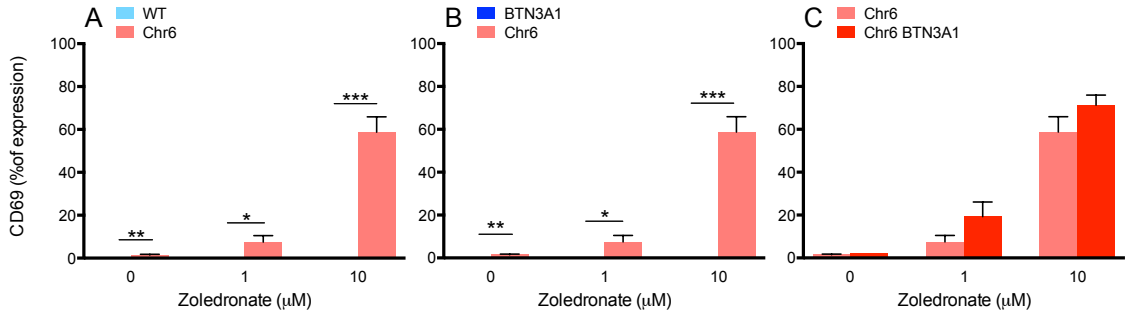


**Figure 9. CHO cell lines expressing BTN3A1 or containing Chr6 differ in their ability to activate primary human Vγ9Vδ2 T cells.** Cultures were performed and analysed as described in figure 8. Stimulations were done in triplicates and pooled in one simple sample for flow cytometry staining. Data are shown as mean ± SEM of four independent experiments. *$p$<0.05, **$p$<0.01, and ***$p$<0.001 (Unpaired Student's $t$-test).

## 3.2 Agonistic anti-BTN3A antibody-induced Vγ9Vδ2 T cell stimulation differs depending on Vγ9Vδ2 TCR clonotype

The finding that clearly demonstrates a high Vγ9Vδ2 T cell activation in response to mAb 20.1 (Figure 3A and 3B) contrasts with the evidence published by Vavassori et al. [137], which described Vγ9Vδ2 activation with PAg but not with mAb 20.1. This discrepancy might be attributed either to differences between the presenting cells or to different responder cell line origin. In their experiments, Vavassori and colleagues used A375 cells (a human melanoma cell line) as APC. As responder cells, they employed transgenic mouse T cells expressing the Vγ9Vδ2 TCR clone D1C55 (TCR D1C55), different to the Vγ9Vδ2 TCR MOP clone expressed by the rat/mouse hybridomas used as responder cells in the present work. Regarding this last point, TCR specificities might also have an important impact on the results obtained. To evaluate the hypothesis regarding the APC, the same A375 melanoma cell line overexpressing BTN3A1 from the aforementioned research were tested as stimulator cells to Vγ9Vδ2 TCR MOP-expressing 53/4 CD28 rat/mouse cells. They were used either as originally provided or after transduction with huCD80. In a pilot experiment, the capability of A375 cells to induce response to PAg was compared to Raji RT1B$^{l}$ cells, which had been used as internal control in the previous

experiments with CHO cell lines. A375 BTN3A1 cells and A375 CD80 BTN3A1 cells provoked a higher response to HMBPP and zoledronate in comparison to Raji RT1B$^{l}$ cells. In presence of sec-butylamine, both A375 cell lines lacked the ability to cause activation, though (Table 4).

**Table 4.** IL-2 levels in 20 h-cultures of Vγ9Vδ2 TCR MOP murine cells stimulated with HMBPP, sec-butylamine, and zoledronate.

| Stimulus | Cell line | | |
|---|---|---|---|
| HMBPP (μM) | Raji RT1B$^{l}$ | A375 BTN3A1 | A375 CD80 BTN3A1 |
| 9 | 88 | ⩾166 | ⩾162 |
| 3 | 62 | ⩾172 | ⩾167 |
| 1 | 33 | ⩾167 | ⩾166 |
| 0,3 | 7 | ⩾155 | ⩾165 |
| 0,1 | 1 | 116 | ⩾157 |
| 0,03 | 1 | 47 | ⩾148 |
| 0,01 | 0 | 6 | 108 |
| 0 | 0 | 0 | 0 |
| Sec-butylamine (mM) | | | |
| 15 | 1 | 0 | 0 |
| 12 | ⩾174 | 0 | 2 |
| 9 | ⩾172 | 0 | 5 |
| 3 | 119 | 0 | 0 |
| 1 | 9 | 0 | 0 |
| 0,3 | 0 | 1 | 0 |
| 0,1 | 0 | 0 | 0 |
| 0 | 0 | 0 | 0 |
| Zoledronate (μM) | | | |
| 90 | 63 | 77 | 132 |
| 30 | 0 | 132 | ⩾164 |
| 10 | 0 | 34 | 119 |
| 3 | 0 | 1 | 12 |
| 1 | 0 | 0 | 2 |
| 0,3 | 0 | 1 | 0 |
| 0,1 | 0 | 0 | 2 |
| 0 | 0 | 0 | 0 |

Raji RT1B$^{l}$ cells, A375 BTN3A1, and A375 CD80 BTN3A1 were used as APC. Data are pg/mL.

After this confirmation of their ability to induce PAg response, A375 cells were used as APC in stimulation assays similar to those performed with CHO cells. A375 BTN3A1 cells displayed an ability to incite Vγ9Vδ2 T cell stimulation with HMBPP, zoledronate, and mAb 20.1 (Figures 10A-10C). This was significantly surpassed by the A375 CD80 BTN3A1 cell line (Figure 10A-10C). These results demonstrate the capability of BTN3A1-overexpressing cancer cells to stimulate Vγ9Vδ2 TCR MOP-transduced T cells. This is comparable with the previous findings obtained with CHO cells. Consequently, it can be concluded that the aforementioned differences concerning the mAb 20.1-elicited activation are not due to the cells used as APC.

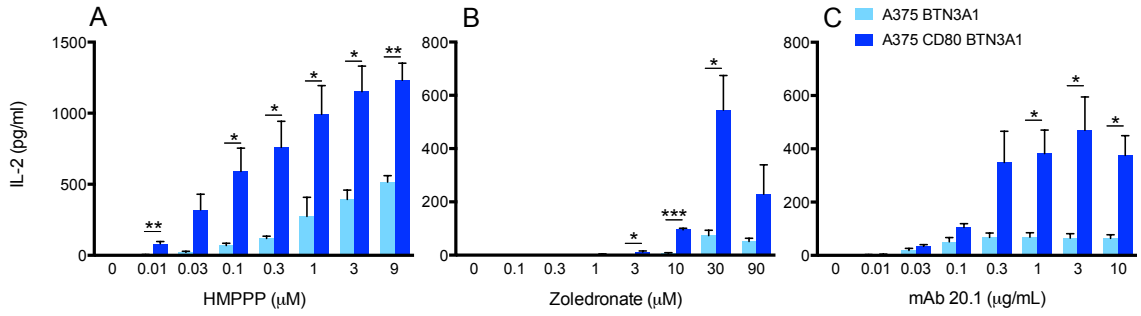


**Figure 10. A375 melanoma cells have the ability to induce both PAg- and mAb 20.1-induced Vγ9Vδ2 activation.** (A) 1x10$^4$ A375 BTN3A1 postive or negative for human CD80 were seeded in 96-well plates and incubated overnight. For 20 h, they were stimulated with HBMPP in co-culture with 5x10$^4$ responder cells. IL-2 production by the latter cells was determined by ELISA. (B and C) Stimulations were performed as previously described with the indicated concentrations of zoledronate or mAb 20.1. Cultures were performed in duplicates for each condition. Data show mean ± SEM of three independent experiments. *$p$<0.05 and **$p$<0.01 (Unpaired Student's $t$-test).

Next, the possibility that Vγ9Vδ2 TCR clonalities might lead to variations in PAg- and mAb 20.1-induced response was evaluated. To this purpose, Raji RT1B$^l$ cells were co-cultured with murine cells lines bearing any of the following Vγ9Vδ2 TCRs: MOP, D1C55, G115, and G115 clone 5 (G115 cl5) (Table 5). The cells were stimulated with HMBPP, zoledronate, mAb 20.1, and single chain versions of mAb

20.1 (sc 20.1) and mAb 103.2 (sc 103.2). Stimulation with mAb 20.1 induced a significant higher IL-2 production compared to its isotype control when TCR MOP-expressing cells were the responder cells but not with TCR D1C55-expressing cells (Figures 11A and 11B), highlighting the inability of cells with this receptor to respond to mAb 20.1. In response to HMBPP and zoledronate, cells with TCR MOP or with TCR D1C55 showed no significant differences in the production of IL-2, although the last one produced higher levels of the cytokine (Figure 12A and 12B). Meanwhile, cells transduced with TCR G115 or TCR G115 cl5 had a lower response to PAg compared to those bearing TCR MOP and TCR D1C55 (Figures 12A and 12B) Stimulation with mAb 20.1 and its single chain equivalent induced significant higher levels of IL-2 with TCR MOP-expressing cells compared to the cells with TCR G115 or TCR G115 cl5 (Figures 12C and 12D). Surprisingly, sc 103.2 induced some degree of activation, only at the highest concentration tested (10 μg/mL). The response to this antibody was significantly increased with TCR MOP compared to both TCR G115 and TCR G115 cl5. The activation elicited by sc 20.1 and sc 103.2 was specific, since TCR MOP cells, TCR G115 cells, and TCR G115 cl5 cells did not respond to sc F10/21A3, the isotype control for the mentioned single chain antibodies.

**Table 5.** CDR3 sequences of Vγ9Vδ2 TCR D1C55, MOP, G115, and G115 cl5.

| gamma | 104 | 105 | 106 | 107 | 108 | 109 | 110 | 111 | 111.1 |
|---|---|---|---|---|---|---|---|---|---|
| D1C55 CDR3 | C | A | L | W | E | W | V | T | Q |
| MOP CDR3 | C | A | L | K | E | L | G | * | * |
| G115 CDR3 | C | A | L | W | E | A | Q | Q | * |

| gamma | 112.2 | 112.1 | 112 | 113 | 114 | 115 | 116 | 117 | 118 |
|---|---|---|---|---|---|---|---|---|---|
| D1C55 CDR3 | E | L | G | K | K | I | K | V | F |
| MOP CDR3 | * | * | * | K | K | I | K | V | F |
| G115 CDR3 | E | L | G | K | K | I | K | V | F |

| delta | 104 | 105 | 106 | 107 | 108 | 109 | 110 | 111 | 111.1 |
|---|---|---|---|---|---|---|---|---|---|
| D1C55 CDR3 | C | A | C | D | T | V | L | G | D |
| MOP CDR3 | C | A | C | D | P | V | V | L | G |
| G115 CDR3 | C | A | C | D | T | L | G | M | G |
| G115 cl5 CDR3 | C | A | C | D | T | L | K | R | * |

| delta | 111.2 | 112.2 | 112.1 | 112 | 113 | 114 | 115 | 116 | 117 | 118 |
|---|---|---|---|---|---|---|---|---|---|---|
| D1C55 CDR3 | * | R | S | R | T | D | K | L | I | F |
| MOP CDR3 | D | T | G | Y | T | D | K | L | I | F |
| G115 CDR3 | G | E | * | Y | T | D | K | L | I | F |
| G115 cl5 CDR3 | * | T | D | * | T | D | K | L | I | F |

Amino acid numbers and CDR3 lengths were determined with IMGT-V quest. Points indicate lack of amino acid. Positions 104 and 118 belong to framework 3 and 4, respectively.

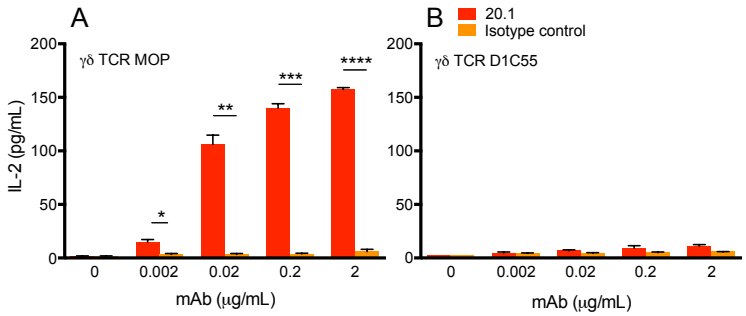


**Figure 11. Vγ9Vδ2 T cells expressing the γδ TCR D1C55 lack the ability to respond to mAb 20.1.** (A) 5x104 Raji RT1B$^{l}$ (used here as APC) were co-cultured with Vγ9Vδ2 TCR MOP-expressing responder cells for 20 h in presence of mAb 20.1 or its isotype control. The amount of IL-2 produced was determined by ELISA. (B) Cultures were done as previously described but using Vγ9Vδ2 TCR D1C55-expressing responder cells. Stimulations were done in duplicates and data represent mean ± SEM of four independent experiments. *$p$<0.05, **$p$<0.01, ***$p$<0.001, and ****$p$<0.0001 (Unpaired Student's $t$-test).

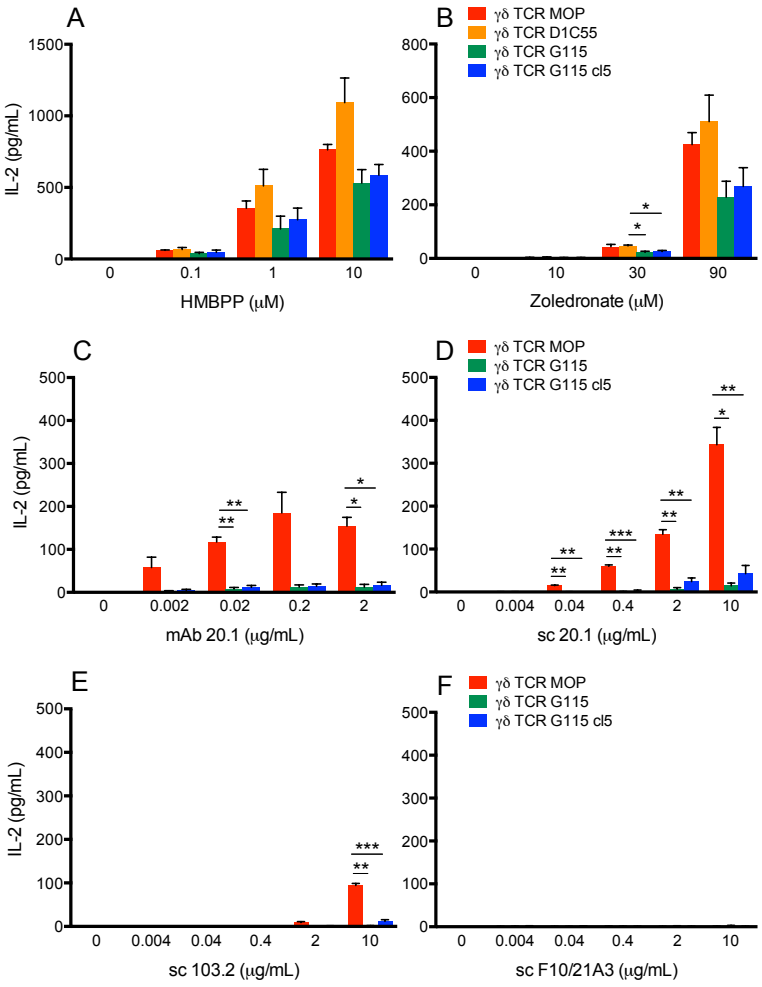


**Figure 12. PAg- and mAb 20.1-induced Vγ9Vδ2 T cell response differs depending on Vγ9Vδ2 TCR clonotype.** (A) 5x10$^4$ Raji RT1B$^l$ cells were seeded in 96-well plates. They were stimulated, during 20 h, with grading doses of HBMPP in presence of 5x10$^4$ responder cells expressing any of four different Vγ9Vδ2 TCRs: MOP, D1C55, G115 and G115 cl5. The amount of IL-2 in the cultures was determined by ELISA. (B-F) Experiments were done as previously described, using the indicated concentrations of zoledronate, mAb 20.1, mAb 20.1 single chain (sc 20.1), mAb 103.2 single chain (sc 103.2), and sc F10/21A3 (sc 20.1 and sc 103.2 isotype control). TCR MOP-, TCR G115- and G115 cl5-bearing cells were used as responder cell. Cultures were performed in duplicates for each condition. Data are presented as mean ± SEM of three independent experiments. *$p$<0.05, **$p$<0.01, and ***$p$<0.001 (Unpaired Student's $t$-test).

Additionally, in an attempt to dissect the individual contribution of the γ and δ chains composing the Vγ9Vδ2 TCR, an assay was performed with responder cells expressing TCR containing a γ chain from one Vγ9Vδ2 TCR and a δ chain from another Vγ9Vδ2 TCR. 53/4 rat/mouse CD28 T cells were transduced with either of

the following constructs: TCR containing the γ chain of TCR D1C55 and the δ chain of TCR MOP (TCR γD1C55 δMOP) or TCR containing the γ chain of TCR MOP and the δ chain of TCR D1C55 (TCR γMOP δD1C55). As APC, Raji RT1B$^{l}$ were included in the cultures. Cells with TCR γMOP δD1C55 showed the lowest IL-2 production compared to TCR D1C55 or TCR γD1C55 δMOP (Figure 13A). This suggests that, in this system, the γ chain of TCR D1C55 gives some small advantage to respond to HMBPP, as seen also from the lower activation of cells with TCR MOP compared to those with TCR D1C55 or TCR γD1C55 δMOP (Figure 13A). Nevertheless, that is not the case concerning the mAb 20.1-induced response. As expected and in line with the results obtained with CHO cells, cells bearing TCR MOP produced significantly more IL-2 compared to TCR D1C55 and the two crossed-chain receptors (Figure 13B). Interestingly, the amount of IL-2 generated by TCR γMOP δD1C55 responder cells was increased in relation to cells with TCR D1C55 and TCR γD1C55 δMOP (Figure 13B). These findings imply that the γ chain of TCR MOP makes a special contribution to the Vγ9Vδ2 T cell activation by means of mAb 20.1.

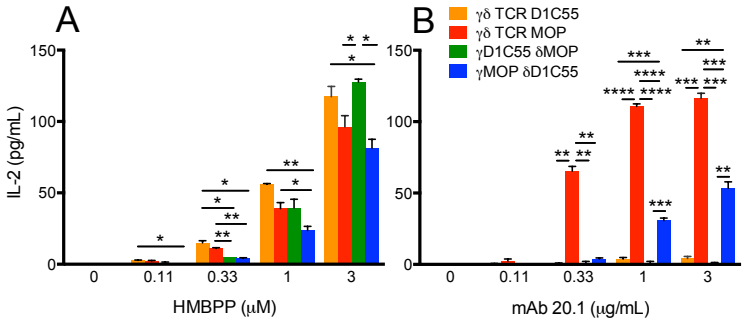


**Figure 13. Differential contributions of the TCR γ chain and the TCR δ chain in the Vγ9Vδ2 T cell response to PAg and mAb 20.1.** (A) 5x10$^{4}$ Raji RT1B$^{l}$ cells were seeded in 96-well plates. They were stimulated, during 20 h, with different doses of HBMPP in presence of 5x10$^{4}$ responder cells expressing any of the following Vγ9Vδ2 TCRs: D1C55, MOP, γ chain D1C55 δ chain MOP (γD1C55 δMOP), and γ chain MOP δ chain D1C55 (γMOP δD1C55). The IL-2 levels were determined by ELISA. (B) Cultures were done as previously described, using the indicated concentrations of mAb 20.1. Samples were cultured in duplicates. Data are presented as mean ± SEM of three independent experiments. *$p$<0.05, **$p$<0.01, ***$p$<0.001, and and ****$p$<0.0001 (Unpaired Student's $t$-test).

