Die Evolution der Vγ9Vδ2 T-Zellen

The Evolution of Vγ9Vδ2 T Cells

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Section of Infection and Immunity

Submitted by

Mohindar Murugesh Karunakaran

From Thiruvannamalai, Tamilnadu, India

Würzburg, 2014

Submitted on:

……..……..

Office stamp

Members of the *Promotionskomitee*:

Chairperson: Prof. Thomas Hünig

Primary Supervisor: Prof. Thomas Herrmann

Supervisor (Second): Prof. Thomas Dandekar

Supervisor (Third): Prof. Lutz Walter

Date of Public Defence: ……..……

Date of Receipt of Certificates:

…….

அம்மா அப்பாவிற்கு எனது வணக்கங்கள். எனது ஆய்வறிக்கையினை என் அம்மா அப்பா ஆசான்கள் மற்றும் இறைவனுக்கு சமர்பிக்கின்றேன்.

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Summary

Human Vγ9Vδ2 T cells are the major subset of blood γδ T cells and account for 1-5% of blood T cells. Pyrophosphorylated metabolites of isoprenoid biosynthesis are recognized by human Vγ9Vδ2 T cells and are called as phosphoantigens (PAg). Isopentenyl pyrophosphate (IPP) and (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) are among the few well studied PAg. IPP is found in all organisms while HMBPP is a precursor of IPP found only in eubacteria, plants and apicomplexaen parasite. Interestingly, the PAg reactive Vγ9Vδ2 T cells are so far identified only in human and higher primates but not in rodents. Hence, Vγ9Vδ2 T cells are believed to be restricted to primates. With regard to PAg recognition, a Vγ9JP recombined TCRγ chain and certain CDR3 motifs of the TCRδ chain are mandatory. The BTN3A1 molecule is essential for a response to PAg. BTN3 is a trans-membrane protein belonging to butyrophilin family of proteins. Though BTN3A1 was found to be essential for PAg presentation, the exact molecular basis of PAg presentation still remains unclear.

This thesis presents new data on the evolution of Vγ9Vδ2 TCR and its ligands (BTN3) as well as the genetic basis of PAg presentation to Vγ9Vδ2 TCR.

The comprehensive analysis of genomic database sequences at NCBI and other public domain databases revealed for the first time that Vγ9, Vδ2 and *BTN3* genes emerged and co-evolved along with the placental mammals. Vγ9, Vδ2 and *BTN3* genes are scattered across mammalian species and not restricted to primates. But interestingly, all three genes are highly conserved between phylogenetically distinct species. Moreover, the distribution pattern of Vγ9, Vδ2 TCR genes and *BTN3* genes suggests a functional association between these genes representing the TCR - ligand relationship. Alpaca (*Vicugna pacos*), a member of the camelid family, is one among the 6 candidate non-primate species which were found to possess functional Vγ9, Vδ2 and *BTN3* genes.

From peripheral lymphocytes of alpaca, Vγ9 chain transcripts with a characteristic JP rearrangement and transcripts of Vδ2 chains with a CDR3 typical for PAg-reactive TCR were identified. The transduction of αβ TCR negative mouse thymoma BW cells with alpaca Vγ9 and Vδ2 TCR chains resulted in surface expression of the TCR complex as it was deduced from detection of cell surface expression of mouse CD3. Cross-linking of alpaca Vγ9Vδ2 TCR transductants with anti-CD3ε led to IL-2 production which confirmed that alpaca Vγ9 and Vδ2 TCR chains pair to form a functional TCR. Besides the conservation of human like Vγ9 and Vδ2 TCR chains, alpaca has conserved an orthologue for human BTN33A1 as well. Interestingly, the predicted PAg binding sites of human BTN3A1 was 100% conserved in deduced amino acid sequence of alpaca BTN3A1. All together alpaca is a promising candidate for further studies as it might have preserved Vγ9Vδ2 T cells to function in surveillance of stress and infections.

This thesis also provides the sequence of Vγ9Vδ2 TCR of African green monkey (*Chlorocebus aethiops*), which was previously unknown. Moreover, our data indicates the lack of any species specific barrier which could hinder the PAg presentation by African monkey derived COS cells to human Vγ9Vδ2 TCR and vice versa of human cells to African green monkey Vγ9Vδ2 TCR which was in contradiction to previously reported findings.

Apart from the above, the thesis also presents new data on the genetic basis of PAg presentation to Vγ9Vδ2 T cells, which revealed that human chromosome 6 is sufficient for the presentation of exogenous and endogenous PAg. By employing human/mouse somatic hybrids, we identified the role of human chromosome 6 in PAg presentation and in addition, we observed the lack of capacity of human chromosome 6 positive hybrids to activate Vγ9Vδ2 TCR transductants in the presence of the alkylamine sec-butylamine (SBA). Investigation of Chinese hamster ovary (CHO) cells containing the human chromosome 6 also yielded similar results. This suggests that aminobisphosphonates (zoledronate) and

alkylamines employ different mechanisms for activation of Vγ9Vδ2 T cells although both have been described to act by inhibition of farnesyl pyrophosphate synthase activity which is known to increase intracellular levels of the IPP.

In conclusion, this thesis suggests that Vγ9, Vδ2 and *BTN3* genes controlling Vγ9Vδ2 TCR-ligand relationship emerged and co-evolved along with placental mammals; and also identified candidate non-primate species which could possess Vγ9Vδ2 T cells. Furthermore, it suggests alpaca as a promising non-primate species to investigate the physiological function of Vγ9Vδ2 T cells. With respect to PAg antigen presentation it was shown that chromosome 6 is essential and sufficient for exogenous and endogenous PAg presentation. Moreover, the alkylamine SBA and aminobisphosphonate zoledronate may engage different cellular mechanism to exert inhibition over IPP consumption. The thesis raises interesting questions which need to be addressed in future: 1) What are the environmental and evolutionary factors involved in preservation of Vγ9Vδ2 T cells only by few species? 2) What could be the functional nature and antigen recognition properties of such a conserved T cell subset? 3) What is the genetic and molecular basis of the differential capacity of human chromosome 6 bearing rodent-human hybridoma cells in activating Vγ9Vδ2 T cells in presence of SBA and aminobisphosphonates?

Zusammenfassung

Vγ9Vδ2 T Zellen stellen im Menschen die größte Population an γδ T Zellen im Blut dar. Ihr Anteil an den Blut-T Zellen beträgt 1-5%. Humane Vγ9Vδ2 T Zellen erkennen als Phosphoantigene (PAg) bezeichnete pyrophosphorylierte Metabolite der Isoprenoidbiosynthese wobei Isopentenylpyrophosphat (IPP) und (E)-4-Hydroxy-3-methyl-but-2-enylpyrophosphat (HMBPP) zu den wenigen gut erforschten PAg gehören. IPP ist in allen Organismen zu finden während HMBPP ein IPP Vorläufer ist, der nur in Eubakterien, Pflanzen und Apikomplexa vorkommt. Interessanterweise wurden PAg-reaktive Vγ9Vδ2 T Zellen bisher nur im Menschen und höheren Primaten gefunden, aber nicht in Nagern. Daher wurde angenommen, dass Vγ9Vδ2 T Zellen eine exklusiv in Primaten vorkommende Population darstellt. Hinsichtlich der PAg-Bindung sind TCR γ Ketten mit einer Rekombination von Vγ9 und JP zwingend notwendig und bestimmte CDR3 Motive der Vδ2 TCRδ Kette, wobei die Erkennung der PAg von der Präsenz des BTN3A1 Moleküls abhängt. BTN3 ist ein Transmembranprotein und gehört zur Butyrophilinfamilie. Obwohl gezeigt wurde, das BTN3A für die PAg-Präsentierung unerlässlich ist, ist deren molekularer Mechanismus noch immer unklar.

Die vorgelegte Arbeit beinhaltet sowohl neue Daten über die Evolution des Vγ9Vδ2 TCR und dessen Liganden (BTN3), als auch über die genetischen Grundlagen der PAg-Präsentierung.

Eine umfassende Analyse genomischer Datenbanksequenzen des NCBI sowie anderer öffentlicher Datenbanken zeigte erstmals, dass Vγ9, Vδ2 und *BTN3* Gene zusammen mit den höheren Säugetieren (Placentalia) entstanden und sich gemeinsam weiter entwickelten. Vγ9, Vδ2 und *BTN3* Gene existieren über die gesamten Placentalia verteilt und nicht allein in Primaten. Erstaunlicherweise sind alle drei Gene auch zwischen phylogenetisch

unterschiedlichen Spezies hoch konserviert und das Verteilungsmuster von Vγ9, Vδ2 und BTN3 Genen lässt auf eine funktionale Verbindung dieser Gene schliessen, wie sie die TCR/Ligand Interaktion darstellt. Weitergehende Analysen resultierten in der Identifizierung von sechs möglichen Kandidatenspezies, die nicht zu den Primaten gehören und funktionelle Vγ9, Vδ2 und *BTN3* Gene besitzen. Hierzu gehört auch das Alpaka (*Vicugna pacos*), ein Mitglied der Famile der Kamele.

Aus periphären Alpakalymphozyten wurden TCR-γ-Kettentranskripte mit charakteristischem Vγ9JP Rearrangement sowie TCR-δ-Kettentranskripte mit für PAg-reaktive Zellen typischen CDR3 amplifiziert. Die Transduktion der Alpaka-Vγ9 und Vδ2 Ketten in die TCR-negativen Maus T-Zell Hybridomlinie BW resultierte in einer Oberflächenexpression des TCR Komplex wie aus der Zelloberflächenexpression von Maus CD3 geschlossen werde konnte. Die Aktivierung dieses TCR Komplexes mittels anti-CD3ε Antikörpern führte zur Produktion von IL-2 durch die TCR-Transduktante, was die funktionelle Paarung der Alpaka Vγ9 und Vδ2 TCR-Ketten bestätigte. Neben den Vγ9 und Vδ2 TCR-Kettengenen existiert im Alpakagenom ebenso ein konserviertes Ortholog des humanen BTN3. Interessanterweise sind die mutmaßlichen PAg-Bindungsstellen des humanen BTN3A1 in dessen abgeleiteter Aminosäuresequenz zu 100% konserviert. Diese Daten machen das Alpaka zu einen vielversprechenden Kandidaten für weitere Untersuchungen, da hier möglicherweise die Population der Vγ9Vδ2 Zellen in ihrer Funktion zur Überwachung von Stress und Infektionen erhalten geblieben ist.

Ebenso liefert diese Arbeit die Sequenz des Vγ9Vδ2 TCR der Grünen Meerkatze (*Chlorocebus aethiops*), welche zuvor nicht bekannt war. Darüber hinaus wurde keine Speziesspezifität in der Präsentierung von PAg durch COS Zellen der Grünen Meerkatze für den humanen Vγ9Vδ2 TCR oder umgekehrt der Präsentierung von PAg durch

Meerkatzenzellen für humane Vγ9Vδ2 TCRs gefunden, was im im Widerspruch zu bisher veröffentlichten Ergebnissen steht.

Zudem liefert diese Arbeit auch neue Ergebnisse zur genetischen Grundlage der PAg-Präsentierung für die Vγ9Vδ2 T Zellen. Hier zeigte sich, dass das humane Chromosom 6 für die Präsentierung exogener sowie endogener PAg ausreicht. Durch die Generierung somatischer Mensch/Maus Hybride konnten wir die Rolle des humanen Chromosom 6 in der Phosphoantigenpräsentierung ermitteln und zudem beobachten, dass Chromosom 6 positive Hybride nicht in der Lage waren, Vγ9Vδ2 TCR Transduktanten in Anwesenheit des Alkylamins sec-Butylamin (SBA) zu aktivieren. Desweiteren brachten Versuche mit Ovarialzellen des chinesischen Hamsters (CHO), die das humane Chromosom 6 enthielten, ähnliche Ergebnisse. Dies legt nahe, dass Aminobisphosphonate (Zoledronat) und Alkylamine unterschiedliche Mechanismen der Aktivierung von Vγ9Vδ2 T Zellen nutzen obwohl für beide beschrieben ist, dass sie durch Inhibition der Farnesylpyrophosphatsynthase wirken, die wiederum zum Anstieg des intrazellulären IPP-Spiegels führt.

Zusammengefasst legt diese Arbeit die Co-Evolution der die Vγ9Vδ2 TCR/Ligand Interaktion kontrollierenden Vγ9, Vδ2 und BTN3 Gene in Placentalia nahe und identifiziert nicht den Primaten zugehörige Spezies als Kandidaten, die Vγ9Vδ2T Zellen besitzen könnten, von denen das Alpaka als vielversprechend für die Untersuchung der physiologischen Rolle von Vγ9Vδ2 T Zellen vorgeschlagen wird. Hinsichtlich der PAg-Präsentierung bestätigen die vorliegenden Ergebnisse, dass das humane Chromosom 6 zugleich nötig und ausreichend ist, endogene sowie exogene PAg zu präsentieren. Zudem könnten das Alkylamin SBA und Aminobiosphonat Zoledronat verschiedene Mechanismen zur Inhibierung des IPP Verbrauchs nutzen. Diese Ergebnisse werfen einige Fragen auf, die es in Zukunft zu beantworten gilt: 1) Was sind die Umwelt- und Evolutionsfaktoren, die dazu geführt haben, dass Vγ9Vδ2 T Zellen nur in wenigen Spezies erhalten blieben? 2) Was

könnte die funktionale Natur und die Antigenbindungseigenschaften einer solchen konservierten T Zell Population sein? 3) Was ist die genetische und molekulare Grundlage für die unterschiedliche Fähigkeit von das humane Chromosom 6 tragenden Mensch-Nager Hybridomazellen, Vγ9Vδ2 T Zellen in Anwesenheit von SBA und Aminobisphosphonaten zu aktivieren?

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1. Introduction

Jawed vertebrates (Gnathostomata) have evolved an adaptive immune system in addition to innate immune system to combat pathogens (1). The cell types of the hallmark adaptive immune system of jawed vertebrates are lymphocytes, which by their characteristic three antigen receptors can be divided into three cell lineages, namely γδ T cells, αβ T cells and B cells. These receptors have been conserved for 400-500 million years (2, 3). Unlike αβ T cells, γδ T cells are MHC independent in antigen recognition. γδ T cells are different from conventional αβ T cells in several aspects, especially with regard to co-receptors. Most of the γδ T cells are negative for CD4 or CD8 which act as co-receptors for MHC class II and MHC class I molecules, respectively (4). Lack of co-receptors by γδ T cells is in line with the MHC independent antigen recognition and response (5).

1.1 Evolution of antigen receptors in jawed vertebrates

T cell receptors (TCR) and immunoglobulins (Ig) are the two major classes of antigen recognition receptors of jawed vertebrates (6). TCR and Ig belong to a large protein family called immunoglobulin super family (IGSF) and they are more closely related to each other than rest of the family members (7). Both TCR and Ig are generated by a process called somatic recombination where variable (V), diversity (D) and joining (J) gene segments become rearranged in a process called somatic recombination or rearrangement, which is guided by the products of recombination-activating genes (RAG1 and RAG2) (Fig 1) (8). This process is critical for the generation of T- and B-lymphocytes and occurs in the primary lymphatic organs. Furthermore, immunoglobulins attain another quality of diversity through somatic hypermutation (SHM), where V regions of B cell receptor are subjected to undergo mutations to achieve high-affinity antigen binding sites. This process occurs during the

immune response in secondary lymphatic organs and is restricted to B cell receptors but is not found in (MHC-restricted) T-cells. (9).

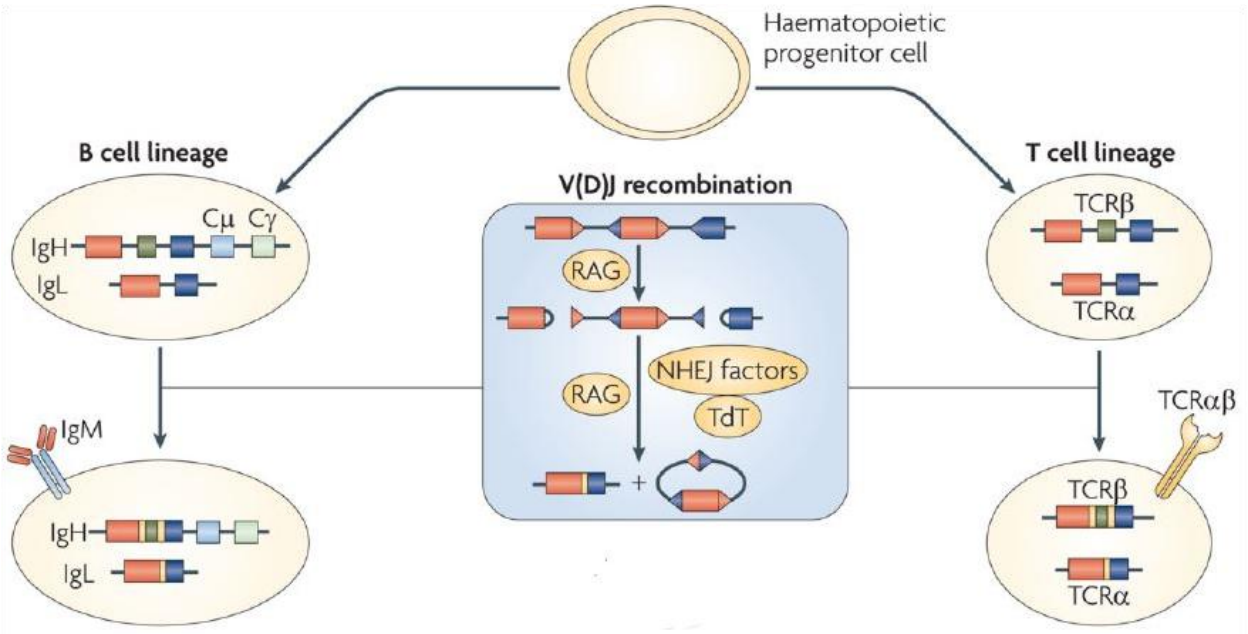

Figure 1: VDJ recombination of lymphocytes antigen receptors

During VDJ recombination, the recombination activation gene (RAG1-RAG2) recognizes the recombination signal sequences (Red and blue triangles) of individual gene segments (red and blue genes). Upon recognition, enzymes introduces two double stranded breaks with blunt end and introduces hair-pin loop to the desired coding sequence ends. During the joining of desired coding sequences, terminal deoxytransferase induces random nucleotide sequences (tiny yellow box) which dramatically raise the diversity of the repertoire. Simultaneously, RSS sequences were joined and excision of the undesired V genes as circles takes place. In this manner V gene (Red box), D gene (green box) and J gene (Blue box) sequences were recombined. The above picture was adapted from a review (6).

Immunoglobulins occur as membrane bound B-cell antigen-receptors (BCR) as well as secreted molecules (antibodies). They recognize antigens directly without the need of antigen presenting cells or molecules (10). In contrast to Ig, there are no soluble forms of TCR. The conventional αβ TCRs are restricted to recognition of MHC-bound peptides (11). Interestingly, γδ TCRs recognizes both membrane bound antigens as well soluble antigens, which is similar to immunoglobulins (5, 12-14). Additionally, γδ TCRs carry specificities for the recognition of ligands and presenting molecules, similar to αβ TCRs (15). Also studies

have shown that antigen recognition of γδ TCR is to an extent similar to immunoglobulins (16). Since γδ TCRs possess structural properties and antigen recognition properties, which are shared by αβ TCRs and immunoglobulins, it has been proposed that all three antigen receptor structures might have arose from a γδ TCR-like antigen recognition receptor (6, 10)

1.2 Gamma and delta antigen receptor genes

Every TCR chain is assembled by splicing of primary transcript composed of VJ or VDJ recombined gene segments and C segments. The γ-chain genes are assembled via VJ rearrangement whereas the δ-chain genes are by V-D-J rearrangement and it commonly involves multiple D segments (Fig 2). There are some unique properties of generic γδ TCR which act like γδ signatures, such as presence of serine residue at position 8 and a IHWY motif at positions 34-37 of Vγ gene (17-19). Interestingly, Vγ gene always carries a negatively charged residue at position 37 whereas Vδ gene carries a positively charged residue. This feature may enable them to have a unique γ/δ chain pairing. Compared to Vα segments, CDR1 or CDR2 of Vδ and Vγ genes are longer which can restrict pairing of Vδ chain to specific Vγ chain. For example, human Vγ9 and Vδ2 have CDR1 which have two extra amino acids, whereas Vγ1.3 and Vδ3 share similar feature in CDR2. Sharing such features predicts the restricted pairing, which proves to be a nature of γδ TCR (20). With regard to CDR3 regions, CDR3δ are highly diverse similar to IgH CDR3s (14) but CDR3δ differs from conventional αβ TCR chain by D-D joining segments and flexibility in reading frame usage. This feature yields higher diversity and variability in CDR3δ lengths and so the length of CDR3δ ranges from 8-21 amino acids (14, 21). This may also enable them to recognize diverse antigens. Whereas Vγ CDR3 regions are not as long as Vδ, but they too show a high degree of variability in length (1-12 amino acids) (14).

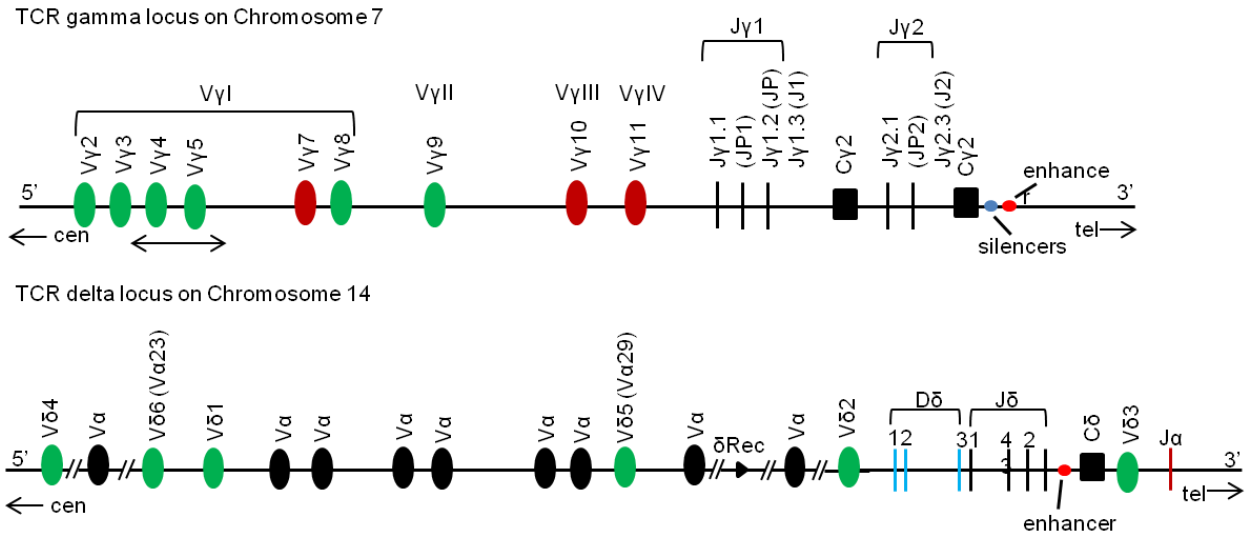

Figure 2: Human TCR gamma delta locus

Human TCR gamma locus lies on chromosome 7 and TCR delta locus lies on chromosome 14. Green oval shaped box indicates functional Vγ and Vδ genes. Red oval boxed indicate pseudo Vγ genes (adapted from (22)).

Though jawed vertebrates have three cell lineages bearing antigen receptor, Ig and TCR V genes can be classified into families sharing 75% identity. Among the TCR V genes, Vα/β/δ confers high similarity between different species for example human and mice, which is not so in the case of Vγ genes (19). Vγ genes have shown remarkable divergence between phylogenetically related and unrelated species. Secondly, Vγ genes show high divergence with respect to genetic complexity. In species like human and mice, they appeared to have only 6 functional Vγ genes whereas in chicken, there are as plenty as 20-30 genes (23). Interestingly, γδ T cells exhibit direct correlation between abundance of γδ T cells and their genetic complexity. Species with fewer Vγ and Vδ genes have fewer γδ cells in periphery (<5% in human and mice) but they are abundant in chicken as well as in the peripheral blood (>70%) of ruminants (24, 25). Another interesting feature of γδ T cells is the remarkable correlation between the usage of TCR genes and their anatomical localization (26).

1.3 Human γδ T cells

Vγ genes are more diverse than other TCR V genes, which is well illustrated by the presence of unique Vγ genes in mouse and human (19). Several unique γδ T cell subsets which possess diverse and unique antigen recognition properties such as Vγ9Vδ2 T cells were identified in humans. In the past two decades, human γδ T cells were found to recognize diverse antigens, peptide and non-peptide, and they play a significant role in anti-infection immunity (27, 28). In humans, the first reported γδ T cells response was towards tetanus taxoid (29) but still underlying mechanism of recognition remains unclear. Later on, γδ T cells have been found to elicit specific response towards soluble bacterial antigens such as toxin listeriolysin and staphylococcal enterotoxin and (30-32) In case of the latter, further examination revealed that these microbial products acted more like super antigens (33). Other than soluble proteins, γδ T cells can recognize non-peptide antigens as well. For example, circulating Vγ9Vδ2 T cells recognize phosphoantigens which are low molecular weight prenylated pyrophosphate molecules in an MHC independent manner (34, 35). $V\delta2^+$ T cell subset has been shown to play role in elimination of microbial pathogens especially intracellular bacteria and has been implicated in killing of tumors (36-38). γδ T cells are not yet reported for any MHC-specific TCR response but have been recorded for response towards members of CD1 family which are structurally MHC- class I like molecule. The group 1 CD1 molecules (CD1a, b, c) presents lipid A to $V\delta1^+$ γδ T cells subset and their activation leads to production of IFN- γ and granulysin (39, 40). It has been suggested that such activation could provide protection against microbial infections prior to development of an antigen specific immune response from αβ T cells (40, 41). Additionally, very closely related group 2 CD1d molecule seems to present a myelin glycophospholipid called sulfatide to Vδ1 expressing γδ T cells in humans. $V\delta1^+$ γδ T cells also respond to stress induced MHC-Class I related molecules MICA/B for

which antigen is not required (42). MICA/B molecules are widely expressed by several tumor cells which might be recognized by tumor infiltrating $V\delta1^+$ γδ T cells (43).

