The Armadillo (Dasypus novemcinctus): A Witness but Not a Functional Example for the Emergence of the Butyrophilin 3/Vγ9Vδ2 System in Placental Mammals

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1–5% of human blood T cells are Vγ9Vδ2 T cells whose T cell receptor (TCR) contain a TRGV9/TRGJP rearrangement and a TRDV2 comprising Vδ2-chain. They respond to phosphoantigens (PAgs) like isopentenyl pyrophosphate or (E)-4-hydroxy-3-methyl-but-2-enyl-pyrophosphate (HMBPP) in a butyrophilin 3 (BTN3)-dependent manner and may contribute to the control of mycobacterial infections. These cells were thought to be restricted to primates, but we demonstrated by analysis of genomic databases that TRGV9, TRDV2, and BTN3 genes coevolved and emerged together with placental mammals. Furthermore, we identified alpaca (Vicugna pacos) as species with typical Vγ9Vδ2 TCR rearrangements and currently aim to directly identify Vγ9Vδ2 T cells and BTN3. Other candidates to study this coevolution are the bottlenose dolphin (Tursiops truncatus) and the nine-banded armadillo (Dasypus novemcinctus) with genomic sequences encoding open reading frames for TRGV9, TRDV2, and the extracellular part of BTN3. Dolphins have been shown to express Vγ9- and Vδ2-like TCR chains and possess a predicted BTN3-like gene homologous to human BTN3A3. The other candidate, the armadillo, is of medical interest since it serves as a natural reservoir for Mycobacterium leprae. In this study, we analyzed the armadillo genome and found evidence for multiple non-functional BTN3 genes including genomic context which closely resembles the organization of the human, alpaca, and dolphin BTN3A3 loci. However, no BTN3 transcript could be detected in armadillo cDNA. Additionally, attempts to identify a functional TRGV9/TRGJP rearrangement via PCR failed. In contrast, complete TRDV2 gene segments preferentially rearranged with a TRDJ4 homolog were cloned and co-expressed with a human Vγ9-chain in murine hybridoma cells. These cells could be stimulated by immobilized anti-mouse CD3 antibody but not with human RAJI-RT1Bl cells and HMBPP. So far, the

Abbreviations: BTN, butyrophilin; BTN3-V, BTN3 IgV-like region; BTN3-C, BTN3 IgC-like region; HMBPP, (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate; PAg, phosphoantigen; wgs, whole genome shotgun contigs.
lack of expression of TRGV9 rearrangements and BTN3 renders the armadillo an unlikely candidate species for PAg-reactive Vγ9Vδ2 T cells. This is in line with the postulated coevolution of the three genes, where occurrence of Vγ9Vδ2 TCRs coincides with a functional BTN3 molecule.

Keywords: Vγ9Vδ2, TRGV9, TRDV2, butyrophilin 3, coevolution, nine-banded armadillo, placental mammals

INTRODUCTION

With up to 5% of T cells, Vγ9Vδ2 T cells constitute a major γδ T cell population in the human blood (1, 2). Their T cell receptor (TCR) is characterized by a pairing of a Vγ chain, encoded by a TRGV9/TRGJP gene rearrangement and a TRGC1 constant region, and a Vδ2 chain using a TRDV2 variable region. This cell subset recognizes and rapidly reacts to endogenous or exogenous phosphoantigens (PAgs) in a MHC-unrestricted fashion (1). PAgs are small molecules with pyrophosphate groups produced during isoprenoid synthesis. The most important naturally occurring PAgs are isopentenyl pyrophosphate and (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP). The importance of the Vγ9Vδ2 T cell subset lies within their multitude of effector functions such as production of cytokines, killing of cells (via TCR, NKG2D, CD16), B cell help and APC-like functions (2). Their reactivity to aminobisphosphonates and PAgs makes them a potential tool for tumor treatment (3) and like functions (2). Their reactivity to aminobisphosphonates and killing of cells (TCR, NKG2D, CD16), B cell help and APC-multiplicity of effector functions such as production of cytokines, 9V2 T cell subset lies within their γδ The importance of the Vγ9Vδ2 γδ2 chain using a TRDV2 γ chain, encoded by TRGV9/TRGJP genes in several species (23, 24). Therefore, an emergence of those genes with Placentalia presence of BTN3. In line with this, our group generated first evidence for PAg-reactive γδ T cells in this species (25).

Apart from that, the bottlenose dolphin (Tursiops truncatus) has recently been found to express TRGV9- and TRDV2-like productive rearrangements (26) and a BTN3A3-like gene was predicted via Gnomon gene prediction tool (GenBank: XM_004332447.2). Another candidate with in-frame TRGV9, TRDV2, and BTN3 extracellular domain genes is the nine-banded armadillo (Dasypus novemcinctus), which belongs to the Xenarthra superorder. Armadillos are a natural reservoir of M. leprae and, therefore, a valuable tool for leprosy research (27, 28). In addition, the neurological involvement and dissemination in armadillos infected with M. leprae is similar to the one observed in humans and could not be reproduced in rodent models, as reviewed elsewhere (29). Karunakaran et al. (23) predicted armadillo TRGV9 and TRDV2 genes with rather high identities to their human homologs as well as a BTN3-V-like domain. In this study, we tested the expression of those genes in armadillo PBMCs. Here, we report the expression of in silico translatable TRDV2 chains but the apparent lack of expression for productive TRGV9 rearrangements and of a complete BTN3-like transcript and discuss the implications of these findings for the coevolution of Vγ9, Vδ2, and BTN3 genes.