To further expand the results obtained with murine transductants, and to determine whether the differences in the PAg- and mAb 20.1-mediated response are also observed in human cells; Vγ9Vδ2 T cell clones were generated by isolation of mononuclear cells from peripheral blood, Vγ9Vδ2 negative selection by MACS, and single cell dilution culture. The cells were cultured for two weeks in presence of anti-CD3 (clone OKT-3), HMBPP, and IL-2 along with irradiated EBV-LCL and irradiated PBMCs as feeder cells. Three clones were selected based on their purity as Vγ9Vδ2 T cell population (designated as clone A, clone B and clone C). They were further expanded, for two weeks, in the same culture conditions mentioned before and then stimulated for 20 h with HMBPP or three different concentrations of mAb 20.1, with Raji RT1B$^{l}$ as APC. The cell activation in terms of CD25 upregulation was measured by flow cytometry. To obtain the proportion of activated Vγ9Vδ2 T cells, the lymphocyte region was gated on a SSC-FSC dot plot and a new dot plot was created with the parameters FSC and anti-Vδ2 TCR chain. The Vδ2$^{+}$ cell population was gated and the expression of the activation marker CD25 was determined (Figure 14). The upregulation of CD25 showed some clear differences between the clones. Clone A exhibited a higher response to HMBPP and to the three tested concentrations of mAb 20.1 (2, 0.2, 0.02 μg/mL) compared to clones B and C (Figure 15). These results undoubtedly depict a hierarchy between the clones in the response to both PAg and mAb 20.1, dividing them between two low responders and a high responder for the stimuli tested. There are, however, different profiles of PAg- and mAb 20.1-mediated stimulation by the Vγ9Vδ2 T clones. Although clone A had the highest CD25 upregulation, its response to HMBPP was lower compared to the mAb 20.1-induced activation. On the other hand, clones B and C had a relative higher response to HMBPP than to mAb 20.1 (Figure 16). These findings complement those obtained with the murine cell lines expressing the different Vγ9Vδ2 TCRs and provide evidence that there is clonal heterogeneity in response to PAg and mAb 20.1 among the Vγ9Vδ2 T cell population.

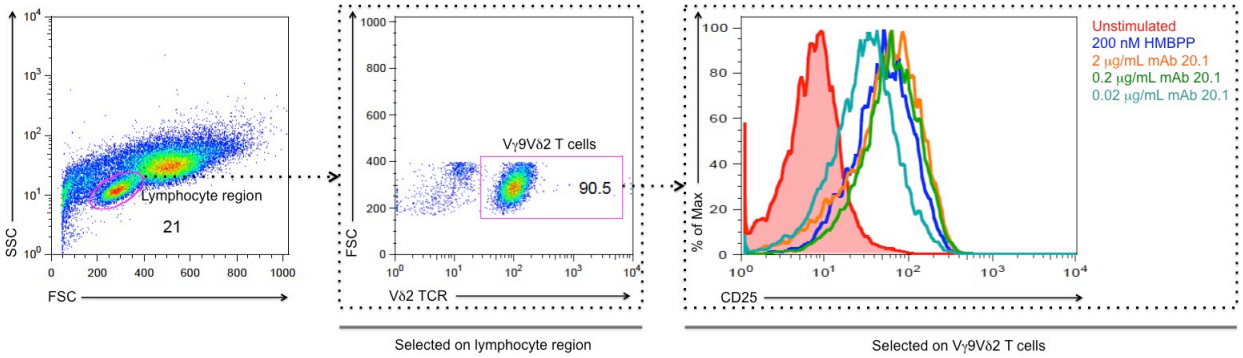


**Figure 14. Strategy to determine the level of CD25 expression on PBMCs-generated Vγ9Vδ2 T cell clones.** 2x10$^5$ Raji RT1B$^l$ cells were seeded in 48-well plates and stimulated, for 20 h, with 200 nM HMBPP, 2 µg/mL, 0.2 µg/mL, or 0.02 µg/mL mAb 20.1 in co-culture with 1x10$^5$ PBMCs-generated Vγ9Vδ2 T cell clones. The cells were collected and stained with anti-Vδ2 (clone 4G6), washed, and stained with FITC-labeled F(ab')$_2$ fragment donkey anti-mouse IgG. To measure CD25 upregulation, cells were stained with anti-CD25 PE. The percentage of CD25 on Vδ2$^+$ cells was assessed by flow cytometry. The analysis was done on the lymphocyte region, Vγ9Vδ2 T cells were gated, and histograms with CD25 expression on these cells were generated.

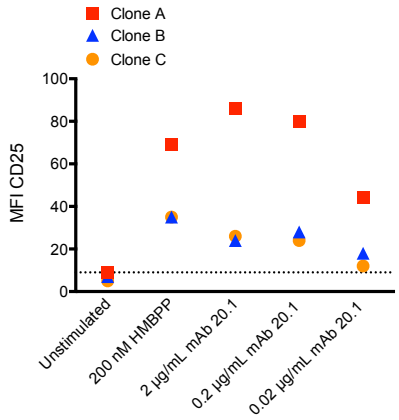


**Figure 15. Response of PBMCs-generated Vγ9Vδ2 T cell clones to HMBPP and mAb 20.1.** Cultures were performed and analyzed as described in figure 14. Stimulations were done in triplicates and pooled as a single sample for flow cytometry staining. Data are the CD25 MFI showed by each clone in response to the tested stimuli. The dashed horizontal line represents the threshold to consider a positive CD25 expression and is the MFI average of the three clones in the unstimulated culture condition (MFI=7).

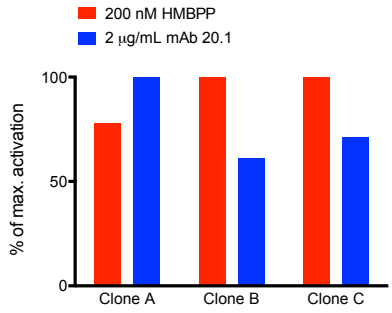


**Figure 16. PBMCs-generated Vγ9Vδ2 T cell clones differ in their profiles of response to HMBPP and mAb 20.1.** MFI values of CD25 expression (figure 15), on each clone, in response to 200 nM HMBPP and 2 μg/mL mAb 20.1 were normalized to obtain the percentage of maximum activation. For each single clone, 0% was defined as its CD25 MFI value in the unstimulated condition; and 100% was defined as the highest value of CD25 found in the stimulated conditions aforecited.

## 3.3 PAg- and mAb 20.1-induced Vγ9Vδ2 T cell responses are not synergistic and interfere with each other

Since HMBPP, zoledronate, and mAb 20.1 induced a strong activation in Vγ9Vδ2 TCR MOP responder cells, it is plausible that PAg and the agonist antibody together could potentiate the Vγ9Vδ2 TCR-mediated response. To test whether such synergism exists, Raji RT1B$^{l}$ cells were stimulated with any of the following antibodies, either alone or in presence of 1μM HMBPP: mAb 103.2, sc 103.2, mAb 20.1, or sc 20.1. 53/4 CD28 rat/mouse T cells bearing either Vγ9Vδ2 TCR MOP or Vγ9Vδ2 TCR D1C55 were the selected responder cells. As anticipated from the previous results obtained with CHO cells, mAb 103.2 completely abolished the HMBPP-mediated activation of reporter cells bearing the TCR MOP, yielding 99% of inhibition (determined as the difference of percentage of maximum activation between 0 ng/mL or 0 μg/mL of antibody plus 1 μM HMBPP and the highest antibody concentration plus 1 μM HMBPP). According to the antagonistic nature of this antibody, it did not induce any activation (defined as the percentage of maximum activation at the highest antibody concentration) (Figure 17A and Table 6).

In contrast, sc 103.2 did not have any inhibitory capacity since the percentage of inhibition was 1% and the levels of IL-2 obtained at 1 μM HMBPP remained basically unchanged at the different antibody concentrations. This antibody induced residual levels of IL-2, which translated into 5% of activation (Figure 17B and Table 6). Remarkably, mAb 20.1 when combined with HMBPP inhibited the response provoked by this PAg, leading to a 38% of inhibition. Its stimulatory capability translated into 63% activation, related to that obtained in the absence of antibody (Figure 17C and Table 6). In the case of sc 20.1, the percentage of inhibition at the highest concentration (2 μg/mL) was 20%, slightly lower compared to mAb 20.1; and although the inhibition was not statistically significant, it somehow resembles the inhibition shown by mAb 20.1. Like mAb 20.1, sc 20.1 has an effective activation capacity, leading to 63% of activation (Figure 17D and Table 6). Noteworthy is the slight increase of the percentage of activation at the highest concentration of this antibody in presence of HMBPP (Figure 17D), a behavior different to that observed with mAb 103.2 and mAb 20.1 (Figures 17A and 17C). In the particular case of TCR D1C55-expressing cells, mAb 103.2 performed in a similar way it did with TCR MOP-transduced cells. With the former cells as responder, this antibody showed 100% inhibition of the HMBPP-triggered activation and no activation (Figure 17E and Table 6). Its single chain equivalent, sc 103.2, did not show any inhibitory or stimulatory ability with percentages of 0% and 1%, respectively (Figure 17F and Table 6).

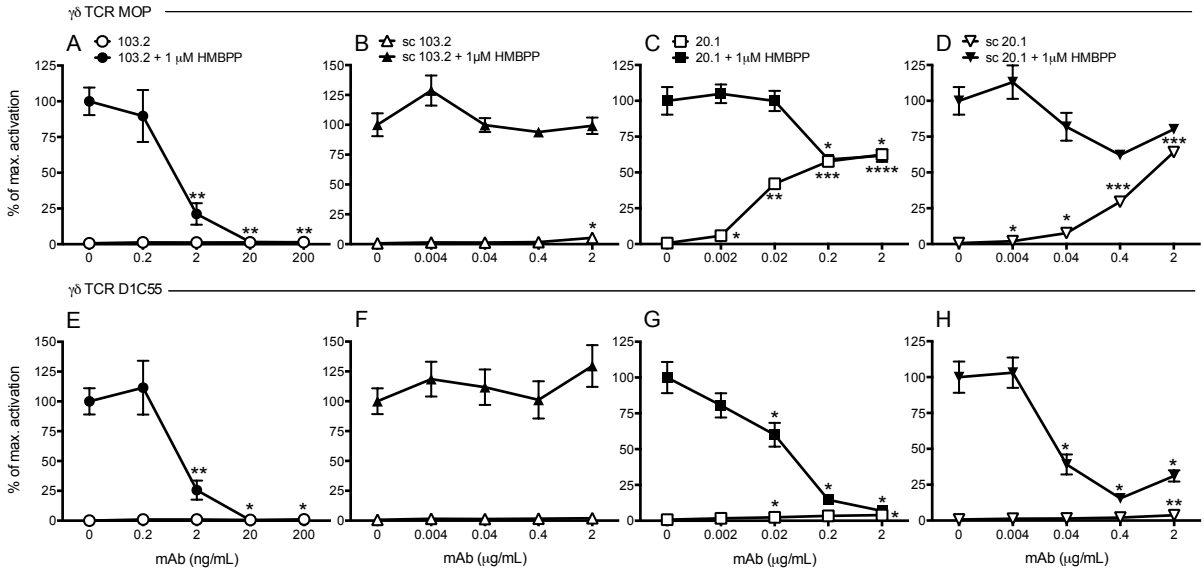


**Figure 17. PAg- and mAb 20.1-induced Vγ9Vδ2 T cell responses are not synergistic and interfere with each other.** (A) 5x10$^4$ Raji RT1B$^l$ were co-cultured, in 96-well plates, with 5x10$^4$ Vγ9Vδ2 TCR MOP-expressing reporter cells, and stimulated for 20 h with either mAb 103.2 alone or in combination with 1 μM HMBPP. IL-2 produced by the responder cell lines was measured by ELISA. (B-D) Stimulations were performed as described in "A" but using sc 103.2 (B), mAb 20.1 (C), or sc 20.1 (D). (E-F) Cultures were done as explained in "A-D", but employing Vγ9Vδ2 TCR D1C55-expressing responder cells instead. All of the experiments were performed in duplicate. Data are presented as mean ± SEM of three independent experiments and normalized to percentage of maximum activation, where 100% is the concentration of IL-2 produced in response to 1μM HMBPP in absence (0 ng/mL or 0 μg/mL) of mAb. Each experimental condition (antibody alone or antibody with HMBPP) was evaluated separately. Thus, statistical significances (where they arise) refer to the difference of IL-2 production between the untreated cultures and those treated with the different concentrations of the antibody. *$p$<0.05, **$p$<0.01, ***$p$<0.001, and ****$p$<0.0001 (Unpaired Student's $t$-test).

Conversely, the profile of response of TCR D1C55-transduced cells, compared to the cells expressing TCR MOP, clearly changed with mAb 20.1 and sc 20.1. The first antibody almost completely abrogated the PAg-driven activation leading to 93% inhibition and exhibited 3% activation (Figure 17G and Table 6), a picture that resembles that of mAb 103.2 (Figures 17A and 17E). In addition, sc 20.1 led to 69% inhibition of activation with 1μM HMBPP and no more than 3% of activation (Figure 17H and Table 6), sharing the inaptitude of mAb 103.2, sc 103.2, and mAb 20.1 to incite activation in responder cells expressing TCR D1C55.

Taken together, these results demonstrate that the effects of PAg and the agonistic antibodies, mAb 20.1 and sc 20.1, on BTN3A1 are not synergistic but

interfere with each other, suggesting mutually exclusive mechanisms for PAg- and antibody-dependent Vγ9Vδ2 T cell activation. They also reiterate the different impact that these antibodies have on Vγ9Vδ2 TCR with distinctive clonotypes.

**Table 6.** Percentages of activation and inhibition of 1μM HMBPP-elicited response yielded by agonistic and antagonistic antibodies.

| | Percentages of activation | | Percentages of inhibition of 1 μM HMBPP-induced activation | |
|---|---|---|---|---|
| Antibody | TCR MOP | TCR D1C55 | TCR MOP | TCR D1C55 |
| mAb 103.2 | 1% | 1% | 99% | 100% |
| sc 103.2 | 5% | 1% | 1% | 0% |
| mAb 20.1 | 61% | 3% | 38% | 93% |
| sc 20.1 | 63% | 3% | 20% | 69% |

Vγ9Vδ2 TCR MOP or Vγ9Vδ2 TCR D1C55 were co-coculured with Raji RT1B$^{l}$ as APC, and treated with different concentrations of agonistic and antagonistic antibodies. IL-2 levels obtained by ELISA were normalized to percentage of maximum activation. Percentage of activation was defined as the percentage of maximum activation at the highest antibody concentration. Percentage of inhibition was determined as the difference of percentage of maximum activation between 0 ng/mL or 0 μg/mL of antibody plus 1 μM HMBPP and the highest antibody concentration plus 1μM HMBPP.

## 3.4 BTN3A2 but not BTN3A3 supports PAg-mediated activation, whereas both isoforms support mAb-induced Vγ9Vδ2 T cell activation

Besides BTN3A1, another two isoforms constitute the BTN3A family, BTN3A2 and BTN3A3. They share a high degree of similarity in their extracellular domain, but differently to BTN3A1, BTN3A2 lacks the intracellular domain and in BTN3A3 this is longer. In an effort to understand the relative contribution of these other two isoforms in the Vγ9Vδ2 T cell activation, CHO cells and CHO Chr6 cells overexpressing either BTN3A2 or BTN3A3 were evaluated as stimulator cells to TCR MOP-transduced rat/mouse responder cells. Since BTN3A2- and BTN3A3-overexpressing CHO Chr6 cells contain endogenous BTN3A1 derived

from Chr6, CHO Chr6 cells were used as baseline control cells. Additionally, the response mediated by BTNA2- or BTN3A3-overexpressing APC was also compared to BTN3A1-overexpressing CHO and CHO Chr6 cell lines.

First, the cells were stained with both mAb 20.1 and mAb 103.2 to measure expression of BTN3A proteins. These two antibodies recognize the three isoforms. In general, the pattern of cell staining was similar for the two antibodies, with mAb 103.2 yielding higher levels of mean fluorescence intensity (MFI) than mAb 20.1 (Figures 18A and 18B). Consequently with previous results, CHO Chr6 cells showed marginal levels of staining with these two antibodies (Figures 18A and 18B). CHO cell lines showed a hierarchy in the staining, with BTN3A1 cells in the first place, followed by CHO BTN3A2 cells (designated as BTN3A2 cells), and CHO BTN3A3 cells (designated as BTN3A3 cells) in the last position (Figures 18A and 18B). Similarly, Chr6 BTN3A1 cells showed the strongest staining capability in the group of cells containing Chr6, but the levels of MFI of either antibody were comparable in the case of CHO Chr6 BTN3A2 cells (designated as Chr6 BTN3A2), and CHO Chr6 BTN3A3 cells (designated as Chr6 BTN3A3) (Figures 18A and 18B).

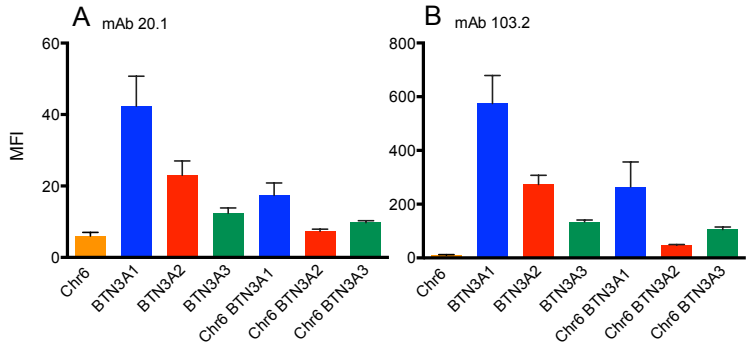


**Figure 18. Cell membrane staining with mAb 20.1 and mAb 103.2 on CHO cell lines overexpressing BTN3A1, BTN3A2, or BTN3A3.** $2x10^5$ CHO or CHO Chr6 cells transduced with BTN3A1, BTN3A2, or BTN3A3 were stained with (A) mAb 20.1 or (B) mAb 103.2, washed and stained with PE-labeled $F(ab')_2$ fragment donkey anti-mouse IgG. The MFI was determined by flow cytometry. Data are shown as mean ± SEM of three independent experiments.

