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INVITED REVIEW

A glance over the fence: Using phylogeny and species comparison for a better understanding of antigen recognition by human γδ T-cells

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Abstract

Both, jawless and jawed vertebrates possess three lymphocyte lineages defined by highly diverse antigen receptors: Two T-cell- and one B-cell-like lineage. In both phylogenetic groups, the theoretically possible number of individual antigen receptor specificities can even outnumber that of lymphocytes of a whole organism. Despite fundamental differences in structure and genetics of these antigen receptors, convergent evolution led to functional similarities between the lineages. Jawed vertebrates possess αβ and γδ T-cells defined by eponymous αβ and γδ T-cell antigen receptors (TCRs). "Conventional" αβ T-cells recognize complexes of Major Histocompatibility Complex (MHC) class I and II molecules and peptides. Non-conventional T-cells, which can be αβ or γδ T-cells, recognize a large variety of ligands and differ strongly in phenotype and function between species and within an organism. This review describes similarities and differences of non-conventional T-cells of various species and discusses ligands and functions of their TCRs. A special focus is laid on Vγ9Vδ2 T-cells whose TCRs act as sensors for phosphorylated isoprenoid metabolites, so-called phosphoantigens (PAg), associated with microbial infections or altered host metabolism in cancer or after drug treatment. We discuss the role of butyrophilin (BTN)3A and BTN2A1 in PAg-sensing and how species comparison can help in a better understanding of this human Vγ9Vδ2 T-cell subset.

KEYWORDS
antigen presentation, BTN2, BTN3, butyrophilin, evolution, γδ TCR

1 | INTRODUCTION

The discovery that butyrophilins (BTNs) control the development and activity of human and mouse γδ T-cell subsets,[1-3] the increasing knowledge on γδ T-cell antigen receptor (TCR)-ligand interaction[4,5] together with the exploding interest in cell-based therapeutics stimulated a general interest in γδ T-cells during the last years.[6] One fascinating aspect of γδ T-cells is their Janus-faced nature. Cells with γδ TCRs are found in all classes of jawed vertebrates, yet they can be highly divergent between species and specialized within an organism. There are clear indications for functions in the adaptive immune response, yet they also show features of an innate immune cell.[7]

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Some TCRs and their ligands seem to co-evolve and are conserved between some species but are completely lost in others and give examples for "Birth-And-Death Evolution" of multigene families.[8,9] Finally, although not subject of this review, some cells develop capabilities involved in processes such as sensing of mechanical stress,[10] thermoregulation,[11] or meningeal development [12] which raises the question on the role of their TCR in these processes. In other cases, TCRs seem to act as sensors of danger or alterations which indicate a need for surveillance and maintenance of tissue integrity.[13-15]

This review describes some of the specialized features of γδ T-cells and other non-conventional T-cells and illustrates how a comparison between species and additional phylogenetic considerations may promote a better understanding of these cells. A special emphasis will be placed on Vγ9Vδ2 T-cells. They were originally discovered in humans and have been intensively studied for their role in infection and tumor surveillance and are promising targets for cellular therapies. The TCR of the Vγ9Vδ2 T-cell subset senses phosphorylated metabolites of isoprenoid synthesis found in microbial infections and in some host T-cells, especially in tumors or after treatment with drugs such as aminobisphosphonates (ABP).[16-18] After a short overview on how species comparison and the discovery on the first non-primate species possessing these cells helps to understand the molecular basis of PAg-sensing, we discuss the discovery of BTN2A1 as new player in PAg sensing by Vγ9Vδ2 T-cells and general implications on models of γδ TCR specificity and for the development of small animal models to study physiology and develop preclinical models of human γδ T-cells.

2 | VERTEBRATE ANTIGEN RECEPTORS

2.1 | Multigene families as basis of vertebrate antigen receptor diversity

Both, jawless (Agnatha) and jawed vertebrates (Gnathostomata) possess lymphocytes with clonally expressed antigen receptors. These are encoded by recombined genes belonging to different multigene families which are rearranged by different molecular mechanisms. These rearrangements are a prerequisite for the expression of cell surface antigen receptors and mandatory for the maturation of lymphocytes and their contribution to the adaptive immune response.

The genes which are subject of the rearrangement belong to multigene families of homologous genes or gene fragments. Multigene families are found in clusters formed by cis-duplication events, and their existence allows the adoption of new functions while maintaining others or the loss of their original function. They are found for many immunologically relevant molecules and include the adaptive immune receptors of both vertebrate lineages. Members of such gene families can also be subjected to homogenization events resulting from homologous recombination as part of concerted evolution.[19] Sometimes, it is difficult to discriminate between orthologs, meaning that genes have a direct common ancestor, and paralogs, defined as genes that were created by duplication which took place after speciation. Although of interest when discussing phylogenetic relationships, sometimes we may only use the term homolog, indicating that genes have a common ancestor.

2.2 | Variable lymphocyte receptors of jawless vertebrates

Cyclostomata such as lamprey (Petromyzontidae) or hagfish (Myxinidae) as extant representatives of Agnatha possess three lymphocyte lineages defined by their variable lymphocyte receptors (VLR)A, B, or C. VLRs are composed of leucine-rich repeats (LRR) containing domains. LRRs are evolutionary conserved and found in innate immune receptors of vertebrates and invertebrates. Prominent examples are toll and toll-like receptors (TLRs) which sense structurally diverse bacterial, fungal, parasite, and viral components. The LRR domain of VLRs consists of segments with the consensus sequence LxxLxLxxN/CxL with secondary structures of a β-strand and an α-helix connected by a loop. In VLRs, they form a solenoid structure that binds antigen at its concave surface (Figure 1A). The *VLR* loci contain a central LRR region with multiple exons encoding for VLR variable sequences (LLRV) which can be combined by gene conversion-like events mediated by activation-induced cytidine deaminases

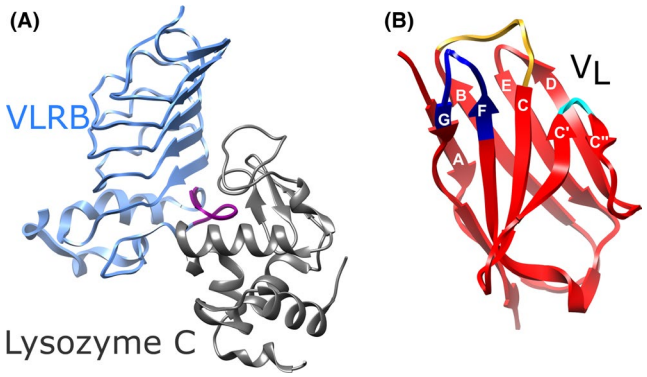


FIGURE 1 Jawless (A) and jawed antigen receptors (B). A, The variable lymphocyte receptors (VLRs) are composed of multiple leucine-rich repeats (LRRs), namely N-terminal LRRNT, LRR1, LRRV, LRRVe, LRRCP, and C-terminal LRRCT. LRR1 and LRRCP form parallel β-strands resulting in a concave surface of hydrophobic nature. LRRNT and LRRCT cap the hydrophobic core unit. The amino acids of these LRRs contact the antigen. VLRB (blue) complexed with hen egg lysozyme (dark gray) was adapted from the PDB structure 3G3A, the LRRCT (purple) loop extends into the antigen, lysozyme. For the interaction of an antibody with the same antigen, see Figure 2C. B, Immunoglobulin V-domain (IgV) is composed of two-β sheets build by short anti-parallel β-strands A-G. The loops connecting the strands B-C, C′-C″, and G-F are designated CDR1 (gold), CDR2 (cyan), and CDR3 (dark blue), respectively. The Ig-light chain (V_L) structure was adapted from the PDB structure 1HZH

(AID), like cytosine deaminase (CDA). The combination of these central LRRVs that determine the antigen specificity with a C- and an N-terminal LRR finally build up an intron-less gene.[19-21] Cyclostomata possess B-like lymphocytes producing soluble VLRs (VLRBs) and T-like cells which mature in a thymus-equivalent expressing VLRAs and VLRCs and might be seen as analogs of αβ and γδ T-cells of jawed vertebrates. The genes encoding for VLRBs are assembled by CDA1 and the *VLRA* and *VLRC* genes of the T-like lymphocytes by CDA2. The T-cell like cells may provide "help" for B-like VLRB-expressing cells as indicated by the fact that they produce cytokines such as IL-17 while VLRB-expressing cells carry the IL-17 receptor. VLRC cells are mostly found in the epithelia, analogous to γδ T-cells of jawed vertebrates, and express genes such as *Sox13* which has been connected to the development of subsets of IL-17-producing mouse γδ T-cells and genes for adhesion molecules that might be related to their epithelial localization.[20-23]

Jawed vertebrates also possess three antigen receptor-defined lymphocyte lineages but these antigen receptors differ completely from VLRs and are discussed below. Nevertheless, although both types of vertebrates use vastly different genetic mechanisms to generate their three major lymphocyte antigen receptors with completely different structures, these mechanisms were preserved over a 450 million years after separation of the phylogenetic groups.[19,24]

Such convergent evolution is common for diverging immune receptors as seen for rapidly evolving classes of mammalian NK-cell receptors. These NK-cell receptors bind to polymorphic Major Histocompatibility Complex (MHC) molecules although they were observed to be very different in structure, for example murine Ly49 molecules which are C-type lectin-like type II membrane glycoproteins and primate killer inhibitory receptor (KIRs) which belong to the immunoglobulin superfamily (IgSF).[19,25]

2.3 | The variable immunoglobulin-like domain (V-domain)

Like Cyclostomata, Gnathostomata also possesses three lineages of lymphocytes which are defined by their antigen receptors. The membrane-bound αβ or γδ TCR is expressed by αβ and γδ T-cells, respectively, and immunoglobulins which consist of an Ig-heavy and Ig-light chain and serve as antigen receptors of B-cells but are also secreted as antibodies. The antigen receptors belong to the IgSF and use a variable type immunoglobulin domain (V-domain) for antigen binding. The antigen receptors are single-pass membrane protein heterodimers consisting of a membrane-distal antigen-binding variable (V) domain and one or more constant (C) domains. These domains consist of two β-sheets, each made up of short anti-parallel β-strands usually joined by a disulfide linkage. V-domains form the antigen-binding site of antigen receptors but are also functional domains of other immunologically relevant molecules such as members of the B7 family, including butyrophilins. Figure 1B gives a general view of a single V-domain of an antigen receptor. It contains A to G β-strands forming two opposing surfaces; one composed by the A-B and E-D strands and the other by G-F, C, and C′-C″ strands. The antigen-binding sites are formed by the complementary determining regions (CDRs). The loop connecting the B and C strand is called CDR1, the one linking C′ and C″ CDR2 and CDR3 connects F and G strand. The loop between the E-strand and F-strand is also referred to as the hypervariable loop 4 (HV4). Usually, two V-domains with altogether six CDRs form the antigen-binding site with the CDR3s positioned in the center.[19]