1.4 Human Vγ9Vδ2 T cells

Human γδ T cells approximately accounts for 5% of total $CD3^+$ T cell population in peripheral blood of a healthy individual (26). Vγ9Vδ2 T cells are major subset of human γδ T cells and their T cell receptor (TCR) is formed by pairing of Vγ9-containing γ chain with Vδ2-containing-δ chain. Vγ9Vδ2 T cell population ranges from 50% to more than 95% of γδ T cells in blood. Interestingly, such dominance was not present at the time of birth but gained during childhood and preserved throughout adulthood (26, 44-46) and is finally declining with age (47) . Initially, it was identified that broad range of bacteria including *Mycobacterium tuberculosis* could rapidly activate human Vγ9Vδ2 T cells (48). Later it was discovered that Vγ9Vδ2 TCRs recognize pyrophosphorylated metabolites of 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway of isoprenoid synthesis, such as isopentenyl pyrophosphate (IPP) and E-4-4hydroxy-3-methyl-byt-2-enyl pyrophosphate (HMBPP) as microbial antigens (35, 49). Such pyrophosphate non-peptide metabolites/ligands are collectively called as phosphoantigens (PAg). HMBPP is the precursor of IPP in 2-*C*-methyl-D-erythritol 4-phosphate pathway (MEP) of isoprenoid synthesis in several eubacteria, plants and apicomplexa parasites plants (Fig 3) whereas mevalonate pathway of isoprenoid synthesis found in most animals including all vertebrates lacks HMBPP (44, 50). But still, human Vγ9Vδ2 T cells recognize endogenous mevalonate pathway intermediates accumulated in tumor cells (51) which was later found to be IPP (52). Compared to IPP, HMBPP activates Vγ9Vδ2 T cells at least 1000 folds stronger than IPP and is suggested that it could be because of its microbial origin (53). Interestingly, accumulation of endogenous IPP can be achieved by blocking the consumption of IPP by downstream enzymes such as

farnesyl diphosphate synthase (FPPS). Treatment of cells with aminobisphosphonates (such as pamidronate or zoledronate) (54), alkylamines (such as sec-butylamine) or even siRNA against FPPS has resulted in the accumulation and presentation of IPP, which eventually led to the activation of Vγ9Vδ2 T cells (55, 56). Recognition of PAg by Vγ9Vδ2 T cells require cell – cell contact or surface receptor mediated presentation of PAg (34, 57). But Vγ9Vδ2 T cells recognize antigens in an MHC-independent manner (15) and antigen processing is not required (34). Antigen mediated activation of Vγ9Vδ2 TCR, leads to rapid release of cytokines such as IFN-γ and TNF-α by these T cells (58, 59). Direct recognition of molecules without antigen processing is an advantage of Vγ9Vδ2 T cells over αβ T cells, which makes them an attractive tool for anti-tumor therapeutics. *In vivo* activation of Vγ9Vδ2 T cells has been achieved either by direct administration of synthetic phospho-antigens such as phosphorylated Bromohydrin (BrHPP) or by administration of aminobisphosphonates (54, 60). Such a mechanism of activation may enhance their anti-tumor activity and leads to eradication of tumor (61).

Apart from direct activation of Vγ9Vδ2 T cells through PAg, Vγ9Vδ2 T cells can be activated or modulated through various molecules such as NK receptors (NKG2D), costimulatory and inhibitory molecules expressed by these T cells. NKG2D is a NK cell activating receptor which is widely expressed by Vγ9Vδ2 T cells and it is a receptor for MHC class 1 related A/B (MICA/B). MICA/B molecules were known as stress induced and tumor associated ligands (62). NKG2D mediated activation of Vγ9Vδ2 T cells in a TCR independent manner has been reported for the killing of tumor cells by cytotoxic effector activity of Vγ9Vδ2 T cells (63). Apart from MICA/B, UL16-binding proteins (ULBP)1-4 also acts as ligand for NKG2D and several epithelial cancer cells express ULBPs (42). Interestingly, association of NKG2D with its ligand (ULBP1) has been reported to promote anti-infectious activity of Vγ9Vδ2 T cells against intracellular bacteria (64). Thus human

Vγ9Vδ2 T cells play a significant role in providing first line of defense against infectious agents as well as pivotal role in immune and stress surveillance.

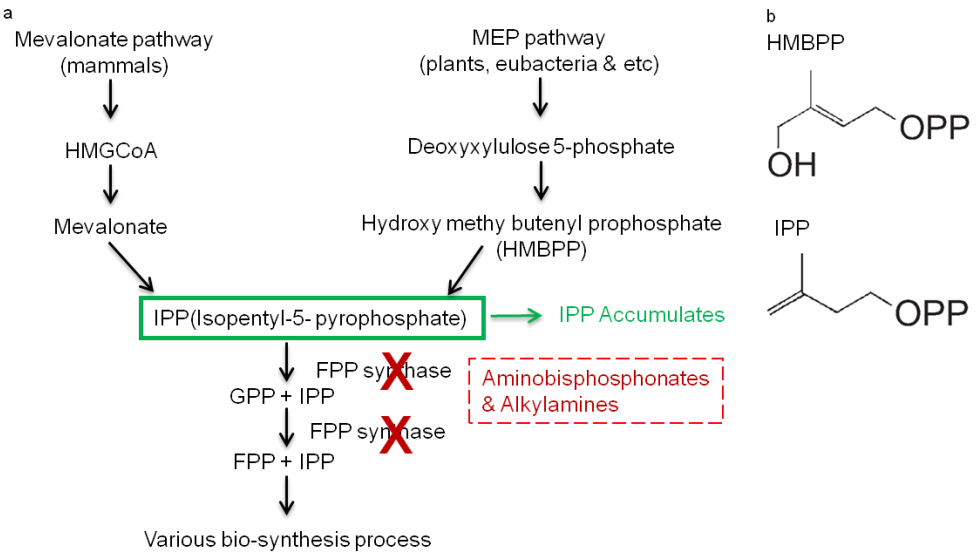

Figure 3: Mevalonate and MEP pathway of isoprenoid synthesis

a) Mevalonate pathway in vertebrates and 2-C-methyl-D-erythritol 4-phosphate pathway in plants and eubacteria are involved in isoprenoid biosynthesis. Isopentenyl pyrophosphate (IPP) is an intermediate found in both the pathways. Farnesyl diphosphate synthase (FPPS) is the downstream enzyme which consumes IPP. Aminobisphosphonates and alkylamines blocks FPPS which lead to accumulation of IPP. b) Structure of phosphoantigens: HMBPP and IPP adapted from (44).

1.5 Phosphoantigen presentation and Vγ9Vδ2 TCR

Vγ9Vδ2 T cells are so far identified only in human and primates (60, 65-67) and genes homologous to Vγ9 and Vδ2 are absent in mice (4). Unlike conventional αβ T cells, Vγ9Vδ2 T cells recognize PAg in an MHC independent fashion, but the antigen presenting molecule was unknown at the start of this thesis. Most of the human cells of various tissue origins are able to elicit PAg mediated activation of human Vγ9Vδ2 T cells but none of the murine cell lines can do so. Therefore, it is believed that the antigen presenting molecule is widely expressed in human cells but absent in rodents. Several studies have shown that monkey cells can present PAg and activate monkey Vγ9Vδ2 T cells (60, 65, 67). Thus, it is widely accepted that Vγ9Vδ2 T cell-PAg recognition system is restricted to primates. Ever since the discovery of antigen recognized by Vγ9Vδ2 TCRs, identification of the antigen presenting

molecule has been a great challenge for the scientific community. In 2012 Harly *et al.* have shown that BTN3A1, a member of butyrophilin family is mandatory for the antigen mediated activation of Vγ9Vδ2 T cells (68). *BTN3A1* gene is localized at the telomeric region of human Chromosome 6 (69) and encodes for a transmembrane protein. It is composed of an extracellular domain and an intracellular B30.2 domain. The extracellular domain comprises of IgV and IgC domain. BTN3 exists in three isoforms BTN3A1, A2 and A3, and they differ largely only with the possession of intracellular B30.2 domain, notably it is absent in BTN3A2 (70). In 2013, Vavassori *et al.* have confirmed that *BTN3A1* from Chromosome 6p is mandatory for PAg presentation. They also suggested the possibility of BTN3A1 being the putative antigen presenting molecule for PAg by demonstrating HMBPP and IPP binding to purified BTN3A1, and analysis of co-crystals of BTN3 with these two PAg (71). Recently, Wang *et al.* failed to show binding of HMBPP to the extracellular domain of BTN3A1 and suggested, but did not demonstrate, the binding of PAg to the intracellular B30.2 domain of BTN3A1. In their study, they also suggested a mechanism for molecular basis of antigen presentation, which would involve BTN3A1 and other unidentified molecule(s), for the proper loading of PAg and presentation of PAg to Vγ9Vδ2 TCR (Fig 4) (66). So, the molecules involved in PAg presentation and the exact mechanism of PAg presentation to Vγ9Vδ2 TCR still remain unclear.

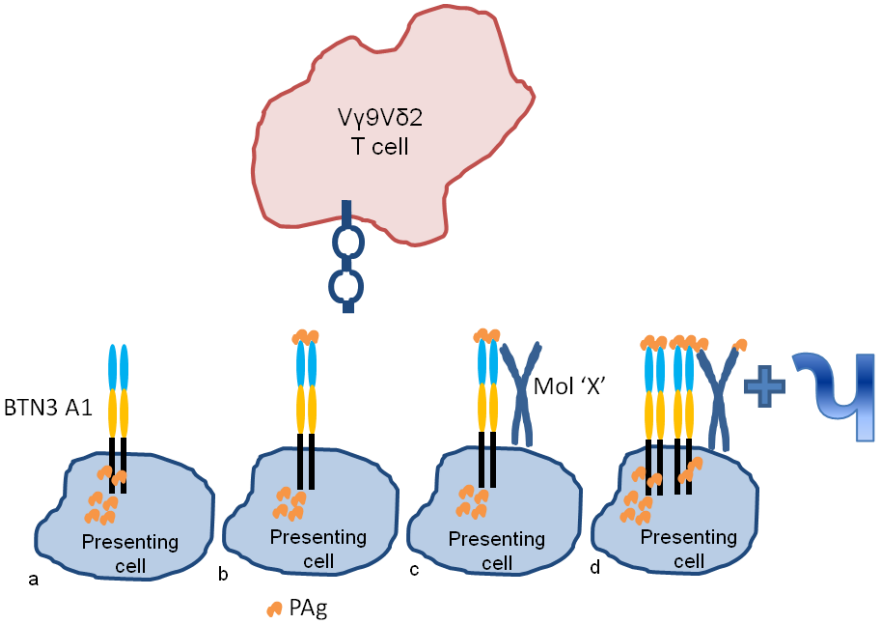

Figure 4: Model of PAg presentation to Vγ9Vδ2 T cells

The Vγ9Vδ2 TCRs recognize PAg presented by presenting cell/target cell. The PAg accumulated endogenously is presented to Vγ9Vδ2 TCRs in a BTN3A1 dependent manner but exact mechanism is unclear. Different model of PAg presentation has been suggested a) BTN3A1 alone independently activate TCR upon intracellular binding of PAg. b) BTN3A1 alone independently presenting PAg. c) Involvement of unknown molecule Mol 'X' in loading presentation of PAg by BTN3A1. d) Endogenously accumulated PAg, bind to intracellular domain of BTN3A1 and with/without of involvement of Mol 'X', the PAg is loaded and presented by BTN3A1. For all the above mode of PAg presentation and activation of TCR other unknown molecules 'ɥ' could be involved.

1.6 Human/mouse somatic cell hybrids

Somatic cell hybrids have a long history of being used as a remarkable and an efficient tool for the mapping of genes and chromosome as well for the characterization of mechanism that regulate gene expression (72). The very first report on somatic hybrids came from Barsky *et al.* who then constructed intra-specific somatic hybrids of mouse origin. Somatic hybrids arise as a result of spontaneous cell fusion between cells (73). Three most widely used methods to induce fusion of cells were virus induced, chemically mediated or electrical field induced fusion (74-76). Human-mouse somatic hybrids were widely used as a tool to characterize the phenotype of human chromosomes because of the following reasons; 1) in such hybrids the chromosomes from two different parental cells remain segregated. 2)

Human/mouse somatic hybrids have the tendency to lose human chromosomes and remain stable after retaining 10-20 percent of human chromosome content (77). Thus, such hybrids enabled to correlate the functional phenotype of hybrids to human chromosomes, but it was necessary to determine the human chromosome being retained by such hybrids. Earlier, karyotyping of human chromosome was achieved by staining of chromosomes. The technique developed by Caspersson *et al.* where cells were stained with an alkaline dye, quinacrine, which particularly stains chromosomes at specific chromatic regions, and the resulting image shows a banding pattern which is called Q-banding (78, 79). This techniques paved way to genetic characterization of human chromosome where functional phenotype of somatic cell hybrids has been positively correlated to human chromosomes and thereby the gene loci for a particular functional phenotype has been assigned (78). In this manner, several human genes and their loci on human chromosome have been identified (80-83). But the drawback with that technique was difficulty in identification of translocated human chromosomes and truncated chromosomes. So, human chromosome specific PCR based karyotyping was later developed (84, 85) and has been successfully employed in identification of human chromosomes in such somatic hybrids.

1.7 Aims of study

Vγ9Vδ2 T cells are the major subset of the circulating γδ T cell population and recognize PAg. Up to the present moment, Vγ9Vδ2 T cells are identified only in human and higher primates. Hence, these cells are believed to be primate specific T cell population and so knowledge about Vγ9Vδ2 T cells is limited only to primate models. Identification of other species which harbor Vγ9Vδ2 T cells would unravel more on the functional significance and antigen recognition properties of these T cells. Hence, we aimed to understand the evolution of PAg reactive Vγ9Vδ2 T cells. Therefore, we targeted to investigate the distribution pattern

of Vγ9Vδ2 TCR genes and their ligands by performing comprehensive analysis of the genomic database for sequences homologous to human Vγ9, Vδ2 and *BTN3* genes. If genes controlling Vγ9Vδ2 T cell population were to be identified in non-primate species, then those were to be further analyzed for the presence of Vγ9Vδ2 T cells and PAg-reactivity.

Another objective of the thesis is to investigate the molecular basis of PAg presentation which could clear up the mystery behind the molecular mechanism of PAg presentation to Vγ9Vδ2 T cells. Vγ9Vδ2 TCRs recognize PAg in a BTN3 dependent manner but the exact molecular basis of PAg presentation and recognition still remains unclear. To delineate the same, we targeted to identify the genes which could be involved in PAg presentation. So the primary aim was to identify the chromosome(s) which carry genes necessary for PAg presentation. It was proposed that human/rodent somatic hybrids would be used as presenting cells to identify the chromosomes involved in PAg presentation. The reason behind the use of such hybrids is that they were well known for their ability to lose human chromosomes over a period of time. This special feature of these hybrids will enable us to point out the chromosomes by loss or gain of function (PAg presentation) of the somatic hybrids. Altogether, this thesis aimed to provide valuable data in understanding the evolutionary paradigm of Vγ9Vδ2 T cells and to unravel more on the basis of PAg presentation to Vγ9Vδ2 T cells.

2 Material and Methods

2.1 Materials

2.1.1 Chemical reagents

Agar-agar	Roth (Karlsruhe, Germany)
Ampicilin	Gibco BLR (Eggenstein, Germany)
Betaine (5M)	Sigma (Germany)
B-mercaptoethanol	Gibco BLR (Eggenstein, Germany)
Calcium Chloride ($CaCl_2$)	Roth (Karlsruhe, Germany)
DMSO (Dimethyle sulfoxide)	Sigma (Taufkirchen, Germany)
dNTP	NEB (Frankfurt, Germany)
Boric acid	Roth (Karlsruhe, Germany)
EDTA	AppliChem GmbH (Darmstadt, Germany)
Ethanol	AppliChem GmbH (Darmstadt, Germany)
Ficoll paque (LSM1077)	PAA laboratories GmbH (Austria)
Formaldehyde	Roth (Karlsruhe, Germany)
Glycerol	Sigma (Schnelldorf, Germany)
Hypozanthine-Aminopterin-Thymidine (HAT)	Sigma (Steinheim, Germany)
Hypozanthine-Thymidine (HT)	Sigma (Steinheim, Germany)
HMBPP	Dr. Sabine Amslinger, University of Regensburg
Hygromycin	Sigma
IPP	Sigma (Diesenhofen, Germany)

Isopropanol	AppliChem GmbH (Darmstadt, Germany)
LB (Broth Base medium)	Gibco BLR (Eggenstein, Germany)
Methanol	AppliChem GmbH (Darmstadt, Germany)
Polyethyleneglycol (PEG)	Boehringer Mannheim (Germany)
Penicillin	Gibco BLR (Eggenstein, Germany)
Polybrene	Sigma (Diesenhofen, Germany)
Recombinant human IL-2 (rhuIL-2)	Miltenyi Biotec (Germany)
Sodium Butyrate (Na-Butyrate)	Sigma (Diesenhofen, Germany)
Sodium Azide (NaN_3)	Merck (Darmstadt, Germany)
Sodium Chloride	Roth (Karlsruhe, Germany)
Tris (hybroxymethyl)-aminomethan (Tris)	AppliChem GmbH (Darmstadt, Germany)
Trypan blue	Sigma (Diesenhofen, Germany)
Tween20	Sigma (Diesenhofen, Germany)
Zeocine	Cayla (Toulouse Cedex, France)
Zoledronate	Novortis (Basel, Switzerland)
8-Azaguanine	Sigma (Steinheim, Germany)

2.1.2 Media, solutions and buffers

Media used were supplied by Gibco-BRL (Eggenstein, Germany)

Dulbeccos's Modified Eagles's Medium (DMEM)

- With pyruvate, without HEPES
- Without pyruvate, with HEPES

RPMI	RPMI 1640 + L-Glutamine
Supplement complete	500 ml heat deactivated FCS
(SC; 50ml/500 ml Medium)	100 ml Na pyruvates 100mM
	100 ml non-essential amino acids
	5 ml β-mecrcaptoethanol 50 mM
	100 ml Penicllin – Streptomycin (1000U/ml)
	58.4 ml L-Glutamine Solution 5%
ATV	0.05% Trypsin, 0.02% EDTA in PBS
2 x HBS (pH 7.05)	50 mM HEPES pH 7.05, 10 mM KCL, 12 mM Glucose,
	280 mM NaCl, 1.5 mM $NaHPO_4$
LB medium	20 g LB in 1000 ml dH_2O, autoclaved and stored at 4 ^{o}C
Phosphate buffered saline (PBS)	4 mM KH_2PO_4, 16 mM Na_2HPO_4, 115 mM NaCl, pH
	7.3
PBS/BSA/Azide	0.2% BSA, 0.02 % Na-Azide in PBS
10x TBE	890 mM Tris pH 8.0, 890 mM Boric acid, 20 mM EDTA
FACS buffer	0.1% BSA, 0.05% NaN_3 in PBS
Cell freezing solution	50% FCS, 40% RPMI, 10% DMSO

Buffers for ELISA

Coating Buffer (100ml)	0.84 g $NaHCO_3$, 0.356 g $Na2CO_3$ ddH_2O pH 9.5
Assay Dilute	PBS with 10% FCS, pH 7.0
Wash Buffer	PBS with 0.05% Tween20
Stop Solution	2M H_2SO_4

2.1.3 Cell lines

RAJI	Human Burkitt lymphoma cell line (ATCC CCl-86)
Jurkat	Human T cell leukemia cell line (ATCC TIB-153)
293T	Human embryonic kidney cell line (ATCC CRL-1573)
A20	Mouse B lymphoma cell line (ATCC TIB-208)
BW5417	Mouse thymoma cell line (negative for expression of αβ TCR)
BW58	Mouse hybridomas derived AKR/J mouse αβ TCR-negative cell lymphoma (86)
BW58r/mCD28	BW58 cells transduced with r/m CD28 (87)
L929	Mouse fibroblast cells line (ATCC CCL-1)
COS	Fibroblast cell line derived from monkey kidney tissue (ATCC CRL-1651)

2.1.4 Vectors

pVSV-g	pCZVSV-g containing *env* form Vesicular Stomatitis Virus (88)
pHIT-60	CMV-MCC-*gag-pol*-SV40ori; vector carries human cytomegalovirus (CMV) promoter under which *gag* and *pol* genes from Moloney Murine Leukemia Virus (MoMLV) were controlled (89)
pczCG5IEGZ	Retroviral vector for the MuLV driven constitutive expression of gene of interest and IRES mediated expression of EGFP – zeocine resistant fusion protein
pIH	Retroviral vector for the MuLV driven constitute expression of gene of interest with hygromycin resistant gene expression
pIZ	Retroviral vector for the MuLV driven constitute expression of gene of interest with zeocine resistant gene expression

2.1.5 Antibodies

Antigen	Clone	Conjugated	Isotype	Manufacturer
mCD3ε	145-2C11	Bio	Hamster IgG1,κ	BD Pharmingen
hCD71	M-A712	FITC	mIgG2a, κ	BD Pharmingen
HLA-ABC	G46-2.6	FITC	mIgG1, κ	BD Pharmingen
rCD80	3H5	Purified	mIgG1, κ	BD Pharmingen
huVδ2 chain	B6	PE	mIgG1, κ	BD Pharmingen

Secondary antibodies commonly used for the staining were against mouse IgG (H+L). Antibody used was donkey $(Fab')_2$ fragments with minimal cross reactivity towards rat and other serum proteins. Antibody was either conjugated with FITC/PE and was supplied by Jackson Immunoresearch Laboratories.

2.1.6 Cloning reagents

Taq DNA polymerase 2x PCR mix	Fermentas (St.Leon-Rot, Germany)
One *Taq* Hot start DNA polymerase	NEB (Frankfurt, Germany)
One *Taq* 2x master mix	NEB (Frankfurt, Germany)
Pfu-DNA polymerase	Finnzymes (Espoo, Finnland)
Restrictions enzymes (EcoRI, BamHI, BglII, XhoI, Xba1)	Fermentas (St.Leon-Rot, Germany)

2.1.7 Kits

Mini Elute Gel Extraction Kit	Qiagen (Hilden, Germany)
Big Dye 3.1	AB applied biosystems (California, USA)
GeneJET Plasmind Minipret Kit	Fermentas (St.Leon-Rot, Germany)

JET quick DNA clean Up Spin Kit	Genomed (Löhne, Germany)
Midi JETSTAR Kits	Genomed (Löhne, Germany)
QIAamp DNA mini Kit	Qiagen (Hilden, Germany)
RNAeasy Mini Kit	Qiagen (Hilden, Germany)
QIAshredder	Qiagen (Hilden, Germany)
OPTEIA mouse IL-2 Set	BD (Heidelberg, Germany)
GeneRacer® Kit with AMV RT and TOPO TA Cloning® Kit for Sequencing	Life technologies (Germany)

2.1.8 Consumables

6 well flat bottom culture plates	Greiner Bio-One (Germany)
12 well flat bottom culture plates	Greiner Bio-One (Germany)
24 well flat bottom culture plates	Greiner Bio-One (Germany)
48 well flat bottom culture plates	Greiner Bio-One (Germany)
96 well U bottom culture plates	Greiner Bio-One (Germany)
6 cm tissue culture dish	Greiner Bio-One (Germany)
50 ml cell culture flask	Greiner Bio-One (Germany)
5, 15 and 25 ml single use pipettes	Greiner Bio-One (Germany)
1.5 ml Eppendorf centrifuge tube	Eppendorf (Eppendorf, Germany)
15 ml centrifuge tube 50 ml centrifuge tube	Greiner Bio-One (Germany)
Cuvette	Bio-Rad (Munich, Germany)
10 µl tips	Molecular Bioproducts (USA)
200 µl yellow tips	Roth (Karlsruhe, Germany)
1000 µl blue tips	Roth (Karlsruhe, Germany)
5ml single-use syringe	BD Biosciences Pharmingen (San Diego, USA)

2.1.9 Software

Cell Quest and FlowJo: For the analysis of flow cytometry data

MEGA: For the construction of Phylogenetic tree to understand the phylogenetic relationship between different sequences and species

Endnote: Tool for the management of bibliographies

2.1.10 Primers used for PCR and RACE

Horse Vδ2 Rev	GAAGGAAGAACGATGGAACATTTATTGAC
Horse Vγ9 Rev	GAGCGGATGTGCTAACAGTGGTGAGGAC
Horse Vδ2 Fwd	GCAAACGCCCTCTTGGGGAACAAAACAGCAG
Horse Vδ2 Rev	GCAGAAGAGTGACCGAGGCTGAAGGAAAGG
Alpaca Vγ9 Fwd	GTGCAGGTCATCTAGAGCAACCGC
Alpaca Vγ9C Rev	TCCATTGACTTTTCAGGCACGGTCA
Alpaca Vδ2 Fwd	GTCAGCAGATGTGTTGGTGCCTCA
Alpaca Vδ2 C Rev	CCAGCACCGAGAGGGACATCATGT
Alpaca JC Fwd	CGGCTCATTATTACAGACAGAAAGCTTGATG
3'RACEQt	CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCTTTTTTTTTTTTTTTTTVN
3'RACEQo Rev	CCAGTGAGCAGAGTGACGAGGAC
3'RACEQi Rev	TGACGAGGACTCGAGCTCAAGC
Alpaca Vγ9 5'UTREcoRI	TTCCGAATTCGCCGCCACCACAGTGCGGTCCATCCAGACATG
Alpaca Cγ9 3'UTR	ATCCGGATCCGAGAAGTTCAGAGCAACAGACGAT
Alpaca Vδ2Ldr	ATGCAGAGGGTCTGCTCCCTCATCC
Alpaca Vδ2 Ldr EcoRI	CAGTGAATTCGCCGCCACCATGCAGAGGGTCTGCTCCCTCATCC

Alpaca Cδ2 3'UTR	TCACGGATCCCCTCACCATTCCTAAATTCCTTT
AGMγ9EcoRIKozakFwd	CTACGAATTCGCCGCCACCATGCTGTCACTGCTGCTCCACGC
AGMγ9BglIIRev	CCACAGATCTTTACGGTCTCTCTCCATTGCAGC
AGMδ2EcoRIKozakFwd	CTACGAATTCGCCGCCACCATGATCTCCTCCCTCATCCATCT
AGMδ2BglIIRev	CCACAGATCTTTACAAGAAAAATAACTTGGCAG
Generacer5'fwd	CGACTGGAGCACGAGGACACTGA
AlpacaBTN3stopRev3	CTAGGCAGGGACAAGCAAGGATG
VpBTN3RaceQoRev1	CTGTCTTCTACTTCCTTCCCACCTGCGT
CowVγ9Fp1	GCTTGTAAGTTGTTCTTTTCCCCCAATTTC
CowVγ9Rp1	GTGGTAACTGTGGTTTCCTAACTCACGATA
CowVδ2Fp1	GGACCAGGCAAGAACGAGTCAGGAGGCAGAGC
CowVδ2Rp1	AGGGATTGCCTATCTGAGCACCACGGGAG