Next, the cells were stimulated with grading doses of HMBPP, zoledronate, and mAb 20.1. In response to HMBPP and zoledronate, Chr6 BTN3A1 cells and Chr6 BTN3A2 cells induced significantly increased levels of IL-2 compared to Chr6 cells, but without differences between them (Figure 19A and 19B). Nevertheless, after HMBPP and zoledronate stimulation, IL-2 production in presence of Chr6 BTN3A3 and Chr6 cells showed no significant differences, and was for both significantly lower than for Chr6 BTN3A1 cells (Figure 19C and 19D). Completely different was the picture for stimulation by mAb 20.1. When treated with this agonistic antibody, both Chr6 BTN3A2 cells and Chr6 BTN3A3 cells induced significantly increased concentrations of IL-2 by the responder cells, in comparison to Chr6 cells. This ability to induce IL-2 was comparable to that of Chr6 BTN3A1 cells (Figures 20A and 20B). This was not the case, however, when CHO cells were used as APC. Neither BTN3A2 cells nor BTN3A3 cells showed a strong ability to support mAb 20.1-induced activation. Although both isoforms induced some degree of responder cell activation, this was significantly lower compared to BTN3A1 cells and not significantly different in relation to Chr6 cells (Figures 20C and 20D).

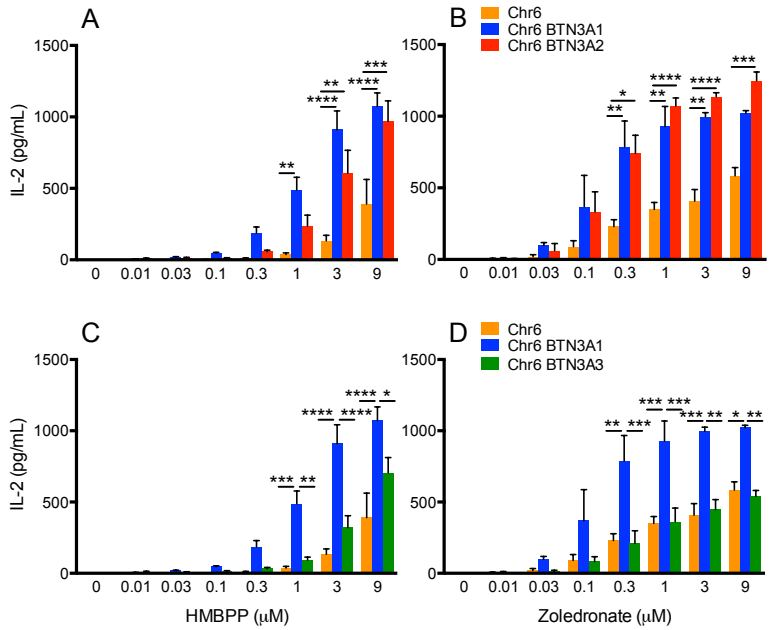


**Figure 19. BTN3A3 does not support PAg-mediated Vγ9Vδ2 T cell activation.** (A) 1x10$^4$ CHO Chr6 cells transduced with either BTN3A1 or BTNA32 were seeded in 96-well plates and incubated overnight. They were then co-cultured with 5x10$^4$ responder cells during 20 h and stimulated with different concentrations of HMBPP. The amount of IL-2 in the culture was measured by ELISA. CHO Chr6 cells were used as control cells to consider the participation of endogenous Chr6-derived BTN3A in the BTN3A-transduced cells. (B) Cultures were done as in „A“, but using zoledronate as the stimulant. (C and D). Cultures were performed as in „A“ and „B“, but using CHO Chr6 BTN3A1 and CHO Chr6 BTN3A3 as APC. (A-D) Experiments were done in duplicates for each condition. Data are presented as mean ± SEM of three independent experiments. *$p$<0.05, **$p$<0.01, ***$p$<0.001, and ****$p$<0.0001 (Unpaired Student's $t$-test).

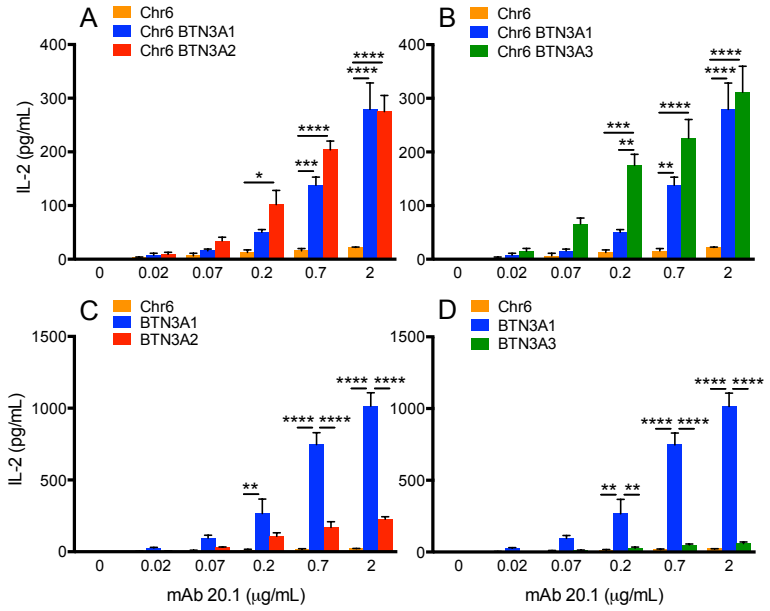


**Figure 20. BTN3A2 and BTN3A3 contribute to the mAb 20.1-mediated Vγ9Vδ2 T cell activation.** (A) 1x10$^4$ CHO Chr6 cells transduced with BTN3A1 or BTN3A2 were seeded in 96-well plates and incubated overnight. They were co-cultured with 5x10$^4$ responder cells and treated with grading doses of mAb 20.1 for 20 h. IL-2 in the cultures was determined by ELISA. CHO Chr6 cells were used as control to assess the contribution of the Chr6-derived endogenous BTN3A molecules in the BTN3A-transduced cells. (B) Stimulations were performed as in „A“ but using CHO Chr6 BTN3A1 and CHO Chr6 BTN3A3 as APC. (C and D) Cultures were done as in „A“ and „B“, but using CHO BTN3A1, CHO BTN3A2 or CHO BTN3A3 cells instead. (A-D). All samples were cultured in triplicates and data represent mean ± SEM of three independent experiments. *$p$<0.05, **$p$<0.01, ***$p$<0.001, and ****$p$<0.0001 (Unpaired Student's $t$-test).

Since the levels of mAb 20.1 and mAb103.2 staining were different between the CHO cell lines (Figures 18A and 18B), it might be argued that the differences in PAg- and mAb-induced activation are due to variations in BTN3A expression. To exclude this possibility, the levels of IL-2 obtained at the highest mAb 20.1 concentration (2 μg/mL) were compared between CHO cell lines with comparable anti-BTN3A staining (Figures 18A and 18B). Thus, the amount of IL-2 in presence of Chr6 BTN3A1 cells were compared to those obtained with BTN3A2 cells as APC, without any significant differences (Figure 21A). Conversely, although Chr6 BTN3A3 cells and BTN3A3 cells had similar levels of anti-BTN3A staining, they significantly differ in their ability to induce IL-2 production by the responder cells (Figure 21B). This simple analysis is an indication that the capacity of CHO cells to

induce Vγ9Vδ2 T cell activation is independent of the levels of anti-BTN3A staining. In summary, these results suggest that BTN3A1 and BTN3A2 play the major role in the PAg-triggered stimulation, with the contribution of BTN3A3 being dispensable to some degree. Nonetheless, both isoforms contribute to activate Vγ9Vδ2 T cells by means of mAb 20.1. Hence, the contribution of other molecules encoded by genes on Chr6 might be needed for the correct function of BTN3A2 and BTN3A3 even in the case of PAg-independent stimulation.

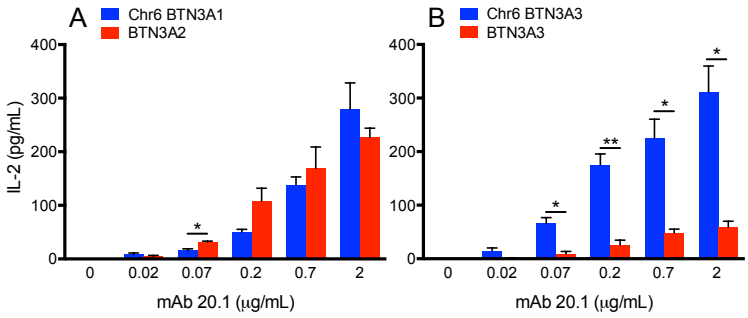


**Figure 21. mAb 20.1-evoked Vγ9Vδ2 IL-2 production mediated by CHO cells with similar levels of mAb 103.2 staining.** (A) Values of mAb 20.1-induced IL-2 production in presence of CHO Chr6 BTN3A1 and CHO BTN3A2 as APC (figures 19 and 20) were graphed together and compared. (B) Procedure was the same as in „A“, but comparing CHO Chr6 BTN3A3 and CHO BTN3A3. Data show mean ± SEM of three independent experiments. **p*<0.05 and ***p*<0.01 (Unpaired Student's *t*-test).

## 3.5 Mutations in the BTN3A1 extracellular domain revoke the mAb 20.1-induced Vγ9Vδ2 T cell activation but not that induced by PAg

The importance of both the extracellular and intracellular domains of BTN3A1 as PAg-binding sites has been highlighted by two models which are based on research findings that will be addressed in greater detail later in the discussion section of this thesis [134, 137]. The first model proposes that BTN3A1 acts as a bona fide antigen-presenting molecule, able to bind PAg at the extracellular domain and directly presents it to the Vγ9Vδ2 TCR. The second argues that PAg bind to the

intracellular BTN3A1 domain, inducing changes in the structure or membrane cell distribution of BTN3A1. To evaluate the prominence of the extracellular BTN3A1 domain in the V$\gamma$9V$\delta$2 T cell response to PAg and mAb 20.1, the ability of CHO cells and CHO Chr6 cells bearing mutated versions of BTN3A1 as APC was tested. By means of site-directed mutagenesis, the following mutations were induced in the coding sequence of this domain: a) a substitution of an arginine by an alanine at position 65 (R65A), b) a replacement of a lysine by an aspartic acid at position 37 (K37D), and c) a double change containing the previous one and a replacement of a glutamine by an alanine at position 100 (K37D:Q100A). Mutation „a)“ is located at the site where mAb 20.1 contacts BTN3A1, whereas mutations „b)“ and „c)“ are placed at the presumed PAg-binding site (Figures 22A and 22B). Constructs with these mutations were made and retrovirally-transduced into CHO cells and CHO Chr6 cells. After transduction, the cells showed heterogeneous levels of plasmid expression as indicated by green fluorescence of the reporter gene (EGZ). The expression of the mutated BTN3A1 versions was assessed by staining with mAb 103.2 and with mAb 20.1. The first was not expected to be affected by the mutations, while the R65A mutation has been predicted to impair mAb 20.1 binding on the basis of the molecular model shown in Fig. 22.

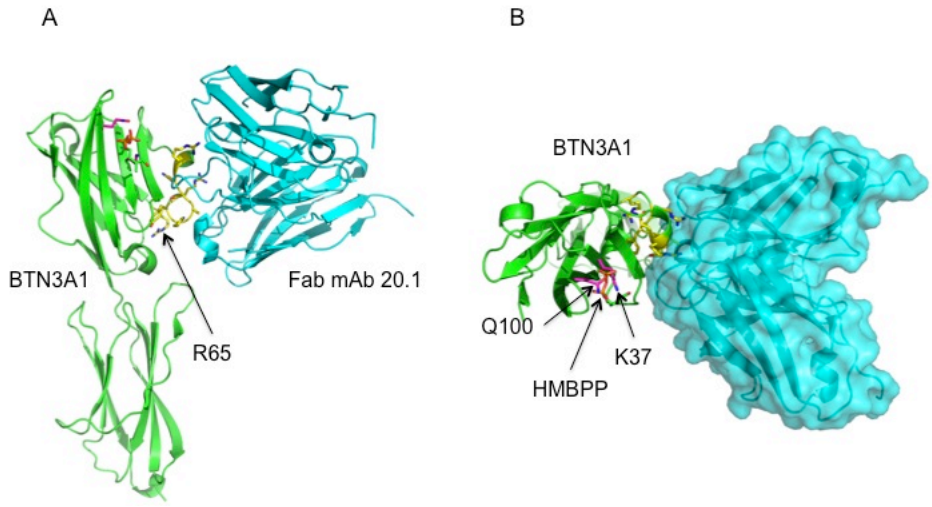


**Figure 22. Depiction of BTN3A1 interaction with mAb 20.1 and HMBPP.** (A) Side view of ribbon representation of complex between BTN3A1 and $F(ab)_2$ 20.1 mAb, with the regions of both molecules involved in the activation. Black arrow remarks an arginine at position 65 (R65) of BTN3A1 structure, which seems to be important for mAb 20.1 binding. (B). Upper view of ribbon representation of interaction between BTN3A1 and HMBPP (highlighted by the central arrow). Glutamine at position 100 (Q100) and lysine at position 37 (K37) are marked on BTN3A1 by black arrows. Done by Thomas Müller, Department of Botany I - Plant Physiology and Biophysics, Würzburg University, Würzburg, Germany.

Staining with mAb 20.1 revealed heterogeneity of BTN3A1 expression —both wild type and mutated— among the cell lines (Figure 23). The MFI values obtained with mAb 20.1 were considerably lower than those obtained with mAb 103.2 (Figures 23 and 24). Thus, the MFI of the reporter gene and of mAb 103.2 were compared to correlate the level of expression of the transduced BTN3A1 gene and the expression of the protein itself. Some cell lines like BTN3A1 and BTN3A1 R65A showed high levels of staining with mAb 103.2 with lower BTN3A1 gene expression, whereas others like BTN3A1 K37D:Q100A and Chr6 BTN3A1 K37D:Q100A displayed the opposite pattern (Figures 24). These results suggest that the mutations would have altered the structure of BTN3A1, thereby the binding of mAb 20.1, of mAb 103.2 and the staining profile compared to the cells with wild type BTN3A1.

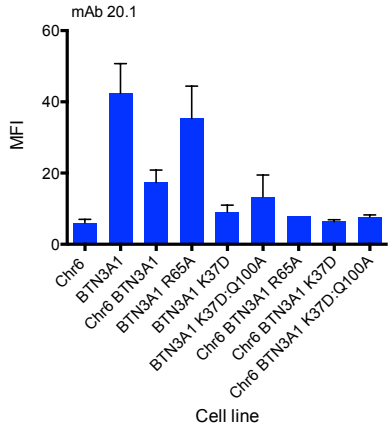


**Figure 23. Cell membrane staining with mAb 20.1 on CHO cell lines overexpressing either wild type BTN3A1 or mutated BTN3A1 versions.** $2x10^5$ CHO or CHO Chr6 cells transduced with either wild type BTN3A1 or mutated BTN3A1 were stained with mAb 20.1, washed and stained with PE-labeled $F(ab')_2$ fragment donkey anti-mouse IgG. The MFI was determined by flow cytometry. Data are shown as mean ± SEM of three independent experiments.

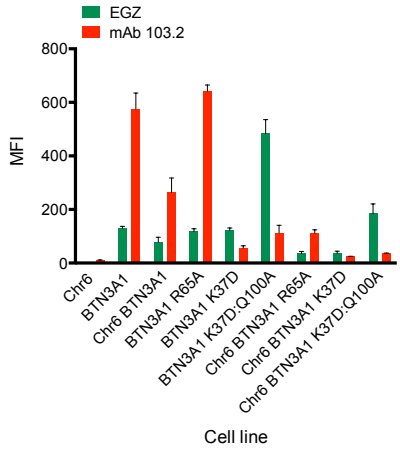


**Figure 24. Comparison of BTN3A1-containing EGZ plasmid fluorescence and cell surface staining by mAb 103.2 of CHO cells and CHO cells transduced with mutated BTN3A1.** $2x10^5$ CHO Chr6 cells and CHO BTN3A1 cells, along with CHO and CHO Chr6 cells transduced with mutated versions of BTN3A1, were stained with mAb 103.2, washed, and stained with PE-labeled F(ab')2 fragment donkey anti-mouse IgG. The plasmid containing both WT BTN3A1 gene and the mutated ones has EGZ as reporter gene, which allows the direct detection of green fluorescence. The MFI was determined by flow cytometry. Data are shown as mean ± SEM of three independent experiments.

The mutated BTN3A1-expressing cells were then co-cultured with Vγ9Vδ2 TCR MOP rat/mouse reporter cells in presence of HMBPP, zoledronate, and mAb 20.1. To account for the contribution of endogenous BTN3A1 in the case of the cell lines containing Chr6, Chr6 cells were included as baseline controls. R65A mutation did not alter the response to HMBPP nor to zoledronate as compared to the wild type BTN3A1, since the levels of IL-2 induced by Chr6 BTN3A1 R65A cells were comparable to those induced by Chr6 BTN3A1 and significantly higher compared to the activation induced by Chr6 cells (Figures 25A and 25B). Likewise, K37D mutation did not significantly affect the response to HMBPP assisted by Chr6 BTN3A1 K37D cells, compared to Chr6 BTN3A1 cells (Figure 25C). The zoledronate-provoked activation was modified, however, since Chr6 BTN3A1 K37D cells significantly induced higher levels of IL-2 in relation to Chr6 cells or Chr6 BTN3A1 cells (Figure 25D). Strikingly, the double mutation at the PAg binding site, K37D:Q100A, significantly increased the Vγ9Vδ2 T cell activation after both HMBPP and zoledronate treatment, since Chr6 BTN3A1 K37D:Q100A displayed a strong ability to stimulate in comparison to the control cells (Figures 25E and 25F); although staining by both mAb 20.1 and mAb 103.2 was considerably lower as compared to Chr6 BTN3A1 cells (Figures 23 and 24).

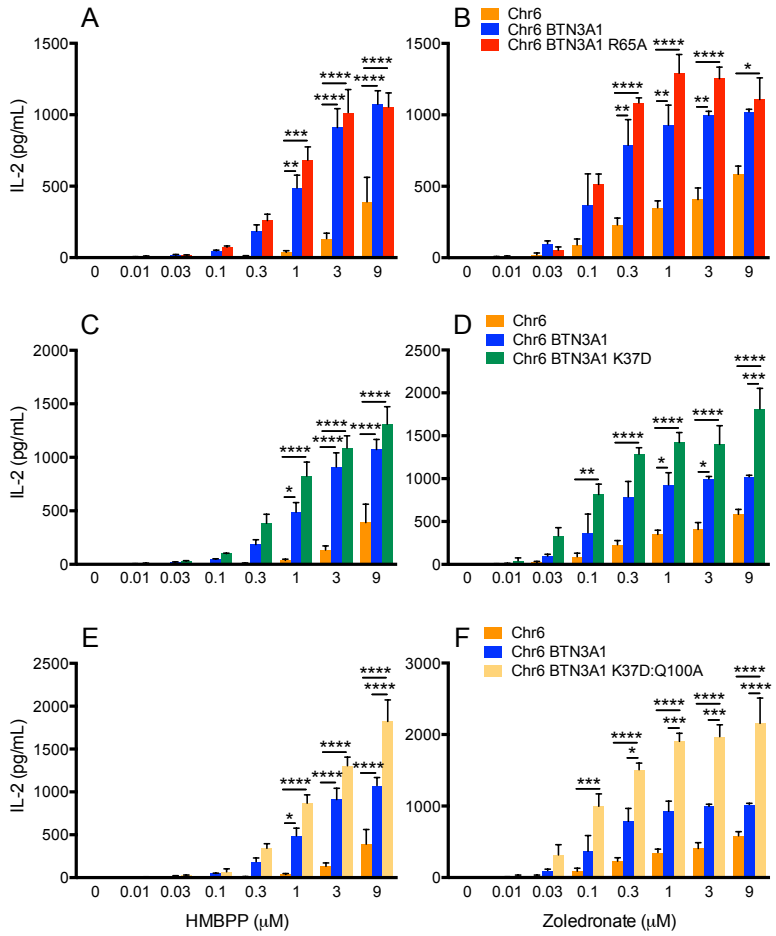


**Figure 25. Mutations in the PAg-binding site of the BTN3A1 extracellular domain do not impair the PAg-mediated Vγ9Vδ2 T cell activation. (**A) 1x10$^4$ CHO Chr6 cells, CHO BTN3A1 cells, and CHO were seeded in 96-well plates and incubated overnight. After that, they were co-cultured with 5x10$^4$ responder cells and treated with grading doses of HMBPP. Levels of IL-2 were estimated after 20 h of incubation by ELISA. (B) Cultures were done as in „A“, but replacing zoledronate as the stimulus. (C and D). Cultures were undertaken as in „A“ and „B“, but using CHO Chr6 BTN3A1 K37D as the mutant cell line to compare with CHO Chr6 and CHO Chr6 BTN3A1. (E and F). Stimulations were performed as in „A“ and „B“, but employing CHO Chr6 BTN3A1 K37:Q100A as the compared mutant cell line. (A-F) All samples were cultured in triplicates and data represent mean ± SEM of three independent experiments. *$p$<0.05, **$p$<0.01, ***$p$<0.001, and ****$p$<0.0001 (Unpaired Student's $t$-test).