MATERIALS AND METHODS

Armadillo/Alpaca/Dolphin Homologs for TRGV9, TRDV2, and BTN3

Dasypus novemcinctus (taxid 9361) whole genomic shotgun sequences (wgs) were taken from the National Center for Biotechnology Information (NCBI) databases (BioProject: PRJNA12594/PRJNA196486; BioSample: SAMN02953623; GenBank: gb|AAGV00000000.3). Homologous sequences to human Vγ9Vδ2 TCR MOP (GenBank: KC170727.1/KC196073.1) or G115 (PDB: 1HXM_A) (30) and BTN3A1/2/3 (GenBank: NM_007048.5/NM_007047.4/NM_006994.4) were predicted using the NCBI Basic Local Alignment Tool (BLAST) (31). Accession numbers of identified armadillo homologs are: TRGV9 AAVG03121505.1 nt402-695; TRGC-A Ex1 AAVG03121543.1 nt3646-3947; TRGC-B Ex1 AAVG03121550.1 nt3170-3471; TRGC-C Ex1 AAVG03121548.1 nt6289-6590; TRGD-C Ex1 AAVG03173223.1 nt672-373; TRDV2 AAVG03208792.1 nt2277-1994; TRDC Ex1/2 AAVG03208782.1 nt782-510/nt95-27;
Amplification of Armadillo TRGV9, TRDV2 Rearrangements, and BTN3 Transcripts

Armadillo PBMCs in RNALater and genomic liver DNA were provided by the National Hansen’s Disease Program, Baton Rouge, LA, USA. Armadillos were maintained and samples collected in accordance with all ethical guidelines of the U.S. Public Health Service under protocols approved by the IACUC of the National Hansen’s Disease Program, assurance number A3021-1.

RNA isolation was performed with RNeasy Mini Kit (Qiagen) and First Strand cDNA Synthesis (Thermo Fisher Scientific) was performed with Oligo dT primer after DNase digestion with DNase I (Thermo Fisher Scientific). Unknown 5’ and 3’ ends of transcripts were determined using the GeneRacer Kit with SuperScript III RT (Invitrogen) according to the manufacturer’s instructions. Touchdown PCR with RACE-ready cDNA was performed with Q5 Hot Start Polymerase (NEB) and Phusion Polymerase (Thermo Fisher Scientific) was used for other PCR experiments. TOPO TA cloning set for sequencing with pCR4-TOPO vector (Thermo Fisher Scientific) was used for cloning and sequencing of PCR products. Armadillo genomic liver DNA was used as a control for PCR amplifications. Primer sequences are given in Supplementary Table S1 in Supplementary Material.

TRDV2

TRDV2/TRDC amplification was performed with the primers A21 and A72, nested PCR with A71 and A73. The 5’ end of TRDV2 was determined via 5’ RACE PCR with the primer A118 and nested primer A119. The primers A94 and A95 were applied for 3’ RACE PCR starting from TRDV2. The PCR products of those amplifications were subsequently cloned and clones were analyzed.

TRGV9

Attempts to amplify a TRGV9 rearrangement included amplification of TRGV9/TRGC with different primer combinations and 3’ RACE PCR starting from TRGV9. The 5’ end of TRGC transcripts was, therefore, amplified using 5’ RACE PCR and the primers A86 and A87, and the PCR product was cloned with the TOPO TA cloning kit. The 3’ sequence of TRGC was analyzed with 3’ RACE PCR using the primers A103 and A104.

Butyrophilin 3

Expression of a BTN3 homolog in armadillo PBMCs was analyzed with the partial amplification of BTN3 from the BTN3-V to BTN3-C domain with primers specific for all three armadillo homologs (A122 + A123). Furthermore, RACE PCR to obtain the 5’ sequence of BTN3-V (A165, A166) and the 3’ sequence from BTN3-V (A163, A164) and BTN3-C (A167, A168) was conducted.

Sequence Analysis

Sequence analysis of genomic sequence data or PCR amplifications was performed with NCBI BLAST and Clustal Omega software. Alignments were calculated with Clustal Omega and BioEdit software was used for editing of alignments.