2.1.11 Accession number of genomic sequences

VGamma9:
ABRR01244317.1 - Alpaca (Vicugna pacos), AAGV03121505.1 – Armadillo (Dasypus novemcinctus), AGTM011099715.1 – Aye-aye(Daubentonia madagascariensis), AGVR01031390.1 – Wild bactrian camel (Camelus bactrianus), gb|GADY01006660.1 - Dromedary (Camelus dromedarius), AGCE01067087.1 – Black capped squirrel monkey (Saimiri boliviensis), AJFE01038666.1 – Bonobo (Pan paniscus), ABRN02028896.1 – Bottle nose dolphin (Tursiops truncates), AACZ03050974.1 - Chimpanzee (Pan troglodytes), ACFV01091880.1 – Common marmoset (Callithrix jacchus), DAAA02011292.1 – Cow (Bos Taurus), ABRP01014493.1 – Flying fox (Pteropus vampyrus), ADFV01046571.1 – Gibbon (Nomascus leucogenys),CABD02337330.1 – Gorilla (Gorilla gorilla gorilla), ABDC01371517.1 – Grey mouse lemur (Microcebus murinus), AAWR02031701.1 – Horse (Equus caballus), AEHL01292420.1 – Japanese Macaque (Macaca fascicularis), ANOL02003608.1 – Killer whales (Orcinus orca), AAQR03057839.1- Northern greater Galago (Otolemur garnettii), ABGA01290208.1 – Oranguttan (Pongo abelii), ABRT010177380.1 – Philippine tarsier (Tarsius syrichta), AANU01218521.1 - Rhesus monkey (Macaca mulatta), AMGL01088491.1 - Sheep (Ovis aries), ABVD01168789.1 – Sloth (Choloepus hoffmanni), AGTP01012576.1 - Thirteen lined ground squirrel (Ictidomys tridecemlineatus), AGTT01041789.1 – Tibetan antelope (Pantholops hodgsonii), AKZM01048251.1 – White rhinoceros (Ceratotherium simum simum), AOCR01006132.1 – Wild boar (Sus scrofa), AJPT01047514.1 - Yunnan black goat (Capra hircus)
Alpaca (*Vicugna pacos*) Genomic JP segment - gb|ABRR02020993.1

VDelta2:
AAGU03084666.1 – African elephant (Loxodonta Africana), ABRR01280573.1- Alpaca (Vicugna pacos), AAGV03208792.1– Armadillo (Dasypus novemcinctus), AGTM011787934.1– Aye-aye(Daubentonia madagascariensis), AHZZ01035404.1 – Baboon (Papio anubis), AGCE01026517.1 – Black capped squirrel monkey (Saimiri boliviensis), ALWS01051822.1 - Black Flying Fox (Pteropus alecto), ALEH01136097.1 - Big Brown Bat (Eptesicus fuscus), AJFE01024273.1– Bonobo (Pan paniscus), ABRN02535067.1– Bottle nose dolphin (Tursiops truncates), AMDV01053035.1 - Cape golden mole (Chrysochloris asiatica), ACBE01202348.1 - Cat (Felis catus), AACZ03095321.1- Chimpanzee (Pan troglodytes), ACFV01031202.1– Common marmoset (Callithrix jacchus), DAAA02028054.1– Cow (Bos Taurus), ALWT01226188.1 -David's Mouse eared bat (Myotis davidii), AAPE02039938.1 - Fruit bat (Myotis lucifugus), ABRP01238689.1– Flying fox (Pteropus vampyrus), ACTA01003783.1 - Giant Panda (Ailuropoda melanoleuca, ADFV01192055.1– Gibbon (Nomascus leucogenys), CABD02105635.1– Gorilla (Gorilla gorilla gorilla), ABDC01133592.1– Grey mouse lemur (Microcebus murinus), AAWR02001688.1– Horse (Equus caballus), AEHL01378925.1– Japanese Macaque (Macaca fascicularis), ANOL02066598.1– Killer whales (Orcinus orca), AHIN01142883.1 – Manatee (Trichechus manatus latirostris), AAQR03175671.1 - Northern greater Galago (Otolemur garnettii), AAPY01347909.1 – Norther Treeshrew (Tupaia belangeri), AEHK01331688.1- Rhesus monkey (Macaca mulatta), AMGL01102217.1- Sheep (Ovis aries), ABVD01106723.1– Sloth (Choloepus hoffmanni), AJFV01075663.1 - Star nosed mole (Condylura cristata), , AGTT01175372.1– Tibetan antelope (Pantholops hodgsonii), AKZM01006933.1– White rhinoceros (Ceratotherium simum simum), gb|AJKK01135927.1– Wild boar (Sus scrofa), AJPT01105872.1- Yunnan black goat (Capra hircus)

BTN3-ED:
NM_001145008.1 - Human (Homo sapiens); gb|ABRR02153549.1 – Alpaca (Vicugna pacos); tpg|DAAA02055496.1 – Cow (Bos taurus); gb|ABVD01029026.1, gb|ABVD01842615.1 – sloth (Choloepus hoffmanni); gb|ABDC01331604.1- Grey mouse lemur(Microcebus murinus), gb|AGTM011755245.1 - Aye-aye (Daubentonia madagascariensis); gb|AAWR02009770.1, gb|AAWR02009775.1 – Horse (Equus Cabalus), gb|AAGV03145787.1, gb|AAGV03240342.1 - Nine Banded Armadillo (Dasypus novemcinctus); gb|ABRN02268951.1, gb|ABRN02485746.1- Bottlenose Dolphin (Tursiops truncates); gb|ANOL02034815.1Killerwhales (Orcinus Ocra); gb|AGTP01091243.1, gb|AGTP01091244.1 - Thirteen lined squirrel (Ictidomys tridecemlineatus); gb|ABRT010776711.1, gb|ABRT010215784.1 - Philippine tarsier (Tarsius syrichta); gb|AJPT01202731.1 - Yunnan black goat (Capra hircus); gb|GAES01012686.1, gb|GAEX01008159.1 – Wild bactrian camel (Camelus ferus); gb|ACIV011865666.1 – Sheep(Ovis aries), gb|AGTT01201962.1 - Tibetan antelope (Pantholops hodgsonii); gb|AAPE02057860.1 - Fruit bat (Myotis lucifugus); gb|AKZM01051838.1 - White Rhinoceros (Ceratotherium simum simum); gb|ALWT01109578.1 -David's Mouse eared bat (Myotis davidii); gb|ALEH01160890.1 - Big Brown Bat (Eptesicus fuscus).

2.2 Methods

2.2.1 Routine cell culture methods

The mammalian cells were cultured in a CO2 cell incubator in which 37^0C with 5% CO_2 and H_2O saturated atmosphere was maintained. Almost all suspension cells were cultured with RPMI medium (10% SC) whereas adherent cells were maintained with DMEM medium (10% FCS, 1 ml Penicillin – Streptomycin (100mg/ml) per 500ml DMEM). For routine maintenance, suspension cells were cultured in 24/12 well plates and adherent cells were maintained always in 10cm tissue culture dishes. Frozen stocks of cells were always maintained at -80^0 C or -140^0C. $1*10^6$ - $4*10^6$ cells were re-suspended in 1ml of freezing medium (50%FCS, 40% RPMI and 10% DMSO) and stored in a pre-chilled 2ml cryo tubes. Whenever required the stocks were defrost and cells were cultured. Prior to every experiment, cells used for the experiment were counted. Percentage of viable cells was determined by diluting cells with Tryphan-blue solution and counted with Neubauer chamber under the microscope. Those cells remaining unstained are considered as live ones and are counted.

2.2.2 Isolation of peripheral blood mononuclear cells (PBMC)

PBMC were isolated from the fresh blood obtained from healthy donors under informed consent or from blood of alpaca provided by Prof. Thomas Göbel, Institute for animal physiology, Ludwig-Maximilians-University, Munich Separation of PBMC was performed using Ficoll Paque (lymphocyte separation medium (LSM-1077)) supplied by PAA as per manufacturer's protocol. In a 50 ml conical tube, 20 ml of whole blood was transferred and equal volume of PBS was added to it. 10 ml of Ficoll was released at the bottom of the tube gently to avoid the mixing of blood and fecal. Finally, blood will be layer on the Ficoll and the tube was centrifuged at 400x g for 30 min at 20^0C min with brakes applied. After

centrifugation, PBMC concentrated as an interphase layer between plasma and the separation solution was aspirated with a pipette and transferred to a new 15 ml tube and washed twice with 3 volumes of PBS at 400x g for 10 min. Finally, PBMC were resuspend in 1 ml of RPMI (10% SC).

2.2.3 Construction of human/mouse hybridoma

a) Prior to beginning of the fusion, 100ml of RPMI 1640 medium and 50 ml of RPMI 1640 medium with 10% serum complement was warmed up.
b) 5x10*6 cells of Jurkat cells and BW5417 cells were taken for fusion in 1:1 ratio as human and mouse cells, were chosen for fusion.
c) Both cell types were mixed and make up to 50ml with RPMI 1640 medium in a 50ml tube..
d) The cell suspension was centrifuged at 461 x g per minute for 5 minutes.
e) After centrifugation, the supernatant was removed and the cell pellet was generally tapped to break the pellet. Meanwhile, water-bath was warmed to 37^0C for the following steps.
f) The tube was placed in the water-bath with continuous gentle shaking of the tube and 5 ml of RPMI 1640 medium was added slowly drop by drop along the wall for 5 minutes.
g) 10ml of RPMI 1640 medium was added in a similar fashion for 5 minutes.
h) Then 30ml of RPMI 1640 (10% SC) was added slowly and incubated the tube in 37^0C in water-bath for 30 minutes.
i) After incubation, the cell suspension was centrifuged for 461 x g for 5 minutes.
j) After centrifugation, the supernatant was discarded and the cell pellet was resuspended slowly in 25 ml of RPMI 1640 (10% SC). Further cells were diluted to 1 cell/100µl concentration and cells were seeded in 96 well plate with single cell per well dilution.

After 24 hours, 100µl of 2xHAT and antibiotics (Hygromycin - 500µg/mL and Zeocine - 500µg/mL) were added to each well and clones were selected for two weeks.

k) In a similar manner human PBLs ($1x10^7$ cells) and BW 5147 ($5x10^6$) cells were fused to construct hybridomas.

2.2.4 Gene cloning and transfer

2.2.4.1 Genomic DNA isolation from hybridomas

The clones screened for their antigen presentation ability were subjected to PCR Karyotyping. The genomic DNA was isolated from 200,000 cells of each clone by treating them with 200 µl lysis buffer (10mM Tris, 50mM KCl, 2mM $MgCl_2$, 0.1% TritonX100, 0.5% Tween20) containing 10µg/mL proteinase K by incubating at 37^0C for an hour and incubated at 96^0C for 30 minutes to completely lyse the cells. The lysate was used a template for PCR Karyotyping.

2.2.4.2 Genomic DNA isolation and Total RNA extraction from PBMC

Genomic DNA was prepared using the Qiagen genomic DNA isolation kit. RNA was prepared using the Qiagen RNeasy kit. The concentration of eluted DNA and RNA was estimated by with absorbance at 260nm by using Nanodrop.

2.2.4.3 cDNA synthesis

cDNA was prepared from RNA by following the manufacturer's protocol. Fermantas First strand cDNA synthesis kit and oligo dT primers provided with the kit was used for cDNA synthesis.

2.2.4.4 PCR amplification

Different polymerases were used for different purposes. *Taq* DNA polymerase (2xPCR Master Mix, Thermo Scientific) was used for the PCR karyotyping and screening of genes. Phusion high-fidelity DNA polymerase (Thermo Scientific) was used for PCR reactions to amplify the genes for cloning and sequencing. One *Taq* Hot Start DNA polymerases were used for 3' and 5' RACE PCR conditions to amplify the unknown cDNA ends. For all polymerases reaction mix was prepared as per manufacturer's protocol. Below mentioned were the conditions followed for different polymerases.

PCR Conditions	***Taq* DNA polymerase**	**Phusion high-fidelity DNA polymerase**	**One *Taq* Hot Start DNA polymerase**
Initial denaturation	94^0C, 5 mins	98^0C, 30 sec	94^0C, 30 sec
Denaturation	94^0C, 15 – 30 sec	98^0C, 10 sec	94^0C, 15 – 30 sec
Annealing	Variable, 15-60 sec	Variable, 20 sec	Variable, 15-60 sec
Extension	72^0C, 1 min/Kb	72^0C, 30 sec/Kb	68^0C, 1 min/Kb
Final extension	72^0C, 10 mins	72^0C, 10 mins	68^0C, 10 mins

2.2.4.5 5' and 3'RACE PCR

5'RACE PCR was performed to amplify the unknown 5' end of gene sequence by using GeneRacer kit supplied by Life technologies. Similarly 3' RACE PCR was performed using One *Taq* Hot Start polymerase supplied by NEB following the published protocol (90).

2.2.4.6 Gel electrophoresis

5 µl of PCR product will be mixed with 1 µl of 6x loading dye and loaded on 1% or 2% agarose gel with an appropriate DNA ladder (100bp/1Kb ladder). Agarose gels were made

with GelRed nucleic acid gel stain. After electrophoresis, gels were visualized under UV illuminator.

2.2.4.7 Digestion and ligation of PCR products and plasmid

PCR products were either purified by QIAquick PCR purification kit or after gel electrophoresis; the appropriate gel was excised and purified by Qiagen gel extraction kit. The PCR product and vector was digested with suitable restriction enzymes under recommended conditions as per manufacturer's protocol. The digested products were separated by gel electrophoresis and the right size bands were excised and DNA was extracted from gels by Qiagen gel extraction kit. 5 µl of digested PCR product/gene and 1 µl digested vector was added to a mixture containing 1µl of T4 DNA ligase, 1µl of 10X ligation buffer and 2µl of water. Ligation reaction was set at 4^0C overnight.

2.2.4.8 Transformation

1.5ml eppendorf vial containing 50 µl of frozen competent cells was placed on ice for several minutes. Once the competent cells have been thawed, 3 µl of ligation mix was mixed to 50 µl of competent cells and placed on ice for 10 min. After 10 min, competent cells were given a heat shock treatment of 42^0C for 30 sec. Immediately it was placed on ice for 2 min and 250 µl of LB/SOC medium was supplemented to the competent cells and incubated in a shaker incubator for 45 min at 37^0C with 180rpm. Soon after the incubation period, the transformed cells were spread on two LB-Agar plates with appropriate antibiotics and incubated overnight at 37^0C. Resultant colonies were picked and inoculated in 5ml of LB-broth for plasmid mini-prep to characterize the plasmid.

2.2.4.9 Miniprep and identification of positive clones

Several tubes containing 5ml of LB-broth and appropriate antibiotic for selection were inoculated with single colonies and were incubated overnight at 37^0C in a shaker incubator at 180 rpm. After incubation, 2 ml of bacterial culture was used to prepare plasmid DNAs with the Miniprep kit supplied by Genomed. 2 µl of plasmid DNA was digested with appropriate restriction enzymes for an hour and they were tested on the gel by electrophoresis. The positive clones were further purified by plasmid clean up kit supplied by Genomed.

2.2.4.10 Sequencing

The plasmid and PCR products were analyzed by sequencing. The samples to be sequenced were PCR amplified using BigDye® Terminator v3.1 Cycle Sequencing Kit supplied by Life technologies. The amplified PCR sample was in-house sequenced by the Sanger sequencing method using ABI PRISM 310 Genetic Analyzer.

2.2.4.11 Retroviral transduction

To express our gene of interest in mammalian cells, viral particle mediated transduction of the gene was employed. Three-plasmid expression system (retro virus) is used, where 293T cells were transfected with three plasmids by calcium chloride. As a result virus particles were generated by 293T293T cells and released in the supernatant. Which was later, used to infect target cells. The procedure is detailed as below and is adapted from (89),

Day1: $1.5*10^6$ 293T cells were seeded on 6 cm tissue culture dish with 5ml of DMEM. Three vectors (Recombinant vector, pHIT60, pVSV-G), each of 5 µg was mixed and precipitated with 500 µl isopropanol and washed with 500 µl of 70% ethanol. Later air dried in a sterile environment and resuspend in 100 µl of sterile water.

Day 2: Culture medium of 293T cells was replaced with warm, fresh DMEM medium without HEPES and incubated for an hour in incubator to allow the pH to reach equilibrium. Later, 338 µl of water and 62 µl of 2M $CaCl_2$ were added to 100 µl of plasmid DNA. The above mixture of DNA was transferred to 500 µl of 2X HBS solution in a drop by drop manner and mixed simultaneously by bubbling. Immediately, DNA mixture was poured all over the 293T culture dish in a drop by drop manner and mixed gently. The culture dish was incubated for 6-8 hours at 37^0C and the medium was replaced with a fresh warm medium with HEPES. Cells were incubated overnight in same condition.

Day 3: The culture medium was replaced with fresh and warm DMEM medium containing 10mM sodium butyrate. Sodium butyrate activates the CMV promoter, which in turn induces the expression of genes of interest as well as genes necessary for the formation of viral particles. 6-8 hours later, the sodium butyrate medium was replaced with fresh warm medium without sodium butyrate and cells were incubated overnight.

Day 4: Final day of infection, where the supernatant from 293T cell culture dish will be filtered by 0.45µM filter, ensure the filtering of cells but not viral particles. The target cells of $1*10^5$ cells taken in a 15 ml tube will be supplemented with 3ml of viral supernatant and Polybrene (final concentration; 4 µg/ml). The tubes were centrifuged at 871 x g for 150 min at 32^0C. Soon after centrifugation, the supernatant was aspirated and the cell pellet was resuspend in 3ml of fresh medium and cultured in a 12 well plate.

2.2.4.12 Identification and selection of transduced cells

Once the cells were transduced with gene of interest, cells will be cultured with medium supplemented with appropriate antibiotics (puromycin, neomycin or zeocin) for 2-3 days. Later, cells were analyzed by FACS for the expression of fluorescent proteins like GFP, YFP and AsRed or stained with antibodies against the protein of interest. Once the cells were

found to be positive for gene of interest, after a week or two of selection duration, cells will be cultured without antibiotics. If the positive cell population is too less, then positive cells were sorted by a FACS Aria III machine and cultured with selection medium.

2.2.5 Mouse IL-2 assays

Stimulation of TCR transductants mouse cells by antigen presenting cells in presence of antigen is the method to analyze the ability of cells to present antigen and capability of TCR transductants to recognize antigens. For stimulation, TCR transductants were co-cultured with APCs in presence and absence of antigen for overnight at 37^0C. The supernatant of stimulation samples were analyzed for the production of mouse IL-2. Measuring of IL-2 was performed by using mouse IL-2 ELISA kit supplied BD biosciences as per manufacturer's protocol.

2.2.6 PCR Karyotyping

Hybridomas were analyzed for genomic content by PCR; the primers used for the screening were previously reported as human genome specific ones and were used for screening such human/mouse and human/hamster hybrids (84, 85). The PCR conditions to amplify chromosome specific product was below mentioned in the table and were reported previously (84, 85).

Temperature	ProgramA	C	D	E
90^o C	10 sec	10 sec	10 sec	1 min (94^o C)
Annealing Tm	55^o C 30 sec	50^o C 30 sec	48^o C 30 sec	65^o C 2 min
72^o C	30 sec	30 sec	30 sec	3 mins

List of Chromosomes amplified with each PCR condition:

Prog A: 1, 2, 7p, 10p, 12, 13, 17p, 20, X
Prog C: 3, 5, 8, 9p, 11
Prog D: 4, 6, 18
Prog E: 14, 15, 16, 19, 21, 22

2.2.7 Phylogenetic analysis

Human Vγ9, Vδ2 and *BTN3-ED* gene sequences were taken as a query in search of Vγ9, Vδ2 and *BTN3-ED* like genes in other mammalian species. The query sequence was blasted against mammal whole-genome shotgun database using the discontinuous megablast algorithm at NCBI BLAST. Hits which covered at least 90% of V genes in length and with at least 60% sequence identity to the query sequence were chosen for analysis. Other than NCBI BLAST, genomic sequence assembly released by the Broad institute (http://www.broadinstitute.org/science/projects/mammals-models/data-release-mammalian-genome-project, which is now available at the NCBI database) was also used to perform the search for human Vγ9, Vδ2 and *BTN3-ED* like nucleotide sequences. All the sequences obtained from databases were translated *in silico* using Expasy Translate (http://web.expasy.org/translate/) to identify the presence of open reading frames. The amino acid sequences were aligned using the ClustalW2 tool (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Phylogenetic analyses were performed using MEGA software - version 5.2. The nucleotide sequences of human Vγ9, Vδ2 and *BTN3-ED* and their respective homologous sequences identified in several species were analyzed for phylogenetic studies. The phylogenetic trees were constructed by the several methods with bootstrap test and interior branch-test of 10000 replications. Such phylogenetic methods include; maximum parsimony method, maximum likelihood method, neighbor joining method and minimum evolution method. For every method, trees were constructed with bootstrap frequency and branch length as determinants of confidence of each node in a tree.

Each node represents each species branching out from the backbone of the tree. Higher the bootstrap frequency, greater the confidence of node whereas in case of branch length, lower the value higher the strength of node. The values indicate the probability for phylogenetic relationship between species at every node. Since, all three methods yielded similar trees with similar bootstrap confidence for each node in a tree, the phylogenetic trees obtained on maximum likelihood method was finally presented since it is widely accepted for the analysis of phylogenetic relationship between distinct species.

3. Results

3.1 Evolution of Vγ9 and Vδ2 TCR genes and *BTN3* gene

3.1.1 Vγ9 and Vδ2 genes emerged with and are scattered across placental mammal

Vγ9Vδ2 T cells have been found only in higher primates and not been described in rodents. To learn whether such cells may occur in other species than higher primates a database search for genes constitutive for functional Vγ9Vδ2 T cells was performed. A search for Vγ9, Vδ2 and *BTN3* genes (putative antigen presenting molecule) was done across mammalian whole genomic databases *(whole-genome shotgun contigs* and *29 mammals project on Broad Institute database)* by the criteria of more than 60% nucleotide identity and 90% coverage of the query (human) sequences. As a result, we found that Vγ9, Vδ2 and *BTN3* homologous sequences are distributed among species belonging to either of the eutherian magnorders (Fig 5, Table 1). Furthermore, these genes are completely absent in non-eutherian species (data not shown). This is a clear indication that these genes emerged and evolved along with the emergence or placental mammals and that they are not restricted to primates. The gene sequences identified from different species were reverse blasted against human genome and transcript database and human Vγ9, Vδ2 and *BTN3-ED* appeared as the first hit for respective query sequence. Hence the gene sequences which have been identified in the different species were claimed as being homologous to human genes. The interesting revelation from the search is that the three genes are not universally preserved among mammals and exhibit a heterogeneous pattern of distribution. For instance, Vγ9, Vδ2 and *BTN3* like genes are preserved by most of the species of the order Primata and to the contrary, these genes are completely absent in all species belong to the orders like Lagomorpha (Fig 5, Table I).

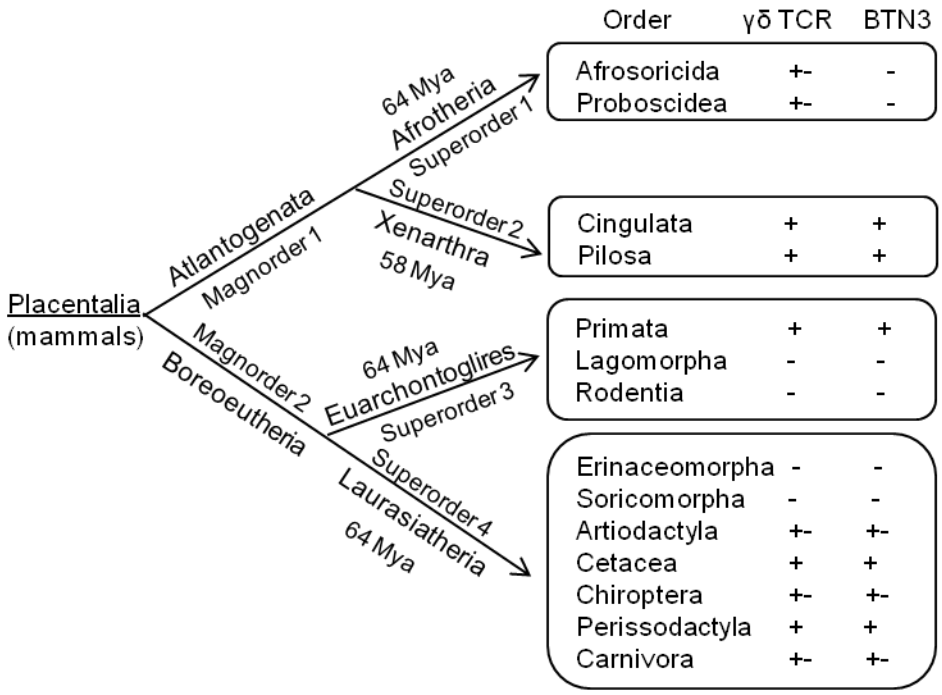

Figure 5: Distribution of Vγ9 and Vδ2 genes among mammals

The figure represents the distribution of Vγ9/Vδ2 and *BTN3* among the different orders belonging to Placentalia. Human Vγ9/Vδ2 and BTN3 IgV-IgC nucleotide sequences were taken as query and blasted against whole-genome shotgun contigs (NCBI) to search for homologous sequences across all organisms available in the NCBI database. The search was performed using the megablast algorithm to obtain even the distinct sequences that are <60% identical to the query sequence. Plus – indicates Collective representation of the presence of genes in all the species belonging to an order. Minus – Indicates the complete absence of genes in the order. Plus/minus- indicates the heterogeneous distribution of genes across the species belonging to the order. Mya – Million years ago represent the period of origin of superorders (91).

The Crown Placentalia splits into the two Magnorders; Atlantogenata and Boreoeutheria. These split into the superorders Afrotheria and Xenarthra belonging to the Atlantogenata and Euarchontoglires and Laurasiatheria belonging to the Boreoeutheria (91-93). Search of Vγ9 and Vδ2 genes across mammalian database revealed that afrotherian species, namely african elephant, manatee and cape golden mole carried only Vδ2 gene but not Vγ9. Still Vδ2 genes found in these species are highly homologous to human Vδ2 gene sequence with at least 77% identity and 93% sequence coverage. (Table I). Whereas both Vγ9 and Vδ2 genes are preserved in species (armadillo and sloth) belonging to the order Cingulata (Table 1).