2.4 | Genes encoding the V-domain antigen receptors

The genes for the V-region of antigen receptors are encoded by two or three exons named variable (*V*), diversity (*D*), and joining (*J*) gene (segments) which are flanked by recognition sites for the recombination-activating gene products RAG-1 and RAG-2. During lymphocyte development in the primary lymphoid organs, they are merged by the RAG-dependent recombination machinery to single genes encoding for a V-domain which is later spliced to the respective constant domains (C) and finally translated to an antigen receptor chain. The loci for the antigen receptor chains contain the respective V- and C-domain-encoding genes.[26] The antigen-binding V-domains are encoded by *V*-, *D*-, and *J*-genes of the Ig-heavy chain locus *IgH*, of the TCR-β chain locus *TRB* and the TCR-δ chain locus *TRD*. The loci for the light chain pairing with the Ig-heavy chain loci are (*IGL*) for λ and (*IGK*) for κ. Those for TCR-α chain and TCR-γ chain pairing with TCR-β or TCR-δ chains are *TRA* and *TRG*. Their variable domains are encoded by *V*-genes and *J*-genes. Especially remarkable with respect to the phylogeny of these gene loci is that in all species (except those few which have two *TRD* loci) the *TRD* locus is inserted into the *TRA* locus with the consequence that the *TRD* locus is lost during recombination of *TRA* and that some of the V-genes can rearrange with *TRDJ* or *TRAJ* genes and consequently be part of either TCR-δ or TCR-α chains.[19] This is analogous to Cyclostomata, where the *VLRC* locus is located within the *VLRA* locus.[23] Peculiarities of *TRG* loci of unknown physiological relevance are their high variability in size and composition and the use of two separate *TRG* loci by some ruminants.[27]

Many Gnathostomata, but not placental mammals, possess additional types of RAG-recombined IgSF antigen receptors which can be considered functional analogs to TCR or BCR and antibodies, respectively. Interestingly, some species such as Xenopus and chicken possess a second *TRD* locus containing *IIGHV* like variable genes.[28,29] Whether these TCR-δ-like chains pair with TCR-γ chains or are part of other antigen receptors is not yet known and also whether cells expressing these alternative types of receptors are functionally and developmentally different from typical B- and T-cells.[28]

Rearranged Ig-heavy and Ig-light chains are further diversified by AID-mediated somatic hypermutation (SHM) during affinity maturation in the germinal centers. AID also increases diversity by gene

conversion in chicken and in rabbit intestinal B-lymphocytes. For very few species, SHM has been described for TCR loci as well: *TRA*, *TRG*, and *TRD* of nurse sharks[30] and *TRG* and *TRD* of dromedary.[31] At least in nurse sharks, SHM happens in the thymus[32] and might serve as a further mechanism of diversity generation of naïve lymphocytes and not as part of an affinity maturation process as in the case of BCR or antibodies, respectively.

2.5 | Antigen-binding sites

Diversity and structure of the antigen-binding site is genetically determined. The CDR1s and CDR2s are encoded by the *V*-genes, and hence, their diversity is limited by the *V*-gene number while CDR3s correspond to a region generated by recombination of *V*-, *(D*-), and *J*-genes. The RAG-dependent recombination mechanism generates CDR3 variability by combining *V(D)J*-genes and additional junctional diversity by excision or insertion of variable numbers of nucleotides at the recombination sites and insertions of N-nucleotides by the terminal-deoxynucleotide transferase (TdT). The lengths of most αβ TCRs are rather restricted. This reflects the general conservation of the ligands for MHC-restricted T-cells whose TCRs bind MHC-peptide complexes. The peptides of these complexes bind to an antigen binding groove on the MHC molecule flanked by two α-helices. TCRs bind with all six CDRs to the MHC-peptide complexes.[33,34] TCRs of non-conventional αβ T-cells bind to complexes of non-polymorphic MHC class I-like (class Ib) molecules and small non-peptide antigens. Those TCRs also cover the surface formed by antigen and α-helices but sometimes as in the case of invariant natural killer T-(iNKT-)cell TCRs, only a few CDRs contribute to ligand-binding.[34] The lengths of the CDRs in Ig can vary considerably which reflects the chemical and topological variety of antigens. The CDR3-length of the TCR-γ chains is rather homogenous while lengths of CDR3s of TCR-δ can vary significantly and be very long. Such long CDR3δs can result from varying numbers of *D*-genes which are used simultaneously by different species (eg, two in mouse, three in humans, four in cow)[35] which increases junctional diversity and potential diversity of antigen receptors in general[26,36] (Figure 2).

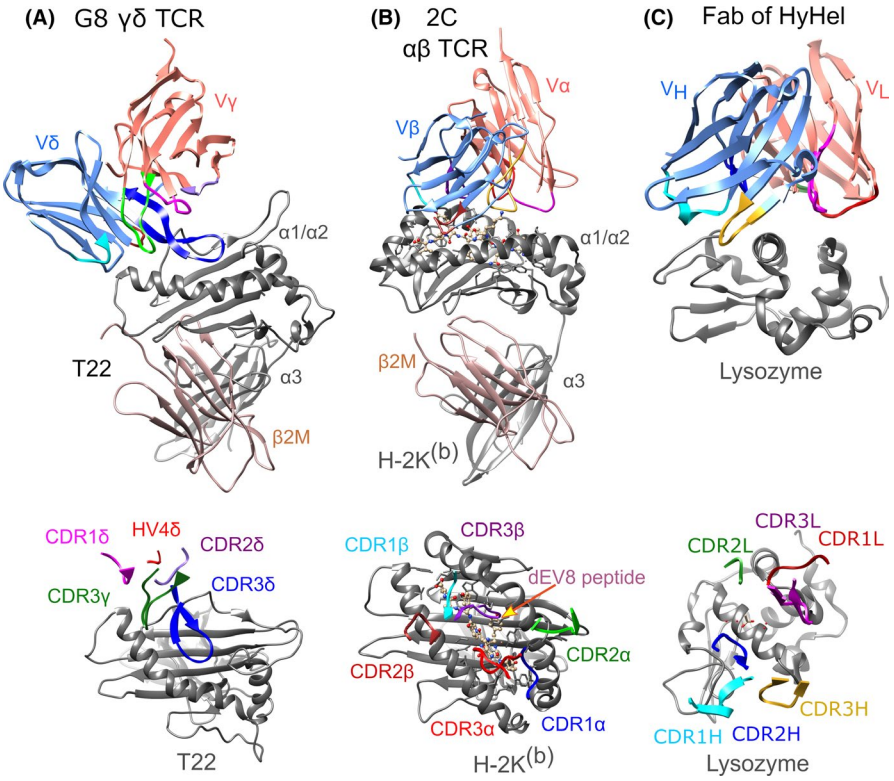


FIGURE 2 Examples for contribution of CDRs to ligand binding in different classes of IgSF antigen receptors. For detailed discussion of interactions, see.[60] Upper panel: A, γδ TCR CDR3 of the δ-chain dominates interaction with its ligand while the other CDRs and HV4 of the δ-chain and CDR3γ make only minor contributions. B, requires the participation of all CDRs of both the TCR chains for the same. C, Comparable interaction was observed with some BCR as well. Noteworthy of all the above types of receptors, is their uniqueness in the mode of antigen recognition. Lower panel: A, G8 γδ TCR-T22 complex (adapted from PDB 1YPZ), CDR3δ loop is relatively longer than the rest and the one that extends into the groove formed between α1/α2 helixes of T22. B, the 2C αβ TCR interacts with H-2K(b)-dEV8 peptide complex (adapted from 2CKB), where CDR3α and CDR3β contact the peptide in the groove. CDR2α and CDR2β interact with α1/α2 helixes, and CDR1α and CDR1β interact partly with α1/α2 helixes and peptide. C, Fab of antibody HyHEL complexed with hen egg white lysozyme, where antibody sits right on the antigen and CDRs had spread over the antigen (PDB: 1NDG)

3 | CONVENTIONAL AND NON-CONVENTIONAL T-CELLS

3.1 | MHC-restricted cells and other cells with highly variable TCRs

Jawed vertebrates express polymorphic MHC class I and II molecules selecting MHC class I-restricted CD8+ T-cells which are precursors of cytotoxic T-lymphocytes and MHC class II-restricted CD4+ T-cells which can differentiate into helper and regulatory T-cells. Although αβ TCR genes are likely to have co-evolved with MHC class I/II molecules, no evidence can be found for an association of certain *TRA* or *TRB* genes with MHC class I or II isotypes or alleles and TCR gene usage does not allow to predict antigen specificity, homing preference, or effector function. As discussed later, this is a principal difference from non-conventional T-cells. Functionally diverse MHC class I- and class II-restricted T-cells are found in (nearly) all jawed vertebrates investigated so far.[29,33] Interestingly, some fish species have lost MHC class II genes but the functional consequences are unclear.[37]

Apart from "classic" peptide-binding MHC molecules, a large number of class Ib exist. Their genes are found in the *MHC* locus on the human chromosome (Chr:)6 but also in *MHC* paralogous regions on other chromosomes. These paralogs result from two rounds of genome duplication during the emergence of vertebrates and are located to a large extent on human Chr:1, 9, and 13. Some of these molecules fulfill functions distinct from antigen presentation such as the neonatal Fc receptor or the iron-transporting HFE molecule. Others, like non-polymorphic CD1 and MR1 molecules, both localized on Chr:1, bind non-peptide ligands.[38] Type I CD1 molecules such as CD1c bind various kinds of lipids and lipid derivatives which can be of microbial or host origin or allergens and present those ligands to αβ and γδ TCRs. Their TCR sequences (TCR repertoire) can be diverse or conserved as in the case of the germline-encoded mycolyl lipid-reactive (GEM) T-cells which recognize CD1b-presenting mycobacterial mycolates formed during infection of the host.[39]