Expression of Armadillo Vδ2 TCR Chains

A murine TCR-negative T cell hybridoma cell line (BW58 r/mCD28) expressing a rat/mouse chimeric CD28 molecule (33, 34) was used to express armadillo Vδ2 TCR chains and test for surface expression, CD3 signaling, and HMBPP-reactivity. Full-length armadillo Vδ2 chains were amplified using the primers A193 and A194 and cloning in pMSCV-IRES-mCherry (a gift from Dario Vignali, Addgene plasmid # 52114) was performed using the In-Fusion HD Cloning Kit (Takara Bio). The clones 7 and 9 were selected for co-expression with the human Vγ9 TCR MOP chain (35). Retroviral transduction of BW58 r/mCD28 cells was used to stably express TCR chains (36) and vector-encoded EGFP (pPEG3 huVγ9) and mCherry (pMSCV dnVδ2 cl7 or cl9) indicated successful transduction. TCR surface expression was confirmed in a flow cytometry staining of human Vγ9 (2.5 μg/ml anti-Vγ9 TCR 4D7 mAb) (37) detected by a secondary antibody [1 μg/ml F(ab’)2 Fragment Donkey α-Mouse IgG (H + L)] (BD Pharmingen) and anti-mouse CD3 (1 μg/ml biotin hamster anti-mouse CD3ε clone I45-2C11) detected by streptavidin [0.4 μg/ml Streptavidin-APC (BD Pharmingen)]. BW58 r/mCD28 cells overexpressing transduced TCR chains can be applied as responder cell lines in various in vitro models of antigen recognition and their activity can be measured by mouse IL-2 ELISA (38, 39). Thus, the human/armadillo TCR transductants (hu/dnTCR cl7 or cl9) were tested for functional TCR signaling by CD3 crosslinking and FAg reactivity (HMBPP, Sigma-Aldrich) in co-culture with Raji RT1B cells (33, 38, 40). TCR-negative BW58 cells expressing r/mCD28 (TCR−), the same cells transduced with only the human Vγ9 chain (hu−/TCR +), and the human TCR MOP
(hu/huTCR) were used as controls for stainings and stimulations. Cells were cultured in 200 μl/well RPMI 1640 supplemented with 5 or 10% FCS, 100 mM sodium pyruvate, 0.05% w/v glutamine, 10 mM nonessential amino acids, and 5 × 10⁻⁵ M mercaptoethanol (Invitrogen). Stimulations were carried out for 22 h with 5 × 10⁴/well responder cells cultured in 96-well round bottom plates (Greiner) in co-culture with 5 × 10⁴/well RAJI-RT1B’ cells. For CD3 crosslinking, 96 well flat bottom plates (Greiner) were coated with anti-mouse CD3ε (clone 145-2C11, BD Pharmingen) in PBS for 24 h at 4°C before stimulations. Mouse IL-2 sandwich ELISA (BD) was used to determine IL-2 secretion in the culture supernatants and appropriate dilutions were measured if the upper detection limit was reached.

RESULTS

Genomic Organization of a Close Homolog of Human BTN3 loci in Armadillo

Previous studies reported armadillo genomic regions homologous to the human BTN3A1 extracellular and intracellular domains (24). After more detailed homology analysis of those armadillo genes, a closer resemblance to human BTN3A3 was confirmed. Through the NCBI Basic local alignment (BLAST) tool (31), we therefore, compared the human BTN3A3 mRNA sequence (GenBank: NM_006994.4) to the D. novemcinctus whole genomic shotgun sequences (wgs) and could identify three homologous regions for the BTN3-V and BTN3-C domains, respectively. To compare these with homologous BTN3 genes in other species, we additionally analyzed the BTN3-like loci of the two other candidate species alpaca (V. pacos) and bottlenose dolphin (Tursiops truncatus). For those species, predicted BTN3A3-like sequences are published in NCBI databases (alpaca: XM_015251744.1, dolphin: XM_004332447.2). Those predicted sequences were compared to the respective wgs databases to analyze BTN3-like loci and isoforms. Whole genome shotgun sequence databases are comprised of contigs with unique accession numbers and contain incomplete non-annotated genomic information. Whole genomic shotgun sequences were taken from the NCBI databases and allow full or partial reconstruction of BTN3 encoding genomic regions (Figure 1). The corresponding nucleotide and amino acid sequence alignments and armadillo locus information are supplied in the Figures S1–S5 in Supplementary Material.

The human BTN3A3 gene is comprised of nine protein-coding exons with exon 2 encoding the BTN3-V region, exon 3 encoding BTN3-C, exon 4 representing part of the transmembrane region, followed by four relatively small exons (5–8) and the B30.2 exon (9) (Figure 1A) (42). The alpaca BTN3-like genomic sequence is organized in a locus strikingly homologous to human BTN3A3 (Figure 1B), showing exons with nucleotide sequence identities to human BTN3A3 ranging from 73 to 93% and conserved intron lengths. The intracellular B30.2 domain is slightly shorter (81 nt) than the human counterpart. In silico splicing and translation of this alpaca BTN3-like gene with an overall nucleotide identity of 81% to human BTN3A3 results in a protein sequence, which shares 72% amino acids with the human homolog. The expression of an alpaca BTN3-like molecule (GenBank: MG029164), with a conservation of 81% on the nucleotide and 73% on the amino acid level to human BTN3A3, has been confirmed before (32). We could, however, identify minor differences between the genomic alpaca BTN3 and the BTN3 transcript amplified from cDNA on the nucleotide and amino acid level (Figures S1 and S2 in Supplementary Material). This can be explained by interindividual polymorphisms that also exist in humans (20). Both available alpaca BTN3 protein sequences carry six conserved amino acids each in the BTN3-N-V (Glu37, Lys39, Arg61, Tyr100, Gln102, and Tyr107) and B30.2 domain (His351, His378, Lys393, Arg412, Arg418, Arg469) (Figure S2 in Supplementary Material) predicted to be involved in PAg recognition in human BTN3A1 (15, 23, 24, 43).