Table I: Distribution of Vγ9, Vδ2 and *BTN3-ED* genes among placental mammals

Super Order	Clade	Order	Family	Species	Vγ9 (Identity/ Cover %)	Vδ2 (Identity/ Cover %)	*BTN3-ED* (Identity/ Cover %)
Afro-theria	Afro-insecti-philia	Afro-soricida	Tenrecidae	Echinops telfari (The Lesser hedgehog tenrec)	-	-	-
			Chryso-cloridae	Chrysochloris asiatica (Cape golden mole)		77/96%	-
	Paen-ungulata	Hyra-coidea	Procaviidae	Procavia capensis (Rock hyrax)	-	-	-
		Probo-scidea	Elephan-tidae	Loxodonta Africana (African elephant)	-	79/96%	-
		Sirinia	Triche-chidae	Trichechus manatus latirostris (The Florida manatee)	-	77/93%	-
Xenar-thra		Cingulata	Dasypodi-dae	Dasypus novemcinctus (Nine-banded armadillo) *	80/98%	77/98%	74/67%
		Pilosa	Megalony-chidae	Choleopus hoffmanni (Sloth) *	82/99%	75/95%	84/78%
Euar-chonto-glires	Euarchon to	Scanden-tia	Tupaiidae	Tupaia belangeri (The northern treeshrew)	-	74/94%	-
		Primata	Cheiro-galeidae	Microcebus murinus (Grey mouse lemur) *	81/99%	81/99%	87/100%
			Dauben-toniidae	Daubentonia madagascariensis (Aye-aye) *	83/99%	83/98%	89/86%
			Tarsiidae	Tarsius syrichta (Phillipine tarsier)	81/99%	?	88/100%
			Hominidae	Homo sapiens (Human) *	100%	100%	100%
	Glires	Lago-morpha	Ocho-tonidae	Ochotona princeps (The America Pika)	-	-	-
			Leporidae	Oryctolagus cuniculus (The European Rabbit)	-	-	-
		Rodentia	Caviidae	Cavia porcellus (Guinea pig)	-	-	-
			Hetero-myidae	Dipodomy ordii (Kangaroo rat)	-	-	-
			Muridae	Mus musculus (Mouse)	-	-	-
				Rattus norvegicus (Rat)	-	-	-
			Sciuridae	Spermophilis tridecemlineatus (Thirteen lined Squirrel)	77/99%	?	83/96%
Laur-asia-theria	Euli-potyphla	Erinaceo-morpha	Erinaceidae	Erinaceus europaeus (European hedgehog)	-	-	-
		Sorico-morpha	Soricidae	Sorex araneus (Common Shrew)	-	-	-
			Talpidae	Condylura cristata (Star nose mole)	-	73/95%	-
	Scroti-fera	Artio-dactyla	Camelidae	Vicugna pacos (Alpaca) *	78/98%	75/95%	84/99%
				Camelus ferus (Wild Bactrian Camel)	78/98%	73/96%	82/99%
				Camelus dromedarius (Dromedary)	78/98%	?	84/99%
			Bovidae	Bos taurus (Cow)	80/98%	77/93%	81/95%
				Ovis aries (Sheep)	75/98%	77/95%	79/81%
				Capra hircus (Yunnan black	76/98%	77/95%	79/78%

				goat)			
				Pantholops hodgsonii (Tibetan antelope)	77/98%	77/95%	80/78%
			Suidae	Sus scrofa (Wild Boar)	73/98%	79/93%	-
		Cetacea	Delphini-dae	Tursiops truncates (Bottle nose Dolphin) *	79/98%	83/94%	84/99%
				Orcinus orca (Killer whales) *	80/98%	83/93%	81/100%
		Chiroptera	Ptero-podidae	Pteropus vampyrus (Flying Fox)	77/97%	79/95%	-
			Vespertilio -nidae	Myotis lucifugus (Fruit bat)	-	78/98%	80/96%
				Myotis davidii (David's mouse eared bat)	-	79/98%	78/96%
				Eptesicus fuscus (Big brown bat)	-	77/98%	80/96%
		Perissodac tyla	Equidae	Equus caballus (Horse) *	80/99%	82/95%	83/99%
			Rhinocero-tidae	Ceratotherium simum (White Rhino)	77/99%	81/95%	81/97%
		Carnivora	Felidae	Felis catus (Cat)	-	79/80%	-
			Canidae	Canis familaiaris (Dog)	-	-	-

The table represents the distribution of Vγ9, Vδ2 and *BTN3-ED* genes across the mammals belonging to each superorders (92). Human genes were taken as query and blasted against the NCBI whole genome database (Whole-genome Shotgun database). The homologous nucleotide sequences from different species were indicated by the percentage of identity and sequence coverage length to that of query sequences. Asterisk represents the species which carries translatable Vγ9, Vδ2 and translatable *BTN3-ED* genes. Question mark represents doubt over the absence of gene. Underlined species indicate those which carry non-translatable gene sequences and their corresponding gene/genes were also underlined. Accession numbers of the genomic sequences retrieved from the database are mentioned in the materials section.

Boroeutheria splits into Euarchotoglires and Laurasiatheria. Euarchotoglires is divided into the clade Euarchonto and Glires. Order Primata belonging to the clade Euarchonto comprises species which carry both Vγ9 and Vδ2 genes. Having said that the higher primates were earlier reported for the presence of PAg responsive Vγ9Vδ2 T cells (60, 65, 67), we searched for these genes in lower primates as well. Our results shows that these genes are preserved by lower and higher primates (Fig 6) indicating that these genes are conserved throughout the primate lineage with exception of *Tarsius syrichata* (Tarsidia) which lacks the Vδ2 gene. It is aimed to test whether this is due to incomplete information in the database, which is conceivable given the low degree of coverage of the genome (twofold), or indeed a lacking gene.

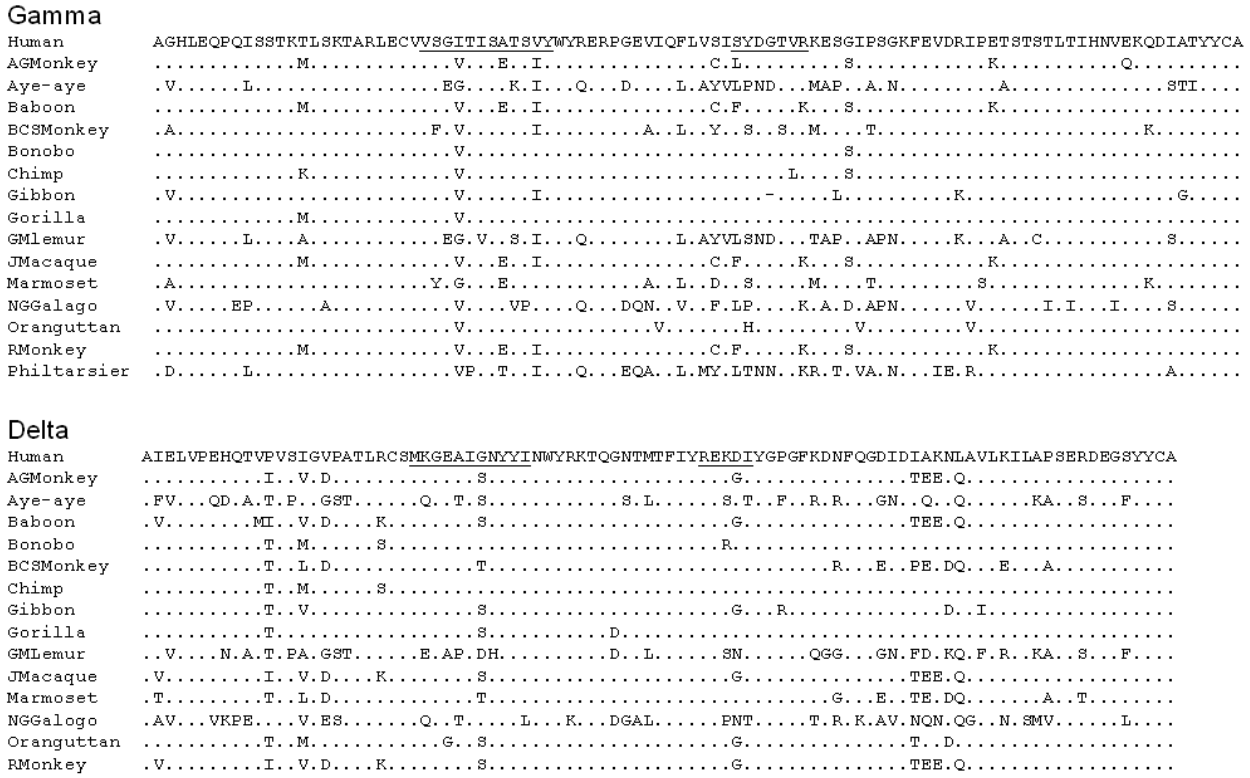

Figure 6: Conservation of Vγ9 and Vδ2 sequence among primates

The figure represents the ClustalW2 alignment of amino acid sequences of human Vγ9 and Vδ2 sequences with Vγ9 and Vδ2 like sequences of primates. Genomic human Vγ9 and Vδ2 sequences and their respective genomic homologous sequences of primates were translated *in-silico* using ExPASy Translate tool. AGMonkey – African green monkey sequences were amplified from its PBMC. Underlined amino acids represent CDR1 and CDR2 of V genes adapted from published crystal structure of human G115 Vγ9Vδ2 TCR (94). The dot represents identity to human sequence and dash indicates gap introduced by the tool for alignment.

Compared to Euarchonto, the clade Glires is in striking contrast with regard to conservation of Vγ9 and Vδ2 genes because most of the families but not all belonging to the clade Glires are negative for these genes. Most representatives of Rodentia and Lagomorpha lack all three genes which correlate well with the lack of reports on PAg T cells in mouse, rats, hamsters and guinea pigs which are classical animal models. An interesting exception might be *Spermophilis tridecemlineatus* representing the family of Sciuridae which lacks the Vδ2 gene but contains translatable Vγ9 and *BTN3-ED* genes.

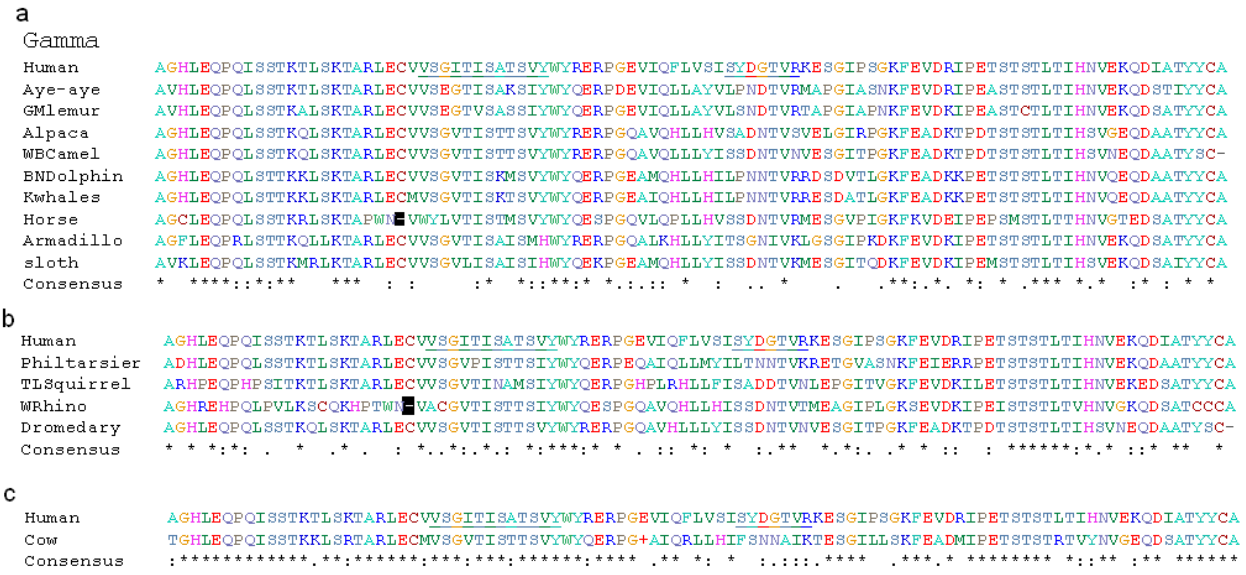

Figure 7: Conservation of Vγ9 sequence among mammals other than simian Primates

ClustalW2 amino acid sequences alignment of human Vγ9 with their respective homologous V segments in several species which carry Vγ9 like sequence. The sequences were retrieved from the NCBI genomic database. a) Species which carry Vγ9 and Vδ2 like sequences b) Species which carry only Vγ9 like sequences. C) Alignment indicating presence of stop codon in cow Vγ9 like sequence. Underlined amino acids represent CDR1 and CDR2 of V genes adapted from published crystal structure of human G115 Vγ9Vδ2 TCR (94). Asterisk represents identity to human sequence, dot represents similarity, plus indicating stop codons, black highlighted fonts represents Ig domain disturbing mutations and dash indicates gap introduced by the tool for alignment.

We also observed a similar pattern of distribution in Laurasiatheria super order, which splits in Eulipotyphyla and Scrotifera. Vγ9 and Vδ2 gene sequences were present in species belonging to Eulipotyphyla but they are absent in nearly all the species of Scrotifera. The above observations clearly suggest that these genes are not universally conserved among placental mammals though they are distributed across them . Another striking feature about Vγ9 and Vδ2 genes are that they are highly conserved among distinct species. Since primates are phylogenetically close, it is very well possible to have such a high degree of homology (>80% identity). But even phylogenetically distinct species also share high degree of homology to human Vγ9 and Vδ2 genes. For example, Sloth and whales are quite distinct from each other and from humans, but still they share >75% similarity at amino acid level (Table 1, Fig 7 and 8).

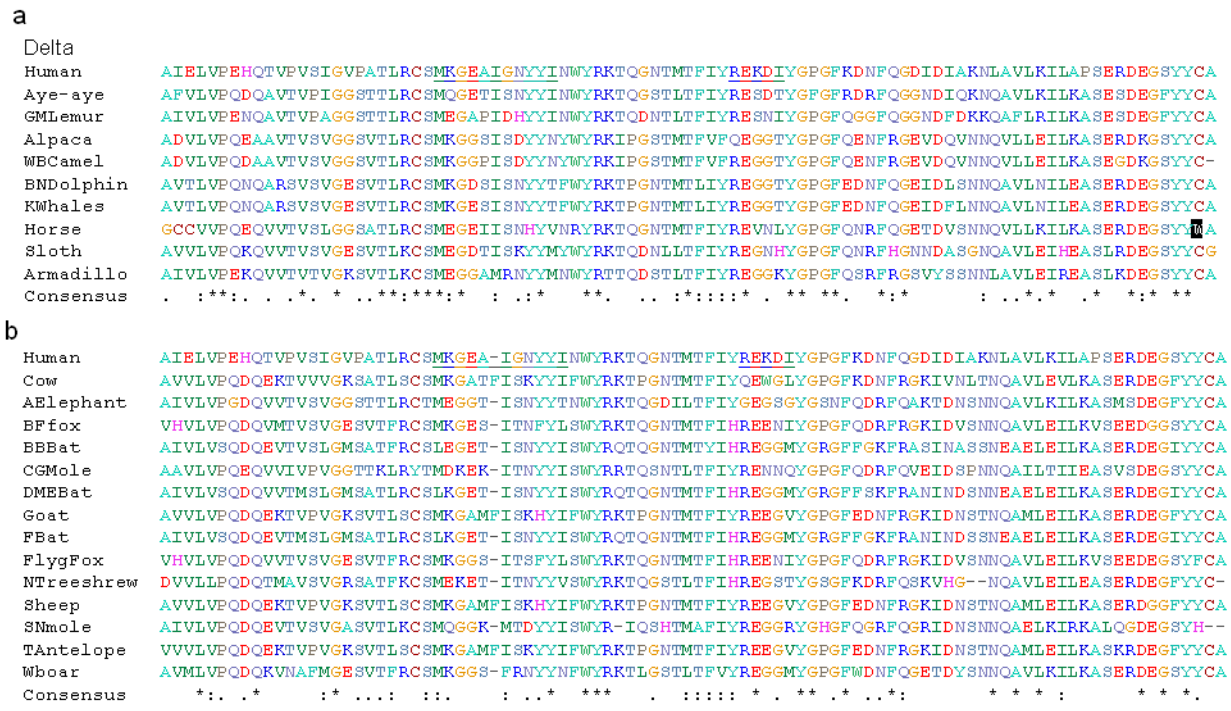

Figure 8: Conservation of Vδ2 sequence among mammals other than simian Primates

ClustalW2 amino acid sequences alignment of human Vδ2 with their respective homologous V segments in several species which Vδ2 like sequence. The sequences were retrieved from the NCBI genomic database. a) . Species which carry Vγ9 and Vδ2 like sequences. b) Species which carry only Vδ2 like sequence. Underlined amino acids represent CDR1 and CDR2 of V genes adapted from the published crystal structure of human G115 Vγ9Vδ2 TCR (94). Asterisk represents identity to human sequence, dot represents similarity, black highlighted fonts represents Ig domain disturbing mutations and dash indicates gap introduced by the tool for alignment.

Collectively, our genomic database search reveals that Vγ9 and Vδ2 genes are not restricted to primates and they are conserved in distinct species as well. Compared to Vγ9, Vδ2 gene has been preserved by more species (Fig 9). However, Vγ9 gene sequences are relatively more conserved than that of Vδ2 genes. Even though these genes are preserved by distinct species they share high homology which is evident from the phylogenetic tree where all these species emerge from a common root which had diverged from an outlier (Fig 9). Another interesting revelation is an indication for the functional association between Vγ9 and Vδ2 genes as suggested by the observation that those species which carry Vγ9 possess Vδ2 gene as well. This suggests that these gene products are preserved to carry out certain functions (Table1).

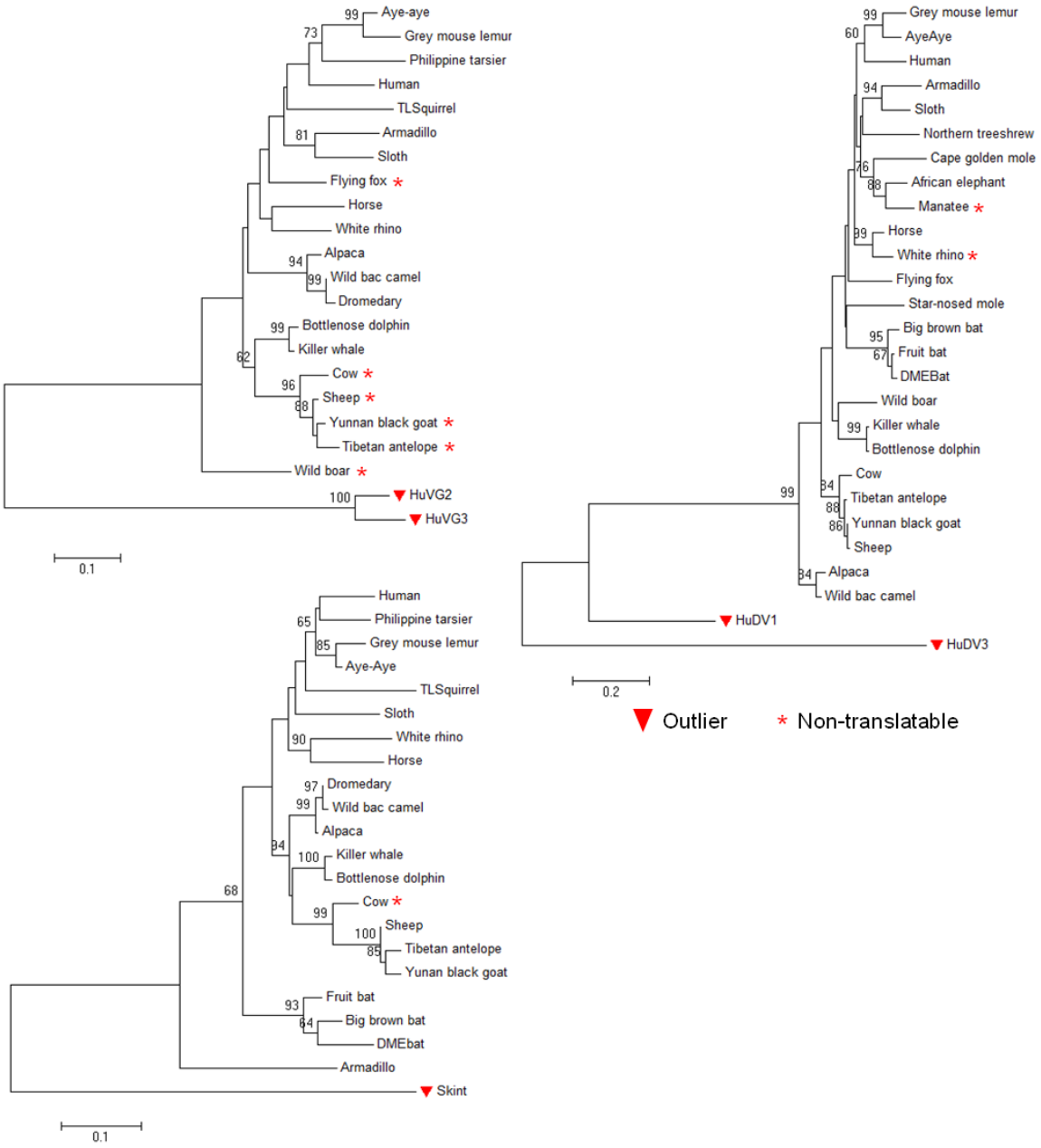

Figure 9: Phylogeny of Vγ9, Vδ2 and *BTN3-ED* among mammals excluding simian primates

The above tree represents phylogeny of Vγ9, Vδ2 and *BTN3-ED* among the non simian primates. Human Vγ9, Vδ2 and *BTN3A1-ED* and their respective homologous nucleotide sequences in several species obtained from genomic database were taken for the construction of a phylogenetic tree. The tree was constructed using MEGA 5.2 software with neighbor-joining algorithm 10000 bootstrap replications and stats less than 60 were hidden. HuVG2, HuVG3 – were human Vgamma1 and Vgamma3 respectively; HuVD1, HuVD3 – were human Vdelta1 and Vdelta3 respectively according to IMGT nomenclature. HuVG2, HuVG3, HuVD1, HuVD3 and *Skint1* of mouse were used as outlier for Vγ9, Vδ2 and *BTN3* phylogenetic tree construction, respectively. a) Phylogeny of Vγ9; b) Phylogeny of Vδ2; c) Phylogeny of *BTN3-ED.*

3.1.2 Coevolution of Vγ9, Vδ2 and *BTN3* genes

Identification of molecules involved in PAg presentation to Vγ9Vδ2 T cells has been a long time quest for the scientific community. In 2010 Harly *C et al.* has identified a molecule named BTN3A1 as a mandatory element for PAg mediated activation of Vγ9Vδ2 T cells (68). It belongs of butyrophilin family of protein. Since BTN3 is associated with the functionality of Vγ9Vδ2 T cells in human, we tried to investigate the evolutionary association between Vγ9 and Vδ2 genes and the gene of putative antigen presenting molecule. BTN3 exists in three isoforms all sharing a highly homologous (>95% amino acid identity) extracellular domain (BTN3-ED) which is composed of IgV and IgC domain (95), but differ by featuring the intracellular B30.2 domain. We performed genomic database search across mammals for *BTN3-ED* gene sequence because species which are absent for BTN3 possess proteins with a B30.2 domain.

Surprisingly like Vγ9 and Vδ2 genes, *BTN3-ED* is absent in pre-eutherian species. Several eutherian species belonging to either of eutherian magnorders possess *BTN3-ED,* but not all. This clearly indicates that *BTN3-ED* also emerged and evolved along with the origin of placental mammals. The most interesting observation was that those species that were negative for Vγ9 and Vδ2 were negative for *BTN3* gene as well. 11 species from 10 different families have lost all three genes completely (Table 1). It implies a strong functional association between Vγ9 and Vδ2 genes and *BTN3* and also indicates that these three genes might have coevolved along with evolution of placental mammals.

Though several distinct species possess Vγ9, Vδ2 and *BTN3-ED* genomic sequences homologous to human genes, it is necessary to evaluate the functional property of those sequences by determining the presence of open reading frame (ORF). Analysis of sequences for the presence of ORF by *in silico* translation revealed that some species possess non-translatable genomic sequences and they are not functional anymore. Interestingly, whenever

a species carries functional Vγ9 and Vδ2 gene, it possesses functional *BTN3-ED* also. Upon keen examination, we found out that whenever Vγ9 gene is functional, *BTN3-ED* is also functional, which again implies a strong positive association between Vγ9 and *BTN3* (Table 1). Furthermore, the *BTN3-ED* gene sequence is highly homologous between primates and non-primate species which is evident from the phylogenetic tree (Fig 9). All these data suggest that Vγ9, Vδ2 and *BTN3* genes emerged along with origin of placental mammals and indicate the functional association between Vγ9, Vδ2 and *BTN3* genes.

Since the intracellular domain of BTN3A1 has been suggested to play role in PAg mediated activation of T cells (68, 95), we looked at the distribution of *BTN3-ID*. The search revealed the presence of *BTN3-ID* like sequence in several placental species as well as monotrometa and marsupials (data not shown). We did not find BTN3-ID like sequences in species which lacked BTN3-ED sequences. Most of the species which possess BTN3-ED like sequences carried BTN3-ID as well and they are orthologous to human BTN3.

After omitting the species which possess stop codons in genomic overlaps of either of Vγ9 or Vδ2 genes, there were 10 species from 7 families which possess Vγ9, Vδ2 and *BTN3-ED in silico* translatable sequences which were determined as functional ones. Those 8 species are: 1. Sloth 2. Armadillo 3. Aye aye 4.Grey mouse lemur 5. Human 6. Alpaca 7. Wild Bactrian camel 8. Bottle nose dolphin 9. Killer whales and 10. Horse. Clustal alignment of Vγ9 and Vδ2 protein sequences indicate that these sequences were very well conserved among these species (Fig 7a and Fig 8a). Especially the conservation at protein level was evident from Vγ9 sequence (Fig 7a). The conservation of quality of genes may be vital to preserve the response against PAg or PAg like antigens. However, deduced amino acid sequences of horse Vγ9 and Vδ2 gene sequences show that they possess substitution mutations replacing conserved cysteines which disturb the Ig domain of these genes. This may lead to loss of function in horse. We also found no transcripts of Vγ9 and Vδ2 containing TCR-chains from

its PBMC (data not shown) and therefore neglected horse for further analysis. Hence, we constructed a phylogenetic tree for Vγ9, Vδ2 and *BTN3-ED* for rest of the species (Fig 10) and by use of an outlier it became obvious that all those sequences are homologous.