3.2 | Invariant or preset T-cells

iNKT-cells and MR1-restricted mucosal-associated invariant T-cells (MAIT) express TCR-α chains with a highly restricted number of *TRAV-TRAJ* rearrangements which pair with β-chains with limited *TRBV* usage.[40] These TCRs bind complexes of non-peptide ligands and the non-polymorphic class Ib molecules CD1d and MR1, respectively. Human iNKT-cells express *TRAV11-TRAJ28* rearrangements, and homologous populations are found in rodents but not in all mammalian orders. They recognize glyco(sphingo)lipids such as α-Galactosylceramide which can be bacterial cell wall components but also other glycolipids of microbial or host origin. Human MAIT-cell TCRs use *TRAV1.2-TRAJ12*, *TRAV1.2-TRAJ20*, or *TRAV1.2-TRAJ12/33* rearrangements and recognize bacterial riboflavin and folate metabolites but also poorly defined host metabolites.[41] Both cell types are positively selected by double-positive thymocytes, have a pre-activated phenotype when leaving the thymus, can be considered as prototypic innate T-cells, and exert effector functions with therapeutic potential.[42] iNKT-cells are mainly found in liver and spleen, while MAIT-cells are more frequent in blood, liver, and mucosal tissues. Quite different are frequencies of those cells among species. In human blood, 1%-10% of T-cells are MAIT-cells and up to 50% of hepatic T-cells while corresponding numbers for mice are about 0.1% and 0.6%. The frequencies for liver iNKT-cells are 30% for C57Bl/6 mice and 0.1%-1% for humans.[43,44] Strong differences are also observed between species with rather homologous CD1d and TCR genes such as mouse (*Mus musculus*) and rat (*Rattus norvegicus*). Rats have very little if any iNKT-cells although in contrast to mice and human they possess up to eight *TRAV11* homologs emphasizing the problem of interpreting high numbers of homologous genes as indicator of their physiological relevance.[45]

CD1d and *MR1* genes emerged with mammals, but many orders lack one of both molecules and their corresponding invariant T-cell populations. Rabbits lack either cell type but possess another type of invariant T-cell characterized by a *TRAV41- TRAJ38* rearrangement and restriction by a MHC class I-like molecule termed MHX.[46] In summary, the genes defining "invariant" αβ T-cells can be highly conserved among species but can also be lost. Thus the numbers of these genes have limited predictive value for frequency and function of these cells.[46] Furthermore, invariant T-cells share some features of innate γδ T-cells such as a specific homing to non-lymphoid organs or expression of transcription factors such as PLZF (encoded by *ZTB16*) or positive selection by double-positive thymocytes.[42,47] At least in mice, (subsets of) innate αβ and γδ T-cells have a different common intra-thymic precursor than MHC-restricted αβ T-cells.[48]

3.3 | Adaptive vs. innate lymphocytes

Lymphocytes can be roughly described as adaptive *vs.* innate subpopulations. Hallmarks of adaptive immunity are antigen specificity and a memory based on antigen-driven clonal expansion of cells. An important feature of "classical" antigen-specific lymphocytes is high specificity and variability of their clonally expressed antigen receptors. Nevertheless, some antigen receptor-bearing cells also have features of innate immune cells, and especially, γδ T-cells (or subsets thereof) have been described as innate or bridging innate and adaptive immunity, a term which sometimes means that they share certain phenotypes or functional features with innate immune cells or that they support both types of immunity, for example, by acting as antigen-presenting cells (innate immune cells) which support the adaptive immune response.[49,50]

During the last years, the borders between innate and adaptive became less sharp and some lymphocytes originally considered as genuinely innate, for example, NK-cells, show adaptive features such as "antigen specificity" and memory for haptens and/or viruses like Cytomegalovirus (CMV). Nevertheless, the range of specificities is so far rather restricted. The "antigen receptors" are either not

known as in the case of hapten-specific NK-cells and if identified they are germline-encoded pattern recognition receptors (PRRs) and variable specificity is generated by varied expression of different receptors by the same cell and therefore fundamentally different from clonally expressed highly specific antigen receptors of classical B- or T-lymphocytes.[51,52] αβ T-cells can also adopt features common to "innate" lymphocytes, either as a result of certain types of antigen-independent activation as in the case of cytokine-induced killer T-cells[53] or as a consequence of their intra-thymic differentiation.[47] This is clearly distinct from MHC-restricted αβ T-cells and does not result in gene silencing leading to the "naïve" phenotype typical for MHC-restricted cells before thymic egress.[50] Furthermore, germline-encoded parts of certain receptors or special *V(D)J*-rearrangements can be specific for molecular patterns and serve as "innate" PRRs as described for B1 B-lymphocytes or unconventional αβ T-cells and many γδ T-cells.[47]

3.4 | Mono- or oligoclonal expansion: Sufficient evidence for antigen-specific immunity?

Hallmarks of an adaptive immune response are clonal diversity of antigen receptors and expansion of unique clones as a consequence of antigen recognition. The emergence of high-throughput and single-cell sequencing technologies allows a rather simple and reliable determination of clonal diversity and outgrowth of single clones.[54] In line with earlier reports of dominance of certain TCR-defined γδ T-cell populations in CMV-infected transplant patients,[55] a change in the γδ TCR repertoire could be determined in detail in patients with reactivation of CMV infection after bone marrow transplantation. These cells were part of the Vδ1 subset of human γδ T-cells, which is clonally diverse[56] and in contrast to innate Vγ9Vδ2 T-cells has a phenotype typical for naïve cells which changes to an effector phenotype in the expanding clones.[57] Altogether, these findings are usually interpreted as an indication of an antigen-specific TCR activation, for example, by viruses. Alternatively, such expansions might reflect a "pseudo-antigen-specific response"; for example, if single clones are activated by antigen-independent signals and expanded by a growth-promoting micro-milieu. In this case, the antigen receptor would just identify a cell clone comparable to a barcode. Clonal differences between activating and inhibitory NK-cell receptors could have a similar effect and even "unspecific" signaling via TCRs might lead to pseudo-adaptive responses if only a few clones are activated beyond a minimal threshold.[58] Such "unspecific" TCR signals could be similar to that of activation by superantigens or other TCR ligands like butyrophilins, as discussed later in this review. Therefore, in an ideal case, defining an immune response as antigen-specific should be demonstrated by testing the TCRs for specificity to the presumed antigen or microorganism initiating a clonal expansion.

3.5 | Sometimes γδ T-cells may be conventional

The lengths of CDRs reflect at least to some extent the nature of the antigen, and it has been noted quite early that the three lineages of antigen receptors differ in their CDR length, especially the CDR3. TCRs of MHC-restricted αβ T-cells show a highly limited length distribution of α- and β-chain CDRs which fits well to the physical constraints of peptide-MHC binding and a contribution of all six CDRs.[36] In the case of immunoglobulins, the CDR3s of both chains vary considerably consistent with the wide array of ligands and the recognition of conformational epitopes. The antigen-binding sites (paratopes) of murine and human antibodies binding to folded proteins tend to have a flat to concave surface. As a result of convergent evolution, the CDR3s of single heavy chain antibodies of camelids and the New Antigen Receptors (NAR) of sharks can form binding sites that can fit into pockets of proteins and can be very long.[59]

The CDR3s of the TCR-δ chain can also be very long, and this length is incompatible with a mode of interaction analogous to the typical ligand binding of MHC-restricted αβ T-cells.[59] The CDR3δ-mediated mode of membrane protein binding has been elucidated for a γδ TCR specific for the non-conventional MHC class Ib T22 and T10 molecules. Around 0.1% to 1% of murine γδ T-cells have such specificity and express TCRs which bind these molecules with a rather high affinity. T10 and T22 molecules do not bind antigenic peptides, but the structure of the γδ TCR G8 complexed with T22 reveals that the CDR3δ binds in an autonomous mode, so that the other CDRs are not involved. Instead, the CDR3δ nuzzles into the molecule similar to the insertion of antigenic peptides into the binding groove of classical MHC molecules[60-62] (Figure 2). The CDR3δ and its flanking regions have also been reported to be essential for binding of human γδ TCRs to other molecules such as MutS and ULBP4.[63] Quite interesting are also other cases of γδ TCR binding without the involvement of "presenting" molecules. Well documented is the binding of mouse and human γδ TCRs to typical B-cell antigens such as the fluorescent algae protein phycoerythrin (PE)[64] and TCRs with specificity for smaller fluorescent molecules like cyanine and the hapten 4-hydroxy-3-nitrophenylacetyl.[65] This may illustrate that γδ TCRs can "recognize" many types of ligand but the physiological meaning is sometimes difficult to demonstrate. However, the ectopic expression of the mitochondrial F1-ATPase by some tumors could indicate involvement in tumor surveillance of the TCR G115 which binds this molecule.[66] The same might apply for specificity to heat-shock proteins such as a GroEL-like protein in Daudi cells.[67] The most convincing disease association is that of the phosphoantigen-unreactive human Vγ3Vδ2 TCR M88 which was isolated from a muscle lesion of a patient with polymyositis. This TCR binds as a single Fv-fragment to a structure found in several amino-acyl tRNA synthases which is remarkable, as one of them is the histidyl tRNA synthase, a target of Jo-1 autoantibodies also detected in myositis patients. This conformational epitope was also found on other structures such as the short helical loop in the elongation initiation factor 1 of E. coli.[68] This wide spectrum of antigens and the link of TCRs to autoimmunity urges questions on central tolerance, γδ T-cell maturation, and control of auto- or cross-reactivity.[69]

Most mouse and human γδ T-cells are usually coreceptor-negative or express the CD8αα coreceptor which is fully consistent with their lack of MHC restriction. Therefore, it was surprising that

about 85% of rat splenic γδ T-cells express CD8 and that lymphocyte kinase (Lck) association of the CD8αβ heterodimers of γδ T-cells was indistinguishable from that of CD8αβ-expressing αβ T-cells. Nevertheless, analysis of CDR3δ lengths of TCRs expressed by the different T-cell types (CD8αβ-positive vs. CD8-negative) showed no difference in length and the average length was even higher than for homologous mouse TCR-δ chains, indicating that these TCRs cannot act in an αβ TCR-like MHC-restricted manner.[70]

More related to classical MHC-restricted αβ T-cells are recently discovered γδ T-cells with specificity for melanoma antigen recognized by T-cells 1 (MART-1) peptides presented by HLA-A*0201.[71] These cells were generated in vitro in cultures of OP9-DL4 cells as stromal cells and cord blood hematopoietic cells as thymocyte precursors. This system allowed the generation of αβ and γδ T-cells. Some clones bound HLA-A*0201 tetramers loaded with the MART-1-derived peptides. These tetramers interacted with CD8αβ-positive αβ T-cells and double-negative or CD8αα-positive γδ T-cells. Co-crystals of αβ and γδ TCRs with the same MHC-peptide complex showed a distinct binding pattern of γδ TCR analogous to typical MHC class I-restricted TCRs and similar footprints on the MHC molecule. The footprints of the δ-chain were close to those of the β chain and that of the γ chain to those of the α chain. Importantly, the MART-1-MHC class I tetramers bound to peripheral blood αβ and γδ T-cells as well, indicating that γδ T-cells expressing TCRs with specificity for MHC-peptide complexes exist in vivo. Not yet known is how these γδ T-cells have been thymically selected, whether they can be part of an adaptive immune response and what effector functions they execute. In any case, these cells provide a unique opportunity to test the role of TCRs, for example, by differential signaling of cells[72] in the definition of lineage-specific features with the help of TCR- and HLA-A*0201-transgenic mice where αβ and γδ T-cells of identical antigen specificity can be compared.