In regard to the mAb 20.1-evoked stimulation, the IL-2 production by the reporter cells was abrogated not only by the mutation R65A but also by the mutations directed at the PAg-binding site. With the three mutations and using either CHO or CHO Chr6 cells, the amount of IL-2 was significantly decreased with reference to Chr6 BTN3A1 cells or BTN3A1 cells (Figures 26A-26F). As with the results obtained with CHO cell lines overexpressing BTN3A isoforms, a comparison was done to exclude the possibility that the degree of IL-2 depends on BTN3A-specific antibody staining. The levels of cytokine produced at 2 μg/mL mAb 20.1 were compared between cell lines with similar anti-BTN3A1 staining (Figures 23 and 24). BTN3A1 cells were compared to BTN3A1 R65A cells. No association was observed between IL-2 production and anti-BTN3A staining (Figure 27). These results indicate that alterations of the extracellular domain, by inducing mutations at the PAg- or mAb 20.1-binding site, do not impair the response to PAg; which suggests that the hypothesized PAg-binding site at the extracellular BTN3A1 domain is unessential for PAg-mediated Vγ9Vδ2 T cell response. In spite of that, any kind of alteration of this BTN3A1 domain does reduce the mAb 20.1-induced response. The inability of the mutations K37D and K37D:Q100A to reduce the PAg-induced response suggests also a crucial role for the intracellular domain as a key mediator of Vγ9Vδ2 T cell activation.

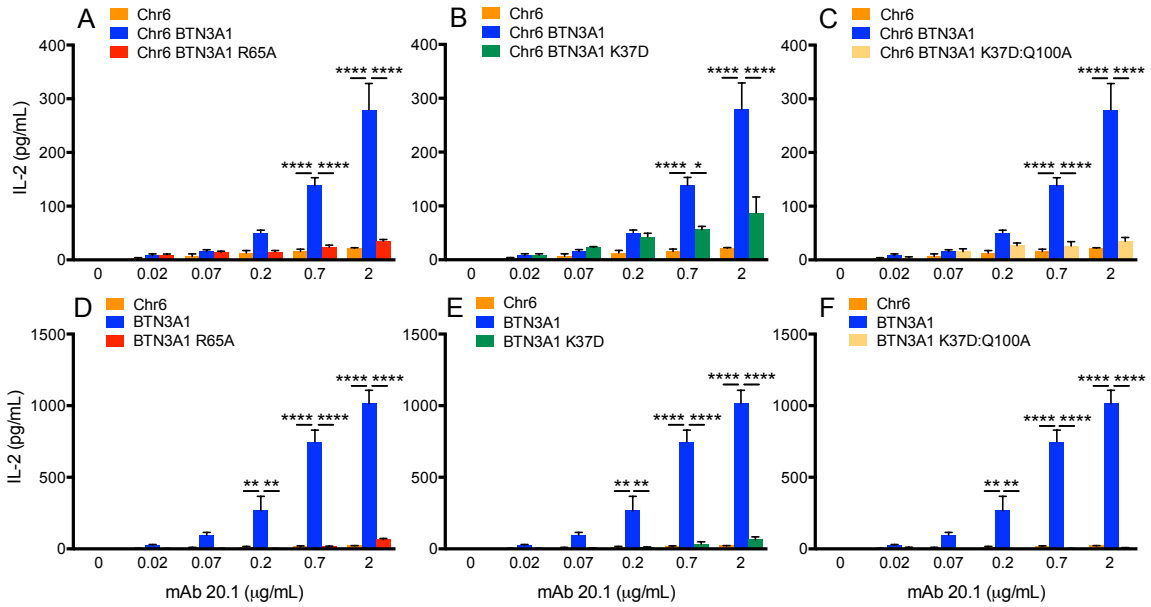


**Figure 26. Mutations in the PAg-binding site of the BTN3A1 extracellular domain impair the mAb 20.1-mediated Vγ9Vδ2 T cell activation.** (A) 1x10$^4$ CHO Chr6 cells, CHO BTN3A1 cells, and CHO Chr6 BTN3A1 R65A were seeded in 96-well plates and incubated overnight. They were then co-cultured with 5x10$^4$ responder cells for 20 h and treated with different concentrations of mAb 20.1. The amount of IL-2 in the cultures was determined by ELISA. (B and C) Cultures were performed as in "A", but using CHO Chr6 BTN3A1 K37D cells and CHO Chr6 BTN3A1 K37D:Q100A as mutant cells lines to compare with the control cells (CHO Chr6 and CHO Chr6 BTN3A1). (D-F) Stimulations were done as explained in "A", however, the cell lines transduced with WT and mutated BTN3A1 were all CHO cells, which were compared to CHO Chr6. (A-F) All samples were cultured in triplicates and data represent mean ± SEM of three independent experiments. *$p$<0.05, **$p$<0.01, and ****$p$<0.0001 (Unpaired Student's $t$-test).

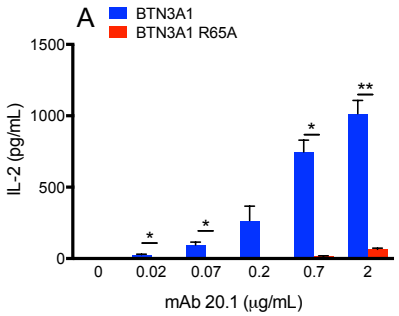


**Figure 27. mAb 20.1-evoked Vγ9Vδ2 IL-2 production mediated by mutated BTN3A1-transduced CHO cells with similar levels of mAb 103.2 staining.** Values of mAb 20.1-induced IL-2 production in presence of CHO BTN3A1 and CHO BTN3A1 R65A as APC (figure 26D) were graphed together and compared. *$p$<0.05 and **$p$<0.01 (Unpaired Student's $t$-test).

## 3.6 BTN3A1 is dispensable for the Vγ9Vδ2 T cell response against influenza A/PR/8/34 virus

Vγ9Vδ2 T cells have shown to contribute to the immune defense against influenza viruses, with an important participation of activating NK-cell receptors such as NKG2D [147], although the contribution of BTN3A1 in this context has not yet been elucidated. To evaluate the role of BTN3A1 in the anti-influenza virus Vγ9Vδ2 T cells immune response; influenza A/PR/8/34 virus strain was replicated in MDCK cells during 5 days. At day 3 of culture supernatants were collected and frozen at -80°C. After 5 days of culture, the cytopathic effect on the cells became visible by the detachment of the cells from the bottom of the wells, the disruption of the cell monolayer and by changes in the cell morphology. At this time point, the virus titers in the culture supernatants were estimated by using a plaque assay with MDCK cells as infection target.

Next, A549 lung epithelial carcinoma cells were infected with influenza A/PR/8/34 virus at different MOI —2, 4, 6, 8, and 10— and co-cultured with Vγ9Vδ2 TCR MOP responder cells during 20 h. Following this time point, the levels of IL-2 produced by the responder cells were determined by ELISA. No activation was observed in response to any of the tested MOI of influenza virus (Figure 28). These results might be explained by the incapability of the murine responder cells to be activated after influeza virus infection, due to the lack of molecules expressed by human cells. Therefore, freshly isolated PBMCs were infected for 10 h with influenza A/PR/8/34 virus at a MOI of 5 and treated or not with 200 ng/mL mAb 103.2. In the pilot experiment depicted in Figure 29, the upregulation of CD69 and CD25 (not showed) was chosen as a marker for Vγ9Vδ2 T cell activation. Infection with influenza led to cell activation, with CD69 showing a higher upregulation than CD25 (Figure 30). Nonetheless, the percentage of cells that upregulated CD69 or CD25 remained unchanged after treatment with mAb 103.2 (Figure 30). These results point out that the Vγ9Vδ2 T cell response to the influenza viral strain used might not be strictly dependent on BTN3A1 and other molecules may have a major role in such scenario.

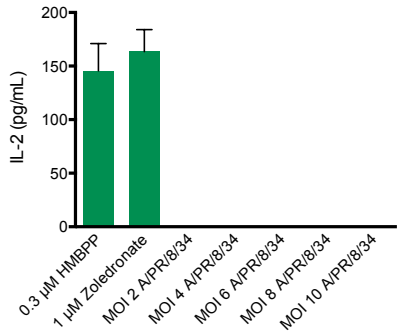


**Figure 28. Influenza A/PR/8/34 does not induce activation of Vγ9Vδ2 TCR-transduced murine cells.** 1x10$^4$ A549 cells were seeded in 96-well plates in incubated overnight. They were co-cultured with 5x10$^4$ responder cells for 20 h and stimulated with HMBPP, zoledronate or influenza A/PR/8/34 virus. The IL-2 produced in the cultures was determined by ELISA. All samples were cultured in triplicates. Data are shown as mean ± SEM of two independent experiments.

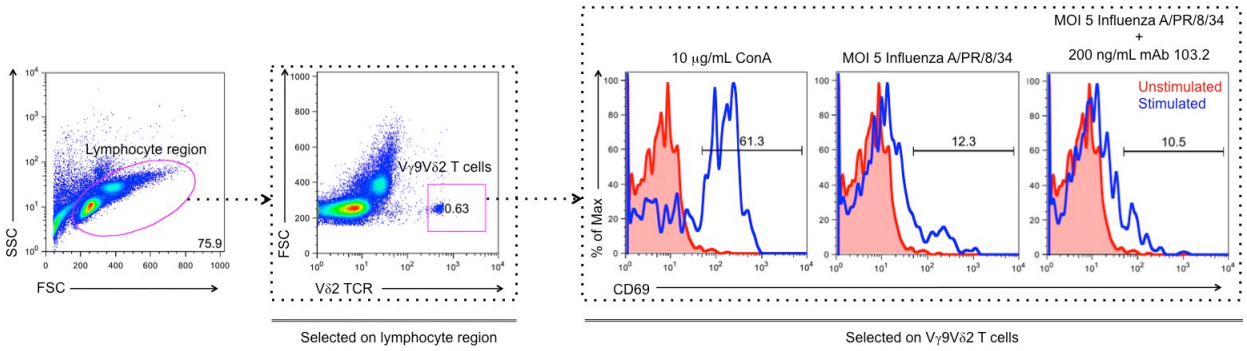


**Figure 29. Strategy to determine CD69 upregulation on PBMCs infected with A/PR/8/34 virus.** 2x10$^5$ freshly isolated PBMCs were treated with concanavalin A (ConA), influenza A/PR/8/34 virus, or influenza A/PR/8/34 in combination with mAb 103.2 for 10 h. Cells were collected and stained with anti-Vδ2 (clone 4G6), washed, and stained with FITC-labeled F(ab')$_2$ fragment donkey anti-mouse IgG. Finally, cells were stained with anti-CD69 PE. The percentage of CD69 on Vδ2$^+$ cells was determined by flow cytometry. Analyses were done on the lymphocyte region, where the Vγ9Vδ2 T cells were gated to generate histograms with the frequencies of CD69$^+$ or CD25$^+$ (not shown) cells for any of the stimuli used.

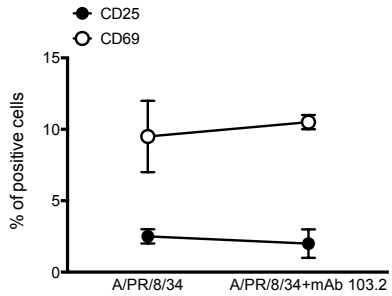


**Figure 30. mAb 103.2 does not inhibit the influenza-induced activation of Vγ9Vδ2 T cells.** Cultures were performed and analyzed as described in figure 29. Stimulations were done in triplicates and pooled as a single sample for flow cytometry staining. Data are CD25 and CD69 MFI on Vγ9Vδ2 T cells, shown as mean ± SEM of two independent experiments.

# 4. DISCUSSION

Modulation of T cell function has been crucial for the development of therapies for infectious diseases and cancer. Vγ9Vδ2 T cells are a special type of lymphocytes with features of cells from both the innate and adaptive immunity. Their rapid activation after antigen exposure and lack of MHC restriction makes this T cell subset an interesting immune component for therapeutic modulation. Nevertheless, several issues about Vγ9Vδ2 T cells are not completely clear: the nature of antigens or ligands that induce their response, the mechanisms of recognition of such antigens/ligands, and their roles in host defense and homeostasis. This knowledge is required not only for a better understanding of the physiology of Vγ9Vδ2 T cells, but also for the development of therapies that allow to regulate the activity of these cells.

This research work provides evidence that: i) BTN3A1 is not sufficient to mediate Vγ9Vδ2 T cell activation; ii) differences in the CDR3 sequences of Vγ9Vδ2 TCR lead to different profiles of response to PAg and mAb 20.1; iii) mAb 20.1-induced response does not synergize with the response to PAg but inhibits this response; iv) BTN3A2 supports response to both PAg and mAb 20.1, whereas BTN3A3 supports mainly the mAb 20.1-mediated response; v) mutations in the BTN3A1 extracellular domain impair the response to mAb 20.1, but not to PAg; and vi) BTN3A1 is dispensable for the Vγ9Vδ2 T cells response to influenza A virus. These findings constitute a contribution to the body of knowledge on Vγ9Vδ2 T cells and to better understand this interesting lymphocyte subset.

## 4.1 BTN3A1 and other genes on Chr6 for Vγ9Vδ2 T cell activation

One of the biggest obstacles that hinders the progress in the study of the role of BTN3A1 in PAg-mediated activation is the ubiquitous nature of this molecule. Most

human cells, including Vγ9Vδ2 T cells express BTN3A molecules and can present PAg. This can be avoided by using Vγ9Vδ2 TCR-transduced murine cells, because rodents do not express BTN3A molecules and also lack the Vγ9Vδ2 T cell population [102, 135]. In here, by transducing the Vγ9Vδ2 TCR genes into rat/mouse thymoma cells acting as responder cells, and the BTN3A genes into CHO cells functioning as APC, it was possible to measure the relative contribution of this protein in the γδ T cell response to PAg. The results of this research demonstrate that activation by PAg requires both BTN3A1 and Chr6, which implies that full activation of Vγ9Vδ2 T cells by antigenic compounds with phosphate moieties such as HMBPP or IPP need additional genes. On the other hand, expression of BTN3A1 alone is sufficient for mAb 20.1-evoked activation and is independent of the presence of Chr6 within the APC.

Previous work in the research group of Thomas Hermann suggested a role of Chr6 in the PAg-induced activation. The reporter cell line employed hereof was used to evaluate the ability of mouse-human hybrid APC lines to activate it in presence of HMBPP, zoledronate, and sec-butylamine [146]. Considering that mouse-human hybridomas lose human chromosomes over the time in culture, retaining few or a single chromosome, it is possible to test chromosome contribution in a particular phenomenon. In a first attempt to identify the human chromosomes required for PAg presentation, the capacity of those hybridomas to induce Vγ9Vδ2 T cell after HMBPP treatment was tested [146]. Loss of human chromosomes 2,3,7,8,9,10,11,13, 17, 18, 20, 21, and X did not affect the ability of the hybrid APC to support Vγ9Vδ2 reporter cell activation [146].

The present work expands the previous results, since they demonstrate that the rat/mouse Vγ9Vδ2 T reporter cells respond to HMBPP and zoledronate with Chr6-containing CHO cells as APC (Figure 2). These data are consistent with the previous reports proving requirement of this human chromosome for PAg-mediated activation [137]. Increased PAg-induced activation of Vγ9Vδ2 T cells, after over-expression of BTN3A1 has also been reported for human cells [136, 137]. The need for Chr6 is further supported by the finding that CHO cells lacking this human chromosome have no ability to sustain Vγ9Vδ2 T cell response to PAg, regardless

of their BTN3A1 expression (Figures 2 and 5). In spite of the need of Chr6 to obtain reporter cell response to PAg, the mAb-provoked response was independent of Chr6 presence; because CHO cells with BTN3A1 over-expression were able to induce a high stimulation of the reporter cell line with mAb 20.1 as stimulant. This stimulation was remarkably higher than that obtained with stimulator cells with Chr6 (Figure 3). Several factors may have contributed to these differences in stimulation response. To some extent, this might be attributed to the higher BTN3A expression by BTN3A1 cells compared to Chr6 BTN3A1 cells (Figure 5). In addition, a negative effect of Chr6, within the CHO cells, on mAb 20.1-induced activation cannot be excluded.

In any event, the strong response to mAb 20.1 conflicts with the results published by Vavassori and colleagues that show activation of Vγ9Vδ2 TCR-transgenic mouse reporter cells with PAg, but not with mAb 20.1 [137]. This discrepancy might be explained by differences in the cell nature or cell origin of both the APC and the reporter cells, in the latter case, TCR specificities may play an important role. These points will be addressed in a later section of this discussion.
The proposed mechanism of action of ABP and alkylamines is the inhibition of the enzyme FPPS, which subsequently cause intracellular IPP accumulation within the APC or target cell [113, 115]. Therefore, it might be expected the sec-butylamine had the same effect as zoledronate. In spite of that, CHO cells, even those with Chr6, did not demonstrate any ability to stimulate the reporter Vγ9Vδ2 T cells in presence of sec-butylamine (Figure 7). Thus, it is possible that the mechanism of action of these amine-containing compounds is slightly different to that of ABP or that they rely on additional cellular molecules or compounds, not present in rodent cells or in human chromosomes different to Chr6. Additionally, the ability to induce response to sec-butylamine might be a feature specific of a particular cell type, since Raji RT1B$^{l}$ cells induced response to this alkylamine, while A375 cells do not (Table 4).