Dolphins have been found to express TRGV9- and TRDV2-like mRNA transcripts (26), however, BTN3 expression has not yet been proven. Here, we report the existence of one locus in the dolphin wgs database that comprises a full-length BTN3-like sequence predicted by NCBI via Gnomon (GenBank: XM_004332447.2) and a remarkably conserved locus organization (Figure 1C). Comparable to the alpaca BTN3-like locus, the dolphin locus features nine exons with nucleotide (nt) identities from 72 to 93% compared to human BTN3A3 and intron lengths similar to the one in the human BTN3A3 locus. However, the intron between exon 6 and 7 is only about half in size compared to the human intron at this location and the intracellular B30.2 exon (9) is 33 nucleotides shorter. The dolphin BTN3-like sequence is in silico translatable and exhibits a nucleotide identity of 81% and an amino acid (aa) identity of 73% with human BTN3A3 (Figure S1 and S2 in Supplementary Material). This BTN3A3-like gene carries five out of six conserved amino acids in the BTN3-V domain and a substitution (Lys391Thr) (Figure S2 in Supplementary Material). All six predicted PAg-binding residues in the B30.2 domain (15, 43) are identical. Interestingly, we report the existence of another BTN3-like partial locus in the dolphin genomic sequences (Figure 1D). This contig is only long enough to comprise exons 1 to 3 of a BTN3-like gene structure. The BTN3-V (exon 2) of this locus (Figure 1D) is 92% identical to and shorter than the other BTN3-V found for the dolphin (Figure 1C), which indicates possible deletions in this exon. Consequently, this locus seems to code for a BTN3-like pseudogene.

Database analysis of the armadillo wgs database resulted in a total of three BTN3-V, three BTN3-C homologous regions, and one exon similar to the human BTN3A3 B30.2 domain. One pair of BTN3-V and BTN3-C is comprised in one single contig of the nine-banded armadillo wgs database (AAGV03145787.1), which also includes a partial hit for the transmembrane region in exon 4 of BTN3A3, three small exons, homologous to human exons 6–8, and a downstream B30.2-like region (Figure 1E; Figure S5A in Supplementary Material). All those homologous regions show a nucleotide conservation of more than 70% compared to human BTN3A3 domains and are also remarkably similar to human BTN3A3 with respect to intron lengths and genomic organization. Two other BTN3-V domains (AAGV03287843.1 and AAGV03240336.1) were found as well as two other BTN3-C domains (AAGV03240337.1 and AAGV03010207.1). However, the BTN3-C containing contig AAGV03240337.1 does not seem to include a B30.2-like region and shows a truncated
Homo sapiens BTN3A3, chromosome 6, alternate assembly CHM1 1.1 (NC_018917.2)

|    | L     | IgV | IgC | TM  | *   | *   | B30.2 (PRYSRY) |
|----|-------|-----|-----|-----|-----|-----|----------------|
| A  |       |     |     |     |     |     |                |
| Exon # | Intron length |
|       | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   |
| NT Location | 26445978- | 26445967- | 26445967- | 26445967- | 26445967- | 26445967- | 26445967- | 26445967- | 26445967- |
| Location | 264459662 | 264459657 | 264459656 | 264459655 | 264459654 | 264459653 | 264459652 | 264459651 | 264459650 |
| Nucleotide identity | 78% | 86% | 80% | 73% | 31% | 93% | 81% | 85% | 82% |

Vicugna pacos (taxid:30538) BTN3 homologous region, whole-genome shotgun contig ABRRO02153549.1

|    | Exon # | Intron length |
|----|-------|---------------|
| NT Location | 1-85 | 86-433        | 343-715        | 716-916        | 917-917        | 938-986        | 985-991        | 992-1018       | 1019-1756       |
| Location | 269207- | 239307- | 27154        | 24821-        | 24446-        | 24331-        | 23447-        | 22996-        | 21360-        |
| Nucleotide identity | 78% | 86% | 80% | 73% | 31% | 93% | 81% | 85% | 82% |