3.6 | Butyrophilins enter the stage

Butyrophilins (BTNs) have originally attracted immunologists´ attention for their immunomodulatory capacity,[73-76] but in recent years, they gained interest for their contribution to γδ T-cell biology.[75,76] The name-giving protein of the gene family is BTN1A1 which is involved in the transport of fat droplets to maternal milk,[77] but has genetic homologs in all jawed vertebrates, for example, in birds where the BTN1A1 homolog Tvc acts as receptor for subgroup C avian sarcoma and leukosis viruses.[78] BTN1A1 and many other BTN and BTN-like (BTNL) molecules modulate activity of immune cells.[75,76,79] In humans, the *BTN1A1* gene is part of the *BTN* gene cluster localized at the telomeric end of the MHC on the short arm of human chromosome 6 (Chr:6p).[80] BTNs are members of the extended B7 family which is named after the costimulatory molecules CD80 (B7-1) and CD86 (B7-2). BTNs and the closely related BTNL molecules are single-pass membrane molecules whose extracellular domain consists of an N-terminal V-domain followed by a C-domain and a peptide connecting it to the transmembrane domain (TM) (Figure 3). The cytoplasmic juxtamembrane domain (JM) is encoded by several exons for heptad amino acid (aa) sequences, and the C-terminus is a PRY/SPRY or B30.2 domain.[74,75] B30.2 domains are part of many other immune response-associated molecules such as tripartite motif-containing proteins (TRIMs)[81] and are already found in the ancestral proto-MHC.[82] Some TRIMs are involved in the antiviral immune defense and a role in cell-autonomous antiviral responses has also been reported for the BTN3A1 molecule.[83] Neither the B7 molecules nor the three-times-membrane passing selection and upkeep of intraepithelial T-cells protein 1 (SKINT1) possess a B30.2 domain.

SKINT1 was the first molecule with BTN-like features reported to affect γδ T-cell development and function since a missense mutation leads to an impaired development of dendritic epidermal T-cells (DETC) in the mouse FVB strain.[1] DETCs are named after their shape and morphology and carry a uniform TCR which is largely conserved in mice and rats.[84] These cells mature in the fetal thymus and egress to the epidermis before birth where they fulfill functions in tissue repair and tumor surveillance.[13,15] DETC maintenance requires SKINT1 expression by the neighboring keratinocytes. Despite the functional link between DETC TCR and SKINT1, biochemical evidence for direct SKINT1-TCR binding is still missing. A knock-out of the entire murine SKINT gene family (11 members) shows, apart from effects on DETCs, no phenotype not even on other fetal-developing epithelial mouse γδ T-cells with uniform TCR.[85] Hominoid species lack SKINT1 but Old world monkeys possess a functional *SKINT1L* gene and cells with DETC localization and morphology whose TCRs are variable and use mostly *TRGV10* which is not a homolog of murine Vγ5 (Vγ3 according to Garman nomenclature).[86] A remarkable case of convergent evolution is found in the skin of lampreys where VLRC cells with dendritic morphology outnumber VLRA cells eightfold.[86]

More recently, BTNL molecules, which are genetically and structurally very similar to classical BTN molecules, were found to shape γδ T-cell repertoire and function. Heteromers of murine BTNL1/6 and human BTNL3/8 are necessary for the maintenance of murine Vγ7 and human Vγ4 T-cells in the gut epithelium and transductants expressing these heteromers stimulate TCRs containing the respective γ-chains.[2] Site-directed mutagenesis experiments suggest interaction of the BTNL molecules with TCR regions and involvement of the HV4 of the γ-chain analogous to binding of some superantigens to the αβ TCR.[87] For BTNL3, direct binding to human Vγ4 chains was reported.[88]

4 | Vγ9Vδ2 T-CELLS: EVOLUTION AND ANTIGEN RECOGNITION

4.1 | Vγ9Vδ2 T-cells: The TCR as metabolic sensor

Most human blood γδ T-cells are Vγ9Vδ2 T-cells (1%-5% of blood T-cells in healthy individuals) and respond to so-called "phosphoantigens" (PAgs). They are defined by eponymous TCRs composed of a γ-chain with Vγ9JP (*TRGV9-TRGJP*) rearrangements (alternatively designated as Vγ2-Jγ1.2) and Vδ2-containing TCR-δ chains. Freshly isolated Vγ9Vδ2 T-cells have transcriptional profiles comparable to

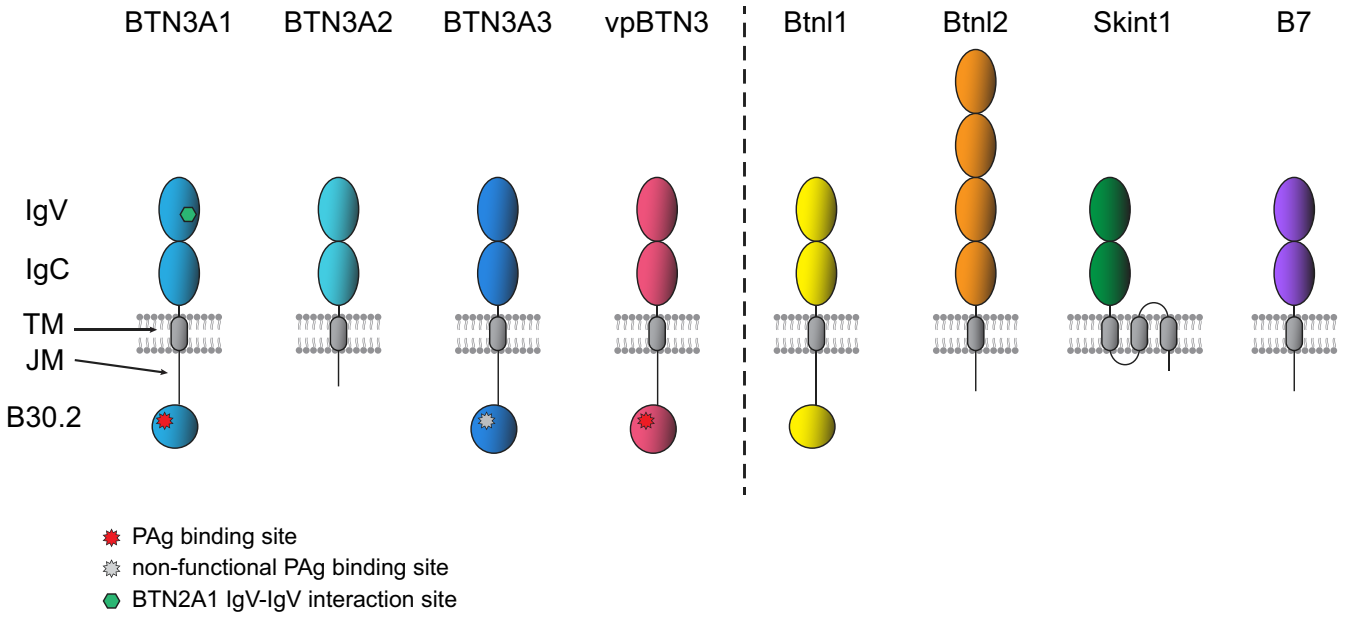


FIGURE 3 Protein-domain composition of butyrophilins and other members of the extended B7 family. The schematic protein structure of members of the human BTN3 family (BTN3A1-3 in blue shades), the alpaca BTN3 homolog (pink), and representative molecules of other members of the extended B7 receptor family namely Btnl1 (yellow), Btnl2 (orange), Skint1 (green), and B7 (purple) are shown. Immunoglobulin-like domains (IgV and IgC), the transmembrane (TM), juxtamembrane (JM), and intracellular B30.2 domain are labeled. PAg-binding sites shown for BTN3s are depicted by a red (functional) or gray (non-functional) star.[102] The recently shown BTN2A1 interaction site of BTN3A1 is represented by a green hexagon[119]

CD8 T-cells and NK-cells,[89] but under some pathological conditions, IL-17-producing Vγ9Vδ2 T-cells have been observed.[90] Furthermore, at least in vitro, they exhibit a remarkable degree of plasticity and multi-functionality such as regulating immune responses by cross-talk with B cells, dendritic cells, NK-cells, and monocytes and the capacity to differentiate into professional antigen-presenting or phagocytosing cells.[49] Comparable to many innate cells, they express inhibitory and activating NK-cell receptors and some effector functions like target cell killing or TNFα secretion can be directly triggered via ligation of NKG2D.[49,91]

The building blocks of isoprenoid synthesis are the weak PAgs isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate, which are barely detectable in normal cells but accumulate in transformed cells such as Daudi[92] and after reduction in activity of the metabolizing enzyme farnesyl pyrophosphate synthase (FPPS) by (aminobisphosphonate) drugs like Zoledronate[93] or reduction in expression with short-hairpin RNA.[94] The microbial PAg (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) stimulates Vγ9Vδ2 T-cells about 10,000-fold more efficiently than IPP.[95-97] HMBPP is the direct precursor of IPP in the non-mevalonate pathway of IPP synthesis also known as the 2-C-methyl-D-erythritol 4-phosphate (MEP)/1-deoxy-D-xylulose 5-phosphate (DOXP) or Rohmer pathway which is found only in eubacteria, cyanobacteria, chloroplasts, and apicomplexan protozoa.[96] It is the driving force of a massive Vγ9Vδ2 T-cell expansion or activation in many infections such as malaria, toxoplasmosis, and tuberculosis.[98] Sensing of HMBPP and other PAgs by the Vγ9Vδ2-TCR requires "presentation" by human or primate cells.[99,100]