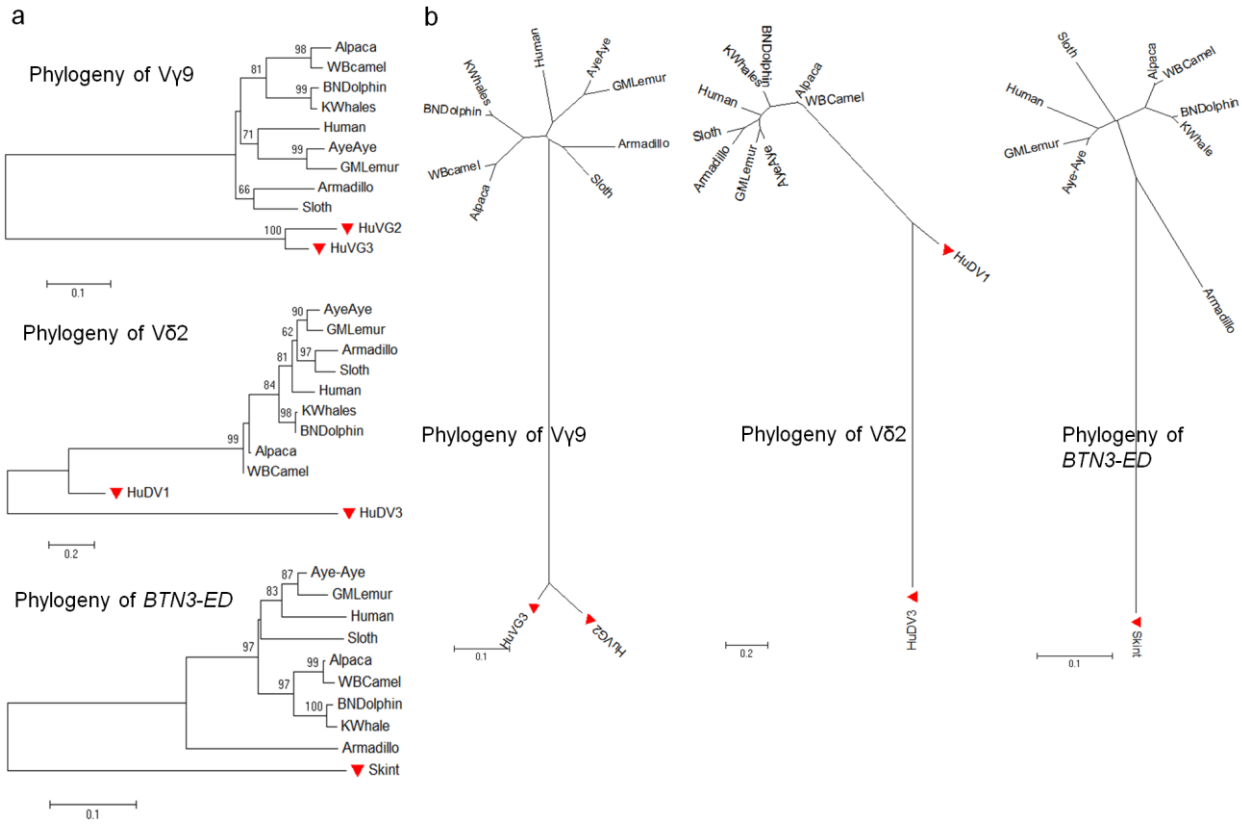

Figure 10: Co-evolution of Vγ9, Vδ2 and *BTN3* sequences among double positive species

a) The figure represents phylogeny of Vγ9, Vδ2 and *BTN3-ED* (extracellular domain) for 9 species which possess all three *in silico* translatable genes. Human Vγ9, Vδ2 and *BTN3-ED* and their respective homologous nucleotide sequences in these species obtained from genomic database were taken for the construction of a phylogenetic tree. The tree was constructed using MEGA 5.2 software by maximum likelihood algorithm with 10000 bootstrap replications and stats less than 60 were hidden. HuVG2, HuVG3 – were human Vgamma1 and Vgamma3 respectively; HuVD1, HuVD3 – were human Vdelta1 and Vdelta3 respectively according to IMGT nomenclature. HuVG2, HuVG3, HuVD1, HuVD3 *Skint1* of mouse were used as outlier for Vγ9, Vδ2 and *BTN3* phylogenetic tree construction, respectively. Accession numbers of the sequences retrieved from the database are mentioned in materials section. b) Representation of phylogeny as unrooted tree and stats frequency were hidden.

Construction of phylogenetic un-rooted tree has validated that all gene sequences are homologous indicated by the formation of clustering of species distinct from an outlier. The phylogenetic trees were constructed using different methods namely maximum likelihood,

neighbor joining and minimum evolution, but all the trees looks similar with comparable statistical frequencies (data not shown). Considering all the above data, it is evident that all three genes; Vγ9, Vδ2 and *BTN3-ED* have coevolved along with placental mammals. The quality of sequence is conserved among closely related species as well as distinct species, which strongly indicates the necessity of preservation of quality to maintain TCR-ligand like functional relationship and suggests that it might be under the control of the three genes.

3.1.3 Alpaca Vγ9 recombines preferentially with JP segment

Eight non-primate species are probable carriers of Vγ9 and Vδ2 genes. Only for alpaca we had access to lymphoid cells (PBMC) which may contain Vγ9Vδ2-TCR expressing cells. The homology of alpaca and human Vγ9 and Vδ2 genes (Fig 11a and Fig 12a) allowed the search for rearranged and expressed Vγ9-γ and Vδ2-δ TCR chains. We obtained PBMC from two animals and amplified Vγ9 and Vδ2 chains from cDNA from both the animals to analyze VDJ recombination and features which are typical PAg responsive Vγ9 and Vδ2 chains of human. Partial length Vγ9 and Vδ2 chains, comprising complete V segment and partial C segment were amplified using *Taq* DNA polymerases and analyzed by TA cloning.

Analysis of alpaca partial length Vγ9 (V-C) chains revealed that different Jγ segment recombines with alpaca Vγ9 chain (Fig 11 and Table II). Interestingly, among different Jγ segments human JP like segment also found to be recombined with Vγ9 in alpaca. Even though, Vγ9 chains with different Jγ segment exists in alpaca, the frequency of Vγ9-JP recombined Vγ9 chain was more than that of other Vγ9-J recombination. Out of 34 clones analyzed 24 clones are unique based on their unique CDR3 nucleotide sequences. Sequencing of Vγ9 chains of 34 clones revealed 20 clones carry a fully translatable Vγ9 chain. Surprisingly, 18 (13 unique) out of those 20 (14 unique) clones carry Vγ9-JP recombined

Vγ9 chain (Fig 11a). This strongly suggests the preference towards the selection of Vγ9 chain with Vγ9-JP recombination in alpaca.

Table II: Vγ9J recombination found in alpaca

Type of Jγ in sequenced clones	No. of clones (34)	
	Translatable (20)	Non-translatable (14)
DGRTIKVFGSGTRLIVT (Genomic) DG**R**TIKVFGSGTRLI**I**T (JP)	14	4
DG**K**TIKVFGSGTRLI**I**T (JP)	4	4
HRVFDGGTKLIVT (JP1)	2	1
WIKIFGEGTKLIVIPP (JP2, mouse J4)	-	4
WATTIKVFGSGTRLIIT (J1)	-	1

The table represents the usage of different J segment by alpaca Vγ9 chains. The partial length alpaca Vγ9Cγ chain was amplified and analyzed by TA cloning. Sequencing reveals 20 out of these 34 TA clones contains VDJ recombined Vγ9 chains without any stop codons. The table indicates the proportion of translatable and non-translatable Vγ9 chains for each J segment.

From the analysis of TA clones with alpaca Vγ9 chains, we also found the existence of a polymorphism in a JP like segment. We found three different JP like segments, which are provisionally assigned as JPA, JPB and JPC as we are were unable to define them as three genes or alleles (Fig 11b). From the genomic database, we could retrieve a sequence only for VpJPA (Accession number - GB|ABRR02020993.1) and not for other two forms of JP segment. In animal 1 we identified VpJPA and B and in animal 2 VpJPB and C were identified. By alanine scanning mutagenesis, germ line encoded lysine residues K (1-3) of human JP (Jγ1.2) were identified as important for the PAg reactive $Vγ9^+$ T cell clones in humans (67). K_3 was reported as essential for the pairing of Vγ9 with Vδ2 chain which was 100% conserved in all JP segments of alpaca (Fig 11b). K_1 and K_2 were reported to be important for PAg reactivity and K_1 was conserved or replaced by arginine ‘R’ which is similar to lysine (basic residue) in alpaca. In place of K_2, alpaca JP segments carry a neutral

charged threonine ‘T’ amino acid (Fig 11b). In summary, alpaca JP segment was very much similar to JP segment of γ-chain found in PAg responsive human Vγ9Vδ2 T cells.

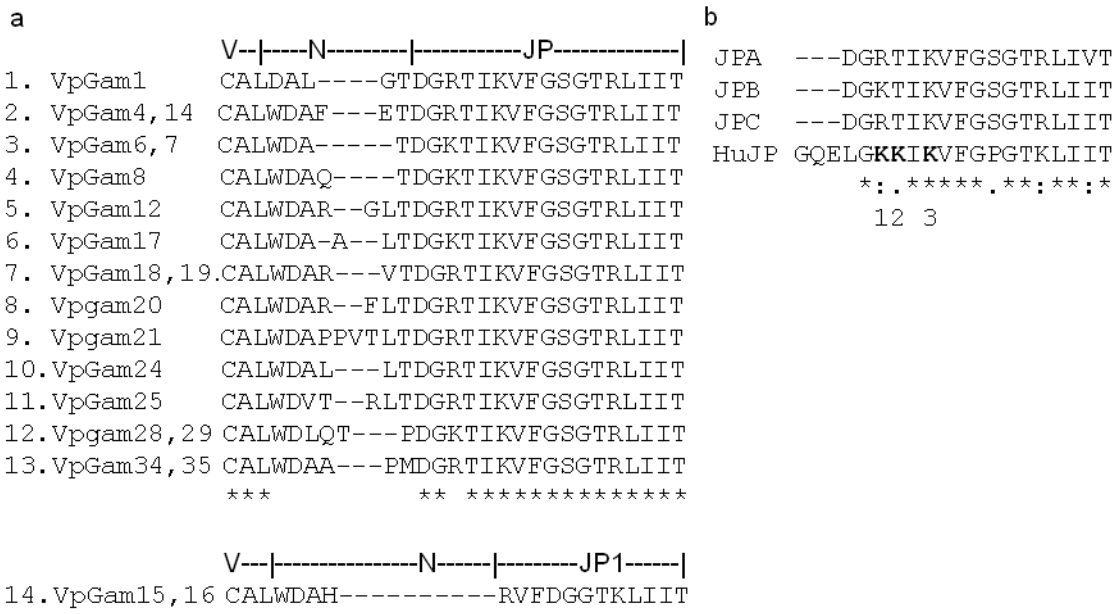

Figure 11: Characterization of Alpaca Vγ9 chains

a) Alignment of VJ recombination sequences found in TA clones bearing VDJ recombined, translatable partial length Vγ9Cγ chain. Alignment 1-13 represents Vγ9 JP recombination with different CDR3 sequences and sequence 14 represents a Vγ9 JP1 recombination found in translatable sequences. TA clones named as VpGam (clone no) and were generated in a single experiment with PBMC of animal 2. b) The figure represents polymorphism observed in JP like segments found in alpaca PBMC (HuJP – Human JP, JP – Alpaca JP). JPA, JPB and JPC were different copies of JP like J segments. JPA and JPB were found in full length chains amplified from animal1 and JPB and JPC were found in partial length chains amplified from animal 2. Bold fonts in human JP segments were important residues necessary for PAg recognition.

3.1.4 Hydrophobic residue at δ97 and diverse CDR3δ length in alpaca Vδ2 chain

Similar to alpaca Vγ9 analysis, partial length Vδ2 chains comprising complete V and partial length (V-C) were amplified and cloned into TA vector. Twenty five TA clones carrying partial length Vδ2-TCRδ chains were analyzed by sequencing to characterize the features of alpaca Vδ2 chain. Seventeen out of 25 Vδ2-TCRδ specific clones were VDJ rearranged and translatable without any stop codons (Fig 12). The salient feature of PAg responsive human Vδ2 chain is the acquisition of aliphatic hydrophobic amino acid like leucine, isoleucine, valine and glycine at position δ97 (94, 96). Analysis of VDJ rearranged translatable alpaca

Vδ2 reveals the presence of such hydrophobic residues at δ97 in at least eight clones. Another striking similarity between human and alpaca Vδ2 chains was variability in length of CDR3δ (96). Like humans; alpaca possesses CDR3δ length ranging from 11-18 amino acids. Unlike alpaca Vγ9 chains, all VDJ rearranged Vδ2 chains carry identical Jδ segment, which is highly homologous to human Jδ4 (TRDJ*04) (Fig 12)

	94	97	N/D/N	Jδ
1. VpDel2	CAA TT	M	WLGDSAGRG	PLIFGKGTYLNVEP
2. Vpdel3,16	CAA ST	**I**	YGRGRH	PLIFGKGTYLNVEP
3. VpDel4	CAA AS	**I**	RTGGIGVGFGKG	PLIFGKGTYLNVEP
4. VpDel5	CAA TY	Y	DIRTGVGLGE	PLIFGKGTYLNVEP
5. VpDel6	CAA SE	A	ISRGDVGDRG	PLIFGKGTYLNVEP
6. VpDel7	CAA TG	**G**	GGFGY	PLIFGKGTYLNVEP
7. VpDel8	CAA TP	T	THDRRSG	PLIFGKGTQLIVEP
8. VpDel10,24	CAA GV	A	GVLRHR	PLIFGKGTYLNVEP
9. VpDel11	CAA TE	**G**	GGFGY	PLIFGKGTYLNVEP
10.Vpdel14	CAA TV	D	IRGTIWLGDY	PLIFGKGTYLNVEP
11.VpDel15	CAA TH	A	GGVAVSRGG	PLIFGKGTYLNVEP
12.VpDel17	CAA SR	**V**	GVGGREG	PLIFGKGTYLNVEP
13.VpDel20	CAA TK	M	GRGWSRGIRD	PLIFGKGTYLNVEP
14.VpDel21	RAA TT	**G**	WDTGAGR	PLIFGKGTYLNVEP
15.VpDel25	CAA TL	Y	GVVGVGLGD	PLIFGKGTYLNVEP
	**			**********

Figure 12: Characterization of alpaca Vδ2 chain

The alignment represents the CDR3 amino acid sequence of Vδ2 clones with unique CDR3 sequence. The partial length alpaca Vδ2 chain was amplified and analyzed by TA cloning. Sequencing reveals 17 out of these 25 TA clones contains VDJ recombined Vδ2 chains without any stop codons. Of those 17 clones, 15 carry different CDR3 nucleotide sequences. TA-clones named as VpDel (clone no). Bold fonts indicate residues I, L, V and G which are conserved by PAg responsive human Vδ2 chains. Clones were generated in a single experiment using PBMC of animal 2.

Since we observed the presence of non-translatable VDJ rearranged Vγ9 and Vδ2 chains amplified from cDNA of alpaca PBMC, we tested the significance of such an event by analyzing the cDNA of human PBMC. Similar to alpaca, we were also able to find the presence of non-translatable VDJ rearranged Vγ9 and Vδ2 chains in human cDNA (data not shown).

3.1.5 Cloning of alpaca Vγ9 and Vδ2 like chains

The analysis of partial length Vγ9 and Vδ2 reveals striking similarities towards to PAg responsive human Vγ9 and Vδ2 TCR chains. Hence, we aimed to amplify full length Vγ9 and Vδ2 TCR-chains in order to clone and express them, and to examine their ability to pair and form a TCR at the cell surface. Since the complete sequence of Cγ and Cδ were unavailable at NCBI database, we performed 3'RACE PCR to amplify the unknown cDNA ends of Cγ and Cδ. As a result, we managed to obtain complete sequence information of Vγ9 and Vδ2 TCR chains expressed in PBMC. Thereafter, VDJ rearranged full length Vγ9 and Vδ2 TCR chains of alpaca were amplified from cDNA and were cloned into pEGZ and pIH vectors, respectively. pEGZ and pIH carried green fluorescent protein and mammalian selection markers respectively, which allowed us to track and select cells transduced with those genes of interest. The cloned TCR chains were sequenced and the alignment of alpaca Vγ9 (VpVγ9; GenBank accession number - KF734082) and Vδ2(VpVδ2) with human Vγ9Vδ2 G115 TCR clearly indicates the partial conservation of human PAg reactive features (94, 96) in alpaca TCR chains. Full length VpVγ9 possesses Vγ9JP recombination (Fig 13) which is mandatory for the PAg recognition. For analysis of alpaca chains to form TCR, we chose two alpaca Vδ2 (GenBank accession number - KF734083 and KF734084) which carry leucine (L) and glycine (G) as aliphatic hydrophobic residue at position δ97 (Fig 13) which is required for PAg reactive human Vδ2 TCR chain (94, 96). Collectively, analysis of full length Vγ9 and Vδ2 chains of alpaca reveals that they share high similarity towards human Vγ9 and Vδ2. They also carry human PAg responsive Vγ9 and Vδ2 like features which indicate a high likelihood of alpaca Vγ9 and Vδ2 TCR chains to exhibit PAg reactivity.

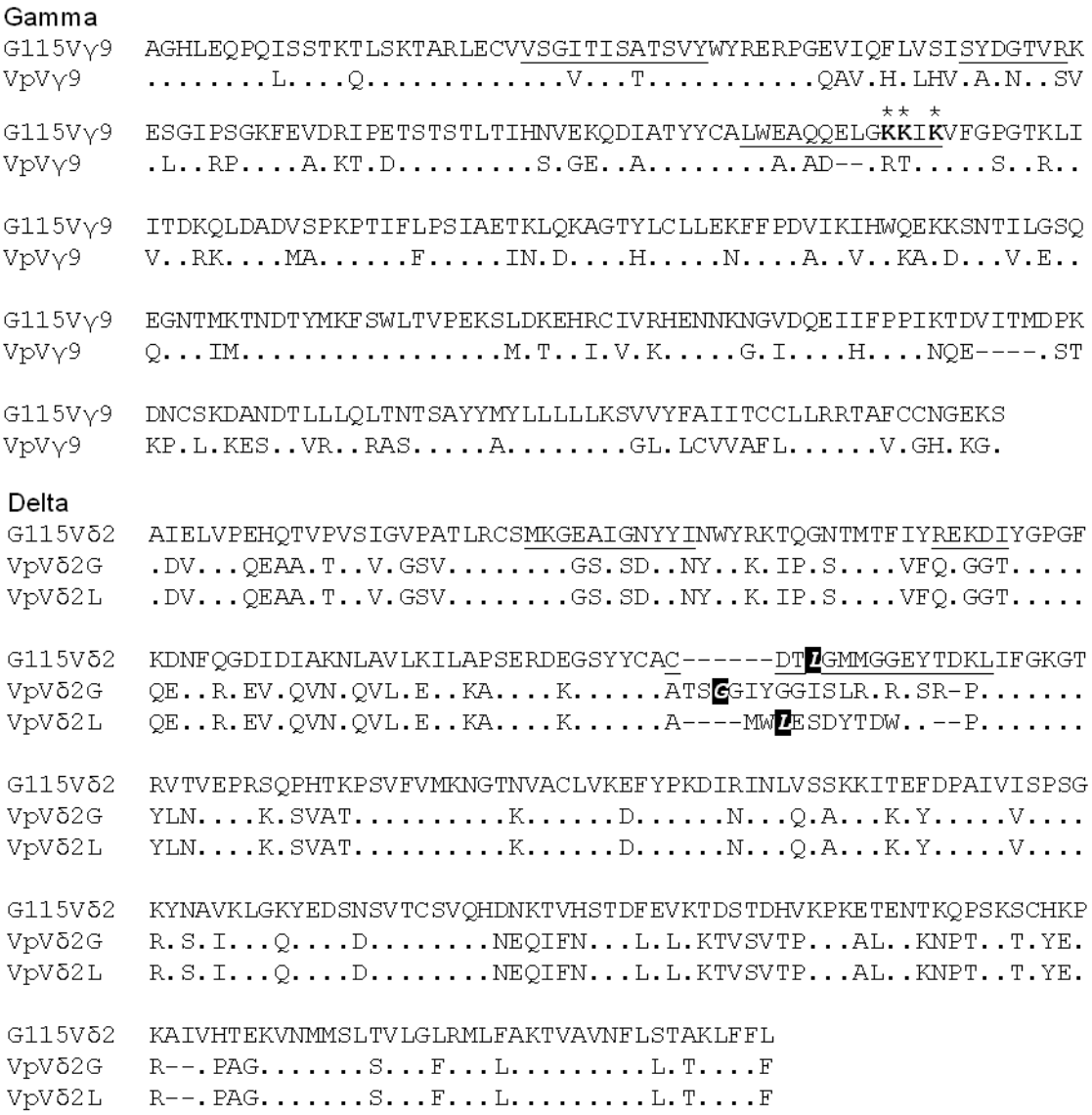

Figure 13: Alignment of alpaca Vγ9 and Vδ2 chain with human G115 Vγ9Vδ2 TCR

VpVγ9 and VpVδ2G/VpVδ2L are Vγ9 and Vδ2 chains amplified from alpaca PBMC. Alpaca Vγ9 and Vδ2 chains are aligned with human G115 γδ TCR chains. Bold fonts with asterisk - the germline encoded lysine 'K' residues in CDR3 of human Vγ9; black highlighted italics – hydrophobic residues at δ97 of Vδ2 chain. VpVδ2G and VpVδ2L are two alpaca Vδ2 chains which carry glycine 'G' and leucine 'L' at δ97 position. CDRs of Vγ9 and Vδ2 chains are underlined and are adapted from published crystal structure of human G115 Vγ9Vδ2 TCR (94). Accession numbers of full length chains are VpVγ9- KF734082; VpVδ2G - KF734083; VpVδ2L- KF734084 Clones were generated from PBMC of animal 1.

3.1.6 Expression of alpaca Vγ9Vδ2 TCR

As the sequence analysis of alpaca full length Vγ9 and Vδ2 were promising, we proceeded to test their ability to form functional TCR. To achieve the same, we transduced alpaca Vγ9 and Vδ2 chains into TCR negative mouse T cell hybridomas (Fig 14). The alpaca Vγ9(VpVγ9) was co-expressed with either of alpaca Vδ2 chains (VpVδ2G and VpVδ2L). The expression of alpaca Vγ9Vδ2 TCR was analyzed by staining with mouse CD3ε, which is expected to be expressed at the cell surface if alpaca Vγ9Vδ2 TCR chains are pairing and become associated with the mouse CD3 complex. Figure 14a shows that this was the case. The functionality of the TCR was assessed by its ability to produce IL-2 upon CD3 crosslinking. The activation of either of the alpaca Vγ9Vδ2 TCR complex (Vγ9Vδ2L TCR and Vγ9Vδ2G TCR) by CD3 crosslinking resulted in production of IL-2 which confirmed that alpaca Vγ9 and Vδ2 TCR chains can pair together to form a functional TCR (Fig 14b). In order to analyze the compatibility between distinct human and alpaca TCR chains, human Vγ9 and alpaca Vδ2 TCR chains as well as alpaca Vγ9 and human Vδ2 TCR chains were transduced. We observed surface expression of TCR in cells transduced with human Vγ9 and alpaca Vδ2 TCR chains, but not in the case of alpaca Vγ9 and human Vδ2 TCR transductants (data not shown). The above result suggests that these two species share a degree of compatibility between them to form chimeric Vγ9Vδ2 TCR. Since alpaca Vγ9 and Vδ2 TCR formed functional TCR, we accessed the possibility of existence of PAg reactive T cells in alpaca PBMC. So we performed a preliminary experiment where alpaca PBMC was cultured with HMBPP and agonist BTN3 antibody (mAb 20.1) for 24 hours. We did not observe any activation/proliferation of cells in PAg cultured PBMC, but observed the activation of cells in one of two culture-plate wells treated with agonist (mAb 20.1) antibody (data not shown).

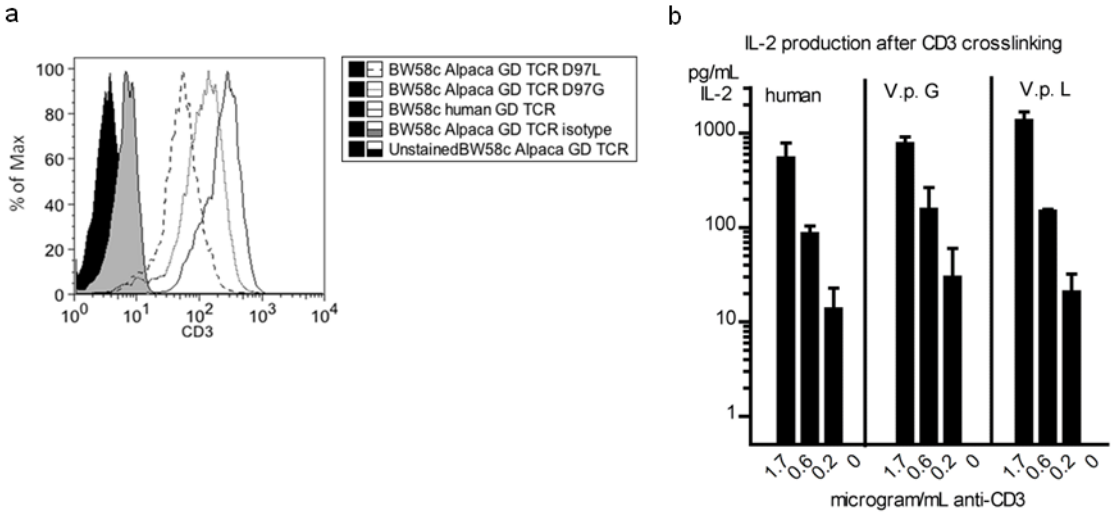

Figure 14: Alpaca Vγ9 and Vδ2 chains form functional TCR

a) Surface expression of alpaca Vγ9Vδ2TCR in BW58 r/mCD28. Histogram represents CD3 expression on the surface of cells transduced with Vγ9 and Vδ2 chains. Solid black - unstained cells, solid tinted lines - isotype staining. Solid and dashed lines represent the anti-CD3 staining of cells transduced with different Vγ9 and Vδ2 chains. Black line – human γδ TCR; grey line - Vp TCR D97G (Alpaca γδ TCR, δ97G), dashed line – Vp TCR D97L (alpaca γδ TCR, δ97L). b) IL2 production by BW58c r/mCD28 Vγ9Vδ2 TCR transductants: Human - Human γδ TCR; V.p G – Vp TCR δ97G, V.p L – Vp TCR δ97L. Error bar indicates standard deviations recorded in three individual experiments. IL-2 concentration indicated by pg/mL and anti-CD3 antibody concentration used for assay indicated in microgram/mL.