Tursiops truncatus (taxid:9739) BTN3 homologous region, whole-genome shotgun contigs

|    | Exon # | Intron length |
|----|-------|---------------|
| NT Location | 1-85 | 86-433        | 343-715        | 716-916        | 917-917        | 938-986        | 985-991        | 992-1018       | 1016-1722       |
| Location | 19668241- | 1968563- | 19670022- | 19872440- | 19872822- | 19872842- | 19872956- | 19873359- | 19873835- |
| Nucleotide identity | 79% | 86% | 81% | 72% | 70% | 93% | 81% | 81% | 81% |

Dasypus novemcinctus (taxid:9361) BTN3 homologous regions, whole-genome shotgun contigs

|    | Exon # | Intron length |
|----|-------|---------------|
| NT Location | 88-433 | 1941-2288 | 3676-3934 | 5511-5620 | 938-984 | 985-991 | 992-1019 | 1020-1696 | 816-18829 |
| Location | 12279-12826 | 12279-12826 | 12279-12826 | 12279-12826 | 12279-12826 | 12279-12826 | 12279-12826 | 12279-12826 | 12279-12826 |
| Nucleotide identity | 77% | 73% | 75% | 89% | 87% | 78% | 70% |

FIGURE 1 | Continued.
transmembrane homolog directly followed by another exon similar to the transmembrane region of human BTN3A3 (Figure 1F; Figure S5B in Supplementary Material). No homologous intracellular regions could be found in AAGV03010207.1 due to the short contig length (Figure 1G). Owing to the abundant use of SPRY/B30.2 domains in several families of molecules (44), prediction of BTN3-related B30.2 regions is difficult, except for the one found in contig AAGV03145787.1 (Figure 1E; Figure S4 in Supplementary Material). Additionally, conserved leader sequences encoded by exon 1 and another part of human BTN3A3 encoded by exon 5 could not be predicted in all contigs through Blast using BTN3A3 and it is noteworthy that gene prediction tools like Gnomon used for a predicted armadillo BTN3A3 entry (GenBank: XM_012528284.1) or FGENESH+ (reference protein: huBTN3A1/2/3; GenBank: NM_007048.5/NM_007047.4/ NM_006994.4) (45) also fail to predict a leader sequence in the AAGV03145787.1 contig. The published predicted BTN3A3 homolog calculated by Gnomon software and our own calculations with FGENESH+ locate the start codon within the BTN3-V region (Figure 1E). In silico translation was successful for two BTN3-V-like regions (Figure S3A in Supplementary Material). The third BTN3-V homolog in AAGV03287843.1 (Figure 1G) carries a stop codon, if translated in the same frame. All three BTN3-C homologs were translatable; however, the respective region in AAGV03145787.1, although not having any stop codons, exhibits one nucleotide deletion leading to a frameshift (Figure 1E; Figure S3B in Supplementary Material). The only intracellular B30.2 domain found in this setting in the armadillo is identical with the previously reported one (24), but reexamination of the nucleotide to protein translation reveals several stop codons if the human B30.2 frame is used (Figure S4 in Supplementary Material). Yet, nucleotide alignments show the conservation of codons encoding all of the six conserved PAg-binding residues in the B30.2 domain of BTN3A1 described by Sandstrom et al. (15) including His351. Six extracellular PAg-binding residues have been proposed for the BTN3-V domain of BTN3A1 (43) and codons for these amino acids are partially conserved in the armadillo. Here, four out of six codons are conserved in the BTN3-V exons found in AAGV03240336.1 and AAGV03287843.1, and three out of six in AAGV03145787.1.

In addition to database analysis, we tested for expression of potential BTN3 isoforms, as well as TRGV9 and TRDV2 transcripts, in cDNA of armadillo PBMCs. D. novemcinctus PBMCs dissolved in RNAlater were provided by the National Hansen's Disease Program, Baton Rouge, LA, USA and tested for transcripts of BTN3, TRGV9, and TRDV2. These PCR approaches included the amplifications of BTN3 performed with primers specific for all BTN3-V and BTN3-C regions and the RACE PCR amplification of the 5' and 3' sequences starting in several domains of the predicted genes (Table S1 in Supplementary Material). No transcripts of BTN3 were found, but we were able to amplify BTN3-V to BTN3-C including a corresponding intron from genomic liver DNA using the same primers. TOPO TA cloning of this PCR product resulted in five clones of apparently two distinct types (GenBank: cl1: MG600558; cl3: MG600559; cl5: MG600560; cl4/6: MG600561). One type was strikingly like the BTN3-V containing contig AAGV03240336.1 and the BTN3-C comprising contig AAGV03240337.1, which lead us to link those two contigs together (Figure 1F). However, the three TOPO clones of this subtype were not nucleotide-identical (cl1, cl3, cl5). The two remaining TOPO clones (cl4, cl6) were identical but could not be mapped to an armadillo wgs database contig and those clones were only 92–95% identical to the previously predicted BTN3 loci. This could indicate the existence of even more loci for BTN3 homologs in the armadillo. Closer comparison of the two predicted BTN3 loci in the armadillo showed an apparent deletion in the AAGV03240337.1 contig when blasted with AAGV03145787.1 (Figure 1F). The first deletion results from a fusion of a truncated exon 4 with exon 6, the second deletion includes exon 8 and the B30.2 domain encoded by exon 9. In summary, no evidence was found for the expression of a BTN3 homolog and even in the unlikely case that expression of such a gene was missed, we do not expect that these transcripts yield functional proteins. This is especially evident compared to the loci of alpaca and dolphin BTN3-like genomic regions, which feature not only homologous regions to all nine BTN3A3 exons, but are also in silico translatable and in the case of alpaca also expressed on cDNA level.