4.2 | The human BTN3A family and stimulation of Vγ9Vδ2 T-cells

A breakthrough for understanding the molecular basis of Vγ9Vδ2 T-cell activation by PAgs was the identification of human BTN3A molecules as key compounds in PAg-induced Vγ9Vδ2 T-cell activation[101] (For a more detailed view on BTN3-specific and PAg-mediated activation, see also[97,102]). In humans, the *BTN3A* gene family consists of *BTN3A1*, *BTN3A2*, and *BTN3A3* (Figure 3) which have been generated by gene duplication events during primate evolution and are part of a BTN gene cluster at the telomeric end of the MHC complex on human Chr:6.[80] The EDs of BTN3A1 (CD277), A2, and A3 are highly homologous. Their V-domains differ only by a single, conservative substitution (R37K), and their C-domains are more than 90% identical on the aa level.[102] The differences in TMs and IDs of BTN3As are pronounced and insertion of an Alu sequence truncates BTN3A2 within the JM.[103] BTN3A-specific antibodies act as co-stimulators for αβ T-cells, but BTN3 has also been implicated in negative and positive regulation of NK-cell and monocyte responses.[104-107] Counter-receptors for BTN3 have been proposed,[108-110] but it is unclear whether and how they affect immune responses. Instrumental for the discovery of BTN3A1 as player in Vγ9Vδ2 T-cell activation were BTN3-specific monoclonal antibodies. In cultures of peripheral blood mononuclear cells (PBMCs), the agonistic BTN3-specific mAb 20.1 induces Vγ9Vδ2 T-cell-specific proliferation while the antagonistic mAb 103.2. inhibits PAg-induced activation. BTN3A1 is expressed on essentially all cell types although at different levels. Its expression on lymphocytes which includes Vγ9Vδ2 T-cells raises the possibility of

direct signaling into the Vγ9Vδ2 T-cells and mutual presentation between those cells.[101] To circumvent this problem, many experiments with primary cells have been done with ABP-pulsed cells as "presenters" and purified Vγ9Vδ2 T-cells or Vγ9Vδ2 T-cell lines as responders. As an alternative, murine reporter cells can be used.[101,102,111,112] These cells do not express BTN3A1 but require overexpression by transduction of the costimulatory molecule CD28 on the reporter cell and of counter ligands such as CD80 on the presenting cells for efficient activation.[101,102,112]

Already the first report on Vγ9Vδ2 T-cell activation established the pivotal role of BTN3A1 in PAg-mediated Vγ9Vδ2 T-cell activation and the importance of its ID by BTN3A knockdown and reconstitution experiments. Reports on the contribution of BTN3A2 and BTN3A3 to PAg-mediated stimulation led to different results but in most experimental settings both molecules clearly contribute to PAg-mediated stimulation while their contribution to stimulation by the agonistic mAb 20.1 is comparably minor. Later work established a PAg-binding site in the B30.2 domain of BTN3A1 which is missing in BTN3A3 as a result of a single substitution (H351R) and loss of the domain in case of BTN3A2.[102]

The structural characterization of crystalized recombinant BTN3A1-EDs identified two types of BTN3A1 homodimers: One dimer with a symmetric parallel V-shaped structure with interacting C-domains and another asymmetric head-to-tail conformation with contact of V- and C-domain (Figure 4A).[113] The antagonistic mAb 103.2 binds to the B-C, C′-C″, and D-E connecting loops of the V-domain but inhibits only as intact antibody and not as Fv-fragment, suggesting that these loops are not directly involved in phosphoantigen-mediated T-cell activation. The agonistic mAb 20.1 stimulates as intact antibody and as Fv-fragment. It binds to the C, C′, and C″ strands of the V-domain, prevents the formation of the head-to-tail conformation, and promotes clusters of BTN3 molecules. Therefore, the

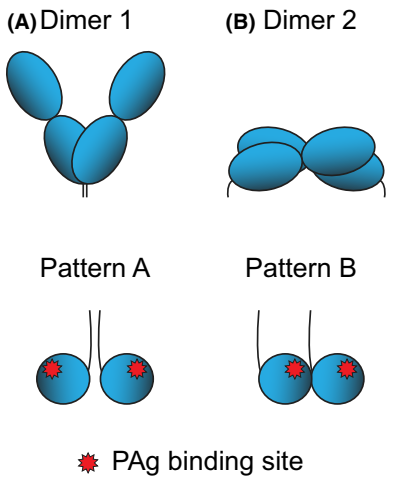


FIGURE 4 Dimers of extra- and intracellular domains of the BTN3A1 molecule. A, V-shaped and head-to-tail dimers found in crystal structures of extracellular BTN3A1 domains. B, Orientation of PAg-binding B30.2 dimers. Pattern B has been suggested to be the active conformation. The PAg-binding site is indicated by a red star[102]

V-shaped dimer has been suggested as an active conformation of the molecule.[101,113] Furthermore, FRET experiments of ABP-stimulated cells suggested a switch to a conformation with the V-domain pointing away from the membrane.[114] In contrast, introduction of a disulfide bridge between the C-domains, which fixes the V-conformation, diminished ABP-induced stimulation but did not affect ABP-induced mobility reduction in these BTN3A1 constructs, casting doubts on the significance of mobility changes in ABP-induced activation.[115]

The B30.2 domain also forms dimers, the symmetric dimer A (or type II) and the asymmetric dimer B (type I) (Figure 4B). In dimer A, both PAg-binding sites of the B30.2 domains point outward while in the asymmetric dimer B one binding site is located at the interface between both domains and the other one points outward.[115,116] HMBPP derivatives with bulky hydrophobic side chain and good binding to the PAg-binding sites prevent the formation of dimer B and show no activating properties, supporting the asymmetric dimer B as the active conformation.[116] The authors argue also that the orientation of B30.2 domains in dimer B eventually prevents the formation of a V-shaped extracellular domain and suggests the head-to-tail formation of the extracellular domain as the active form.[116] Other important aspects are that binding of PAg alone is not sufficient to induce activation and that active PAg induces changes distant from the binding site in the B30.2 but also in the JM.[116] This led to the hypothesis that PAg-induced conformational changes in the ID either transmit into changes in the ED which are somehow sensed by the TCR or allow other molecules to interact with BTN3A and/or the TCR.[117,118] How can we reconcile the apparently contradicting findings on the "active" forms of BTN3A? We propose that both types of dimers might be required for an optimal BTN3A function demanding either reversibility or coexistence of different conformations. Such coexistence or conformational transition would also allow the different BTN3 domains to fulfill different functions, for example, a dimer B conformation may facilitate the interaction of other molecules with the JM while a V-shaped conformation would permit interaction of the V-domain with other molecules like BTN2A1 which will be discussed later.[102,119]

While there is no doubt on the existence of BTN3A1-ED and BTN3A1-B30.2 dimers, the intact molecule might behave differently.[102] Purified BTN3A1 does not form homodimers but co-expression with BTN3A2 facilitates heterodimers which adapt a V-shaped conformation if inserted in artificial membranes (nano-disks).[115] A preference for heteromers was also found in co-immunoprecipitation experiments as well as different cellular trafficking of the isoforms.[120] Cells expressing these heteromers were far better in promoting PAg-mediated stimulation than cells expressing BTN3A1 alone.[102,120,121] A key role in this improved stimulation plays the JM of BTN3A2/A3 as shown by constructs with swapped JMs between BTN3s and mutagenesis experiments.[122,123]

4.3 | Vγ9Vδ2 TCRs and BTN3s in different species

Vγ9Vδ2 T-cells have long been considered to be of primate origin. The increasing availability of mammalian genome sequences

allowed us to test for the existence of the triad of Vγ9 (*TRGV9*), Vδ2 (*TRDV2*), and the exons of the ED of BTN3.[124,125] A large number of non-primate orders lacked at least two genes of the triad. Some species such as cows (*Bos bovis*) and horses (*Equus caballus*) possessed all three but those were unlikely to be functional due to stop codons and other mutations such as the loss of intermolecular disulfide bridges. Non-primate species with functional open reading frames (ORFs) of the PAg-sensing triad were found among camelids and whales but not in rodents and lagomorphs. An important observation for understanding the evolution of *TRGV9*-, *TRDV2*-, and *BTN3* genes was their detection in armadillo (*Dasypus novemcinctus*) and sloth (*Choloepus hoffmanni*). Both species belong to the order of Xenarthra which is part of the Eutherian magnaorder Atlantogenata, while primates, camelids, and whales are Boreoeutheria, the other magnaorder of Eutheria which implies that the common ancestor of Eutheria and therefore of all placental mammals possessed those genes. Striking was that nearly all species with *BTN3* carried also *TRGV9*- and *TRVD2*-like genes and if BTN3 was likely to be non-functional, the same was the case for the relevant TCR genes.[124,125]

The armadillo genome revealed ORFs for *TRGV9*, *TRDV2*, and *BTN3-ED* and was analyzed in greater detail,[126] not only because of its phylogenetic position, but also for being a natural reservoir and model organism for infections with *Mycobacterium leprae*.[127] Furthermore, human Vγ9Vδ2 T-cells have been implicated in the defense to mycobacterial diseases[128] and *M leprae*-infected animals were reported to show an increased frequency of circulating lymphocytes stained by an antibody specific for human TCR-δ chains.[129] The analysis of cDNA from armadillo PBMCs revealed neither *BTN3* nor *TRGV9* transcripts, although genes were present. A more detailed analysis of published and newly generated sequence data revealed three *BTN3* genes. None of them possessed an exon for a leader peptide but all exons for the V- and C-domain. Two *BTN3* genes contained exon 4, which encodes the connecting peptide between C-domain and TM of BTN3. Exons encoding the aa heptad sequences of the JM were mostly lost but the respective introns were identified. The most complete *BTN3* gene contained an exon for the B30.2 domain with an internal stop codon. In the B30.2 domain, codons for aa forming the PAg-binding site of human BTN3A1 (huBTN3A1) and alpaca BTN3 (vpBTN3) B30.2 domains were conserved, making it likely that the common ancestor of placental mammals possessed BTN3(s) with PAg-binding properties. Furthermore, armadillo Vδ2 chains were transduced together with a human *TRGV-TRG9JP*-containing TCR-γ chain and paired to surface-expressed Vγ9Vδ2 TCRs in a TCR-negative mouse T-cell hybridoma, demonstrating conservation of Vγ9Vδ2 TCRs.[126]