3.1.7 Predicted PAg binding sites of human BTN3A1 are conserved in alpaca BTN3-ED

The exact mechanism behind PAg presentation to Vγ9Vδ2 TCR still remains unclear, but in the last two years, BTN3A1 has been proven to be mandatory for the presentation of PAg and PAg mediated activation of Vγ9Vδ2 T cells (66, 68, 71). BTN3A1 comprises of extracellular domain (IgV-IgC) and intracellular B30.2 domain. Recently, Vavassori *et al.* work suggested the role of BTN3A1 as antigen presenting molecule to which PAg binds to its extracellular domain (BTN3-ED) (71). Hence, we searched for the presence of human *BTN3-ED* like sequence in alpaca genomic sequence. Since we retrieved such a sequence from alpaca genomic database, we tried to retrieve the complete sequence for alpaca BTN3 sequence. But we were not able to retrieve a complete sequence from the database, especially the leader

sequence which is necessary for the localization of the mature protein. So we performed 5'RACE PCR to amplify unknown cDNA ends and managed to identify the 5'end sequence. Further, we amplified the complete alpaca BTN3 like sequence comprising BTN3-ED as well as intracellular B30.2 domain. Since BTN3-ED was believed to the binding site of PAg, we analyzed the alpaca sequence by aligning with human BTN3-ED. Interestingly, the amino acids which are predicted as the binding site for PAg in human BTN3-ED were completely conserved in alpaca (Fig 15). Above results suggests the presence of PAg interactive human BTN3A1 like protein in alpaca suggesting the possibility for the existence of PAg responsive Vγ9Vδ2 T cell population in alpaca as well.

```
          --Signal peptide-----------=------------------IgV--------------------
HsBTN3A1  MKMASFLAFLLLNFRVCLLLLQLLMPHSAQFSVLGPSGPILAMVGEDADLPCHLFPTMSAETMELKWVSS
VpBTN     ..T.RS.DSPR.YLL...V.VE..T.C....A.I..P..V.VI.....E.....S.K..........Q.
          ------------------------------------------------------------------
HsBTN3A1  SLRQVVNVYADGKEVEDRQSAPYRGRTSILRDGITAGKAALRIHNVTASDSGKYLCYFQDGDFYEKALVE
VpBTN     ......FM..G........I.E.....E....D.....V....R..R.....N........N........
          ----=-----------------------IgC-------------------------------------
HsBTN3A1  LKVAALGSDLHVDVKGYKDGGIHLECRSTGWYPQPQIQWSNNKGENIPTVEAPVVADGVGLYAVAASVIM
VpBTN     ...........IEM..H.......G.T.S..........RDV..Q.M.A.A..LA...A...T.TS.L.V
          ----------------------------------=          =------TM-------=
HsBTN3A1  RGSSGEGVSCTIRSSLLGLEKTASISIADPFFRSAQRWIAALAGTLPVLLLLLGGAGYFLWQQQEEKKTQ
VpBTN     KD.A..E...IVKNP..NQ....R.............V.VF......C....T.......L.KK..EAL
                                                                 =-----------
HsBTN3A1  FRKKKREQELREMAWSTMKQEQSTRVKLLEELRWRSIQYASRGERHSAYNEWKKALFKPADVILDPKTAN
VpBTN     .LE.E.AK.EK.I.QTEKE...RIKET.QY..K..K...MA...KSQ..A.......Q........N...
          ---------------------------B30.2-----------------------------------
HsBTN3A1  PILLVSEDQRSVQRAKEPQDLPDNPERFNWHYCVLGCESFISGRHYWEVEVGDRKEWHIGVCSKNVQRKG
VpBTN     ......D....L...D.R.N........D........K..T.....................QE..E..C
          ------------------------------------------------------------------
HsBTN3A1  WVKMTPENGFWTMGLTDGNKYRTLTEPRTNLKLPKPPKKVGVFLDYETGDISFYNAVDGSHIHTFLDVSF
VpBTN     ............V.....S...A.SD...K.TVAN..QR..........EV.....M.....Y..PHTF.
          ----------------------=
HsBTN3A1  SEALYPVFRILTLEPTALTICPA-------------------------------------------
VpBTN     .GP.W.................LTGEGSSIVPDLVPDLSLENTVAVGPADENGEPQAEVTSLLVPA
```

Figure 15: Sequence alignment of human BTN3A1 and alpaca BTN3

The figure represents alignment of human BTN3A1 (HsBTN3A1) with its homologue found in alpaca (VpBTN). Bold and underlined residues of human BTN3A1 ED contribute to hydrogen bond interactions and van der Waals interactions with PAg (71). The dots represent identity to the human sequence; dash represents gaps introduced for alignments. Domain of the corresponding amino acids was mentioned above the sequence.

3.1.8 Alpaca Vγ9 and Vδ2 TCR lack Somatic hyper-mutation

Recent studies on *Camelus dromedarius* (the dromedary) reported the presence of somatic hypermutation (SHM) in γδ TCR-chains comprising Vγ and Vδ genes other than Vγ9 and Vδ2 (97, 98). Since alpaca is phylogenetically very close to the dromedary, it was of interest to look for SHM in Vγ9 and Vδ2 like genes of alpaca. The frequencies of mutations we observed in V genes of Vγ9 and Vδ2 TCR-chains are negligible and were equivalent to the frequencies observed in C segments of Vγ9 and Vδ2 chains (Table III). We conclude that Vγ9 and Vδ2 TCR-chains of alpaca lack SHM. Though alpaca Vγ9 and Vδ2 genes lack SHM, we took the effort to characterize other Vγ and Vδ genes which are reported to possess SHM in dromedary. Those genes are TRGV1, TRGV2, TRDV1, TRDV2 and TRDV4 and analysis of these genes revealed that TRDV1 and TRDV4 possess mutations but not the rest of the V genes (Table IV). But further analysis of database sequence of dromedary revealed the presence of several homologues for TRDV1 and TRDV4, which carry slight variations between them at nucleotide level. Moreover, comparison of available genomic sequences of TRDV1 and TRDV4 with cDNA clones indicated that those cDNA sequences carry mutations. The above results suggest the possible presence of SHM in TRDV1 and TRDV4 of alpaca and also the presence of duplicated genes. Therefore, in-depth investigations are required to validate whether mutations observed in TRDV1 and TRDV4 are somatic hyper-mutations or if they are a result of duplication events, because presence of enormous duplication events in TCRG and TCRD locus of ruminant have been previously reported (99-101).

Table III: Absence of somatic hypermutation in Vγ9 and Vδ2 chains of alpaca

Chain	Clones analyzed	Total no of bases	Mutations observed	Mutation Frequency
V gene of Vγ9 chain	34	9996	11	$1.1*10^{-3}$
C segment of Vγ9 chain	34	8432	9	$1.1*10^{-3}$
V gene of Vδ2 chain	25	7000	7	$1.0*10^{-3}$
C segment of Vδ2 chain	25	9675	17	$1.7*10^{-3}$

The table represents the frequency of mutations observed in V gene and C segment of Vγ9 and Vδ2 chain of alpaca.

Table IV: Search for somatic hypermutation in other V genes of γ and δ TCR genes of alpaca

Chain	Clones analyzed	Total no of bases	Mutations observed	Mutation Frequency
TRDV1	2	559	17	3/100 bases
C segment of D		610	NIL	NIL
TRDV2	3	837	NIL	NIL
C segment of D		810	NIL	NIL
TRDV4	3	864	13	1/100
C segment of D		825	NIL	NIL
TRGV1	3	777	NIL	NIL
C segment of GV1		720	NIL	NIL
TRGV2	3	927	NIL	NIL
C segment of GV2		825	NIL	NIL

The table represents the frequency of mutations observed in V gene and C segment of Vδ1, Vδ2, Vδ4, Vγ1 and Vγ2 chain of alpaca.

3.1.9 Vγ9 and Vδ2 chains are homologous between human and African green monkey (AGM)

The presence of PAg responsive Vγ9Vδ2 T cell population in African green monkeys was earlier reported (60), but sequences of Vγ9Vδ2 TCRs of AGM were not known. Interestingly, it was also found that COS-7 cells derived from AGM were unable to activate human Vγ9Vδ2 T cells (96). This raised question over the chances for the existence of species specificity in regard to PAg presentation and activation of T cells within primate lineage. Hence, cloning and testing AGM Vγ9Vδ2 TCRs will yield interesting data to understand more on species specificity with regard to PAg presentation. From mRNA of AGM, we amplified Vγ9 and Vδ2 chains. They both share a high degree of similarity towards human Vγ9 and Vδ2 chains. Like in humans, AGM Vγ9 chains do possess Vγ9 recombined JP segment which is typical feature for PAg reactive human Vγ9Vδ2 T cells. Similarly in case of Vδ2 chains, conservation of aliphatic hydrophilic amino acid at δ97 is mandatory for PAg reactivity and this feature has been conserved in the AGM Vδ2 chain as well (Fig 16). So at sequence level, AGM Vγ9 and Vδ2 chains that carry all significant features which contribute

to PAg recognition has been well preserved. Hence, AGM TCR can be tested for their ability to recognize PAg cross presented by human antigen presenting cells.

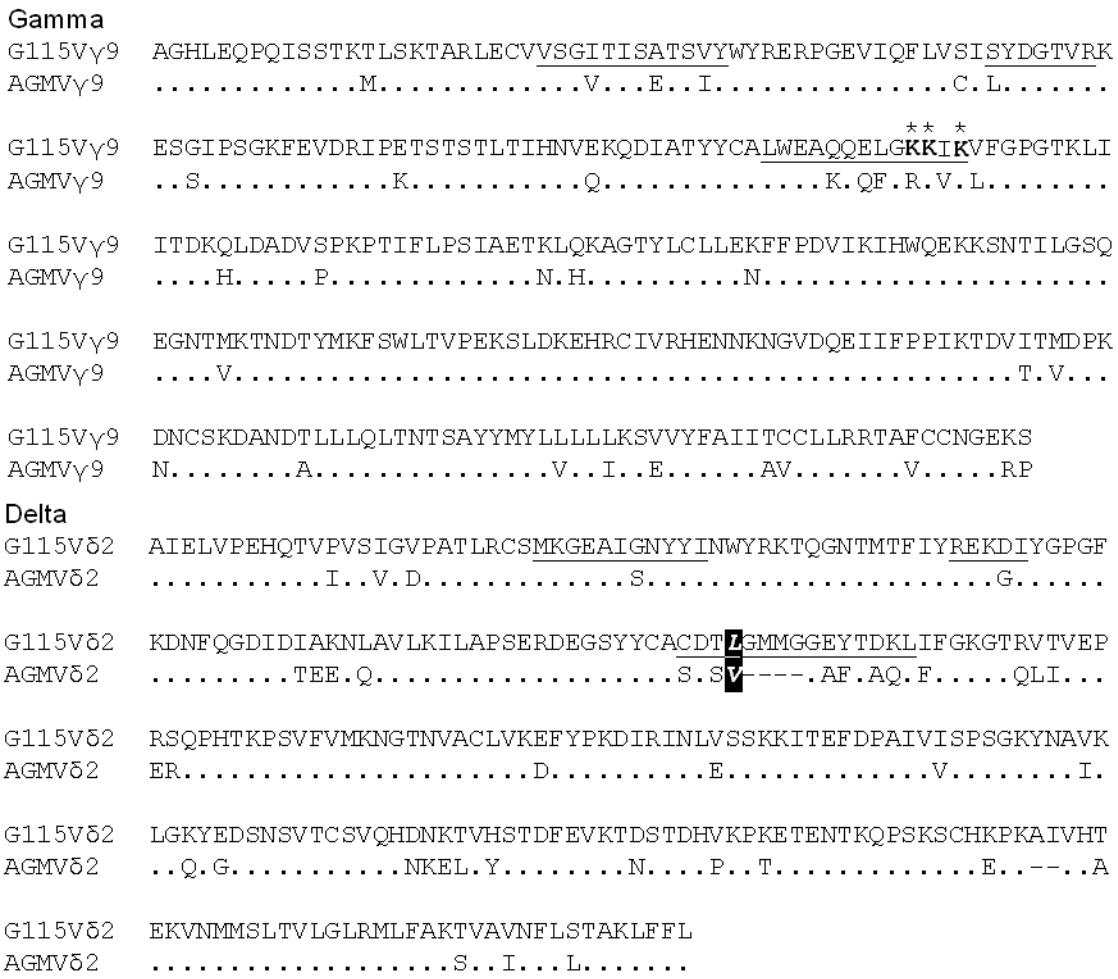

Figure 16: Sequences of AGM Vγ9 and Vδ2 TCR chains

AGMVγ9 and AGMVδ2G are Vγ9 and Vδ2 chains amplified from African green monkey PBMC. AGM Vγ9 and Vδ2 chains align with human G115 γδ TCR chains. Bold fonts with asterisks - the germline encoded lysine 'K' residues in CDR3 of human Vγ9; black highlighted bold italic font – hydrophobic residues at δ97 of Vδ2 chain. CDRs of Vγ9 and Vδ2 chains are underlined and are adapted from the published crystal structure of human G115 Vγ9Vδ2 TCR (94).

3.1.10 Interspecies interaction between human and AGM

Since it was earlier reported that COS cells were not able to activate human Vγ9Vδ2 TCR, we made an attempt to examine the ability of COS cells in presenting PAg to human and AGM Vγ9Vδ2 TCR. So we transduced COS cells with rat CD80 *(rCD80)* gene for proper signaling of co-stimulation required for the activation of our reporter Vγ9Vδ2 TCR

transductants in presence of PAg. We found that COS rCD80 cells were able to present PAg to human and AGM Vγ9Vδ2 TCR transductants (Fig 17a), but they were unable to present PAg as good as RAJI cells to human Vγ9Vδ2 TCRs. AGM Vγ9Vδ2 TCRs show auto-reactivity to RAJI cells even in absence of PAg. As it was well established that BTN3 is mandatory for PAg presentation, from our results we can conclude the BTN3 (endogenous) of COS cells can present PAg to human TCR. Interestingly, human BTN3 transduced COS cells activated both human and AGM Vγ9Vδ2 TCR several fold stronger than untransduced COS cells (Fig 17b). Hence it is evident that human BTN3 can very well present PAg to AGM Vγ9Vδ2 TCR and COS BTN3 can present to human Vγ9Vδ2 TCR (Fig 17a). From the above results, we conclude that AGM and Human can cross present PAg and activate Vγ9Vδ2 TCR of the both the species.

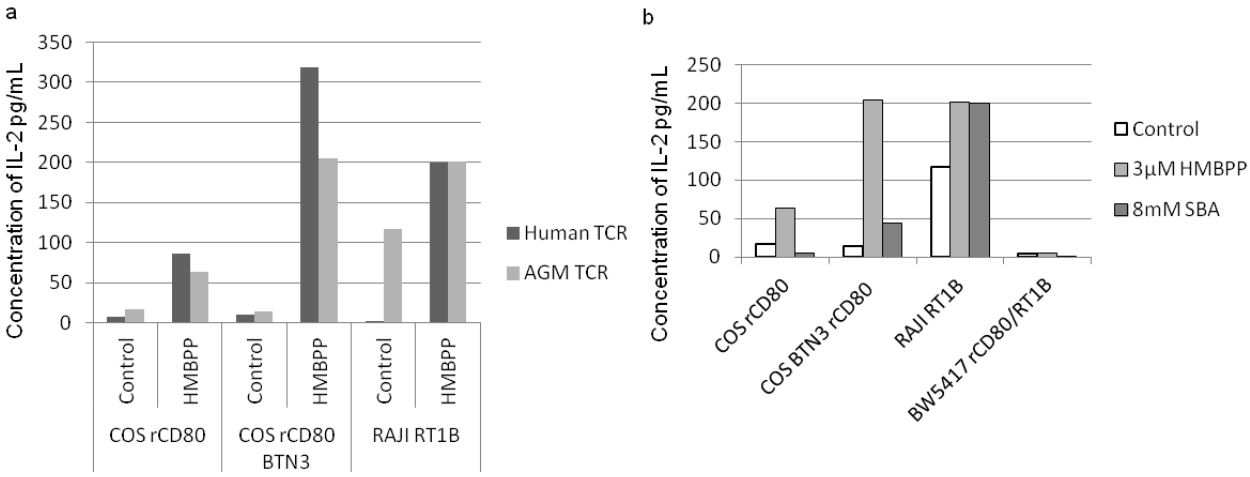

Figure 17: Cross species interaction between human and AGM

a) COS cells transduced with and without human BTN3A1 were tested for their ability to activate Human and AGM Vγ9Vδ2 TCR transductants in the presence of 3μM HMBPP. Raji cells were used as positive control. b) Represents the reactivity of AGM Vγ9Vδ2 TCR transductant to human BTN3A1 COS transductant. COS rCD80, COS rCD80/BTN3, RAJI and BW cells are antigen presenters in presence of 3μM HMBPP and 8mM SBA. RAJI and BW cells are positive and negative controls.

3.2 Identification of chromosome(s) involved in phosphoantigen presentation

3.2.1 Jurkat cells and PBL for human/mouse hybrids with BW 5147 Cells

The recognition of PAg by Vγ9Vδ2 TCR requires cell-cell contact and most of the tissues of human origin can present PAg to human Vγ9Vδ2 T cells (34, 57, 102). Though BTN3A1 was shown to be mandatory for the presentation of PAg, the exact mechanism of PAg presentation and other molecules involved in PAg presentation were unknown (66, 68, 71). Hence, we made an attempt to identify the chromosome(s) which carry the genes necessary for antigen presentation by using human/mouse somatic hybridomas. Interestingly, rodents are suitable partners as they lack Vγ9, Vδ2 TCR and *BTN3* genes, as previously shown by us (103) and also reported in this thesis. Therefore, they lack mechanism for PAg mediated presentation and activation of Vγ9Vδ2 T cells (4, 68). Human/mouse somatic hybridomas have a long history of having been used for mapping human genes (78, 80, 82, 104) as well as identification of functional human genes. Several human origin cell lines, such as Jurkat, RAJI, HEK293T cells, etc. have been reported as good presenters of PAg to Vγ9Vδ2 TCRs (56, 68). Hence, we tried to construct hybridomas of cells of human origin in combination with HAT-sensitive mouse T cell hybridoma cells line (BW5417 cell line). From the above cell lines, we constructed stable Jurkat/BW hybridomas (J/BW). Similarly, Human peripheral blood lymphocytes (PBL) were also fused with BW cells and were successfully constructed (PBL/BW).

3.2.2 Characterization of hybridomas for human and mouse markers

Soon after HAT selection, hybridomas were tested for the expression of human (Jurkat) and mouse (BW) markers to ensure they were true hybridomas. The hybridomas were analyzed for the expression of human MHC class I, human transferrin receptor (CD71) and ratCD80. MHC and transferrin represents human, whereas rCD80 represent BW mouse cells because

BW cells were transduced with *rCD80* gene. FACS staining confirmed the expression of hCD71, human MHC class I molecules and rCD80; and indicated that the hybridomas were derived from true fusion of human and mouse cells (Fig 18).

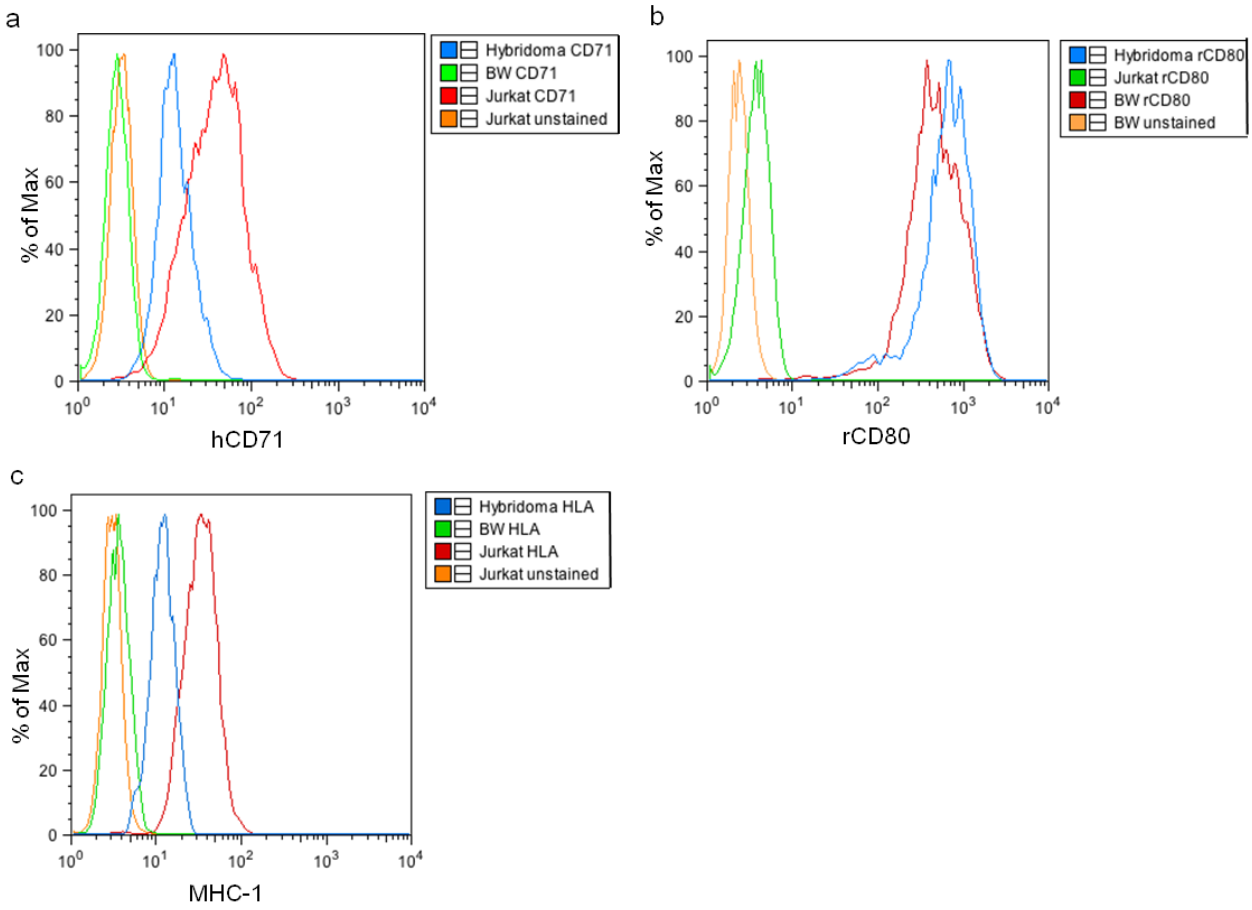

Figure 18: Characterization of Jurkat/BW hybrids

The hybridomas were characterized for the expression of human and mouse markers. Hybridomas were stained for a) human CD71, b) rat CD80, c) human MHC Class I. Jurkat and BW cells were used as control cell lines.

3.2.3 Antigen presentation by human/mouse hybridomas

Once the Jurkat and PBL based hybridomas were successfully constructed, we aimed to test their ability to present PAg. Few single cell clones which appeared after HAT/antibiotic selection of J/BW and PBL/BW hybridomas were tested for their ability to present PAg to Vγ9Vδ2 TCR transductants. The hybridomas were co-cultured with Vγ9Vδ2 TCR transductants in the presence of HMBPP and SBA. The stimulation clearly indicated the obvious differences between J/BW and PBL/BW clones in their ability to stimulate the TCR

transductants in the presence of antigen. Two clones of Jurkat/BW hybridomas (D3 and C6) (Fig 19a) and a clone (C12) of PBL/BW hybridomas (Fig 19b) were able to present HMBPP to Vγ9Vδ2TCR transductants. But none of them were able to activate TCRs in presence of SBA which already indicates the absence of compounds necessary for endogenous antigen presentation (Fig 19), which could be due to loss of chromosome encoding containing the respective genes or of expression of these genes. Though Jurkat/BW and PBL/BW hybrids present antigen, Jurkat/BW hybrids present relatively better which is indicated by the increased production of IL-2 by Vγ9Vδ2 TCR transductants (Fig 18a). Since it is known that loss of chromosome is a heterogeneous pattern (105), we performed single cell dilution of the first appeared PAg presentable hybrid clones. In summary, we proved for the first time that human/mouse somatic hybrids do present the PAg to Vγ9Vδ2 TCR transductants and might serve as a tool to map genes important for that feature.

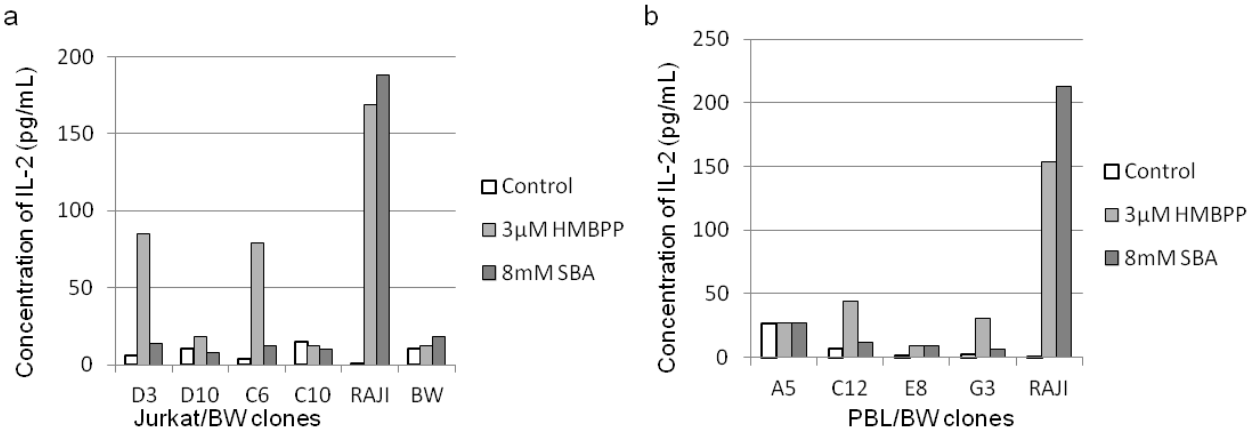

Figure 19: PAg presentation by Jurkat/BW hybrids to Vγ9Vδ2 TCR transductant

Hybridomas were tested for their ability to present PAg to Vγ9Vδ2 TCR transductant. Hybridomas and Vγ9Vδ2 TCR transductant were co-cultured along with HMBPP and SBA overnight. RAJI and BW cells were positive and negative control respectively, for the experiment. a) Analysis of Jurkat/BW clones b) Analysis of PBL/BW clones

3.2.4 Loss of chromosome by human/mouse hybridomas

Since hybridomas were able to present PAg, we aimed to analyze the stability of human genomic content in J/BW hybrids and PBL/BW hybrids by PCR karyotyping using previously reported human chromosome specific primers (84, 85) and identified loss of human chromosomes by Jurkat/BW (Fig 20) and PBL/BW clones (Table V and VI). PCR karyotyping also revealed some interesting difference between Jurkat and PBL hybridomas. In general, Jurkat/BW clones relatively lost more human chromosomes than PBL/BW clones. Within the span of 3 months Jurkat/BW clones have lost 13 human chromosomes where PBL/BW clones have lost only 1 or 2 human chromosomes (Table V). It is evident that PBL/BW hybridomas were more stable than Jurkat/BW Hybridomas (Table V) and it further strengthens that Jurkat/BW hybridomas were suitable to proceed further as they show better activation of Vγ9Vδ2 TCR transductants in presence of PAg.