**In Silico Translatable TRDV2 Rearrangements Are Expressed in Armadillo**

In contrast to the lack of expression of a BTN3-like gene by D. novemcinctus, we demonstrate the expression of *in silico* translatable TRDV2 TCR chains (IMGT nomenclature if not otherwise indicated). Full-length armadillo TRDV2-like variable regions preferentially recombined with a TRD4 homolog could be assembled through the amplifications of TRDV2/TRDC from armadillo PBMCs, RACE PCR and cloning of full-length TRDV2 chains into the pMSCV-IRESmCherry FP plasmid. The overall amino acid identities of two clones carrying TRDV2/TRD4...
homologs to the human G115 Vδ2 chain were 65% for both clones (Figure 2A). The armadillo V region shares a 59% aa identity with the human G115 Vδ2 chain, the J region is 86% (nt) and 86% (aa) identical to the human TRDJ4, TRDC of armadillo and human show a conservation of 82% (nt) and 69% (aa). A single clone was found to carry a TRDV2 rearrangement with a TRDJ region homologous to human TRDJ3, with 88% (nt) and 89% (aa) identity (Figure 2B). PAg-reactive Vδ2 chains in humans commonly use TRDJ1, 2 or 3 (46), however, preferential but not exclusive rearrangement of TRDV2 with a TRDJ4-like J segment has been shown in V. pacos (23). Other conserved features of PAg-reactive Vδ2 chains are varying CDR3 lengths (46), the residues Arg51 (30, 46, 47) and Glu52 (30), and the presence of a hydrophobic amino acid (Leu, Ile, Val) at position δ97 (46, 48). Partial armadillo TRDV2-like rearrangements were amplified either through 3′ RACE (8 clones) or TRDV2/TRDC amplification (10 clones) and PCR products were cloned with the TOPO TA cloning set for sequencing with pCR4-TOPO vector (Thermo Fisher Scientific). Another eight unique TRDV2 clones were obtained from cloning of full-length rearranged armadillo TRDV2 transcripts into the pMSCV-IRES-mCherry FP vector. All those partial clones were in frame with CDR3 lengths of 9-23 aa (Figure 2B). The positions Arg51 and Glu52 are conserved in all our armadillo clones and 5 out of 15 unique CDR3 sequences carry valine or isoleucine at δ97. Two armadillo Vδ2 chains amplified by PCR from cDNA were co-expressed with a human Vγ9 chain (TCR MOP) in a TCR-negative mouse cell line (BW58 r/mCD28) (33, 34). Surface expression of heterodimeric TCRs was confirmed by flow cytometry staining of the Vγ9 and Vδ2 chain and mouse CD3, as well as vector-encoded EGFP (human Vγ9) and mCherry (armadillo Vδ2) (Figure 3). CD28 expression of all cell lines was confirmed to be equal. The Vγ9 and CD3 expression of both cell lines overexpressing human/armadillo TCRs (hu/dnTCR cl7 or cl9) was significant but lower in comparison with human Vγ9Vδ2 TCR (huTCR) overexpressed in the same cell line. Thus, structural features important for pairing of armadillo Vδ2 chains with human Vγ9 chains seem to be conserved. Transduction of only the human Vγ9 chain did not

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**Figure 2** In silico translatable Vδ2 T cell receptor chains are expressed in Dasypus novemcinctus PBMCs. (A) Alignment of human G115 Vδ2 chain (PDB: 1HXM_A) (30) and two representative armadillo Vδ2 chains (obtained from cloning of full-length armadillo Vδ2 chains into pMSCV-IRES-mCherry FP). CDR regions appear underscored and positions δ51/52 and δ97 (gray) are highlighted. (B) CDR3 regions of TRDV2 clones obtained by TRDV2/TRDC PCR (clVC1, 3, 4 and 6–12), TRDV2 3′RACE PCR (cl2-7, 9, 10), and cloning (clpMSCV2, 4–10). CDR3 lengths and TRDJ-usage are indicated on the right. Alignments were calculated with Clustal Omega webtool, identical amino acids (dots), J region (underscored), and positions δ51/52 and δ97 (gray) are highlighted. The GenBank Accession numbers of unique clones are: cl2 (MG021118); clVC1 (MG021131); clVC4 (MG021132); cl3 (MG021127); cl4 (MG021128); cl5 (MG021129); cl6 (MG021130); clpMSCV2 (MG807648); clpMSCV4 (MG807649); clpMSCV5 (MG807650); clpMSCV6 (MG807651); clpMSCV7 (MG807652); clpMSCV8 (MG807653); clpMSCV9 (MG807654); clpMSCV10 (MG807655).
result in surface expression of Vγ9 or CD3. Signal transduction of huVγ9/dnVδ2 TCRs was studied with in vitro stimulation assays. Crosslinking of CD3 by plate-bound anti-mouse CD3 mAb was performed as described before (23, 40) and resulted in a substantial mIL-2 production of TCR transductants but no detectable IL-2 secretion of TCR cells or cells transduced with the human Vγ9 chain only (Figure 3B). Anti-CD3 mediated stimulation of hu/huTCR reached saturation at 3 µg/ml anti-CD3 as indicated by stimulation with 10 µg/ml. Reactivity to the PAg HMBPP was not observed in a stimulation assay with RAJI-RT1B1 cells, although human TCR transductants (hu/huTCR) readily recognized HMBPP in this context (Figure 3B). In summary, we report functional V62 chains in the armadillo, that pair with TCR γ chains and show no crossreactivity to human BTN3.