In contrast, the dolphin genome possesses two *BTN3*-like genes. One non-functional gene covering the BTN3-ED exon and one full-length BTN3 ORF which showed the highest aa identity with human BTN3A3 and vpBTN3 and a fully conserved PAg-binding site.[126] This, together with the detection of in-frame *TRGV9-TRGJP* rearrangements and *TRDV2* genes, makes the bottle nose dolphin (*Tursiops truncates*) another prime candidate for PAg-reactive Vγ9Vδ2 T-cells,[130] together with the dromedary (*Camelus dromedaris*) which expresses typical Vγ9Vδ2TCR rearrangements.[131]

4.4 | Alpaca (*Vicugna pacos*): The first non-primate species with PAg-reactive cells

Genomic data and access to fresh blood allowed a more detailed analysis of alpaca as candidate species for PAg-reactive Vγ9Vδ2 T-cells. Analysis of PBMCs confirmed database sequences and the expression of the *TRGV9*, *TRDV2*, and *BTN3* genes. As in armadillo, nearly all *TRDV2* sequences were rearranged with *TRDJ4* gene segments which contrasts the preferential *TRDV2-TRDJ1* rearrangements of humans.[54,132] *TRGV9* was nearly exclusively rearranged with *TRGJP*, and the frequency of productive in-frame rearrangements was similar to humans. Three, probably allelic, *TRGJP* sequences (*JPA*, *JPB*, *JPC*) were identified with a lysine/arginine threonine isoleucine lysine (R/KTIK) sequence motive similar to the human KKIK sequence containing K109 known to be important for the PAg -response of human Vγ9Vδ2 T-cells.[121,124,133,134] An instructive difference to humans is that *vpBTN3* is a singleton gene with overall higher sequence similarity to *BTN3A3* compared with *BTN3A1* but a complete identity in the amino acids defining the PAg-binding pocket of BTN3A1.[124,125] Isothermal calorimetry (ITC) studies of recombinant wild-type and mutant vpBTN3 B30.2 domains showed very similar binding characteristics for HMBPP and IPP to huBTN3A1 and vpBTN3.[121]

To directly test for a BTN3-dependent PAg response of alpaca Vγ9Vδ2 T-cells,[121] monoclonal antibodies were generated against alpaca Vγ9Vδ2TCRs and vpBTN3. The BTN3-specific antibody WTH-5 bound with high affinity to vpBTN3 transductants and primary cells. We also found some cross-reactivity of mAb 103.2 and 20.1 to BTN3-negative cells transduced with vpBTN3 and of WTH-5 with BTN3A1 transductants but neither the inhibitory mAbs 103.2 nor WTH-5 showed cross-species reactivity on primary cells. These findings were contrasted by rather good staining of alpaca cells with PE-labeled agonistic mAb 20.1. Also noticed was a differential binding of mAb 103.2 and 20.1 to human monocytes. PE-labeled mAb 20.1 bound to human monocytes slightly better than to lymphocytes, while mAb 103.2 bound well to human lymphocytes but hardly if at all to monocytes. The differential staining patterns of the mAbs to the transductants, primary cells, and to primary cell subpopulations of both species warrant further investigation and suggest differential conformation of the molecules or covering of epitopes by associated molecules.[121]

The alpaca TCR-specific mAb WTH-4 bound to transductants expressing *TRDV2-TRDJ4* but not the rare *TRDV2-TRDJ2* rearrangement. Frequencies of WTH-4-positive cells ranged from 0.2% to 1.2% of CD3-positive lymphocytes and varied between individuals but also time points of blood sampling.[121] WTH-4-positive cells expanded in six-day cultures of alpaca PBMCs with HMBPP and IL-2, and this was inhibited by the vpBTN3-specific mAb WTH-5. Despite cross-reactivity of mAb 20.1, no expansion of WTH-4-positive cells was detected.[121]

For a more detailed analysis of PAg reactivity of alpaca Vγ9Vδ2TCRs, TCR sequences were cloned and transduced into murine reporter cells. TCRs cloned from single WTH-4-positive cells contained *TRGV9-TRJP* and *TRDV2-TRDJ4* rearrangements, while WTH-4-negative HMBPP-expanded cells possessed *TRGV9-TRJP* and *TRDV2-TRDJ2* sequences. Both TCR transductants were compared for their response to PAg and mAb 20.1 with transductants expressing human TCRs (TCR MOP). TCR MOP responded well to HMBPP and mAb 20.1 in the presence of 293T cells but not in the presence of BTN3A1-A2-A3-deficient 293T cells (BTN3KO) transduced with *vpBTN3*. The alpaca TCRs cloned from HMBPP-expanded cells responded to HMBPP presented by 293T or vpBTN3-expressing BTN3KO 293T cells. Randomly combined Vγ9 and Vδ2 TCRs from unstimulated PBMCs[124] showed no PAg response at all, emphasizing the importance of the right combinations of Vγ9 and Vδ2 TCRs for PAg reactivity.[121] Despite reactivity of the alpaca Vγ9Vδ2TCR-transduced cells to PAg presented by wild-type 293T cells, stimulation by 293T cells plus mAb 20.1 was not observed. So far it is not possible to decide whether this lacking response results from the specific CDR3s of these alpaca Vγ9Vδ2TCRs or is common to alpaca Vγ9Vδ2 TCRs in general.[121] The differential specificity of alpaca Vγ9Vδ2TCR transductants which recognize PAg in the context of alpaca as well as human BTN3, and human TCR MOP transductants which respond exclusively to cells expressing human BTN3 should allow identification of BTN3 and TCR regions controlling PAg-mediated activation by comparing interspecies chimeras or single aa mutants.[121] This TCR-dependent differential response to vpBTN3 vs. human BTN3A argues, at least for this experimental system, against a role of BTN3 as PAg-sensing ligand whose interaction with a counter-receptor on the reporter cells mediates PAg reactivity of the Vγ9Vδ2 TCR as recently suggested by the Kuball group.[109]

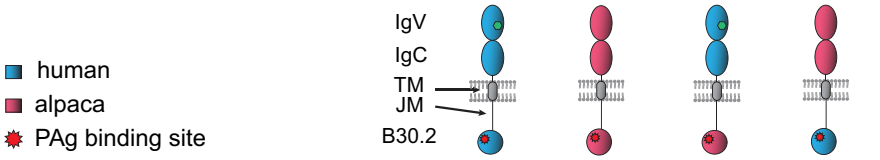
4.5 | Alpaca BTN3: An All-in-One Solution

In contrast to humans with several *BTN2* and *BTN3* genes, the alpaca *BTN* cluster carries only single copies of *BTN1*, *BTN2*, and *BTN3*. The singleton nature of vpBTN3 implies that the capacity of PAg-sensing and γδ T-cell activation is merged in vpBTN3, while humans require the cooperation of two to three BTN3As. To identify which parts of the BTN3 molecules "help" during PAg sensing, chimeras of human BTN3A1 and alpaca BTN3 were expressed in BTN3KO- or BTN3A1-deficient 293T cells (BTNA1KO) and tested for PAg-dependent activation of human and alpaca TCR transductants leading to a complex picture of synergism and interference of the different BTN3 molecules (Figure 5). It confirmed that the expression of BTN3A1 in BTN3KO cells rescued PAg reactivity only poorly while the response of human TCRs to BTN3A1KO cells transduced with BTN3A1 was fully reconstituted. Transduction of BTN3KO cells with alpaca BTN3 induced a PAg response of vpTCR but not of huTCR transductants, indicating a species specificity of the TCR-BTN3 or TCR-BTN3 + factor X interaction. Surprisingly, BTN3A1KO cells transduced with vpBTN3 stimulated neither human nor alpaca TCR-transduced cells which may indicate an interference of human BTN3A2 and BTN3A3 molecules with other molecules mandatory for effective PAg sensing, for example, human BTN2A1, as discussed in the next paragraph. BTN3KO and BTN3A1 KO cells transduced with a human BTN3A1-ED/alpaca BTN3-TM/ID chimera stimulated human and alpaca TCRs even better than wild-type 293T cells, suggesting that this molecule is superior to complexes of BTN3A1 and BTN3A2 and/or BTN3A3. Quite dramatic differences were seen for chimeras of alpaca BTN3-ED and human BTN3A1-TM/ID. If transduced in BTN3KO cells, they stimulated neither human nor alpaca TCR transductants but after expression in BTN3A1KO cells, they activated human as well as alpaca TCR transductants. This suggests that PAg binding to the human ID of this chimera does not suffice to induce an "activating" conformation of the alpaca BTN3-ED, but this capacity somehow translates to associated BTN3A2 and/or BTN3A3 molecules which are then sensed directly or indirectly by either the human or the alpaca TCR.[121]

An aa sequence comparison of vpBTN3 with the human BTN3A1/A2 or A3 revealed a very similar degree of identical aa for the three molecules (without leader sequence). Only for vpBTN3-TM similarity to BTN3A1 (88%) was clearly higher than for BTN3A2 and BTN3A3 (both 76%), while the respective percentage of identity for the JM domain was 64% for BTN3A3 and 49% for BTN3A1. This and unpublished data (Karunakaran et al) with human BTN3 chimeras led us to hypothesize that the BTN3A3-like nature of the alpaca JM domain enables efficient PAg sensing by the single molecule. Whether this acts as a dimer and in which conformation remains to be elucidated but should help to better understand the general principles underlying BTN3A function in PAg sensing.[121]

4.6 | BTN2A1: A Vγ9-binding molecule as player in PAg sensing

One drawback of the original report of BTN3A1 as a key compound in PAg sensing and γδ T-cell stimulation was that ABP-pulsed rodent T-cells transduced with BTN3A1 did not stimulate human Vγ9Vδ2 T-cells.[101] However, such a negative result could reflect impaired costimulatory signals or cell-cell adhesion as a consequence of the species barrier between stimulating and responding cells. As discussed in greater detail elsewhere,[102] our murine reporter cell system allows to (partially) overcome this obstacle. Using these lines as reporter cells and various human Chr:6-containing cell lines (rodent-human hybridoma) as presenters, we showed that in addition to BTN3A1, other gene(s) on human Chr:6 are mandatory for HMBPP- and Zoledronate- but not for mAb 20.1-induced stimulation.[135] Furthermore, unpublished data showed that these molecules were not BTN3A2 and BTN3A3, albeit expression of all molecules increased the PAg-independent "background" stimulation (Paletta, Fichtner, Karunakaran, Herrmann, unpublished data). In summary, rodent-human hybrid lines identified human Chr:6 as a carrier of the species-specific gene(s) which, in addition to BTN3A1, controlled PAg sensing by the Vγ9Vδ2TCR.[135]



mIL-2 response of				BTN3KO/BTN3A1KO +			
hu TCR	vp TCR	hu TCR	vp TCR	hu	vp	hu/vp	vp/hu
+	+	++	++	+	-	-	-
-	++	-	-	-	+	-	-
+++	+++	+++	+++	-	-	+	-
-	-	++	++	-	-	-	+
-	-	-	-	-	-	-	-
BTN3KO		BTN3A1KO					