The data reported in here also shows independent evidence that Chr6 is necessary to make rodent cells mediators of human Vγ9Vδ2 T cell response to PAg. Similar to the results with the murine responder cells, zoledronate-pulsed

Chr6-containing CHO cells induced activation of the Vγ9Vδ2 T cell fraction within PBMCs (Figure 9). The high frequencies of cell activation among this γδ T cell subset indicates that the observations made with the TCR transductants are characteristic of human Vγ9Vδ2 T cells. The contribution of BTN3A1 in the Vγ9Vδ2 T cell activation has been highlighted using primary Vγ9Vδ2 T cells [136-138], nevertheless the present work reinforces not only the role of BTN3A1, but also the need of other genes in Chr6 to activate these cells. In summary, the experiments with the rat/mouse reporter cell line, along with the results obtained with primary γδ T cells indicate the need of BTN3A together with additional molecules that are the products of genes in Chr6.

Since BTN3A1 loaded with PAg binds to recombinant TCR in a cell-free system [137], it is conceivable that the additional Chr6-derived proteins perform duties related either to PAg loading onto BTN3A1 molecules or to regulation of its cell-membrane distribution and cellular compartmentalization. This possible co-localization of BTN3A genes with other genes of proteins associated with PAg presentation may resemble the events occurring in MHC-dependent antigen presentation [148]. Indeed, the complexity of PAg sensing has become clearer as other proteins have been involved in this process. Rhodes et al. demonstrated specific interaction of BTN3A1 with periplakin, a cytoskeletal adaptor protein, via a membrane-proximal di-leucine motif present in BTN3A1, but not in BTN3A2 and BTN3A3 [149]. The researchers could restore PAg-induced responses after re-expression of wild type BTN3A1 in BTN3A-knocked down cell lines, but not with BTN3A1 variants that lacked the periplakin interaction motif. Additionally, they suggest that expression of periplakin is coordinated with the expression of BTN3A1, since BTN3A1 knock down cells have reduced levels of periplakin which are comparable with those observed with periplakin-depleting reagents. With these main findings, they propose a model in which communication of intracellular PAg increase requires not only BTN3A1, but also periplakin and other cytolinker proteins that mediate anchoring or redistribution of BTN3A in the cell membrane as well as receptor clustering or recruitment of additional molecules to a signaling complex.

Further evidence of the participation of other proteins in the PAg-mediated Vγ9Vδ2 T cell activation has been provided by Sebestyen and colleagues [150]. These authors highlight the critical role of RhoB, a small GTPase, in the spatial redistribution and conformational change of BTN3A1 to mediate γδ T cell response. In their research, stimulation with aminobisphosphonates leads to RhoB activation and compartmentalization to a membrane proximal connector region of BTN3A1 in its cytoplasmic tail. This evidence supports a direct connection between increased intracellular levels of IPP, cytoskeletal reorganization, and tumor cell-induced Vγ9Vδ2 T cell activation. In spite of these findings, they agree that additional factors must be involved in PAg recognition and propose that the gene(s) still to be identified might be GTPase activating proteins or guanine nucleotide exchange factors, which coordinate the balance between the active and inactive state of RhoB In conclusion, the results of the present work support the importance of BTN3A1 in the PAg- and mAb 20.1-mediated activation. Furthermore, it provides data that indirectly highlights the need for additional proteins whose genes are located on Chr6. Identifying the additional genes is mandatory to understand the mechanism of PAg presentation and to generate animal models for Vγ9Vδ2 T cell physiology that could allow the evaluation of their therapeutic potential.

## 4.2 Differences in the response to PAg and mAb 20.1 according to Vγ9Vδ2 TCR clonotype

The CDR of immunoreceptors such as αβ TCR and BCR establish the specificity and affinity for a given antigen. Via V(D)J recombination T and B cells create an extremely variable repertoire that ensures the recognition of an enormous amount of antigens [8, 9]. The expression of randomly rearranged TCR is also a feature of Vγ9Vδ2 T cells. The results of this dissertation clearly show that differences in the CDR3 sequences of the Vγ9Vδ2 TCR lead to variations in the response to both PAg and the agonistic mAb 20.1.

As mentioned in the preceding section, Vavassori and colleagues did not find stimulation with mAb 20.1 using transgenic Vγ9Vδ2 $TCR^+$ mouse cells [137]. The

discrepancy between their results and those exposed in this dissertation might be attributable to differences in the nature of the APC (A375 melanoma cells vs. CHO cells) or in the origin of the reporter cells (Vγ9Vδ2 TCR transgenic cells isolated from RAG deficient mice vs. rat/mouse Vγ9Vδ2 $TCR^+$ hybridomas). The conjecture regarding the APC is ruled out by the finding that A375 cells support 53/4 r/m CD28 Vγ9Vδ2 T cell reporter cell stimulation (Figure 10). Therefore the discrepancies found in the mAb 20.1-provoked response cannot be related to the APC. Turning the focus of the approach to the reporter cells, clear differences in the mAb 20.1 response are revealed, defining responder and non-responder TCR clones for this murine system. Rat/mouse reporter cells bearing the γδ TCR D1C55, G115 and G115 cl5 are practically non-responder in relation to reporter cells bearing the γδ TCR MOP (Figures 11 and 12C). These results provide proof and an explanation of the differences in the mAb 20.1-dependent activation that have been previously published [137]. This evidence is a confirmation that the lack of activation of γδ TCR D1C55 transgenic cells by mAb 20.1 is not a feature of this type of cells, but rather an inherent characteristic of this particular Vγ9Vδ2 TCR. Moreover, it is clear that this unresponsiveness is not limited to TCR D1C55, but is also a feature of other Vγ9Vδ2 TCR such as G115 and G115 cl5. This finding places TCR MOP-bearing cells as an exceptionally reactive TCR to mAb 20.1.

These γδ TCR-dependent differences in response to mAb 20.1 were also observed, by means of CD69 upregulation, with Jurkat cells transduced with TCR MOP, TCR D1C55, or TCR G115 (Thomas Hermann and Lisa Starick, unpublished results). In these conditions, however, the differences were smaller compared to those found with the murine cells. Besides that, there was some degree of stimulation with Jurkat cells expressing γδ TCR that were unresponsive in the murine reporter cells (TCR D1C55 and TCR G115), which implies that human cells have other factors or molecules that favor this kind of Vγ9Vδ2 T cell stimulation.

Differences between PAg- and mAb 20.1-elicited activation have been explained with effects associated to the APC: the loss of ABP-induced activation after mevastatin treatment, without affecting the response to mAb 20.1; or the ability of mutated BTN3A1-intracellular domain-expressing cells to respond to mAb 20.1,

but not to PAg [138]. The findings of the current work demonstrate that differences in the reactivity to both PAg and mAb 20.1 are also linked to variations in the CDR3 of the Vγ9Vδ2 TCR, indicating distinctive modes of TCR-ligand interaction. The sequence comparison of the mAb 20.1-responder and non-responder Vγ9Vδ2 TCR used herein shows unusual features in the γ chain of TCR MOP (Table 5): a substitution of a tryptophan for lysine at position 107 and a shorter CDR3 compared to TCR G115, TCR D1C55, and other PAg-responsive clones listed elsewhere [151]. These characteristics of the TCR MOP γ chain might enable the activation by mAb 20.1 as evidenced by the clear, although weak response of the reporter cells with γMOP δD1C55 TCR (Figure 13B). Nonetheless, a critical participation of the CDR3δ is also implied by the significant reduction of the mAb 20.1-elicited response of these cells in comparison to TCR MOP cells (Figure 13B). These Vγ9Vδ2 TCR-dependent differences are also observed with sc 20.1 and sc 103.2; these antibodies induce a higher response with cells expressing the TCR MOP (Figure 12D and 12E), supporting the results obtained with mAb 20.1.

The PAg-induced response is also different between the Vγ9Vδ2 TCR tested, and together with the mAb 20.1-induced activation shows contrasting reactivity profiles or hierarchies. Response to PAg by TCR MOP reporter cells is slightly lower compared to TCR D1C55 reporter cells, whereas in response to mAb 20.1 the situation is the opposite (Figures 11, 12A and 12B). This means that the strong activation with mAb 20.1 is not just a consequence of a higher sensitivity to all stimuli by the responsive transductants, but a selective affinity for a given stimulant. PBMCs-derived Vγ9Vδ2 T cell clones also show such hierarchy (Figures 15 and 16), which indicates that the TCR-determined differences in response to both PAg and mAb 20.1 are actual characteristics between the Vγ9Vδ2 T cell population.

Differences in the Vγ9Vδ2 T cell response to PAg have been demonstrated by Bukowski and colleagues using a TCR gene transfer approach [152]. In their results, a substitution of the entire TCRγ junctional segment, from the PAg-reactive Vγ2/W/Jγ1.2 TCR for another from the PAg-nonreactive Vγ9/GN/Jγ1.3 TCR, results in non-responsiveness to ethyl pyrophosphate and IPP, yet preserving reactivity to PAg in a culture supernatant of *Mycobacterium fortuitum*. Moreover, a change in the

sequence of the N nucleotide region within the CDR3 of the γ chain abrogates response to both ethyl pyrophosphate and the mycobacterial supernatant. This dependence on the sequence of the CDR3 for PAg reactivity has also been proven in the Vγ9Vδ2 T cell response to tumor cells [151]. Mutations in the CDR3 region of the Vγ9Vδ2 TCR DG.SF13 render Jurkat cells unable to respond to the tumor cell line RPMI 8226, as well as to HMBPP, IPP, other prenyl pyrophosphates, bisphosphonates, and alkylamines.

Additional evidence has been provided by Gründer et al. by means of site-directed mutagenesis of tumor responsive Vγ9Vδ2 TCR [140]. They prove that individual Vγ9Vδ2 T cell clones show differences in their reactivity to tumors, in terms of specificity and functional avidity of the TCR. These events are regulated by the CDR3 of a specific Vγ9Vδ2 TCR, as single amino acid substitutions in the CDR3 can strongly influence the interaction between the TCR and its ligand. Therefore, the potential of Vγ9Vδ2 T cells to react to PAg and the degree of such reactivity are not universal aspects within the cell population, but are differential features among the Vγ9Vδ2 clones. Consequently, in spite of the notion that Vγ9Vδ2 TCR seem to behave more like pattern recognition receptors (recognizing invariant hallmarks of infection and cell stress or transformation), it does not mean that they do not undergo selection during γδ T cell development or stimulation, as T and B cells do. This flexibility in the PAg recognition by Vγ9Vδ2 TCR might be a way to adjust the response to a variable antigenic cell surface or to an antigen that is exposed in different ways; as it has been demonstrated for T22-reactive mouse γδ T cells with variable CDR3 [153]. To conclude, the results presented hereof do not only give an additional evidence of the marked CDR3-influenced clonotypic differences in the Vγ9Vδ2 T cell response to PAg, but provide proof that this is also the case for the response to mAb 20.1, something that has not been described so far. The findings also make the Vγ9Vδ2 TCR MOP a very interesting tool in the study of Vγ9Vδ2 T cell activation.

## 4.3 Non-synergistic effect of mAb 20.1 on the PAg-induced response

The discovery that mAb 20.1 could expand and lead to proliferation of γδ T cells in PBMCs cultures, stands as a key factor in the study of the role of BTN3A1 in Vγ9Vδ2 T cell activation. mAb 20.1 induces production of IFNγ, TNF, and upregulation of CD69 by Vγ9Vδ2 T cells [136]. While mAb 20.1 can strongly activate Vγ9Vδ2 T cells, mAb 103.2 has an antagonistic behavior on these cells, inhibiting their PAg-mediated activation [136]. Some knowledge has been gained on the mechanism of interaction between these antibodies and BTN3A1. Nonetheless, the picture of such mechanism is still not complete. The results presented herein demonstrate that mAb 20.1 has not synergistic effect in the PAg-induced response but inhibits it instead.

The findings confirm the mAb 20.1-induced stimulation of Vγ9Vδ2 TCR MOP reporter cells and also that of sc 20.1. The latter antibody requires a higher concentration to stimulate the reporter cells, compared to mAb 20.1, which may be a consequence of a different valence and therefore, different avidity. Additionally, the efficient inhibition of the response to PAg by mAb 103.2 was also confirmed. In contrast, this was not the case of sc 103.2. It is possible that the antagonistic antibody, in this monovalent state, is not able to block the association of other molecules or proteins that function together with BTN3A to activate γδ T cells, or that the size of the divalent antibody is a requisite for the inhibition of the PAg response.

Activation by sc 20.1 does not seem to reach saturation, as in the case of mAb 20.1. Indeed, activation by the agonistic single chain antibody, at the highest concentration, is slightly higher than the activation at the saturating concentration of mAb 20.1 (Figures 17C and 17D). This might be a consequence of the larger size or the bivalency of mAb 20.1, whose binding hinders stimulatory cells to achieve fully activating state. The weak but significant response of Vγ9Vδ2 TCR MOP cells induced by sc 103.2 was an unexpected finding (Figure 17B). It is conceivable that, in this murine system, even a low degree of engagement of BTN3A molecules is sufficient to convert BTN3A-expressing cells into Vγ9Vδ2 T cell stimulators, without needing to form the proposed lattice induced by mAb 20.1 [134, 136].

Regarding TCR D1C55 cells, the results confirm the lack of responsiveness to mAb 20.1 by this Vγ9Vδ2 TCR and reveal a complete inhibition of PAg-induced activation by this antibody (Figure 17G), which in the case of TCR MOP cells was partial (Figure 17C). The differences between TCR D1C55 cells and TCR MOP cells are further highlighted by the fact that the activation caused by sc 20.1 is lower with the former reporter cell line (Figures 17D and 17H). This supports the idea that differences in the CDR3 of Vγ9Vδ2 TCR strongly affect the response to PAg and to the agonistic antibodies. Noteworthy is the increase of the Vγ9Vδ2 T cell activation of both D1C55 and MOP cells when sc 20.1 (at 2 μg/mL) is combined with HMBPP (Figures 17D and 17H), which means that the inhibition decreases and activation starts at this point. The sc 20.1-mediated inhibition of PAg response is specific, since it is not seen with sc 103.2, which has been reported to bind with higher affinity to BTN3A1 than sc 20.1 [134].

Initial insights into the mechanism of action and the binding sites of both mAb 20.1 and mAb 103.2 have been revealed by Palakodeti and colleagues through crystal structures of complexes between BTN3A1 and the single chain versions of these antibodies [134]. Their study suggests that mAb 20.1 and mAb 103.2 bind to different epitopes of BTN3A and assumes that the extracellular BTN3A domains are expressed as dimers on the cell surface. Thus, 20.1 antibody has no ability to bind bivalently to one BTN3A dimer, since the binding sites are separated from each other. Consequently, the engagement of two different BTN3A dimers is needed to occupy both 20.1 antibody-binding sites. As a result of this kind of binding, 20.1 antibody leads to cross-linking of BTN3A molecules on the cell surface. Conversely, 103.2 antibody might bind to one BTN3A dimer using both binding sites.

Based on these evidences, the researchers propose a model that focuses on structural modifications of the extracellular domain, whereby two BTN3A dimeric states define the stimulatory potential of the cell that expresses them. In homeostatic, non-stimulatory conditions, BTN3A monodimers would exist in a head-to-tail closed state, with the two monomers lying parallel to the cell membrane. In such state, BTN3A dimers have no ability to convey any stimulatory signal to Vγ9Vδ2 T cells. Upon 20.1 antibody stimulation, BTN3A dimers would be

cross-linked and adopt an open V-shape conformation. In this conformation BTN3A1 dimers turn into stimulatory proteins that permit Vγ9Vδ2 T cell stimulation. For 103.2 antibody, the model proposes two situations: either it blocks a site required by BTN3A to induce Vγ9Vδ2 T cell activation or it stabilizes the closed head-to-tail conformation, inducing the observed inhibition of PAg response [134].

The results presented in here are well explained by the previous model: mAb 20.1 induces a conformational modification of the BTN3A extracellular domain, mimicking PAg-mediated activation as a switch from a non-stimulatory to a stimulatory conformation. Additionally, with the data generated in this work, it is possible to conceive some mechanisms by which mAb 20.1 inhibits the response to PAg. First, mAb 20.1 and PAg might compete for binding to the extracellular domain and mAb might also block the access of a BTN3A-PAg complex to the Vγ9Vδ2 TCR. These two possibilities fit well in the case that BTN3A1 acts as a bona fide antigen-presenting molecule. But in the case that PAg do not directly bind to the extracellular domain of BTN3A1, it is plausible that mAb 20.1 hinders BTN3A1 to take the stimulatory conformation that is initiated by PAg at the intracellular domain. mAb 20.1 may also prevent the association between BTN3A1 and other cell surface molecules that act as Vγ9Vδ2 TCR ligands or function in a complex with BTN3A proteins.

## 4.4 Participation of BTN3A2 and BTN3A3 in the responses to PAg and mAb 20.1

Three BTN3A isoforms, each one coded by an individual gene, have been identified in humans: BTN3A1, BTN3A2, and BTN3A3. The extracellular domains of these proteins share a high homology both in sequence and structure. Minor variations have been observed in the hinge region that connects the Ig-like variable domain and the Ig-like constant domain. The major differences among these three isoforms are in the intracellular B30.2 domain, which is absent in BTN3A2 and in BTN3A3 has an extra extension of 70 amino acids compared to BTN3A1[154]. Furthermore, BTN3A1, but not BTN3A2 and BTN3A3, has a membrane proximal di-leucine motif,

which allows its interaction with the cytoskeletal adaptor periplakin [149]. Although the role of BTN3A1 in the activation of Vγ9Vδ2 T cells is indisputable, the participation of BTN3A2 and BTN3A3 in this context is not completely clear.

The results of the current dissertation show that BTN3A2 contributes to the PAg-mediated Vγ9Vδ2 T cell response together with BTN3A1. In contrast, the role of BTN3A3 seems to be, to some extent, unessential for the PAg response (Figure 19). In spite of this, both BTN3A2 and BTN3A3 participate in the mAb 20.1-elicited response, when Chr6 is present in the APC (Figure 20). This supports the notion of the central role of BTN3A1 and the involvement of other Chr6-encoded molecules to activate Vγ9Vδ2 T cells, since CHO cells without Chr6 and overexpressing either BTN3A2 or BTN3A3 cannot support mAb 20.1-mediated response. That all three BTN3A isoforms mediate activation in response to mAb 20.1 agrees with results from others [136, 138, 155] showing that they are recognized by the 20.1 antibody and mediate Vγ9Vδ2 T cell response to it. This suggests the presence of a shared epitope on these molecules that mediates γδ T cells stimulation by using this agonist antibody.

Regarding the Vγ9Vδ2 T cell PAg response, the picture remains unclear. Few studies have addressed the role of the two isoforms besides BTN3A1 in this process and their results show discrepancies. Harly and colleagues generated cells expressing only one of the BTN3A isoforms by transfecting BTN3A knockdown cells with cDNA encoding BTN3A1, BTN3A2, or BTN3A3 [136]. They co-cultured these cells with a human Vγ9Vδ2 T cell line and measured CD107a expression and TNF production by the latter group of cells. Their results show that the re-expression of BTN3A1 and (to a lower degree) of BTN3A3 restores the capacity, of pamidronate-treated knockdown cells to induce Vγ9Vδ2 T cell activation, when CD107a is the read-out. By measuring TNF production by the γδ T cell line, they prove that the ability to mediate response to HMBPP is regained only after BTN3A1 re-expression and not with BTN3A2 or BTN3A3 re-expression in BTN3A knockdown cells. In this result, the significant difference is only between BTN3A1 and BTN3A3, and although BTN3A2 induces lower levels of TNF compared to BTN3A1, this does not rule out a role for BTN3A2 in the Vγ9Vδ2 T cell activation.