**Functional TRG Chain Rearrangements Lack Homologs to Human TRGV9**

Genomic surveys revealed a TRGV9-like gene (Accession: AAV03121505.1 nt402-695) in D. novemcinctus, which is in silico translatable and shares 80% of its nucleotides and 69% of its amino acids with the human G115 TCR γ. We were, however, not able to amplify a TRGV9 transcript from armadillo PBMCs via PCR of TRGV9/TRGC or 3′RACE PCR from TRGV9. Notably, we found four different regions (TRGC-A, -B, -C, -D) homologous to the first exon of the TCR γ constant region in the armadillo wgs database. Armadillo TRGC-A, -B, and -C (Accession: TRGC-A Ex1 AAV03121543.1; TRGC-B Ex1 AAV03121550.1; TRGC-C Ex1 AAV03121548.1) can be fully translated, however, TRGC-D (Accession: TRGC-D Ex1 AAV0317323.1 nt672-373) contains stop codons and is most likely a TRGC pseudogene. The first exons of TRGC-A and TRGC-B/C share 94% nucleotide identity, TRGC-B, and TRGC-C are 98% identical on the nucleotide level and all of them are 80% identical to exon 1 of the human TRGC1. Amplification of the 3′ end and 5′RACE PCR of TRGC-A/B/C exon 1 confirmed TRGC-A and TRGC-B, but not TRGC-C transcripts on cDNA level. It appears that TRGC-A is encoded by 3 exons, which are all represented in the contig AAV03121543.1 (exon1: nt3646-3953, exon2: nt7499–7548, exon3: nt9731-9871),
whereas TRGC-B and TRGC-C are encoded by 4 exons with exon 1 and 2 in the contigs AAVG03121548.1 (nt6277–6590 and nt7441–7491) and AAGV03121550.1 (nt3170–3478 and nt7125–7178), and exon 3 and 4 in AAVG03121549.1 (nt101-152 and nt2384–2525) and AAGV03121551.1 (nt1733–1784 and nt3978–4119), respectively. However, we were not able to assemble full-length TRGC-like regions from those contigs. Through 5′RACE PCR of TRGC, we can additionally report the existence of several armadillo TRGV transcripts. Of 23 clones used for the analysis (Table S2 in Supplementary Material), only a few were fully translatable, which corresponds to other findings of a multitude of non-productive TCR γ chain rearrangements, which can be expressed by cells that later commit to the αβ lineage (49, 50). The transcripts were compared to the armadillo wgs database and genomic location and accession numbers of contigs indicated the existence of nine different TRGV regions in our clones (Figure 4A). Those regions were found to be homologous to human TRGV1 cluster (TRGV1-8) with amino acid identities ranging from 46% up to 54%. Higher similarities were found with Bos taurus TRGV (43–64%). One particular armadillo V segment could not be assigned to a human TRGV; however, it shares 56% identity with the mouse TRGV6. These V genes were rearranged with three different J regions (TRJ-A, TRJ-B, and TRJ-C) (Figure 4B) sharing amino acid homologies of 63–80% with human TRJ segments. Query cover with human homologs varied from 52 to 84%, which made a definite assignment difficult and lowers amino acid identities. Concerning the translatable clones resulting from the 5′RACE PCR (Figure 4C), it is interesting that TRJ-A and TRJ-B from D. novemcinctus seem to associate with other TRGV than TRJ-C. Additionally, the TRGC usage of TRJ-C is restricted to TRGC-A, the other J segments use either TRGC-B or TRGC-C, which could not be distinguished in this 5′RACE PCR. This apparent bias in C region usage is reminiscent of a cassette structure of the armadillo TRD locus comparable to artiodactyls or the bottlenose dolphin (IMGT-Locus representations) (26). Due to the lack of any evidence for a functional TRGV9 rearrangement in armadillo PBMCs, together with the fact that we found other TRGV in a functional rearrangement with TRJ-B and TRJ-C in the armadillo, we propose the lack of expression of Vy9V82 TCRs in this species.