FIGURE 5 Interplay of BTN3 molecules and domains and species-specific activation of TCR transductants. Pattern of the HMBPP responses of reporter lines transduced with human Vγ9Vδ2TCR MOP (huTCR) or alpaca TCR with *TCRD-TRDV2JD2* rearrangement (vpTCR) co-cultured with 293T cells lacking functional BTN3A1 + A2+A3 (BTN3AKO) or only BTN3A1 (BTN3A1KO) transduced with the indicated BTN3 constructs, as measured by IL-2 production. Pink indicates alpaca protein domains (vp) and blue human BTN3 domains (hu)[54]

To identify these gene(s), we used another cytogenetic method exploiting species differences, the so-called radiation hybrids (RH). RH are hybrids of irradiated (human) cells and a HAT-sensitive (rodent) cell line.[136,137] In these cells, parts of the irradiation-induced chromosomal fragments of the (human) donor genome are integrated into the recipient genome (rodent T-cell line). The hybrid lines were tested for the phenotype "PAg-dependent stimulation" using the same methodology as for CHO-Chr:6 cells[135,138] and for stimulation of reporters by mAb 20.1 as positive control. Nearly all RHs stimulated in the presence of mAb 20.1 but some also in a PAg-dependent fashion. The latter was tested for genomic content, and a common candidate region of 580 kB on Chr:6 was identified. The only membrane protein-encoding genes in this region were those of the *BTN* cluster and the highly conserved MHC class I-like iron transporter HFE. *HFE* and all *BTN* genes with the exception of *BTN1A* were expressed by the stimulatory RHs. We considered BTN2A1 and BTN2A2 as prime candidates for the missing Chr:6-encoded gene(s) contributing to the PAg-presentation phenotype. 293T cells with CRISPR/Cas9-induced deficiency of BTN2A1, BTN2A2, or both were generated. Only cells deficient for BTN2A1 lost the capacity to stimulate in a Zoledronate-, HMBPP- but also in a mAb 20.1-dependent stimulation manner.[119]

Independently, Rigau et al identified BTN2A1 as a mandatory component of PAg presentation by screening cells transfected with a shRNA library for reduction in Vγ9Vδ2 TCR tetramer binding and identified signal peptide peptidase-like 3 (SPPL3) and BTN2A1 as prime candidates. Knock-out of BTN2A1 in different human cells massively reduced binding of TCR tetramers and newly generated BTN2-specific mAbs inhibited tetramer binding as well as stimulation with ABP.[139]

4.7 | BTN2A1: Interaction with TCR and BTN3A1

Both groups found that rodent T-cells transduced with BTN2A1 and BTN3A1 reconstituted the PAg response and bound Vγ9Vδ2 TCR tetramers. Interestingly, this binding relied only on BTN2A1 expression while BTN3A1 showed no direct TCR interaction, and TCR tetramer binding was not increased by treatment of BTN3A1 + BTN2A1 transductants with ABP, suggesting that BTN2A1 binds the TCR autonomously while both BTNs were still required for the induction of a PAg-dependent Vγ9Vδ2 T-cell response.[119,139] The direct binding of BTN2A1 to the Vγ9 TCR was demonstrated by plasmon resonance studies. K_Ds were in the range of 50 μM, and binding was independent of the CDR3s of Vγ9 and the paired δ chain. Molecular modeling and mutagenesis studies mapped the surface formed by the C-F-G strands of BTN2A1 as the contact region of the TCR. This corresponds to the area previously mapped for binding of BTNL3 to the human Vγ4 chain.[88] The V-domains of BTN2A1 and BTN2A2 are rather similar and BTN2A2 bound in plasmon resonance assays even better to Vγ9 TCRs than BTN2A1. Yet, TCR tetramers showed nearly no binding to BTN2A2 transductants which in part may be explained by a differential degree of cell surface expression of both molecules. Of unclear significance is also the physiological consequence of a disulfide bridge formed by C247 which stabilizes BTN2A1 as a homodimer but is not found in alpaca BTN2 or any other BTN and

BTNL molecules which carry a tryptophan at this position. A C247W substitution of BTN2A1 had no negative effect on PAg stimulation or TCR tetramer binding.

The binding of the Vγ9-HV4 to the C-F-G surface of BTN2A1 was confirmed by mutational analysis and tested for effects on PAg or ABP stimulation. A mutation of a negatively charged glutamic acid to alanine in the Vγ9-HV4 (E70A) reduced the PAg response of the TCR MOP transductant[119] and the Zoledronate-induced CD69 expression of TCR-transduced Jurkat T-cells.[139] Mutations to positively charged arginine or lysine largely (E70R) or completely (E70K) abolished the PAg-induced stimulation.[119] These data are fully consistent with binding studies testing BTN2A1 tetramer-binding to a number of TCR mutants.[139] Importantly, mutating the Vγ9 CDR3 and Vδ2 CDR2 and CDR3 had no effect on BTN2A1 binding but stimulation by Zoledronate or HMBPP was lost completely.[119,139]

Both groups demonstrated that no ABP was required for BTN2A1 interaction with BTN3A1 as shown by confocal microscopy and FRET,[139] immunoprecipitation after cross-linking cells with a water-soluble membrane-impermeable chemical crosslinker, and by NMR studies..[119] However, very recent work of the Kuball group suggests an enhancement of molecular interactions after ABP treatment and proposes that BTN3A1 stabilizes an immunological synapse involving the TCR and a BTN3 counter-receptor on the Vγ9Vδ2 T-cell and BTN2, different types of TCR-δ chain ligands and BTN3 on the site of the tumor or presenting cell.[109]

NMR analysis showed an interaction of BTN2A1 with the C-F-G surface of BTN3A1 which together with cross-linking results suggests an interaction of the ED of both molecules which resembles the cis-interaction of PD-L1 (CD274) and B7.1 (CD80).[140] Importantly, mutations of BTN3A1 and BTN3A2 in these areas reduce PAg stimulation, which in the case of BTN3A was originally considered evidence for a potential TCR-binding site[88] but may warrant new interpretation. The same may apply for the inhibition of PAg responses by mAb 20.1 and 20.1 Fv-fragment which binds to the C-C′-C″ strand formed close to the C-F-G surface.[88] It is conceivable that mAb 20.1 binding interferes with BTN2A1-BTN3A1 interactions and consequently Vγ9Vδ2 T-cell responses but also that a conformational change as a consequence of mAb 20.1 binding might propagate TCR binding and activation of certain Vγ9Vδ2TCR clonotypes while also interfering with a stimulatory state after PAg-binding to BTN3-ID. In line with, the hypothesis is that sc-Fv of the non-competing mAb 103.2 shows no inhibitory effects,[112] despite a high affinity for recombinant BTN3A1, 2, and 3 (K_D 8-15 nmol/L). Nonetheless, the whole 103.2 antibody is a very efficient inhibitor[113] perhaps by fixing a conformation of the BTN3A molecules which prevents interaction with TCR, BTN2A1 or other not yet defined molecules.[102]

New insights may come from the comparison of BTN2A1 and its homologs in various species. Figure 6 shows a sequence alignment of BTN2A1 with the probably functional homolog of alpaca (named BTN2 or BTN2A2 in some databases) with human BTN2A2 and mouse BTN2A2. Human BTN2A1 and, although not yet formally proven, alpaca BTN2 support PAg-mediated Vγ9Vδ2 T-cell stimulation while human and mouse BTN2A2 do not. The two non-functional BTN2s differ from the functional ones by pronounced insertions and deletions. The 13 aa insertion in the first half of exon 4 was predicted to convert the molecule from a single-pass to a double-pass membrane protein and might alter its entire function.[103] Unclear is the significance of the N-glycosylation site of BTN2A1 generated by isoleucine to threonine (I122T) substitution located at the start of the F-strand which is missing in the BTN2A2 of human, alpaca, mouse, and armadillo (not shown). The V-domains of huBTN2A2 and huBTN2A1 are very similar (93.9% aa identity) and identical in the proposed TCR contacts consistent with similar binding properties in the plasmon resonance studies, while the repetitive L in the leader of BTN2A2 may affect cell surface expression. Finally, given the importance of the JM for BTN3 function it would be surprising if the 16-18 aa deletion (Figure 6) in the JM of human, mouse, and armadillo BTN2A2 (not shown) would not affect BTN2A1 function, for example, by affecting interactions with the PAg-sensing BTN3A1 molecule while similarity of the ED of both molecules might still allow support of mAb 20.1-induced stimulation of certain TCRs as in the case of TCR MOP reporter cells co-cultured with BTN3A1-transduced rodent T-cells.[112,135] These hypotheses can be tested by comparison of interspecies chimeras and mutational analysis.

4.8 | A composite ligand model of PAg recognition and its physiological consequences

When discussing binding partners of the TCR, it is of interest that BTN2A1- and BTN2A1 + BTN3A1-transduced murine or hamster cells show a robust and statistically significant background stimulation of 1%-10% of the maximum response to HMBPP. This was completely abolished by mutations of the Vγ9-HV4 but also by mutations/deletions in the CDR2δ and CDR3δ[119] despite that these regions of the TCR-δ chain are not involved in BTN2A1 binding.[119,139] This is fully consistent with mutagenesis studies on the ABP and PAg response which showed involvement of the three CDRs of both TCR chains.[141] We suggest that these TCR regions may interact or bind to other proteins than BTN2A1 required for full activation of the Vγ9Vδ2 T-cell (Figure 6) and hypothesize a composite ligand model of PAg recognition. This ligand could be a highly conserved molecule(s) and binding simultaneously with BTNs.