On the other hand, Rhodes et al. highlight a role for all three BTN3A isoforms in the regulation of γδ T cell activation in response to PAg [149]. By using BTN3A, BTN3A1, BTN3A2, and BTN3A3 knockdown cell lines, the researchers test the ability of γδ T cells from PBMCs to produce IFNγ in response to HMBPP or zoledronate-pulsed target cells. In response to zoledronate-treated cells, the IFNγ production of γδ T cells was efficiently blocked when co-cultured with BTN3A1, BTN3A2, and BTN3A3 knock down cells. But in response to HMBPP, BTN3A3 knock down cells still show ability to induce cytokine production by γδ T cells.

In summary, the findings from other research groups and those exposed herein confirm the central role of BTN3A1 in the Vγ9Vδ2 T cell activation, but indicate an additional complexity regarding the contribution of BTN3A2 and BTN3A3, which depends on the chosen γδ T cell activation marker or the stimulus used in the experimental system. It is important to keep in mind that the results presented in the current research work are limited to some extent by the endogenous BTN3A1 in the CHO cell lines containing Chr6, which may mask the actual contribution of BTN3A2 and BTN3A3. Nevertheless, it is possible to imagine a scenario where BTN3A2 and BTN3A3 might modulate BTN3A1 cell compartmentalization, trafficking to the cell membrane, or work together in a protein complex that requires a regulated co-expression of these protein isoforms.

## 4.5 Contribution of the extracellular domain of BTN3A1 in the Vγ9Vδ2 T cell activation

BTN3A molecules belong to the Ig superfamily of proteins and are closely related to the B7 costimulatory receptors CD80 and CD86. Their extracellular domain is composed of an Ig-like variable region and a Ig-like constant region. Beyond the transmembrane portion, BTN3A1 and BTN3A3 have an intracellular B30.2 domain, which is longer in the latter isoform [154]. Both the extracellular and the intracellular domain have been proposed to bind PAg, since putative PAg-binding sites have been described within them [137, 138].

Data in the current dissertation show that the ability of BTN3A1 to mediate PAg reactivity does not rely exclusively in the extracellular domain, as the mutations in the proposed PAg-binding in this domain do not affect the response to HMBPP and zoledronate (Figure 25). Therefore, this evidence allows the conclusion that the B30.2 domain in the intracellular tail is of greater importance for the PAg-mediated activation of Vγ9Vδ2 T cells. On the other hand, the mAb 20.1-mediated response strongly depends on the native molecular structure of BTN3A1, since any alteration of this protein, through mutation in either the mAb 20.1- or PAg-binding site, alters the response of Vγ9Vδ2 T cells to the agonistic antibody (Figure 26).

B30.2 domains are found in the majority of proteins of the butyrophilin family and are closely related to PRY-SPRY domains, which are found in around 15 protein families and are known to mediate protein interactions [156]. Much of the interest in the role that B30.2 domains may play in butyrophilins has come from their function in tripartite motif family (TRIM) proteins, important molecules in the innate immune system. In TRIM molecules, B30.2 domains act as pattern-recognition receptors such as TLRs or NLRs, binding molecules that are associated with infection or cell damage. These domains seem to have evolved to recognize a specific ligand with a high affinity, which in turn induces signaling and effector functions in innate immune cells [154].

Evidence based on binding studies have demonstrated that there is a specific interaction between PAg and the B30.2 domain of BTN3A1, with affinities of less that 1 μM for HMBPP and 0.5-0.6 mM for IPP [138, 149, 157]. Indeed Harly and collaborators have proved that the BTN3A1 B30.2 domain is necessary for PAg stimulation [136]. They show how truncation of this domain abrogates the ability of BTN3A1-expressing cells to mediate stimulation in response to PAg. Moreover, the exchange of the BTN3A3 intracellular tail for that of BTN3A1 and the expression of this chimeric protein (BTN3A3 extracellular domain-BTN3A1 intracellular domain) allow BTN3A1-lacking cells to regain the ability to mediate PAg stimulation [136]. This finding underlines the substantial contribution of the intracellular BTN3A1 domain in the Vγ9Vδ2 T cell response to PAg.

In addition, Sandstrom and colleagues have clearly demonstrated, by using molecular and cellular approaches, that PAg bind to a pocket at the N-terminal part of the BTN3A1 intracellular domain [138]. This pocket has a positive charge and is lined by basic amino acids such as histidine and arginine, something that seems to be a special characteristic of the BTN3A B30.2 domain. Change-reversal mutations of the basic amino acids within this pocket lead to abrogation of PAg binding and of PAg-dependent stimulation of Vγ9Vδ2 T cells. Furthermore, comparative sequence analysis between the B30.2 domains of BTN3A1 and BTN3A3 identifies a histidine at position 352 in BTN3A1, which is replaced by an arginine in BTN3A3. This replacement seems to determine the ability to induce response to PAg, since the exchange of the histidine at this position by an arginine renders BTN3A1 unable to bind and to mediate response to PAg. Strikingly, the reverse change in BTN3A3, i.e. the arginine for the histidine, gives to this isoform not only the ability to bind PAg but also to stimulate Vγ9Vδ2 T cells in response to these compounds [138]. These evidences and those showed herein regarding the mutations in the extracellular BTN3A1 domain are a strong indication that the sensing of intracytoplasmic changes of PAg by BTN3A1 through its B30.2 intracellular domain is a critical step in the Vγ9Vδ2 T cell activation.

## 4.6 Role of BTN3A1 in the response of Vγ9Vδ2 T cells towards influenza A virus

Vγ9Vδ2 T cells have shown beneficial effects against influenza virus infection through both direct cytotoxicity of virus-infected cells and cytokine production. Previous studies have demonstrated that Vγ9Vδ2 T cells have the ability to control infection by several influenza virus strains, including both seasonal and pandemic human H1N1, as well as avian strains such as H5N1 or H9N2 [48-50]. It is also known that the antiviral activities of this γδ T cell stimulation can be improved after stimulation with PAg [48, 147]. Since BTN3A1 play a crucial role in the PAg-dependent and independent activation of Vγ9Vδ2 T cells, it is very interesting to elucidate the contribution of BTN3A1 in influenza viral infections.

The results from this dissertation suggest that BTN3A1 is not the essential factor in the Vγ9Vδ2 T cell-mediated immune response to the influenza A/PR/8/34 virus strain. This conclusion is supported by the lack of response of the Vγ9Vδ2 TCR MOP-transduced cell line towards cells infected with different MOI of virus; although they do respond to the target cells when treated with HMBPP or zoledronate (Figure 28). Additionally, the inability of mAb 130.2 to inhibit the upregulation of CD25 and CD69 on Vγ9Vδ2 T cells from peripheral blood (Figure 30) suggests that BTN3A1 might not play a decisive role in this specific infection model and that other receptors may have a major role in this context.

The upregulation of activation markers by Vγ9Vδ2 T cells in peripheral blood has been previously demonstrated [109]. These cells also secrete IFNγ in response to infection with influenza A virus [49, 109], which suggests that the Vγ9Vδ2 T cell-mediated response is an additional mechanism of defense to viral infection along with those mediated by NK and $CD8^+$ T cells. But in this response, other molecules different to BTN3A1 seem to have the central role. Qin and collaborators have showed that IPP-expanded Vγ9Vδ2 T cells, co-cultured with monocyte-derived macrophages infected with human and avian influenza viruses, express NKG2D and that their cytotoxicity is blocked by a NKG2D neutralizing antibody [48]. Additional evidence on the role of this receptor in the Vγ9Vδ2 T cell-mediated control of influenza infection has come from the results of Li and colleagues, who found that MHC class I polypeptide-related sequence A (MICA) and MICB, ligands of NKG2D, are upregulated in lung alveolar epithelial cells infected with influenza virus [147]. They also demonstrate that pamidronate-expanded Vγ9Vδ2 T cells express NKG2D, and that treatment with a neutralizing antibody specific for this receptor abrogates the cytotoxicity of Vγ9Vδ2 T cells of influenza-infected cells. This evidence indicates that the response of this γδ T cell subpopulation toward cells infected with influenza virus relies mainly on NKG2D or related receptors.

It has been demonstrated that activation of Vγ9Vδ2 T cells (measured by IFNγ production) in response to influenza infection depends on the mevalonate pathway [109] since mevastatin, the HMG-CoA reductase inhibitor, suppresses the Vγ9Vδ2 T cell-mediated respose. This indicates that influenza infection induces intracellular

accumulation of IPP. The high levels of IPP are then transmitted by BTN3A1 to Vγ9Vδ2 T cells, inducing their activation. Despite of the importance of BTN3A1 to mediate Vγ9Vδ2 T cell reponse, it is possible that many molecules with redundant functions are involved in the pursuit of the elimination of influenza virus infection by these cells. Thus, in the absence of BTN3A1 function (after mAb 103.2 treatment), other receptors proteins will still mediate Vγ9Vδ2 T cell activation and the elimination of the infection. More research on this topic will allow to unravel the exact mechanisms of Vγ9Vδ2 T cells response to viral infection.

## 4.7 Conclusions and perspectives

The mechanisms of regulation of Vγ9Vδ2 T cells and several aspects of their physiology in human beings are still an enigma. In the last few years, the work of many groups in this field has started to yield important results that show the complexity of this T cell subset. Indeed, in the last five years the experiments that led to the identification of BTN3A1 as a crucial molecule for Vγ9Vδ2 T cell response permitted an enormous advance to understand the role of these cells in the immune response. The ability of Vγ9Vδ2 T cells to rapidly respond to infection or cell transformation and their ability to recognize molecules associated with cellular stress without MHC restriction makes them an interesting target for immunotherapy. Therefore, it is mandatory to fill the gaps in the knowledge about Vγ9Vδ2 T cells to effectively manage them in the clinic.

The results exposed in the present work support the importance of BTN3A1 in the PAg-dependent and independent activation of Vγ9Vδ2 T cells. Moreover, it shows data that underscores the participation of other genes on Chr6. These data set the base for future work that will focus on the identification of additional genes, findings that may permit understanding of the mechanisms of PAg presentation. The data described in here also gives some hints on how to design animal models for Vγ9Vδ2 T cells. Additional work will be required to validate the observations of direct interaction between BTN3A1 and Vγ9Vδ2 TCR, in order to exclude alternative

interpretations of the findings and to solve the molecular basis of PAg-dependent Vγ9Vδ2 T cell activation and the role of BTN3A1 in this process.

This research also shows additional evidence of marked CDR3-influenced clonotypic differences in the Vγ9Vδ2 T cell response to PAg. Evidence that is important to define clones of Vγ9Vδ2 T cells with higher affinity to recognize tumor or infected cells. In addition, the findings described in here provide new additional proof that mAb 20.1-mediated response is also influenced by CDR3 differences, which makes the Vγ9Vδ2 TCR MOP an interesting tool to study Vγ9Vδ2 T cell activation. In this context, it will be interesting to evaluate whether the engagement of BTN3A1 by mAb 20.1 would improve the activation of Vγ9Vδ2 T cell clones that are unresponsive to PAg- or aminobisphosphonates-induced responses. Another approach would be to transfer selected Vγ9Vδ2 TCR with high affinity for PAg or mAb 20.1 to αβ T cells, which may turn these cells sensitive to a broader range of tumors or infections [140].

The findings presented herein demonstrate that mAb 20.1 does not synergize with the PAg-induced response, but it inhibits the Vγ9Vδ2 activation mediated by these compounds. A strategy to understand how BTN3A1 modulate the activation of Vγ9Vδ2 T cells will be to test if BTN3A molecules promote or inhibit Vγ9Vδ2 T cell activation according to: the molecular configuration they assume at the cell membrane; the interaction they have with antigens or agonistic antibodies; or even both possibilities. Future studies of crystallography of the molecular interaction will bring further insights in this matter.

Besides of the central role of BTN3A1 in the Vγ9Vδ2 T cell activation, the experimental system used in this work provided additional evidence that highlights the role for BTN3A2 and BTN3A3 in the Vγ9Vδ2 T cell activation. BTN3A2 isoform supports activation in response to PAg and mAb 20.1, while the BTN3A3 is mainly involved in the mAb 20.1-evoked activation. Since the importance of these two isoforms seems to vary depending on the experimental system, it is mandatory to establish the actual role of these proteins. Important question to answer in this field will be: Why do human cells have three BTN3A types of molecules? What function they actually have? Do they cooperate together? Or they act independently?

This dissertation provides evidence that mutations in the extracellular domain of BTN3A1 do not impair the PAg-mediated response. These findings support the notion that the sensing of intracytoplasmatic PAg changes by BTN3A1 through its B30.2 intracellular domain is a critical step in the Vγ9Vδ2 T cell activation. The relevance of this domain has become clearer over the time by evidence that confirms binding of PAg to the B30.2 domain and also by its important role in the BTN3A1-mediated Vγ9Vδ2 T cell activation. Future research on this topic should aim to identify molecules, which (like periplakin) have the ability to bind to the B30.2 domain.

The co-cultures of Vγ9Vδ2 TCR transductant rat/mouse cells with influenza A virus-infected cells and the infection of PMBCs with this virus did not show any key role for BTN3A1. This suggests that several molecules with redundant functions are involved in the elimination of the infection caused by influenza A. Research on this topic should aim to determine the Vγ9Vδ2 T cell molecules that are critical during viral infection, and the role of the interaction of these molecules with viral proteins or hosts products induced by viral infection.

In summary, the evidence offered by this thesis, together with the work from others [134, 136, 149, 155], allows one to hypothesize a model in which BTN3A1 detects prenyl pyrophosphates in the cytoplasm via its B30.2 domain. Binding of PAg to BTN3A1 might be direct or may need an associated protein. This protein will first bind PAg and then transfers it to BTN3A1. The compartmentalization of BTN3A1 at the cell membrane might be dependent on the BTN3A2 and BTN3A3 chaperone function. The binding of PAg to the B30.2 domain induces a multimerization of BTN3A1 or a conformational change of its extracellular domain (mimicked by mAb 20.1). These modifications might be recognized by the Vγ9Vδ2 TCR or by an associated T cell protein. In the case that the TCR directly recognizes BTN3A1, the intensity of the response will depend on the Vγ9Vδ2 TCR clonotype and its affinity for the stimulus. Future research will allow the confirmations whether this is the actual mechanism of BTN3A1-mediated activation and how it functions in specific cancer and infection models. A better understanding of BTN3A1, its

interaction with other proteins, and its actual role in the activation of Vγ9Vδ2 T cells will be necessary to transform these cells into effective tools in the clinic.

# REFERENCES


1. Delves, P.J. and I.M. Roitt, *The immune system. First of two parts.* N Engl J Med, 2000. **343**(1): p. 37-49.
2. Parkin, J. and B. Cohen, *An overview of the immune system.* The Lancet, 2001. **357**(9270): p. 1777-1789.
3. Chaplin, D.D., *Overview of the immune response.* J Allergy Clin Immunol, 2010. **125**(2 Suppl 2): p. S3-23.
4. Flajnik, M.F. and M. Kasahara, *Origin and evolution of the adaptive immune system: genetic events and selective pressures.* Nat Rev Genet, 2010. **11**(1): p. 47-59.
5. Pancer, Z. and M.D. Cooper, *The evolution of adaptive immunity.* Annu Rev Immunol, 2006. **24**: p. 497-518.
6. Litman, G.W., J.P. Rast, and S.D. Fugmann, *The origins of vertebrate adaptive immunity.* Nat Rev Immunol, 2010. **10**(8): p. 543-53.
7. Williams, A.F. and A.N. Barclay, *The immunoglobulin superfamily--domains for cell surface recognition.* Annu Rev Immunol, 1988. **6**: p. 381-405.
8. Tonegawa, S., *Somatic generation of antibody diversity.* Nature, 1983. **302**(5909): p. 575-81.
9. Bassing, C.H., W. Swat, and F.W. Alt, *The mechanism and regulation of chromosomal V(D)J recombination.* Cell, 2002. **109 Suppl**: p. S45-55.
10. Bentley, G.A. and R.A. Mariuzza, *The structure of the T cell antigen receptor.* Annu Rev Immunol, 1996. **14**: p. 563-90.
11. Garcia, K.C., et al., *An alphabeta T cell receptor structure at 2.5 A and its orientation in the TCR-MHC complex.* Science, 1996. **274**(5285): p. 209-19.
12. Rossjohn, J., et al., *T cell antigen receptor recognition of antigen-presenting molecules.* Annu Rev Immunol, 2015. **33**: p. 169-200.
13. Zuniga-Pflucker, J.C., *T-cell development made simple.* Nat Rev Immunol, 2004. **4**(1): p. 67-72.
14. Wigzell, H., et al., *T cell V-gene usage in man in some normal and abnormal situations.* Ann N Y Acad Sci, 1991. **636**: p. 9-19.
15. Geursen, A., et al., *Population study of T cell receptor V beta gene usage in peripheral blood lymphocytes: differences in ethnic groups.* Clin Exp Immunol, 1993. **94**(1): p. 201-7.
16. Broere, F., et al., *T cell subsets and T cell-mediated immunity.* 2011: p. 15-27.
17. Smith-Garvin, J.E., G.A. Koretzky, and M.S. Jordan, *T cell activation.* Annu Rev Immunol, 2009. **27**: p. 591-619.
18. Wucherpfennig, K.W., et al., *Structural biology of the T-cell receptor: insights into receptor assembly, ligand recognition, and initiation of signaling.* Cold Spring Harb Perspect Biol, 2010. **2**(4): p. a005140.
19. Barber, E.K., et al., *The CD4 and CD8 antigens are coupled to a*

*protein-tyrosine kinase (p56lck) that phosphorylates the CD3 complex.* Proc Natl Acad Sci U S A, 1989. **86**(9): p. 3277-81.
20. Hayday, A.C., *[gamma][delta] cells: a right time and a right place for a conserved third way of protection.* Annu Rev Immunol, 2000. **18**: p. 975-1026.
21. Carding, S.R. and P.J. Egan, *Gammadelta T cells: functional plasticity and heterogeneity.* Nat Rev Immunol, 2002. **2**(5): p. 336-45.
22. Kalyan, S. and D. Kabelitz, *Defining the nature of human gammadelta T cells: a biographical sketch of the highly empathetic.* Cell Mol Immunol, 2013. **10**(1): p. 21-9.
23. Vantourout, P. and A. Hayday, *Six-of-the-best: unique contributions of gammadelta T cells to immunology.* Nat Rev Immunol, 2013. **13**(2): p. 88-100.
24. Vermijlen, D. and I. Prinz, *Ontogeny of Innate T Lymphocytes - Some Innate Lymphocytes are More Innate than Others.* Front Immunol, 2014. **5**: p. 486.
25. Kazen, A.R. and E.J. Adams, *Evolution of the V, D, and J gene segments used in the primate gammadelta T-cell receptor reveals a dichotomy of conservation and diversity.* Proc Natl Acad Sci U S A, 2011. **108**(29): p. E332-40.
26. Elliott, J.F., et al., *The adult T-cell receptor delta-chain is diverse and distinct from that of fetal thymocytes.* Nature, 1988. **331**(6157): p. 627-31.
27. Hata, S., et al., *Diversity and organization of human T cell receptor delta variable gene segments.* J Exp Med, 1989. **169**(1): p. 41-57.
28. Davis, M.M. and P.J. Bjorkman, *T-cell antigen receptor genes and T-cell recognition.* Nature, 1988. **334**(6181): p. 395-402.
29. Rock, E.P., et al., *CDR3 length in antigen-specific immune receptors.* J Exp Med, 1994. **179**(1): p. 323-8.
30. Asarnow, D.M., et al., *Limited diversity of gamma delta antigen receptor genes of Thy-1+ dendritic epidermal cells.* Cell, 1988. **55**(5): p. 837-47.
31. Six, A., et al., *Characterization of avian T-cell receptor gamma genes.* Proc Natl Acad Sci U S A, 1996. **93**(26): p. 15329-34.
32. Deusch, K., et al., *A major fraction of human intraepithelial lymphocytes simultaneously expresses the gamma/delta T cell receptor, the CD8 accessory molecule and preferentially uses the V delta 1 gene segment.* Eur J Immunol, 1991. **21**(4): p. 1053-9.
33. Kagnoff, M.F., *Current concepts in mucosal immunity. III. Ontogeny and function of gamma delta T cells in the intestine.* Am J Physiol, 1998. **274**(3 Pt 1): p. G455-8.
34. Jameson, J. and W.L. Havran, *Skin gammadelta T-cell functions in homeostasis and wound healing.* Immunol Rev, 2007. **215**: p. 114-22.
35. Kuhnlein, P., et al., *Identification and characterization of rat gamma/delta T lymphocytes in peripheral lymphoid organs, small intestine, and skin with a monoclonal antibody to a constant determinant of the gamma/delta T cell receptor.* J Immunol, 1994. **153**(3): p. 979-86.
36. Straube, F. and T. Herrmann, *Differential modulation of CD8beta by rat gammadelta and alphabeta T cells after activation.* Immunology, 2001. **104**(3): p. 252-8.