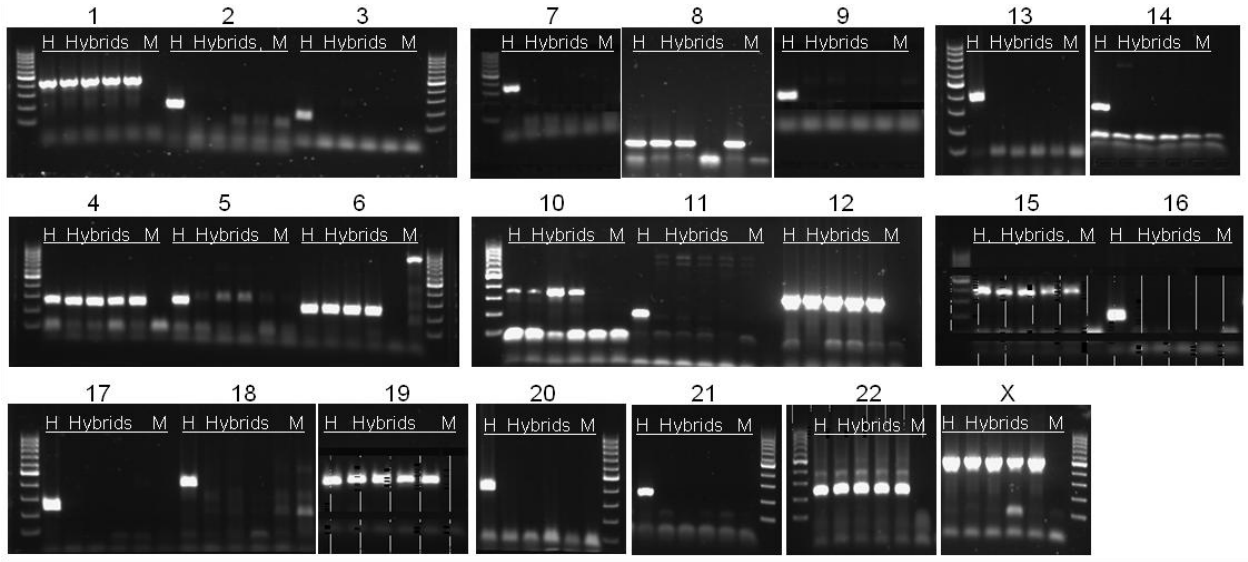

Figure 20: Human chromosome PCR karyotyping of Jurkat/BW hybridomas

Characterization of hybridomas for presence of human chromosomes. RAJI, A4 MC, A2, A11, A10 and BW is the order of samples analyzed. The number above the DNA samples indicates the chromosome tested for. RAJI and Mouse cell line 'BW' acts a positive and negative control for human specific primers.

Another interesting observation from the karyotyping analysis, especially from J/BW clones is the similarity within the clones with regards to preservation of human chromosomes. For e.g., master clone (A4) and its daughter clones (A11, A2 and A10) shares similar human genome content but they confer a very little degree of heterogeneity, which is indicated by the loss of chromosome 3 by daughter clone A2 and loss of chromosome 6 and 10 by A10, apart from the rest of chromosomes which are uniformly lost by master and daughter clones.

Table V: Karyotyping analysis of Jurkat/BW and PBL/BW hybrids

Human chromosomes	Jurkat/BW Clones					PBL/BW Clones		
	A4(+/+)	A11(++/+)	A2(++/+)	A10(-/-)		C6(++/-)	G3(++/+)	G5(++/-)
Chr**1**	+	+	+	+		+	+	+
Chr**2**	-	-	-	-		-	+	+
Chr**3**	+	+	-	+		-	+	-
Chr**4**	+	+	+	+		+	+	+
Chr**5**	+	+	+	+		+	+	+
Chr**6**	+	+	+	-		+	+	+
Chr**7**	-	-	-	-		-	+	-
Chr**8**	-	-	-	-		+	+	+
Chr**9**	-	-	-	-		+	+	+
Chr**10**	+	+	+	-		+	+	+
Chr**11**	-	-	-	-		+	-	+
Chr**12**	+	+	+	+		-	+	+
Chr**13**	-	-	-	-		+	-	-
Chr**14**	+	+	+	+		+	+	-
Chr**15**	+	+	+	+		+	+	+
Chr**16**	-	-	-	-		+	+	+
Chr**17**	-	-	-	-		+	+	+
Chr**18**	-	-	-	-		+	+	+
Chr**19**	+	+	+	+		+	+	+
Chr**20**	-	-	-	-		-	-	-
Chr**21**	+	+	+	+		+	+	+
Chr**22**	+	+	+	+		+	+	+
Chr**X**	+	+	+	+		+	+	+

The table represents the karyotyping of Jurkat/BW (JBW) hybridomas and PBL/BW for human genomic content. A4MC is a master JBW clone; A2, A11 and A10 were daughter clones of A4. C6 is a master PBLBW; G3 and G6 were its daughter clones. The activation of Vγ9Vδ2 TCR transductants by hybridomas in presence of HMBPP/SBA is indicated as + or -. The nature of the human chromosome content in hybridomas was represented as *red plus* and *black minus* sign. *Red plus* indicates presence of chromosome; *black minus* indicates loss of chromosome.

3.2.5 Lack of chromosome 6 correlates with inability to activate TCR transductants

Since the loss of chromosome is a random event, the hybridomas do exhibit heterogeneity in human chromosome content. Hence, we performed single cell dilution of hybridomas at regular intervals and analyzed the clones for their capacity to present PAg and for their genomic content. By this procedure, we found a daughter clone A10 which arose from its master clone A4 which was unable to stimulate the Vγ9Vδ2 TCR transductants in presence of HMBPP (Fig 21a). Immediate PCR karyotyping of master clone along with sub-clones and other antigen presentable clones revealed that the clone A10, which was unable to present PAg, was negative for chromosome 6 (Fig 21b). But the antigen presentable master clone A4, and its daughter clones A2 and A11 were positive for chromosome 6.

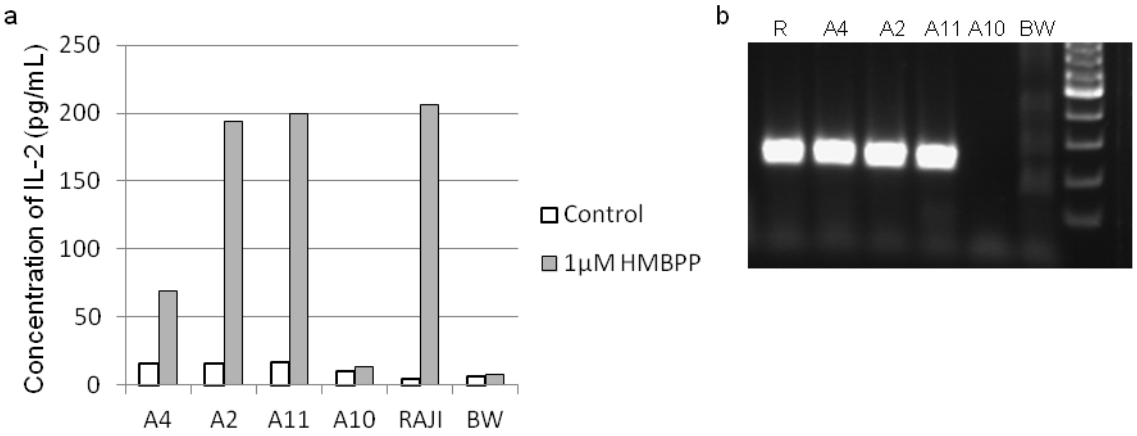

Figure 21: Lack of chromosome 6 - unable to activate Vγ9Vδ2 TCR transductant

a) IL-2 production by Vγ9Vδ2 TCR transductant. Jurkat/BW sub-clones along with their master clone were tested for their ability to present PAg to Vγ9Vδ2 TCR transductant. A4 is the master clone, A2, A11, A10 were its sub/daughter clones. Hybridomas and Vγ9Vδ2 TCR transductant were co-cultured along with HMBPP overnight. RAJI and BW cells were positive and negative control, respectively, for the experiment. b) Figure represents loss of chromosome 6 by a sub clone A10 of its master clone A4. The clone A10 was not able to stimulate the Vγ9Vδ2 TCR transductant. RAJI, A4 MC, A2, A11, A10 and BW are the order of samples analyzed.

Lack of chromosome 6 was the distinct difference between the clone A10 and the rest. Since it was already known that *BTN3A1* gene is located on chromosome 6 (69) and BTN3A1 plays important role in PAg mediated activation of Vγ9Vδ2 T cells, one can correlate the loss of

function with the loss of chromosome. Thus, we correlate the presence of chromosome 6 with the positive nature of antigen presentation by the hybridomas and loss of *BTN3* genes could be the reason behind the failure for the activation of Vγ9Vδ2 TCR transductants by chromosome 6 negative clone. Hence, our data independently validates that chromosome 6 is essential for the presentation of PAg (71).

3.2.6 BTN3 is vital for PAg presentation by human/mouse hybrids

It was earlier shown that human BTN3 plays vital role in presentation of PAg to Vγ9Vδ2 T cells (66, 68, 71). From the hybridomas we could infer the role of chromosome 6 in PAg presentation. Since BTN3 is present in chromosome 6, we validated the function of human BTN3 in PAg by human/mouse hybrids. We cocultured the hybrids with antagonist human BTN3 MAb (BT3 103.2). This resulted in inhibition of the activation of Vγ9Vδ2TCR transductants (Fig 22). The clones were able to activate Vγ9Vδ2 TCR to a great extent, but this activation was completely inhibited by the use of BT3 103.2. This again confirms the involvement of human BTN3 in PAg presentation and also affirms the lack of any such molecule in mice which could complement human BTN3 function.

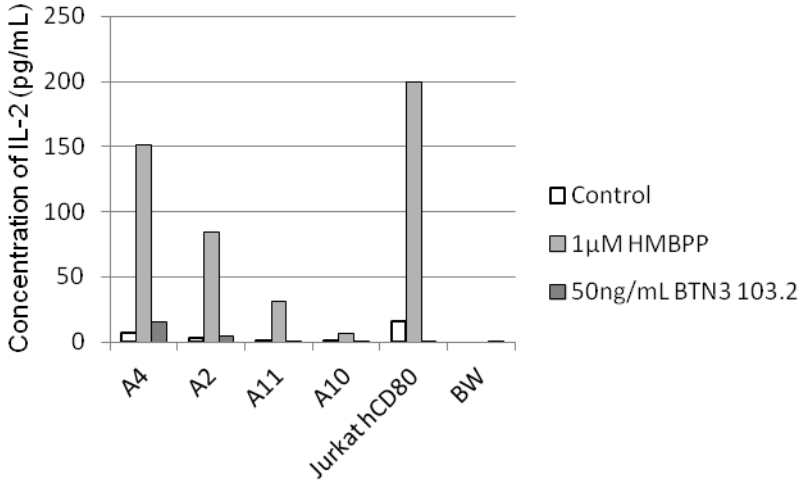

Figure 22: BTN3 is essential for PAg presentation property of Jurkat/BW hybridomas

Analysis of effect of anti-BTN3 (BTN3 103.2) antibody in PAg presentation by hybridomas. Jurkat/BW sub-clones along with their master clone were tested for their ability to activate Vγ9Vδ2 TCR transductant in the presence and absence of antagonist BTN3 antibody (BTN3 103.2). A4 is the master clone, A2, A11, A10 were

its sub/daughter clones. Hybridomas and Vγ9Vδ2 TCR transductant were co-cultured along with HMBPP (with/without BTN3 103.2 antibody) overnight. Jurkat hCD80 and BW cells were positive and negative control, respectively, for the experiment.

3.2.7 Human chromosome 6 is sufficient for presentation of HMBPP and zoledronate mediated PAg

Though our data on human/mouse hybrids supports the necessity of chromosome 6 in PAg presentation, the sufficiency of chromosome 6 to present endogenous antigen remained an open question. Hence, we attempted to evaluate whether chromosome 6 is sufficient for presentation of exogenous and endogenous PAg. Therefore, the hybridomas were co-cultured with alkylamine (Sec-butylamine; SBA), which was well known as a stimulator of Vγ9Vδ2 T cell activation by inhibiting mevalonate pathway (55, 106). Chromosome 6 positive clones were tested for their ability to activate Vγ9Vδ2 TCR transductants in presence of HMBPP and SBA and we found that some clones were unable to activate Vγ9Vδ2 TCR transductants in the presence of SBA (Fig 23). To examine whether those SBA non-responsive clones are limited to present exogenous antigen (HMBPP) only, we tested those clones for their capacity to activate TCR transductants in presence of aminobisphosphonates (Zoledronate; Zol). Interestingly, all those SBA non-responsive clones stimulated Vγ9Vδ2 TCR transductants in the presence of zoledronate (Fig 23b). The above data suggested that these two classes of stimulators, aminobisphosphonates and alkylamines, exert control over mevalonate pathway by inhibiting FPPS through different pathway/mechanisms and it doesn't seem to act via a common mechanism as per present knowledge (55). Moreover, the results clearly indicated that chromosome 6 is essential for the presentation of endogenous antigen, which was accumulated upon aminobisphosphonate treatment. Probable reason behind the lack of SBA mediated response could be extinction of activity of some gene(s) involved in SBA mediated PAg presentation, because somatic hybrids were known for extinction/loss of gene activity (72) and it might also be due to the nature of genetic complexity of mouse/human hybrids.

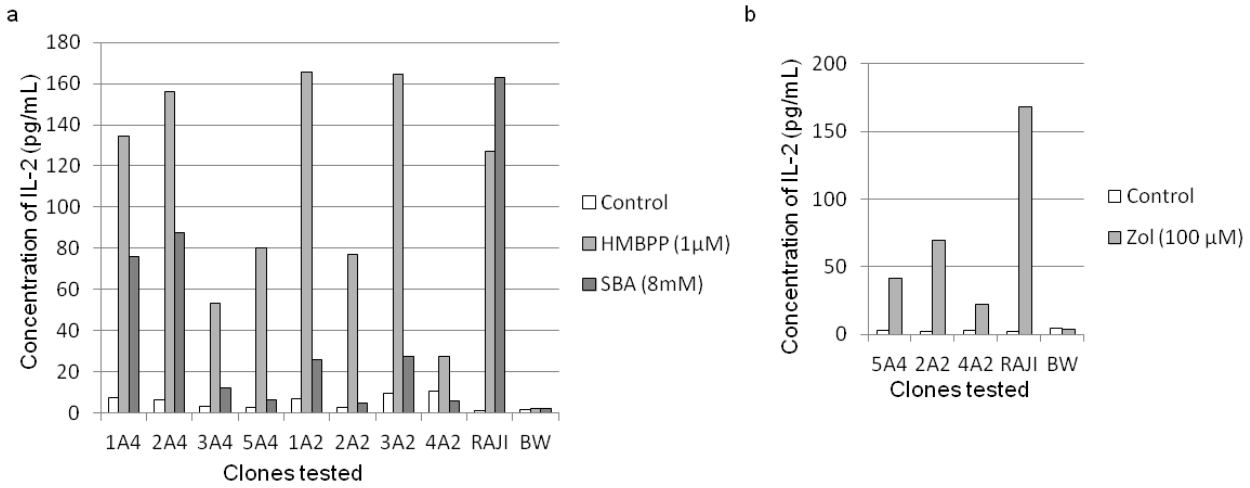

Figure 23: Chromosome 6 positive clones lack SBA mediated PAg presentation

a) Analysis of Jurkat/BW hybridomas for their ability to activate Vγ9Vδ2 TCR transductant in presence of HMBPP (1µM) and SBA (8mM). b) Analysis of SBA non-responsive clones for ability to activate Vγ9Vδ2 TCR transductant in the presence of Zol (100 µM). RAJI and BW cells act as positive and negative controls for both experiments, respectively.

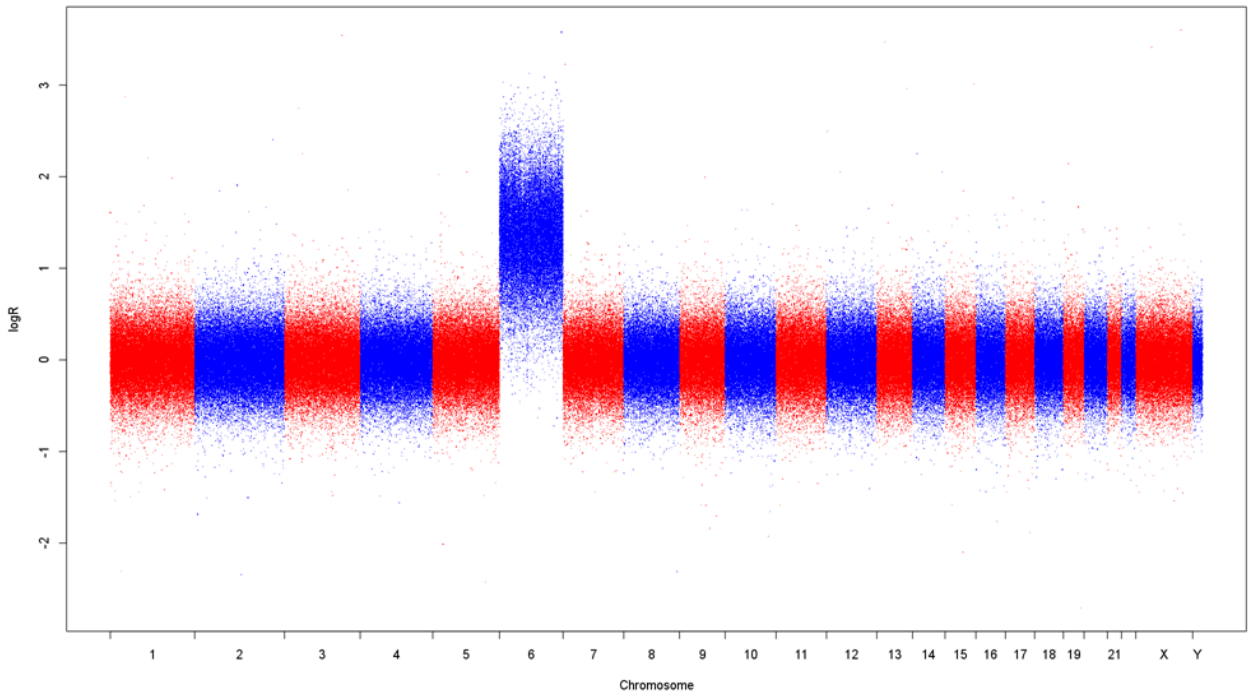

Figure 24: Characterization of CHO-Chr6 somatic hybrid

CHO-Chr6 hybrid was characterized for the presence of human chromosome 6 by comparative genomic hybridization of CHO Chr 6 with CHO cells using affimetrix GenomeWide SNP6.0 microarrays. Analysis was performed by Dr. Claus Jürgen Scholz.

Though chromosome 6 seems to be essential for PAg presentation, the involvement of other chromosomes in PAg presentation cannot be overruled by the above data because chromosome 6 positive clones carry other chromosomes as well. In order to test the sufficiency of chromosome 6 to present exogenous and endogenous antigen, we ought to find genetically simple and stable cell line. Therefore, we decided to analyze hamster/human chromosome 6 somatic hybrid cell line (CHO-chr6 somatic hybrid), which is monosomal for human chromosome 6. Hence, this cell line could act as a perfect tool to evaluate the sufficiency of chromosome 6 in PAg presentation. CHO-chr6 somatic hybrids were analyzed for the presence of chromosome 6 and other human genomic content by chromosomal array analysis. The hybrids were positive only for human chromosome 6 and lack the other chromosomes (Fig 24).

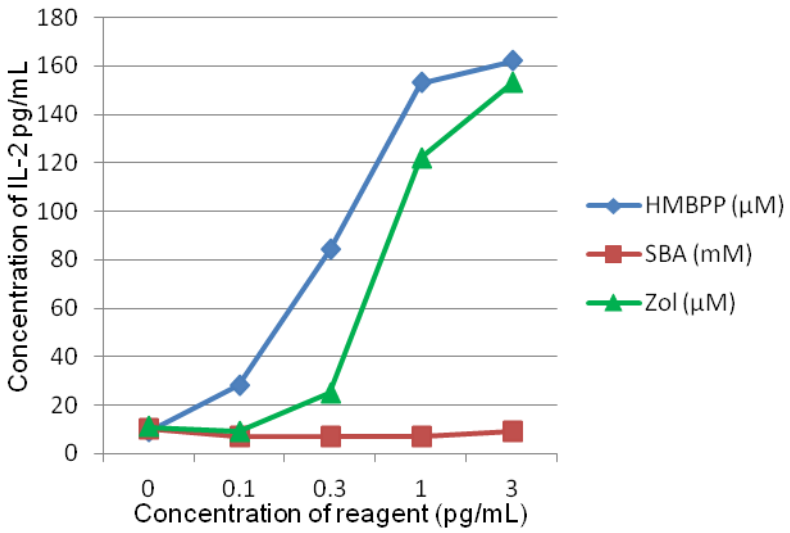

Figure 25: Chromosome 6 is sufficient for PAg presentation but not alkylamines mediated PAg presentation

CHO-chr 6 somatic hybrid cell line was tested for its ability to present PAg to Vγ9Vδ2 TCR transductant. CHO-chr6 somatic hybrid cell line and Vγ9Vδ2 TCR transductant were co-cultured along with different concentration of HMBPP, SBA and Zol overnight. HMBPP- exogenous PAg; SBA – Sec-butyl amine (alkylamine), Zol- Zoledronate (aminobisphosphonate).

The stimulation of Vγ9Vδ2 TCR transductants with CHO-chr 6 somatic hybrid in presence of exogenous antigen, alkylamine and aminobisphosphonates (HMBPP, SBA and Zol) revealed that CHO-chr6 somatic hybrid could activate Vγ9Vδ2 TCR transductants only in presence of exogenous PAg and aminobisphosphonate but not in presence of alkylamine (Fig 25). The above experiment clearly confirmed that chromosome 6 is sufficient for presentation of exogenous PAg and endogenous antigen accumulated due to aminobisphosphonate treatment. But still the reason behind the lack of response to SBA remains unclear. Because of the heterogeneous nature of genetic material, there might be the probable inhibition of genes on chromosome 6 which are involved in SBA response and it cannot be overlooked. Also the possibility for the necessity of genes on other chromosome(s) for successful SBA mediate PAg presentation still remains open. Altogether, the above result confirmed that chromosome 6 is sufficient for presentation of exogenous and endogenous PAg to Vγ9Vδ2 TCR.

4. Discussion

4.1 Identification of alpaca and other non-primate candidate species which probably conserved Vγ9Vδ2 T cells

Human γδ T cells constitute around 5 % of the total T cell population in peripheral blood (26). In which, a major subset expressing Vγ9Vδ2 TCR makes up 95% of the γδ T cell population (44, 45). They recognize pyrophosphate intermediates of the mevalonate pathway of isoprenoid synthesis in all vertebrates and most other animals and the MEP pathway of plants, many eubacteria and apicomplexaen parasites (49, 50). Such pyrophosphate metabolites are called as phosphoantigens (PAg). Vγ9Vδ2 T cells recognizing PAg were conserved in higher primates as well but absent in rodents (60, 65, 67). Hence, Vγ9 and Vδ2 TCR genes and Vγ9Vδ2 T cells were believed to be restricted to primates (107). Vγ9Vδ2 T cells recognize PAgs in MHC independent manner, but BTN3A1 is essential for PAg mediated activation of Vγ9Vδ2 T cells (66, 68, 71). Though BTN3A1 is mandatory for PAg presentation, the exact molecular basis of the PAg presentation is unclear.

Since Vγ9Vδ2 T cells are absent in classical small animal models such as mice, rat and hamster, the study on these cells was so far restricted to primates alone, which has a number of limitations (costs, ethical reasons, the availability of research tools). Hence, we aimed at identifying other possible non-primate species which could carry phosphoantigen responsive Vγ9Vδ2 T cells. To achieve the above, we performed a comprehensive analysis of mammalian genomic database (NCBI), in search of species carrying Vγ9 and Vδ2 TCR genes and *BTN3A1*, the gene of the putative antigen presenting molecule. For the first time, our study clearly shows that Vγ9, Vδ2 and *BTN3* genes emerged and co-evolved along with placental mammals because of the absence of these genes in pre-eutherian species such as marsupials and monotremeta (data not shown). Both magnorders of placental mammals include species carrying all three genes (Fig 5). Thus it is evident that Vγ9, Vδ2 and *BTN3*

genes are not restricted to primates. Secondly, this thesis has provided the first evidence for the existence of Vγ9-γ and Vδ2-δ TCR chains as well as *BTN3* gene in alpaca, with features typical for the PAg responsive Vγ9Vδ2 T cells identified in humans and primates. Furthermore, our study has led to identification of at least 5 more candidate species (non-primates) which could possess Vγ9Vδ2-like T cell population and need to be tested.

The distribution pattern of functional Vγ9, Vδ2 TCR genes and *BTN3* gene among placental mammals clearly indicated the functional association between these genes replicating TCR-ligand relationship. The functional association is obvious from the observation that wherever a species carries functional Vγ9 and Vδ2 genes, it carries a functional BTN3 gene as well. Secondly, a species which lacks Vγ9 and Vδ2 genes lacks *BTN3* gene also. Such a distribution pattern clearly suggests that the functional character of Vγ9Vδ2 T cell could be presumable under the control of Vγ9, Vδ2 and *BTN3* genes.

The presence of PAg responsive Vγ9Vδ2 T cells have been reported only for higher primates (60, 65-67), but search across the mammalian database revealed the presence of in silico translatable Vγ9, Vδ2 genes and *BTN3* genes in aye-aye and grey mouse lemur. So, it is evident that the genes controlling PAg reactive γδ T cell population were conserved throughout the primate lineage not only to higher primates (Fig 6 and 9). Interestingly, philippine tarsier, member of tarsiiformes (Haplorrhini; higher primate), lacks Vδ2 gene. Further analysis of this species is necessary to clarify whether lack of gene is real or due to lack of sequence data. The latter implies that conservation of Vγ9Vδ2 T cell in primate lineage could be a strategy for the effective immune-surveillance (107). The former will represent an exception in primate lineage where one member of the lineage has lost the PAg responsive T cell population. This would probably widen the arena to investigate for the possible mechanism or population to compensate the absence of Vγ9Vδ2 T cell population in primates.