An additional interaction partner would be sterically possible and may also contribute to PAg-sensing or PAg-independent activation reported for other ligands (Figure 7). Some of these ligands may have an affinity to the Vγ9Vδ2TCR that is too low to trigger a response on their own and may need to associate with BTN2 or BTN2-BTN3 complexes to be sensed by the Vγ9Vδ2TCR. Others may have a higher intrinsic affinity for specific TCR clonotypes and might be able to interact with the TCR even in the absence of PAg-induced cellular changes[119] but in some cases still need “help” by the superantigen-like binding of BTN2A1.[58] In any case, it is worth noting that overexpression of BTN2A1 leads only to “background”

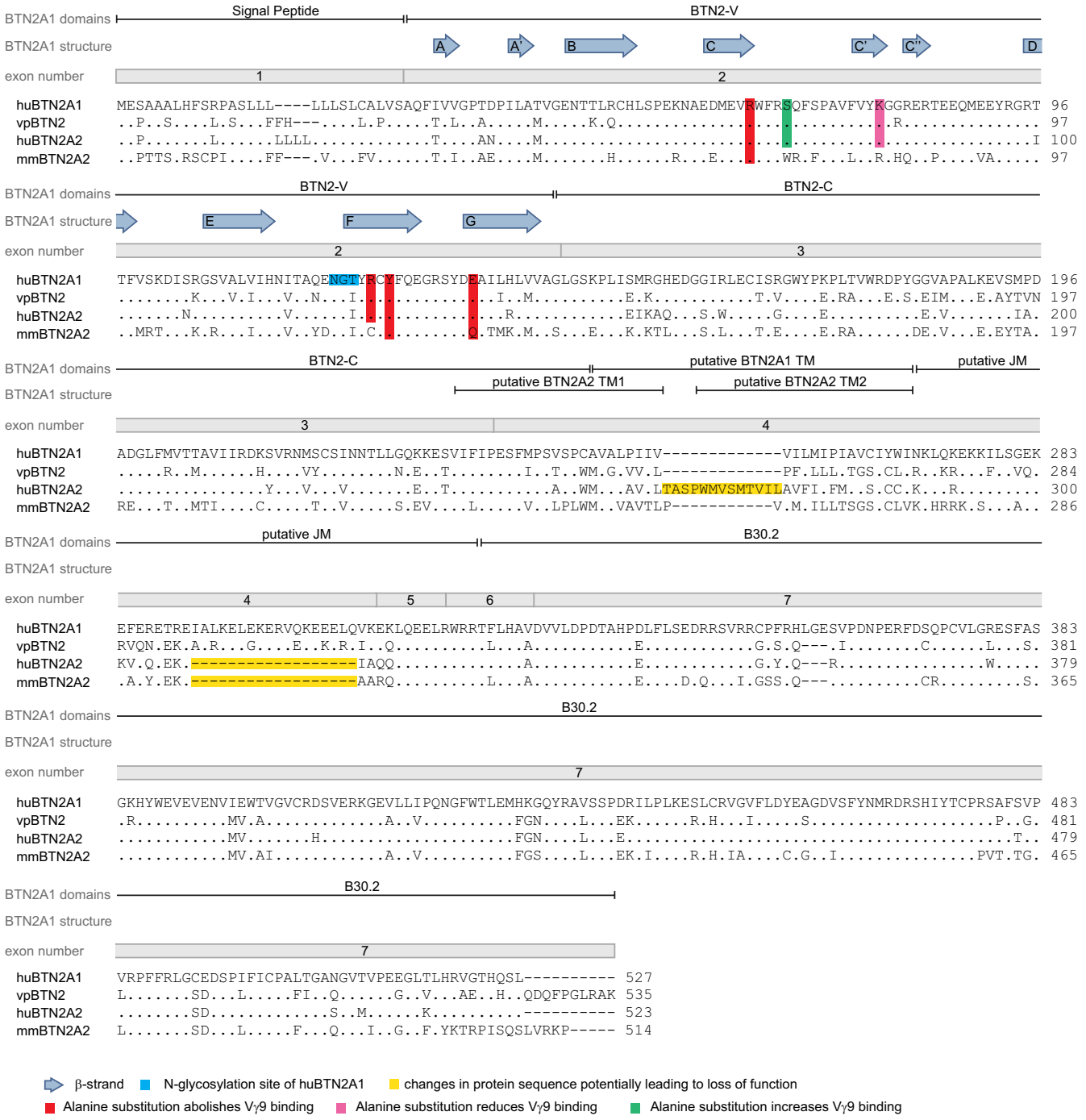


FIGURE 6 Sequence comparison of butyrophilin (BTN)2 isoforms. Protein sequence alignment of human BTN2A1 and A2 isoforms (huBTN2A1/A2), alpaca BTN2 (vpBTN2), and mouse BTN2A2 (mmBTN2A2) (Clustal Omega and BioEdit). Identical amino acids (dots), gaps (dashes), and numbers (right side) are indicated. Amino acid numbers include the signal peptide.[119] The domains of BTN2 (protein domain) and corresponding exons (exon number) are shown according to human BTN2A1 ([119 103] and own database analysis). Some features (β-strands) of the secondary structure of human BTN2-V are visualized by blue arrows.[119] One BTN2A1-specific N-glycosylation site is shown in blue, and deletions/insertions of huBTN2A2 and mouse BTN2A2 are marked in yellow. Changes in site-directed mutagenesis that affect Vγ9 binding to human BTN2A1 (abolishes: red, reduces: pink, increases: green) and their conservation in other BTN2 molecules are shown[119]

stimulation of TCR transductants despite binding of TCR tetramers to these cells was substantially higher than to ABP-pulsed, untransduced cells whose activating properties were far superior.[119] This implies that not only the avidity of TCR-ligand-binding controls the extent and quality of the T-cell response and that a composite ligand containing BTNs or functional equivalents and variable ligands would reduce the required affinity of each ligand to induce for stimulation. Furthermore, the topology of TCR binding and especially

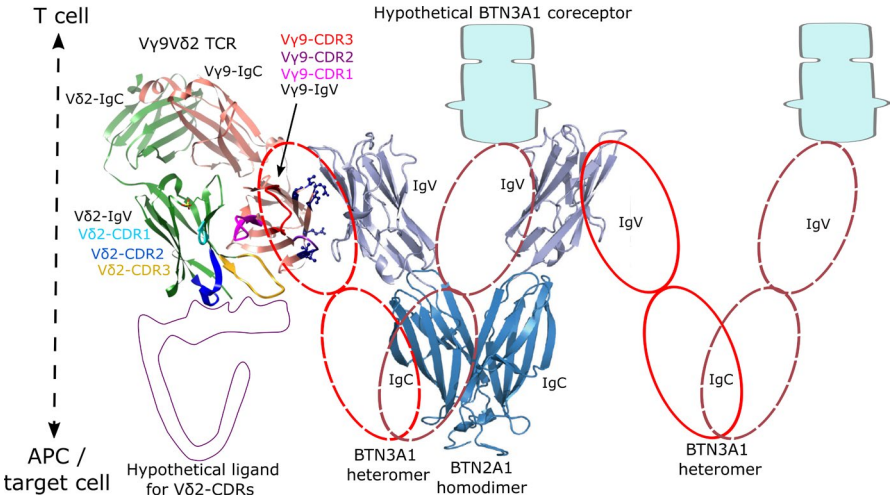


FIGURE 7 Composite ligand model[119]: In this model, BTN2A1 interacts with BTN3A1 as well as with the HV4 and CDR2 of the Vγ9 chain of the Vγ9Vδ2 TCR at steady state. Different changes might be induced upon PAg binding to B30.2-BTN3A1: i) BTN3A1 comes closer to BTN2A1 and TCR allowing its interaction with the CDR3 of Vγ9 and Vδ2 chain. However, this has not been formally proven, and hence, BTN3A heteromers were shown in punctuated lines ii) if not, the PAg-BTN3A1 complex may recruit other unidentified ligand(s) which would probably interact with the CDR2 and CDR3 of Vδ2 chain iii) PAg-BTN3A1 may interact with a counter-receptor of the T-cell, however, the necessity and contribution for activation remains to be shown but would be compatible. The structure of G115 TCR adapted from 1HXM was modified with UCSF Chimera 1,14. CDRs are color-coded, BTN2A1 interacting residues of Vγ9 chain are shown as ball and stick. BTN2A1 was adapted from[119] and the composite ligand model scheme was generated with Inkscape

binding at the "superantigen-binding" site of the Vγ9Vδ2 TCR might lead to other signals than binding of a composite ligand consisting of BTN2A1 and variable ligands which also engages the CDRs of the TCR Vδ2 chain and the CDR3 of the Vγ9 chain. Similar observations have been made for the response of αβ T-cells to viral and bacterial superantigens[142,143] and have recently been discussed as a functional principle of antigen receptors of lymphocytes with innate characteristics.[58]

Such binding might be initiated, facilitated, or stabilized by BTN3A1. In this case, BTN3 would not need to directly interact with TCR which would be in line with most data on (lack of) physical interaction between molecules[102] and the proposed function of BTN3A1 as a coreceptor supporting the immunological synapse of the Vγ9Vδ2 TCR.[109] Nevertheless, the case of direct BTN3-TCR interaction as well as possible binding by other ligands has not yet been settled. Our composite ligand model would even have a place for direct binding of phosphoantigens to the positively charged groove formed by arginine 51 in the CDR2δ and the JP-encoded lysine 109 in CDR3γ chain to the Vγ9Vδ2 TCR. Both amino acids are known to be essential for PAg reactivity, and the groove was identified in the crystal structure of the TCR G115.[133,134] Indeed, binding of both BTN2A1 and BTN3A1 could be imagined either to different (neighboring) TCR molecules but molecular modeling should also allow to test for the possibility of simultaneous binding of BTN3 to the CDR3γ and of BTN2A1 to the TCR-binding sites located at the CDR2 and HV4 of the γ-chain. In any case, despite great progress in understanding biology and the molecular basis of PAg-mediated activation of Vγ9Vδ2 T-cells, the case of what Vγ9Vδ2 T-cells actually "see" is far from being closed but comparative genomics and functional studies should help to develop new perspectives for further studies.

Finally, we would like to speculate that BTNs, BTNLs, and their ligands may steer γδ T-cell development by direct interaction with the TCR although it needs to be acknowledged that such interaction has been firmly documented only for BTNL3 with human Vγ4 and BTN2A1 human Vγ9.[88,119,139] Such missing TCR-BTN interaction could also be the reason why Vγ9Vδ2TCR-transgenic cells show a developmental block in intra-thymic development of the TCR-transgenic cells which can be overcome by anti-CD3 application.[144] Mice transgenic for the relevant BTN(-like) molecules might allow to overcome such developmental block and finally help to establish preclinical mouse models for cells expressing Vγ9Vδ2 other human γδ TCRs.

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