37. Wilson, E., M.K. Aydintug, and M.A. Jutila, *A circulating bovine gamma delta T cell subset, which is found in large numbers in the spleen, accumulates inefficiently in an artificial site of inflammation: correlation with lack of expression of E-selectin ligands and L-selectin.* J Immunol, 1999. **162**(8): p. 4914-9.
38. Berndt, A., J. Pieper, and U. Methner, *Circulating gamma delta T cells in response to Salmonella enterica serovar enteritidis exposure in chickens.* Infect Immun, 2006. **74**(7): p. 3967-78.
39. Bonneville, M., R.L. O'Brien, and W.K. Born, *Gammadelta T cell effector functions: a blend of innate programming and acquired plasticity.* Nat Rev Immunol, 2010. **10**(7): p. 467-78.
40. Kozbor, D., et al., *Human TCR-gamma+/delta+, CD8+ T lymphocytes recognize tetanus toxoid in an MHC-restricted fashion.* J Exp Med, 1989. **169**(5): p. 1847-51.
41. Kozbor, D., et al., *Expression and function of gamma delta- and alpha beta-T cell receptor heterodimers on human somatic T cell hybrids.* J Immunol, 1990. **144**(10): p. 3677-83.
42. Rust, C.J., et al., *Specific recognition of staphylococcal enterotoxin A by human T cells bearing receptors with the V gamma 9 region.* Nature, 1990. **346**(6284): p. 572-4.
43. Guo, Y., et al., *Human T-cell recognition of Listeria monocytogenes: recognition of listeriolysin O by TcR alpha beta + and TcR gamma delta + T cells.* Infect Immun, 1995. **63**(6): p. 2288-94.
44. Kabelitz, D., et al., *A large fraction of human peripheral blood gamma/delta + T cells is activated by Mycobacterium tuberculosis but not by its 65-kD heat shock protein.* J Exp Med, 1990. **171**(3): p. 667-79.
45. Spencer, C.T., et al., *Only a Subset of Phosphoantigen-Responsive 9 2 T Cells Mediate Protective Tuberculosis Immunity.* The Journal of Immunology, 2008. **181**(7): p. 4471-4484.
46. Chen, C.Y., et al., *Phosphoantigen/IL2 expansion and differentiation of Vgamma2Vdelta2 T cells increase resistance to tuberculosis in nonhuman primates.* PLoS Pathog, 2013. **9**(8): p. e1003501.
47. Wang, L., et al., *Antibacterial effect of human Vγ2Vδ2 T cells in vivo.* Journal of Clinical Investigation, 2001. **108**(9): p. 1349-1357.
48. Qin, G., et al., *Phosphoantigen-expanded human gammadelta T cells display potent cytotoxicity against monocyte-derived macrophages infected with human and avian influenza viruses.* J Infect Dis, 2009. **200**(6): p. 858-65.
49. Qin, G., et al., *Type 1 responses of human Vgamma9Vdelta2 T cells to influenza A viruses.* J Virol, 2011. **85**(19): p. 10109-16.
50. Qin, G., et al., *Phenotypic and functional characterization of human gammadelta T-cell subsets in response to influenza A viruses.* J Infect Dis, 2012. **205**(11): p. 1646-53.
51. Meuter, S., M. Eberl, and B. Moser, *Prolonged antigen survival and cytosolic export in cross-presenting human gammadelta T cells.* Proc Natl Acad Sci U S A, 2010. **107**(19): p. 8730-5.
52. He, X., et al., *The potential role of CD16+ Vgamma2Vdelta2 T cell-mediated antibody-dependent cell-mediated cytotoxicity in control of HIV type 1*

*disease.* AIDS Res Hum Retroviruses, 2013. **29**(12): p. 1562-70.
53. Pauza, C.D., et al., *gammadelta T Cells in HIV Disease: Past, Present, and Future.* Front Immunol, 2014. **5**: p. 687.
54. De Paoli, P., et al., *Gamma delta T cell receptor-bearing lymphocytes during Epstein-Barr virus infection.* J Infect Dis, 1990. **161**(5): p. 1013-6.
55. Lafarge, X., et al., *Cytomegalovirus infection in transplant recipients resolves when circulating gammadelta T lymphocytes expand, suggesting a protective antiviral role.* J Infect Dis, 2001. **184**(5): p. 533-41.
56. Tseng, C.T., et al., *Characterization of liver T-cell receptor gammadelta T cells obtained from individuals chronically infected with hepatitis C virus (HCV): evidence for these T cells playing a role in the liver pathology associated with HCV infections.* Hepatology, 2001. **33**(5): p. 1312-20.
57. Russo, D.M., et al., *Antigen-reactive gamma delta T cells in human leishmaniasis.* J Immunol, 1993. **151**(7): p. 3712-8.
58. Subauste, C.S., et al., *Preferential activation and expansion of human peripheral blood gamma delta T cells in response to Toxoplasma gondii in vitro and their cytokine production and cytotoxic activity against T. gondii-infected cells.* J Clin Invest, 1995. **96**(1): p. 610-9.
59. Egan, C.E., et al., *A Requirement for the V 1+ Subset of Peripheral T Cells in the Control of the Systemic Growth of Toxoplasma gondii and Infection-Induced Pathology.* The Journal of Immunology, 2005. **175**(12): p. 8191-8199.
60. D'Ombrain, M.C., et al., *gammadelta-T cells expressing NK receptors predominate over NK cells and conventional T cells in the innate IFN-gamma response to Plasmodium falciparum malaria.* Eur J Immunol, 2007. **37**(7): p. 1864-73.
61. Maher, C.O., et al., *Candida albicans stimulates IL-23 release by human dendritic cells and downstream IL-17 secretion by Vdelta1 T cells.* J Immunol, 2015. **194**(12): p. 5953-60.
62. Zheng, J., et al., *gammadelta-T cells: an unpolished sword in human anti-infection immunity.* Cell Mol Immunol, 2013. **10**(1): p. 50-7.
63. Silva-Santos, B., K. Serre, and H. Norell, *gammadelta T cells in cancer.* Nat Rev Immunol, 2015. **15**(11): p. 683-91.
64. Wilhelm, M., et al., *Gammadelta T cells for immune therapy of patients with lymphoid malignancies.* Blood, 2003. **102**(1): p. 200-6.
65. Dieli, F., et al., *Targeting human {gamma}delta} T cells with zoledronate and interleukin-2 for immunotherapy of hormone-refractory prostate cancer.* Cancer Res, 2007. **67**(15): p. 7450-7.
66. Gomes, A.Q., D.S. Martins, and B. Silva-Santos, *Targeting gammadelta T lymphocytes for cancer immunotherapy: from novel mechanistic insight to clinical application.* Cancer Res, 2010. **70**(24): p. 10024-7.
67. Hannani, D., et al., *Harnessing gammadelta T cells in anticancer immunotherapy.* Trends Immunol, 2012. **33**(5): p. 199-206.
68. Fisch, P., et al., *Recognition by human V gamma 9/V delta 2 T cells of a GroEL homolog on Daudi Burkitt's lymphoma cells.* Science, 1990. **250**(4985): p. 1269-73.
69. Selin, L.K., et al., *Reactivity of gamma delta T cells induced by the tumour*

*cell line RPMI 8226: functional heterogeneity of clonal populations and role of GroEL heat shock proteins.* Scand J Immunol, 1992. **36**(1): p. 107-17.
70. Meraviglia, S., et al., *In vivo manipulation of Vgamma9Vdelta2 T cells with zoledronate and low-dose interleukin-2 for immunotherapy of advanced breast cancer patients.* Clin Exp Immunol, 2010. **161**(2): p. 290-7.
71. Gertner-Dardenne, J., et al., *Bromohydrin pyrophosphate enhances antibody-dependent cell-mediated cytotoxicity induced by therapeutic antibodies.* Blood, 2009. **113**(20): p. 4875-84.
72. Oberg, H.H., et al., *Novel bispecific antibodies increase gammadelta T-cell cytotoxicity against pancreatic cancer cells.* Cancer Res, 2014. **74**(5): p. 1349-60.
73. Oberg, H.H., et al., *gammadelta T cell activation by bispecific antibodies.* Cell Immunol, 2015. **296**(1): p. 41-9.
74. Fisher, J.P., et al., *Effective combination treatment of GD2-expressing neuroblastoma and Ewing's sarcoma using anti-GD2 ch14.18/CHO antibody with Vgamma9Vdelta2+ gammadeltaT cells.* Oncoimmunology, 2016. **5**(1): p. e1025194.
75. Bukowski, J.F., et al., *V gamma 2V delta 2 TCR-dependent recognition of non-peptide antigens and Daudi cells analyzed by TCR gene transfer.* J Immunol, 1995. **154**(3): p. 998-1006.
76. Correia, D.V., A. Lopes, and B. Silva-Santos, *Tumor cell recognition by gammadelta T lymphocytes: T-cell receptor vs. NK-cell receptors.* Oncoimmunology, 2013. **2**(1): p. e22892.
77. Scotet, E., et al., *Tumor recognition following Vgamma9Vdelta2 T cell receptor interactions with a surface F1-ATPase-related structure and apolipoprotein A-I.* Immunity, 2005. **22**(1): p. 71-80.
78. Nedellec, S., M. Bonneville, and E. Scotet, *Human Vgamma9Vdelta2 T cells: from signals to functions.* Semin Immunol, 2010. **22**(4): p. 199-206.
79. Rincon-Orozco, B., et al., *Activation of V 9V 2 T Cells by NKG2D.* The Journal of Immunology, 2005. **175**(4): p. 2144-2151.
80. Lanca, T., et al., *The MHC class Ib protein ULBP1 is a nonredundant determinant of leukemia/lymphoma susceptibility to gammadelta T-cell cytotoxicity.* Blood, 2010. **115**(12): p. 2407-11.
81. Martino, A., et al., *Complementary function of gamma delta T-lymphocytes and dendritic cells in the response to isopentenyl-pyrophosphate and lipopolysaccharide antigens.* J Clin Immunol, 2005. **25**(3): p. 230-7.
82. Shrestha, N., et al., *Regulation of acquired immunity by gamma delta T-cell/dendritic-cell interactions.* Ann N Y Acad Sci, 2005. **1062**: p. 79-94.
83. Devilder, M.C., et al., *Potentiation of antigen-stimulated V gamma 9V delta 2 T cell cytokine production by immature dendritic cells (DC) and reciprocal effect on DC maturation.* J Immunol, 2006. **176**(3): p. 1386-93.
84. Dunne, M.R., et al., *(E)-4-hydroxy-3-methyl-but-2 enyl pyrophosphate-stimulated Vgamma9Vdelta2 T cells possess T helper type 1-promoting adjuvant activity for human monocyte-derived dendritic cells.* Cancer Immunol Immunother, 2010. **59**(7): p. 1109-20.
85. Conti, L., et al., *Reciprocal Activating Interaction Between Dendritic Cells and Pamidronate-Stimulated T Cells: Role of CD86 and Inflammatory*

*Cytokines.* The Journal of Immunology, 2004. **174**(1): p. 252-260.
86. Eberl, M., et al., *A rapid crosstalk of human gammadelta T cells and monocytes drives the acute inflammation in bacterial infections.* PLoS Pathog, 2009. **5**(2): p. e1000308.
87. Davey, M.S., et al., *Microbe-specific unconventional T cells induce human neutrophil differentiation into antigen cross-presenting cells.* J Immunol, 2014. **193**(7): p. 3704-16.
88. Vermijlen, D., et al., *Distinct Cytokine-Driven Responses of Activated Blood T Cells: Insights into Unconventional T Cell Pleiotropy.* The Journal of Immunology, 2007. **178**(7): p. 4304-4314.
89. Brandes, M., et al., *Flexible migration program regulates gamma delta T-cell involvement in humoral immunity.* Blood, 2003. **102**(10): p. 3693-701.
90. Caccamo, N., et al., *CXCR5 Identifies a Subset of V 9V 2 T Cells which Secrete IL-4 and IL-10 and Help B Cells for Antibody Production.* The Journal of Immunology, 2006. **177**(8): p. 5290-5295.
91. Petrasca, A. and D.G. Doherty, *Human Vdelta2(+) gammadelta T Cells Differentially Induce Maturation, Cytokine Production, and Alloreactive T Cell Stimulation by Dendritic Cells and B Cells.* Front Immunol, 2014. **5**: p. 650.
92. Brandes, M., K. Willimann, and B. Moser, *Professional antigen-presentation function by human gammadelta T Cells.* Science, 2005. **309**(5732): p. 264-8.
93. Brandes, M., et al., *Cross-presenting human gammadelta T cells induce robust CD8+ alphabeta T cell responses.* Proc Natl Acad Sci U S A, 2009. **106**(7): p. 2307-12.
94. Wu, Y., et al., *Human gamma delta T cells: a lymphoid lineage cell capable of professional phagocytosis.* J Immunol, 2009. **183**(9): p. 5622-9.
95. Born, W.K., M. Kemal Aydintug, and R.L. O'Brien, *Diversity of gammadelta T-cell antigens.* Cell Mol Immunol, 2013. **10**(1): p. 13-20.
96. Belmant, C., et al., *3-Formyl-1-butyl pyrophosphate A novel mycobacterial metabolite-activating human gammadelta T cells.* J Biol Chem, 1999. **274**(45): p. 32079-84.
97. Jomaa, H., et al., *Vgamma9/Vdelta2 T cell activation induced by bacterial low molecular mass compounds depends on the 1-deoxy-D-xylulose 5-phosphate pathway of isoprenoid biosynthesis.* FEMS Immunol Med Microbiol, 1999. **25**(4): p. 371-8.
98. Espinosa, E., et al., *Y2K+1 state-of-the-art on non-peptide phosphoantigens, a novel category of immunostimulatory molecules.* Microbes Infect, 2001. **3**(8): p. 645-54.
99. Zhang, Y., et al., *Structural studies of Vgamma2Vdelta2 T cell phosphoantigens.* Chem Biol, 2006. **13**(9): p. 985-92.
100. Harly, C., C.M. Peigne, and E. Scotet, *Molecules and Mechanisms Implicated in the Peculiar Antigenic Activation Process of Human Vgamma9Vdelta2 T Cells.* Front Immunol, 2014. **5**: p. 657.
101. Holstein, S.A. and R.J. Hohl, *Isoprenoids: remarkable diversity of form and function.* Lipids, 2004. **39**(4): p. 293-309.
102. Morita, C.T., et al., *Nonpeptide antigens, presentation mechanisms, and immunological memory of human Vgamma2Vdelta2 T cells: discriminating friend from foe through the recognition of prenyl pyrophosphate antigens.*

Immunol Rev, 2007. **215**: p. 59-76.
103. Eberl, M., et al., *Accumulation of a potent gammadelta T-cell stimulator after deletion of the lytB gene in Escherichia coli.* Immunology, 2002. **106**(2): p. 200-11.
104. Hintz, M., et al., *Identification of (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate as a major activator for human gammadelta T cells in Escherichia coli.* FEBS Lett, 2001. **509**(2): p. 317-22.
105. Reichenberg, A., et al., *Replacing the pyrophosphate group of HMB-PP by a diphosphonate function abrogates Its potential to activate human γδ T cells but does not lead to competitive antagonism.* Bioorganic & Medicinal Chemistry Letters, 2003. **13**(7): p. 1257-1260.
106. Kunzmann, V., et al., *Stimulation of gammadelta T cells by aminobisphosphonates and induction of antiplasma cell activity in multiple myeloma.* Blood, 2000. **96**(2): p. 384-92.
107. Gober, H.-J., et al., *Human T Cell Receptor γδ Cells Recognize Endogenous Mevalonate Metabolites in Tumor Cells.* The Journal of Experimental Medicine, 2003. **197**(2): p. 163-168.
108. Kistowska, M., et al., *Dysregulation of the host mevalonate pathway during early bacterial infection activates human TCR gamma delta cells.* Eur J Immunol, 2008. **38**(8): p. 2200-9.
109. Jameson, J.M., et al., *A role for the mevalonate pathway in the induction of subtype cross-reactive immunity to influenza A virus by human gammadelta T lymphocytes.* Cell Immunol, 2010. **264**(1): p. 71-7.
110. Kunzmann, V., E. Bauer, and M. Wilhelm, *Gamma/delta T-cell stimulation by pamidronate.* N Engl J Med, 1999. **340**(9): p. 737-8.
111. Aft, R., et al., *Could targeting bone delay cancer progression? Potential mechanisms of action of bisphosphonates.* Crit Rev Oncol Hematol, 2012. **82**(2): p. 233-48.
112. Gnant, M. and P. Clezardin, *Direct and indirect anticancer activity of bisphosphonates: a brief review of published literature.* Cancer Treat Rev, 2012. **38**(5): p. 407-15.
113. Dunford, J.E., et al., *Structure-activity relationships for inhibition of farnesyl diphosphate synthase in vitro and inhibition of bone resorption in vivo by nitrogen-containing bisphosphonates.* J Pharmacol Exp Ther, 2001. **296**(2): p. 235-42.
114. Das, H., et al., *Vgamma2Vdelta2 T-cell receptor-mediated recognition of aminobisphosphonates.* Blood, 2001. **98**(5): p. 1616-8.
115. Thompson, K., J. Rojas-Navea, and M.J. Rogers, *Alkylamines cause Vgamma9Vdelta2 T-cell activation and proliferation by inhibiting the mevalonate pathway.* Blood, 2006. **107**(2): p. 651-4.
116. Bukowski, J.F., C.T. Morita, and M.B. Brenner, *Human gamma delta T cells recognize alkylamines derived from microbes, edible plants, and tea: implications for innate immunity.* Immunity, 1999. **11**(1): p. 57-65.
117. Kamath, A.B., et al., *Antigens in tea-beverage prime human Vgamma 2Vdelta 2 T cells in vitro and in vivo for memory and nonmemory antibacterial cytokine responses.* Proc Natl Acad Sci U S A, 2003. **100**(10): p. 6009-14.
118. Sturm, E., et al., *Human V gamma 9-V delta 2 T cell receptor-gamma delta*