In contrast to primates, small animal models such as mice and rat were known for the common absence of Vγ9, Vδ2 TCR genes (19) and they lack *BTN3,* but possess a similar homologue called *Skint* which is not associated to PAg response. Moreover, the above knowledge fits well with lack of reports on PAg reactive T cells from rodents and they are believed to lack PAg responsive Vγ9Vδ2 T cell system. However, analysis of public domain database revealed an interesting data, where *Spermophilus tridecemlineatus* (Thirteen lined ground squirrel); a lone member of rodent family was identified to possess functional genes homologous to *Vγ9* and *BTN3* genes. But we could not identify a homologue for *Vδ2* gene from database. It will be interesting and compelling to investigate whether the lack of homologue for Vδ2 gene is due to incomplete database sequence or nature of the organism itself. The former would lead to identification of a first small animal model which can be tested for the presence of Vγ9Vδ2 T cells and will be an exception in the rodent order. In depth investigation of such an organism could be an interesting model to understand the possible physiological reasoning behind the preservation of Vγ9Vδ2 T cells and for possible physiological consequences of PAg responsive Vγ9Vδ2 T cells system as compared with species from other rodent families.

Besides primates, Vγ9 and Vδ2 genes have been identified in several non-primate species but they were lost or remain non-functional in many species as well. Interestingly, compared to Vδ2, Vγ9 gene is relatively more conserved, even in phylogenetically distinct species (human and sloth). The nature of conservation of Vγ9 gene indirectly suggests the existence of evolutionary pressure to retain the quality of sequence which might be necessary for the preservation of its functional property. Despite the fact, among TCR genes Vγ genes are very diverse which was exclusively shown by the presence of unique Vγ genes in mouse and human (19). Furthermore, compared to Vγ9, Vδ2 gene has been preserved relatively by more species, which raises the possibility for compensation of function of Vγ9 gene by other

antigen recognition receptor genes or that function of Vγ9Vδ2 T cells could be dispensable in those species.

Such a differential distribution pattern reminds of another distinct innate T cell population, 'iNKT' cells, which recognizes antigen presented by CD1d. With regard to CD1, there exists five distinct CD1 isoforms in human, whereas rodents bear only CD1d (108). Moreover, innate like T cell population can have a different physiological role in different species, even if they hold a highly conserved genetic organization which is very well demonstrated in iNKT cells. Though iNKT TCR genes and antigen presenting molecules were highly conserved between humans and rodents, dramatic difference in frequency in CD1d restricted iNKT population was observed between human, rat and mice (109). A similar scenario was recorded for another conserved innate T cells, subset 'MAIT' cells which are restricted to MR1 molecule (110).

However, differential pattern of Vγ9Vδ2 T cell, which is indirectly reflected by the distribution pattern of Vγ9, Vδ2 genes and *BTN3* genes, could be an illustrative example of the differential dynamics of evolution for an innate T cell population within different phylogenetic groups. Importantly, it is necessary to identify the presence of distinct Vγ9Vδ2 T cell population in every candidate species in-order to understand the physiological importance of conservation of such T cell population in a few distinct species.

Though Vγ9, Vδ2 and *BTN3*, genes were distributed across placental mammals only few non-primate species namely alpaca, armadillo, sloth, bottle-nose dolphin, killer whales, wild Bactrian camel and horse carry in silico translatable genes. With exception of horse, these species possess functional genes, whereas in case of horse its Vγ9 and Vδ2 genes carry Ig-domain disturbing mutations where conserved cysteine residues were replaced as per NCBI database sequence. The quality of database sequence was validated by sequence analysis of

genomic DNA of horse PBMC. The failure to amplify Vγ9 and Vδ2 TCR chains from cDNA of horse PBMC further strengthened the likely absence of Vγ9Vδ2 T cell population in horse (M.M.K unpublished data). Interestingly, as per NCBI database sequence, cow carries homologous sequence for Vγ9, Vδ2 and *BTN3* genes but it carries stop codons in Vγ9 and BTN3 in silico translatable sequences. Analysis of genomic DNA sequence of cow confirmed the presence of stop codons in Vγ9 like genomic sequence (unpublished data) (Fig 7), which explains the reason for the absence of Vγ9 containing transcripts in circulating bovine γδ T cells (111). This apparently raises question over the mechanism of phosphoantigen responsive γδ T cells in cow (112). But it does not exclude the possibility of presence of T cell population which can confer functions analogous to Vγ9Vδ2 T cell, though it demands a formal demonstration for cognate recognition of PAg by γδ TCR in the cow.

Analysis of alpaca PBMC revealed the presence of Vγ9 and Vδ2 TCR chains with the features typical for PAg responsive Vγ9Vδ2 T cells. Those features are: 1) Presence of Vγ9 with preferential recombination to JP segment indicating a strong selection for Vγ9JP rearrangement, 2) Partial conservation of germ line encoded lysine residues (K_1-K_3) in JP, 3) Diversity in length exhibited by CDR3δ sequences, and restriction in diversity of length by ±2 in CDR3γ and the 4) Presence of aliphatic hydrocarbon residues such as glycine, isoleucine, leucine and valine at δ97 position of alpaca Vδ2-δ TCR chains. Moreover, successful surface expression of alpaca Vγ9Vδ2 TCR chains in αβ TCR negative BW thymoma cells, followed by IL-2 production by alpaca TCR transductants upon CD3 crosslinking clearly indicated the functional nature of alpaca Vγ9Vδ2 TCR. Interestingly, two distinct species human and alpaca exhibited compatibility between their TCR chains. Human and alpaca Vγ9Vδ2 TCR chains co-transduced BW thymoma cells have shown the surface expression of human alpaca chimeric Vγ9Vδ2 TCR. Interestingly, human Vγ9 chains pairs with alpaca Vδ2 chain to form a functional TCR but somehow for unknown reasons alpaca

Vγ9 chains did not pair up with human Vδ2 chains (data not shown). When alpaca TCR transductants were tested for PAg response in presence of RAJI as presenters, they failed to respond to the antigen. But this might be due to species specific barriers. In future, the response of alpaca Vγ9Vδ2 TCR transductants towards PAg can be tested by using rat CD80 and alpaca *BTN3* transduced alpaca or camelid origin cells lines as antigen presenters, which could provide insights on antigen recognition properties of alpaca TCRs. Also for further investigation of alpaca Vγ9Vδ2 T cells, it is necessary to generate necessary reagents and antibodies. These TCR transductants will also be powerful tools for generation of monoclonal antibodies where they can serve as antigen and can be used for the screening of hybridoma producing γδ TCR specific antibodies. Finally, they can be used as a tool to screen and investigate the properties of *in-vivo* Vγ9Vδ2 T cell population of alpaca.

In the recent past, it has been well documented that BTN3A1 is an inevitable associate for the PAg mediated and PAg independent activation of Vγ9Vδ2 T cells by human and primates (66, 68, 71). Identification of *BTN3* gene was mandatory to strengthen the arguable presence of Vγ9Vδ2 T cells in alpaca. Due to lack of complete *BTN3* gene sequence, full length *BTN3* sequence was identified by the 5' RACE PCR analysis. The predicted PAg binding residues of human BTN3A1 were 100% conserved in deduced amino acid sequence of alpaca BTN3 (Fig 15). Furthermore, very recent work from Andrew *et al.* has demonstrated the binding of PAg to basic residues at intracellular B30.2 domain of human BTN3A1 (113). To our surprise, all those predicted residues are conserved 100% in alpaca BTN3 as well (data not shown). In overall, on the basis of features preserved by alpaca Vγ9, Vδ2 TCR chains and *BTN3* gene, alpaca could be a promising non-primate species preserving Vγ9Vδ2 T cells. Therefore, we took an attempt to perform preliminary experiment to test alpaca PBMC with agonist anti-BTN3 and HMBPP. Interestingly, alpaca PBMC treated with agonist anti-BTN3 has shown the activation of PBMC but not in case of PAg treated cells. The above

observation, led to the interpretation for the possible existence of PAg independent activation of Vγ9Vδ2 T cells, as PAg independent BTN3 mediated activation of Vγ9Vδ2 T cells was well established (66, 68). Innate γδ T cells are an established system for recognition of stress related ligands as a mechanism of stress surveillance (42, 62). So our observation could well support the possible mechanism of BTN3 being recognized as a ligand by alpaca Vγ9Vδ2 T cells. Additionally, lack of response to PAg by alpaca PBMC indirectly favors the above hypothesis. However, it is hard to derive a conclusion on functional properties of alpaca Vγ9Vδ2 T cells based on our observation from our preliminary experiment. It is necessary to generate reagents mentioned elsewhere in text, to identify distinct Vγ9Vδ2 T cell population of alpaca to assess their response toward PAg and to understand the physiological significance of these T cells in alpaca.

In conclusion, we report that Vγ9, Vδ2 genes and *BTN3* emerged and co-evolved along with placental mammals. Furthermore, we provide data for the likely presence of Vγ9Vδ2 like T cells in alpaca and suggest alpaca as promising non-primate model to investigate the functional properties and antigen recognition properties of such a population. In-depth studies, addressing these cells are expected to pave way for understanding the functional aspects of Vγ9Vδ2 T cells in different physiological models and may even lead to the development therapeutic strategies based on Vγ9Vδ2 T cells.

4.2 Somatic hypermutation in camelids? An open question.

An interesting facet of the analysis of alpaca TCR γ and δ chains was the search for somatic hypermutation (SHM) and the absence of SHM in Vγ9 and Vδ2 chains of alpaca. Interestingly, such mutations have been reported for V regions of non-Vγ9 and non-Vδ2 like genes in another camelid, the dromedary (97, 98). Since alpaca is phylogenetically related to camel family, we analyzed for the presence of SHM in those V genes which were reported earlier for SHM in dromedary. Analysis of those V genes for SHM revealed accumulation of

mutations at moderate frequency at CDR1 and CDR2 regions, and very sparse mutations in the framework regions of TRDV1 and TRDV4 genes but not other V genes. The frequency of mutations observed in TRDV1 and TRDV4 of the alpaca was far less than that of a dromedary. Moreover, analysis of alpaca genomic database revealed the presence of several genomic homologues (at least 3 homologues) for those V genes which possess variations/mutations at CDR1 and 2, and at framework regions with similar frequency. It indicates the presence of several duplication events in alpaca for those V genes at least. Though our study suggests the possible presence of SHM in TRDV1 and TRDV4 of alpaca, it still requires in depth analysis to validate them as well as to make sure that they are not representation of duplication events. Further investigation in future might involve amplification and sequencing of complete TCRG and TCRD locus, followed by analysis of a larger cohort of cDNA clones carrying these genes for identification of V genes with SHM and to measure the load of mutations at framework regions and CDRs. As a result, two possible outcomes can be expected. One could be the lack of SHM and variation observed in clones could have been due to massively duplicated genes. This is quite possible because ruminants were proven to possess enormous duplication events in γ and δ TCR locus (99-101). Possible reasons for the divergent results could be differences between species in general. Second is an interesting possibility that γδ TCR bearing certain V genes undergo SHM and others do not. In this the case numerous questions arise; such as differential accessibility of gene loci to SHM or whether variable SHM might reflect divergent lifestyle or function of the γδ T cell subpopulation. Hence, investigation for SHM in alpaca V genes might yield better understanding towards the role of SHM in TCR genes specifically in limited species.

Another interesting data on dromedary was identification of homologue for Vγ9 gene. Earlier analysis of gamma and delta locus of dromedary resulted in non-identification of Vγ9 and

Vδ2 genes (97, 98). But from the available genomic sequence of dromedary, we could identify functional homologue for Vγ9 gene but not Vδ2. We speculate that absence of Vδ2 gene sequence could be merely due to incomplete database sequence. Also the presence of orthologous sequences to *BTN3* raises the probability for the presence of functional Vδ2 like gene sequence which might very well fit the conservation of Vγ9Vδ2 TCR-ligand relationship. Therefore, analysis of dromedary PBMC would certainly reveal the true nature of dromedary with regard to the conservation of such a relationship.

4.3 Vγ9Vδ2 TCR in African green monkey

Our analysis of African green monkey (AGM; *Chlorocebus aethiops*) for its ability to present PAg to human Vγ9Vδ2 T cells indicated the absence of any barrier to mediate inter-species presentation and activation. Though several higher primates were tested for PAg response, their ability to cross-present antigen to human Vγ9Vδ2 T cells was sparsely tested. From our study, we confirmed the presence of PAg responsive Vγ9Vδ2 T cells in AGM whose sequences were earlier unknown (60). Interestingly, germline encoded lysine residues of human CDR3γ were partially conserved in AGM, where K_1 was replaced by arginine (Fig 16). Notably, such a substitution was reported earlier in rhesus monkey and was observed in alpaca as well (67). Moreover, AGM Vγ9Vδ2 TCR transductants were activated by COS rCD80 cells as well as by human RAJI cells in presence of PAg, and the activation was increased by several folds in human BTN3A1 transduced COS rCD80 cells (Fig 17). However AGM Vγ9Vδ2 transductants can be activated by human RAJI cells even in the absence of PAg, which could be due to reactivity to some unknown surface molecules. It will be interesting to understand whether the activation of AGM Vγ9Vδ2 TCR transductants is mediated by TCR-BTN3 ligand interaction, probably by testing whether this activation can be inhibited by BTN3 specific antagonistic antibodies or knock down. Interestingly, human

Vγ9Vδ2 TCR transductants also responded to the COS rCD80 and the response was enhanced several folds by COS rCD80-hu BTN3 cells. These results clearly indicate the absence of any such species barrier which could prevent the activation of human Vγ9Vδ2 TCRs by COS cells, which was in contradiction to the inability of COS cells to activate/stimulate human Vγ9Vδ2 TCR as reported earlier (96).

4.4 Chromosome 6 is sufficient for the presentation of PAg not for activation by alkylamines

The second part of this thesis directly demonstrates that chromosome 6 is a mandatory element which carries the essential genes necessary for the presentation of PAg. Also, our data indirectly exemplified the advantage of using human-mouse somatic hybrids to map the chromosome which confers the functional phenotype. The capacity of Jurkat/BW hybrids to activate human Vγ9Vδ2 TCR transductants has directly suggested that genes and molecules are specifically involved in PAg presentation system were not disturbed or deregulated by genetic nature of somatic hybrids. Characterization of human genomic content in such human hybrids, precisely by PCR karyotyping with the use of human chromosome specific primers, had a great advantage in such gene/chromosome mapping studies. Such karyotyping tool has enabled us to predict the functional phenotype of chromosome 6 by correlating the loss of chromosome with loss of capacity of hybrids to activate TCR transductants (Fig 21). Eventually analysis of CHO-human chromosome 6 somatic hybrid has confirmed the fact that human chromosome 6 is sufficient for the presentation of PAg by antigen presenting cells.

Screening of Jurkat/BW hybridomas for their capacity to activate Vγ9Vδ2 TCR transductants were assessed in presence of HMBPP and SBA (alkylamine) where HMBPP represents exogenous PAg presentation and alkylamines accumulates endogenous IPP, which results in presentation of endogenous PAg. Initial screening of hybrids indirectly suggested that

different chromosomes might be involved in executing exogenous and endogenous PAg presentation. We interpret so because the hybrids were able to activate human Vγ9Vδ2 TCR transductants in the presence of exogenous PAg, but not in the presence of endogenous PAg (Fig 19). Since the hybrids have already lost a few chromosomes, it led to a speculation that may be genes involved in SBA mediated response might have been associated with lost chromosomes. It was reinforced by analysis of chromosome 6 positive clones that human Vγ9Vδ2 TCR transductants are activated in the presence of HMBPP, alkylamine (SBA) and aminobisphosphonate (Zol). This revealed that some clones were able to activate TCR transductants in presence of HMBPP and Zol but not in the presence of SBA (Fig 23). Unfortunately, we were not able to correlate the loss of SBA mediated response with any particular chromosome as we could not find out the significant difference in human chromosome content between presenting and non-presenting hybrids (data not shown). Since human/mouse somatic hybrids were earlier reported for their extinction nature of certain tissue specific genes (72), the nature of hybrids could also be a reason behind the specific inability to activate TCR transductants in the presence of the SBA. The above finding clearly indicates the possible existence of different mechanisms for aminobisphosphonate and alkylamine mediated control over IPP consumption or exerting inhibition of IPP consuming enzyme FPPS. Finally, analysis of CHO-chromosome 6 somatic hybrid confirmed that chromosome 6 is essential and sufficient for the presentation of exogenous and endogenous PAg. But still, CHO-Chr 6 somatic hybrid also lacked capacity to activate Vγ9Vδ2 TCR in presence of SBA, which could be due to heterogenetic nature of the hybrid. However, the observation strengthens the speculation of existence of different cellular mechanism for different stimulators to mediate PAg accumulation. At the same time, the probable involvement of other genes from other chromosome(s) engaged in SBA mediated response cannot be excluded.

4.5 Future perspectives

From our data, we show that Vγ9, Vδ2 and *BTN3* genes are not restricted only to primates. Secondly, at least 6 non-primate species were identified as candidate species which could harbor Vγ9Vδ2 T cell population. Particularly analysis of alpaca has certainly yielded promising results and findings that very well support the likely existence of Vγ9Vδ2 T cell population. As a next step, it is worth to make an attempt to we hypothesize to amplify Vγ9 and Vδ2 TCR chains from alpaca PBMC expressing natural Vγ9Vδ2 TCR by using techniques such as emulsion PCR. Further, generation of specific reagents like antibodies specific for alpaca Vγ9Vδ2 TCR specific antibodies will certainly pave way for the identification of distinct Vγ9Vδ2 T cell population.

Meanwhile, we are taking attempts to characterize alpaca BTN3 for its ability to present PAg to alpaca TCR transductants. Since alpaca BTN3 in silico amino acid sequence has conserved the predicted PAg binding amino acid, it is worth to express alpaca BTN3 with specific knockdown of human BTN3 expression. Interestingly, expression of alpaca BTN3 in a rodent background would enable us to investigate PAg independent BTN3 mediate activation of alpaca TCR transductants, as we observed the activation of alpaca PBMC co-cultured with agonist mAb 20.1 in our preliminary experiments. Additionally, expression of alpaca BTN3 could pave way to generate antibodies to unravel more about the physiological role of BTN3 in alpaca. Because, the *Skint-1;* a closest homologue of *BTN3* in mice has been reported for playing a significant role in the selection and development of thymic γδ T cells in mice (114, 115).

The armadillo is another prime candidate among those which carry functional Vγ9, Vδ2 and *BTN3* genes owing to the high similarity towards human Vγ9 and Vδ2 genes despite distant phylogenetic relationship. Most importantly, an armadillo is a well established model organism for *Mycobacterium leprae* studies since it is being a natural host for the intracellular

microbe (116, 117). Moreover, Vγ9Vδ2 T cells are particularly effective against intracellular microbe infections (64). It is highly demanding to investigate this species in detail which would unravel more about the functional significance and modulations in antigen recognition by such T cells. Furthermore, analysis of distinct non-primate species will enable us to understand about possible physiological and environmental fundamentals which drive the preservation of Vγ9Vδ2 T cells.

With regard to identification of locus on chromosome 6 involved in PAg presentation, we are planning to construct radiation hybrids based on CHO-chromosome 6 somatic hybrid. Radiation hybrid could be a useful tool to screen for locus which could carry the genes necessary for PAg presentation (118). Moreover, recent results generated from our lab clearly indicated that BTN3A1 alone is not sufficient for the PAg mediated activation of Vγ9Vδ2 T cells (F.R.A, M.M.K…. T.H unpublished data). In addition, other recent works, though did not directly demonstrate the insufficiency of BTN3A1 alone in PAg presentation but still supported the fact that other molecules could be involved along with BTN3A1 in presentation of PAg (66, 113). Moreover, a recent publication from Andrew *et al.* demonstrated the binding of PAg to intracellular domain of BTN3A1 and the need of other molecules for the transport of PAg to the cell surface was ascertained (113). Hence, it is clear that others genes/locus on chromosome 6 in association with BTN3A1 is essential for the PAg mediated activation of Vγ9Vδ2 T cells.

Furthermore, the lack of capacity of CHO-chromosome 6 somatic hybrid to activate Vγ9Vδ2 TCR transductants in presence of SBA made it compelling to investigate SBA mediated activation of Vγ9Vδ2 TCR by presenting cells of different origin (species) which carry human chromosome-6. Such an experimental condition would enable us to validate whether the lack of SBA mediated response is unanimous to all chromosome 6 positive cells. If that is not the case, then there should have been some unknown species specific regulators which

down-regulate the genes on chromosome 6 that are involved in PAg presentation, which eventually led to lack of SBA mediated response. Interestingly, in our lab, we recently observed that human skin melanoma cells were unable to activate Vγ9Vδ2 TCR (unpublished data) which suggests that the genes involved in PAg could have been down-regulated by unknown cellular mechanisms. So it will be interesting to test the capacity of CHO-chr 6 somatic hybrids to activate Vγ9Vδ2 T cells in the presence of SBA, along with modulators of gene expression such as histone deacetylase (HDAC). However, it will be interesting to continuously monitor hybrids for loss of chromosomes and probably it can result in identification of other chromosome(s) for SBA mediated activation. Such a predicted chromosome can be immediately tested by fusing human chromosome specific hamster hybrid with CHO-chromosome 6 somatic hybrid, if it results in reconstitution of SBA mediated response, then the probable chromosome(s) involved in PAg presentation can be identified.

Here, our data has yielded new findings in the field of Vγ9Vδ2 T cells which we believe would open a new perspective towards the evolution of Vγ9Vδ2 T cells, as well new insights with regard to molecular mechanism of PAg presentation to Vγ9Vδ2 T cells which are: 1) Vγ9, Vδ2 and *BTN3* genes emerged and co-evolved along with evolution of placental mammals and these genes are not restricted only to primates, 2) identification of alpaca as a promising non-primate species to harbor Vγ9Vδ2 T cell population as Vγ9, Vδ2 TCR chains and *BTN3* carry features typical for PAg reactive Vγ9Vδ2 T cells, 3) human chromosome 6 is sufficient for the presentation of exogenous antigen and zoledronate mediated but not for SBA mediated PAg presentation. These findings will pave way in understanding the modalities of Vγ9Vδ2 T cell activation and immune function, as well as to dissect the molecular mechanism involved in PAg presentation to these T cells.

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AGM	African green monkey
APC	Antigen presenting cell
BrHPP	Phosphorylated bromohydrin
BSA	Bovine serum albumin
BTN3	Butyrophylin 3
BTN3-ED	Butyrophylin 3- extracellular domain
CD	Cluster of differentiation
CDR	Complementary determining region
cDNA	Complementary DNA
CHO	Chinese hamster ovary cell
Chr6	Chromosome 6
CMV	Cytomegalovirus
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylendiamintetracetic acid
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FITC	Fluoresceinisothiocyanate
FPPS	Farnesyl pyrophosphate synthase
GFP	Green fluorescent protein
h	Hour
HAT	Hypozanthine, Aminopterin, Thymidine
HMBPP	(E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate
HT	Hypozanthine - Thymidine
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPP	Isopentenyl pyrophosphate
iNKT	Invariant Natural Killer T cells
M	Molar

Abbreviation

mAb	Monoclonal antibody
MEP	2-*C*-methyl-D-erythritol 4-phosphate pathway
MHC	Major histocompatibility complex
MICA/B	MHC class I chain-related gene A/B
min	Minutes
mRNA	Messenger RNA
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PEG	Polyethyleneglycol
RACE	Rapid amplification of cDNA ends
RNA	Ribonucleic acid
RNAase	Ribonuclease
RT	Room temperature
SBA	Sec-butylamine
SiRNA	Short interfering RNAs
TCR	T cell receptor
Tdt	Terminal deoxynucleotidyl transferase
TNF	Tumor necrosis factor
TRIS	N, n, n', n'-tetramethylethylenediamine
ULBPs	UL16-binding proteins
VDJ	Variable, Diversity and Joining segments of the TCR chain
Vp	*Vicugna pacos*
YFP	Yellow fluorescent protein
Zol	Zoledronate

Curriculum vitae

MOHINDAR MURUGESH KARUNAKARAN

Personal details

Father's Name: Karunakaran

Place and date of birth: Thiruvannamalai, India; 26.11.1986

Nationality: India

Contact Information

Address:

Phone:

Email:

Present Position

Doctoral Student at Institute of Virology and Immunobiology, Wuerzburg Since June 2010.

PhD Supervision

Prof. Thomas Herrmann, Institute of Virology and Immunobiology, Wuerzburg

Education

- PhD thesis titled "The Evolution of Vγ9Vδ2 T cells" at University of Wuerzburg, Germany, Since 2010
- M. Sc (Biotechnology): University of Madras, India, 2007 - 2009
- B. Sc (Biotechnology): Vellore Institute of Technology University, India, 2004 - 2007

Publications

Karunakaran MM, Göbel TW, Starick L, Walter L, Herrmann T. Vγ9 and Vδ2 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 April 2014, Volume 66, Issue 4, pp 243-254

Honors and Fellowships

- Awarded travel grant to participate 43rd Annual meeting of German Society of Immunology (DGfI), Sept 2013.
- Awarded travel grant to participate in Gamma Delta T cell conference, May 2012.
- Awarded Fellowship for Ph.D. by Graduate School of Life Sciences, University of Wuerzburg, April 2010
- Awarded "Summer Research Fellowship 2009" for two months from Indian Academy of Sciences, Bangalore, India

Conferences attended

- Poster presentation on "Evolution of Antigen Recognition by Vγ9Vδ2 T cells" at the 43rd Annual meeting of German Society of Immunology (DGfI), Mainz, Germany, Sept 2013
- Oral Presentation on "Evolution of Antigen Recognition by Vγ9Vδ2 T cells" at the 15th International Congress of Immunology, Milan, Italy, Aug 2013
- Poster presentation on "Evolution of Vγ9Vδ2 T cells " at EPOS, Student symposium of GSLS, University of Wuerzburg, Oct 2012
- Poster presentation on "Evolution of Antigen Recognition by Vγ9Vδ2 T cells" at Gamma Delta T cell conference, Freiburg, Germany, May 2012.
- Poster presentation on "Evolution of Vγ9Vδ2 T cells in" at The Bio Bang, Student symposium of GSLS, University of Wuerzburg, Oct 2011
- Attended 3rd Goettingen workshop on immunology and immunogenetics, June 2010.

Curriculum vitae

Research experience

- Junior Research Fellow: Vision Research Foundation, Sankara Nethralaya, Chennai.
    - To study the impact of EpCAM silencing in cancer silencing

- Master's Dissertation: National Institute of Nutrition, Hyderabad.
    - Identification of promoter of *iciA* and protein interaction between IciA and DnaA protein of Mycobacterium tuberculosis.

Technical skills

- Basic cell culture techniques
- PCR, RACE PCR, molecular cloning techniques and retro-viral transduction
- Protein isolation, purification and Western blotting
- Immuno flow-cytometry

Place: Wuerzburg yours truly,

K. Mohindarmurugesh