*lymphocytes show specificity to Daudi Burkitt's lymphoma cells.* J Immunol, 1990. **145**(10): p. 3202-8.
119. Panchamoorthy, G., et al., *A predominance of the T cell receptor V gamma 2/V delta 2 subset in human mycobacteria-responsive T cells suggests germline gene encoded recognition.* J Immunol, 1991. **147**(10): p. 3360-9.
120. Davodeau, F., et al., *Close correlation between Daudi and mycobacterial antigen recognition by human gamma delta T cells and expression of V9JPC1 gamma/V2DJC delta-encoded T cell receptors.* J Immunol, 1993. **151**(3): p. 1214-23.
121. Correia, D.V., et al., *Highly active microbial phosphoantigen induces rapid yet sustained MEK/Erk- and PI-3K/Akt-mediated signal transduction in anti-tumor human gammadelta T-cells.* PLoS One, 2009. **4**(5): p. e5657.
122. Nedellec, S., et al., *NKG2D costimulates human V gamma 9V delta 2 T cell antitumor cytotoxicity through protein kinase C theta-dependent modulation of early TCR-induced calcium and transduction signals.* J Immunol, 2010. **185**(1): p. 55-63.
123. De Libero, G., et al., *Selection by two powerful antigens may account for the presence of the major population of human peripheral gamma/delta T cells.* J Exp Med, 1991. **173**(6): p. 1311-22.
124. Lang, F., et al., *Early activation of human V gamma 9V delta 2 T cell broad cytotoxicity and TNF production by nonpeptidic mycobacterial ligands.* J Immunol, 1995. **154**(11): p. 5986-94.
125. Morita, C.T., et al., *Direct presentation of nonpeptide prenyl pyrophosphate antigens to human gamma delta T cells.* Immunity, 1995. **3**(4): p. 495-507.
126. Kato, Y., et al., *Requirement of Species-Specific Interactions for the Activation of Human T Cells by Pamidronate.* The Journal of Immunology, 2003. **170**(7): p. 3608-3613.
127. Simone, R., et al., *Ligation of the BT3 molecules, members of the B7 family, enhance the proinflammatory responses of human monocytes and monocyte-derived dendritic cells.* Mol Immunol, 2010. **48**(1-3): p. 109-18.
128. Smith, I.A., et al., *BTN1A1, the mammary gland butyrophilin, and BTN2A2 are both inhibitors of T cell activation.* J Immunol, 2010. **184**(7): p. 3514-25.
129. Messal, N., et al., *Differential role for CD277 as a co-regulator of the immune signal in T and NK cells.* Eur J Immunol, 2011. **41**(12): p. 3443-54.
130. Abeler-Dorner, L., et al., *Butyrophilins: an emerging family of immune regulators.* Trends Immunol, 2012. **33**(1): p. 34-41.
131. Arnett, H.A. and J.L. Viney, *Immune modulation by butyrophilins.* Nat Rev Immunol, 2014. **14**(8): p. 559-69.
132. Rhodes, D.A., et al., *The cluster of BTN genes in the extended major histocompatibility complex.* Genomics, 2001. **71**(3): p. 351-62.
133. Compte, E., et al., *Frontline: Characterization of BT3 molecules belonging to the B7 family expressed on immune cells.* Eur J Immunol, 2004. **34**(8): p. 2089-99.
134. Palakodeti, A., et al., *The molecular basis for modulation of human Vgamma9Vdelta2 T cell responses by CD277/butyrophilin-3 (BTN3A)-specific antibodies.* J Biol Chem, 2012. **287**(39): p. 32780-90.
135. Karunakaran, M.M., et al., *Vgamma9 and Vdelta2 T cell antigen receptor*

*genes and butyrophilin 3 (BTN3) emerged with placental mammals and are concomitantly preserved in selected species like alpaca (Vicugna pacos).* Immunogenetics, 2014. **66**(4): p. 243-54.
136. Harly, C., et al., *Key implication of CD277/butyrophilin-3 (BTN3A) in cellular stress sensing by a major human gammadelta T-cell subset.* Blood, 2012. **120**(11): p. 2269-79.
137. Vavassori, S., et al., *Butyrophilin 3A1 binds phosphorylated antigens and stimulates human gammadelta T cells.* Nat Immunol, 2013. **14**(9): p. 908-16.
138. Sandstrom, A., et al., *The intracellular B30.2 domain of butyrophilin 3A1 binds phosphoantigens to mediate activation of human Vgamma9Vdelta2 T cells.* Immunity, 2014. **40**(4): p. 490-500.
139. Luhder, F., et al., *Topological requirements and signaling properties of T cell-activating, anti-CD28 antibody superagonists.* J Exp Med, 2003. **197**(8): p. 955-66.
140. Grunder, C., et al., *gamma9 and delta2CDR3 domains regulate functional avidity of T cells harboring gamma9delta2TCRs.* Blood, 2012. **120**(26): p. 5153-62.
141. Kreiss, M., et al., *Contrasting contributions of complementarity-determining region 2 and hypervariable region 4 of rat BV8S2+ (Vbeta8.2) TCR to the recognition of myelin basic protein and different types of bacterial superantigens.* Int Immunol, 2004. **16**(5): p. 655-63.
142. Knodel, M., et al., *Reversal of Blimp-1-mediated apoptosis by A1, a member of the Bcl-2 family.* Eur J Immunol, 1999. **29**(9): p. 2988-98.
143. Soneoka, Y., et al., *A transient three-plasmid expression system for the production of high titer retroviral vectors.* Nucleic Acids Res, 1995. **23**(4): p. 628-33.
144. Pietschmann, T., et al., *Foamy virus capsids require the cognate envelope protein for particle export.* J Virol, 1999. **73**(4): p. 2613-21.
145. Bueler, H. and R.C. Mulligan, *Induction of antigen-specific tumor immunity by genetic and cellular vaccines against MAGE: enhanced tumor protection by coexpression of granulocyte-macrophage colony-stimulating factor and B7-1.* Mol Med, 1996. **2**(5): p. 545-55.
146. Riano, F., et al., *Vgamma9Vdelta2 TCR-activation by phosphorylated antigens requires butyrophilin 3 A1 (BTN3A1) and additional genes on human chromosome 6.* Eur J Immunol, 2014. **44**(9): p. 2571-6.
147. Li, H., et al., *Human Vgamma9Vdelta2-T cells efficiently kill influenza virus-infected lung alveolar epithelial cells.* Cell Mol Immunol, 2013. **10**(2): p. 159-64.
148. Kaufman, J., *Antigen processing and presentation: evolution from a bird's eye view.* Mol Immunol, 2013. **55**(2): p. 159-61.
149. Rhodes, D.A., et al., *Activation of human gammadelta T cells by cytosolic interactions of BTN3A1 with soluble phosphoantigens and the cytoskeletal adaptor periplakin.* J Immunol, 2015. **194**(5): p. 2390-8.
150. Sebestyen, Z., et al., *RhoB Mediates Phosphoantigen Recognition by Vgamma9Vdelta2 T Cell Receptor.* Cell Rep, 2016. **15**(9): p. 1973-85.
151. Wang, H., Z. Fang, and C.T. Morita, *Vgamma2Vdelta2 T Cell Receptor recognition of prenyl pyrophosphates is dependent on all CDRs.* J Immunol,

2010. **184**(11): p. 6209-22.
152. Bukowski, J.F., et al., *Crucial role of TCR gamma chain junctional region in prenyl pyrophosphate antigen recognition by gamma delta T cells.* J Immunol, 1998. **161**(1): p. 286-93.
153. Adams, E.J., et al., *An autonomous CDR3delta is sufficient for recognition of the nonclassical MHC class I molecules T10 and T22 by gammadelta T cells.* Nat Immunol, 2008. **9**(7): p. 777-84.
154. Rhodes, D.A., W. Reith, and J. Trowsdale, *Regulation of Immunity by Butyrophilins.* Annu Rev Immunol, 2016. **34**: p. 151-72.
155. Wang, H., et al., *Butyrophilin 3A1 plays an essential role in prenyl pyrophosphate stimulation of human Vgamma2Vdelta2 T cells.* J Immunol, 2013. **191**(3): p. 1029-42.
156. Rhodes, D.A., B. de Bono, and J. Trowsdale, *Relationship between SPRY and B30.2 protein domains. Evolution of a component of immune defence?* Immunology, 2005. **116**(4): p. 411-7.
157. Hsiao, C.H., et al., *Synthesis of a phosphoantigen prodrug that potently activates Vgamma9Vdelta2 T-lymphocytes.* Chem Biol, 2014. **21**(8): p. 945-54.

# ABBREVIATIONS

| | |
|---|---|
| A (amino acid) | Alanine |
| ABP | Aminobisphosphonate |
| ADCC | Antibody-dependent cell-mediated cytotoxicity |
| APC | Antigen presenting cell |
| BCR | B cell receptor |
| BSA | Bovine serum albumin |
| BSS | Buffered sodium salts |
| BTN3A | Butyrophilin 3A |
| CD | Cluster of differentiation |
| CDR | Complementarity-determining region |
| Chr6 | Human chromosome 6 |
| D (amino acid) | Aspartic acid |
| D (gene segment) | Diverse |
| DC | Dentritic cells |
| DMEM | Dulbecco's modified eagle medium |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| DOXP | 1-deoxy-D-xylulose 5-phosphate |
| EBV-LCL | Epstein-Barr virus-transformed B lymphoblastoid cell lines |
| EDTA | Ethylenediaminetetraacetic acid |
| EMEM | Eagle's minimum essential medium |
| FACS | Fluorescence-activated cell sorting |
| FSC | Forward scatter |
| FCS | Fethal calf serum |
| FPPS | Farnesyl pyrophosphate synthase |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| HLA | Human leucocyte antigen |
| HMBPP | (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate |
| HMG-CoA | 3-hydroxy-3 methyl-glutaryl CoA |
| IFN$\gamma$ | Interferon $\gamma$ |
| Ig | Immunoglobulin |
| IL | Interleukin |
| IPP | Isopentenyl pyrophosphate |
| J (gene segment) | Joining |
| K (amino acid) | Lysine |
| LB | Lysogenic broth |
| mAb 103.2 | Monoclonal antibody (clone) 103.2 |
| mAb 20.1 | Monoclonal antibody (clone) 20.1 |
| MACS | Magnetic-activated cell sorting |
| MDCK | Madin-Darby canine kidney (cells) |

| | |
|---|---|
| MEM | Minimum essential medium |
| MFI | Mean of fluorescence intensity |
| MHC | Major histocompatibility complex |
| MOI | Multiplicity of infection |
| MVA | Mevalonate (pathway) |
| NLRs | Nod-like receptors |
| NK | Natural killer cell |
| NKG2D | NK group 2, member D |
| PAg | Phosphoantigen |
| PBMCs | Peripheral blood monocluear cells |
| PBS | Phosphate-buffered saline |
| PCR | Polymerase chain reaction |
| Pen-strep | Penicilin-streptomycin |
| Q (amino acid) | Glutamine |
| R (amino acid) | Arginine |
| RAG | Recombination-activating gene |
| rpm | revolution per minute |
| RPMI | Roswell Park Memorial Institute |
| RT | Room temperature |
| SOB | Super optimal broth |
| SSC | Side scatter |
| TBE | Tris-borate-EDTA |
| TCR | T cell receptor |
| TE | Tris-EDTA |
| TLRs | Toll-llike receptors |
| TNF | Tumor necrosis factor |
| TPCK | L-(tosylamido-2-phenyl) ethyl chloromethyl ketone |
| TRIM | Tripartite motif family |
| ULBP | UL16-binding protein |
| V (gene segment) | Variable |

# SYMBOLS

| | |
|---|---|
| Centimeter | cm |
| Diameter | Ø |
| Grade Celsius | °C |
| Gram | g |
| Gray | Gy |
| Hour | h |
| Infinity | ∞ |
| Kilo units per milliliter | kU/mL |
| Liter | L |
| Microgram | μg |
| Microliter | μL |
| Micromolar | μM |
| Milligram | mg |
| Milliliter | mL |
| Millimolar | mM |
| Minute | min |
| Molar | M |
| Nanogram | ng |
| Nanometer | nm |
| Nanomolar | nM |
| Percentage | % |
| Plaque-forming units per millimeter | PFU/mL |
| Registered | ® |
| Second | sec |
| Trademark | ™ |
| Units per milliliter | U/mL |
| Volt | V |

# CURRICULUM VITAE

## PUBLICATIONS

Soraya Zorro, Mauricio Arias, **Felipe Riaño**, Sara Paris, Luis A Ramírez, Oscar Uribe, Luis F García, Gloria Vásquez. 2009. Response to ODN-CpG by B Cells from patients with Systemic Lupus Erythematosus (SLE) correlates with disease activity**.** Lupus, Vol. 18, No. 8, 718-726**.**

Helena del Corral, Sara C. París, Nancy D. Marín, Diana M. Marín, Lucelly López, Hanna M. Henao, Teresita Martínez, Liliana Villa, Luis F. Barrera, Blanca L. Ortiz, María E. Ramírez, Carlos J. Montes, María C. Oquendo, Lisandra M. Arango, **Felipe Riaño**, Carlos Aguirre, Alberto Bustamante, John T. Belisle, Karen Dobos, Gloria I. Mejía, Margarita R. Giraldo, Patrick J. Brennan, Jaime Robledo, María P. Arbeláez, Carlos A. Rojas, Luis F. García. 2009. IFNγ response to Mycobacterium tuberculosis, risk of infection and disease in household contacts of tuberculosis patients in Colombia. PLoS ONE 4(12): e8257.

**Riaño F**, Arroyo L, Paris S, Rojas M, Friggen AH, van Meijgaarden KE, Franken KL, Ottenhoff TH, Garcia LF, Barrera LF. T cell responses to DosR and Rpf proteins in actively and latently infected individuals from Colombia. Tuberculosis (Edinb) 2012 Mar, 92(2):148-59. Epub 2012 Jan 5.

**Riaño F**, Karunakaran MM, Starick L, Li J, Scholz CJ, Kunzmann V, Olive D, Amslinger S, Herrmann T. Vγ9Vδ2 TCR-activation by phosphorylated antigens requires butyrophilin 3 A1 (BTN3A1) and additional genes on human chromosome 6. European Journal of Immunology. 44(9): p. 2571-6. 2014 May.

## PARTICIPATIONS IN MEETINGS (Abstracts)

Jiménez JN, Correa MM, Rúa A, Zapata M, **Riaño R**, Báez P, Ramirez A. Detección Molecular de Staphylococcus aureus resistente a meticilina (MRSA) colonizando manos de individuos de población general. In: VI Encuentro Nacional de Investigación en Enfermedades Infecciosas. July 23rd-26th de 2008, Santa Marta. Colombia.

**Felipe Riaño**, Sara C. París, Tom Ottenhoff, Luis F. García, Luis F. Barrera. Response to latency (DosR) and resuscitation (Rpf) antigens of Mycobacterium tuberculosis in a Colombian population of household contacts of pulmonary tuberculosis patients. In: Mykobak 09 German-Colombian Innovation Forum. November 23rd-26th, 2009. Medellín, Colombia.

**Felipe Riaño**, Sara C. París, Tom Ottenhoff, Luis F. García, Luis F. Barrera. Response to latency antigens of Mycobacterium tuberculosis in a Colombian population of household contacts of pulmonary tuberculosis patients. In: 40th Keystone Symposia on Molecular and Cellular Biology. January 15th-20th, 2011. Vancouver, Canada. Abstract 113, pg. 86.

**F. Riaño**, M.M. Karunakaran, L. Starick, J. Li, T. Herrmann. Role of BTN3A/CD277 in the Vγ9Vδ2 T cell-mediated immune response. In: 8th International Symposium organized by the Students of the Graduate School of Life Sciences. October 9th-10th, 2013. Würzburg, Germany.

**F. Riaño**, M.M. Karunakaran, L. Starick, S. Amslinger, D. Olive, C.J. Scholz, V. Kuzmann, T. Herrmann. Minimal genetic requirements for presentation of phosphorylated antigens to Vγ9Vδ2 T cells. In: Translational Immunology-From Target to Therapy II. Else-Kröner-Forschungskolleg. April 11th-12th, 2014. Würzburg, Germany.

**F. Riaño**, M.M. Karunakaran, L. Starick, J. Li, C.J. Scholz, D. Olive, S. Amslinger, T. Müller, V. Kuzmann, T. Herrmann. Different molecular and genetic requirements for activation of Vγ9Vδ2 T cells by phosphoantigens, alkylamines and agonistic BNT3 specific antibody 20.1. In: 44th Annual Meeting of the German Society for Immunology. September17th -20th, 2014. Bonn, Germany.

**F. Riaño**, M.M. Karunakaran, L. Starick, J. Li, C.J. Scholz, D. Olive, S. Amslinger, T. Müller, V. Kuzmann, T. Herrmann. Different molecular and genetic requirements for activation of Vγ9Vδ2 T cells by phosphoantigens, alkylamines and agonistic BNT3 specific antibody 20.1. In: 9th International Symposium organized by the Students of the Graduate School of Life Sciences. October 14th-15th, 2014. Würzburg, Germany.

**F. Riaño**, L. Starick, M.M. Karunakaran, A.S. Fichtner, S. Amslinger, D. Olive, V Kunzmann, G. De Libero, S. Gu, E. Adams, T. Herrmann. Vγ9Vδ2 T cell activation: a story with different molecular and genetic requirements. In: 18th International Summer School of Immunology. Immune System: Genes, Receptors and Regulation. September 12th -19th , 2015, Rabac, Croatia.

## ACADEMIC HONORS AND AWARDS

Institutional recognition to the highest academic record during the following periods: 2006-I (first semester of the year) and 2006-II (second semester of the year). School of Microbiology, Universidad de Antioquia. 2007.

Institutional recognition for the 3rd place obtained in the scores of the Examenes de Calidad de la Educación Superior (ECAES) – Tests of Higher Education Quality – at the national level. School of Microbiology, Universidad de Antioquia. 2007.

Scholarship for young Scientists. German Academic Exchange Service. 2012-2016.

## LANGUAGES

Spanish: native speaker.

English: Qualified by the internet-based TOEFL: score 83. 2010.

German: B2 level course at Goethe Institut.

French: 400 hours-course in the Multilingua Program at the Language School. Universidad de Antioquia. 2004-2006.

Portuguese: Good speaking, reading, writing and listening.

_____

Rubén Felipe Riaño Arias

# Affidavit

I hereby confirm that my thesis entitled BTN3A1 in the immune response of Vγ9Vδ2 T cells is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Place, Date Signature

# Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation BTN3A1 in der Immunantwort der Vγ9Vδ2 T Zellen eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Ort, Datum Unterschrift