**DISCUSSION**

In this study, we report for the first time, an analysis of the expression of the essential components of the BTN3/Vy9V82 TCR system in the nine-banded armadillo (D. novemcinctus) and a comparison with homologous genes of other mammalian species. Studies of the distribution of the TRGV9, TRDV2, and BTN3 genes identified this animal as a candidate for a functional Vy9V82 T cell population with a corresponding BTN3 molecule, which is essential for PAg recognition. However, we observed that aside from expression of in silico translatable TRDV2 chains, the armadillo does most likely not express a functional TRGV9 rearrangement. Surface expression of armadillo V82 and human Vy9 chains was achieved and signaling after CD3 stimulation was observed. This is an interesting finding, as apparently structural features, which allow pairing of armadillo V62 with
Vγ9 are conserved, although no evidence for TRGV9 expression was found. However, pairing of V82 chains is not restricted to Vγ9 TCR chains in humans (51) even though there are certain pairings of γ6 chains in mice that fail to be expressed (52). PAg-reactivity of human/armadillo heterodimeric γδ TCRs could not be shown. This was not surprising given that previous alanine-scanning mutagenesis showed contribution of all six CDR3 to PAg-reactivity (46). Nevertheless, armadillo V82 chains might become a valuable model for future mutagenesis and structural studies, e.g., by transplanting human CDR into the armadillo V62 chain. Moreover, the fact that in a species, which lacks bona fide PAg-reactive Vγ9V62 TCR a third of the clones expresses the amino acids isoleucine or valine at position 97 suggest that the common use of these amino acids might not be taken as an indicator for a certain PAg-reactivity but may be largely random or a result of selection by structural requirements or other ligands (23, 48).

In addition to the lack of evidence for TRGV9 rearrangements, no full-length BTN3 transcript seems to be expressed in the armadillo. Based on genomic data, we report evidence for the existence of a multigene family of BTN3-like genes in the armadillo. Assessment of numbers of genes and their structural analysis is not possible to this date due to lack of genomic data and transcripts. We identified one locus that closely resembles the human BTN3A3 locus and another one carrying deletions of transmembrane domains and the B30.2 domain, which could be more like a BTN3A2 gene. However, the lack of signal sequences and multiple deletions and frameshifts as well as the overall lack of transcripts of a BTN3-like molecule speaks against functional BTN3 molecules in armadillo. The lack of leader sequences for all identified BTN3-V segments might indicate that loss of function preceded the duplication events. In contrast, in primates a duplication of the BTN3 loci occurred (20) and led to new BTN3 molecules such as BTN3A1. This isoform is not only essential for the mediation of PAg-dependent stimulation of Vγ9V62 T cells but also contributes to signaling to induce type I interferon transcription (15, 53). The fact that the non-functional armadillo B30.2 domain has preserved the codons for all six amino acids contacting the PAg in the proposed PAg binding sites and the existence of a translatable, although not expressed, TRGV9 homolog may indicate the loss of functional elements for PAg sensing by γ6 T cells in the armadillo ancestor. With the armadillo as an animal model for *M. leprae* in mind (27), one could speculate that a non-functional Vγ9V62 T cell subset leads to higher susceptibility for this pathogen. In armadillos, however, low core body temperatures of 33–35°C could be seen as a factor that favors *M. leprae* proliferation in vivo (54, 55). Furthermore, other species like rodents, which have lost the BTN3/Vγ9V62 system do not exhibit higher susceptibility to leprosy manifestations (29, 56). Regarding our observations of lacking transcripts of the BTN3/Vγ9V62 system, we can only state that the armadillo cannot be used as a model for this T cell subset.

So far, there are two other non-primate species that can be considered prime candidates for possessing PAg-sensing Vγ9V62 T cells. First, the alpaca (*V. pacos*), which not only expresses transcripts but also possesses a Vγ9V62-like cell population that expands upon HMBPP stimulation (25). This species shows not only functional rearrangements of TRGV9 and TRDV2 but additionally a single BTN3 molecule (23, 24). Interestingly, this more primordial BTN3 possesses the predicted PAg-binding sites of both BTN3-V and B30.2 domain of the human BTN3A1 within a protein more closely related to human BTN3A3. The second species, the bottlenose dolphin shows functional TRDV2 rearrangements as well as TRG rearrangements homolog to human TRGV9/TRGJP containing TCR-chains and a single BTN3-like gene. With these candidates in mind, it seems even more likely that *D. novemcinctus* cannot be considered a model organism for PAg-reactive Vγ9V62 T cells, but stands as a witness for the emergence of this system with placental mammals.

**ETHICS STATEMENT**

Armadillos were maintained and samples collected in accordance with all ethical guidelines of the U.S. Public Health Service under protocols approved by the IACUC of the National Hansen's Disease Program, assurance number A3032-1.

**AUTHOR CONTRIBUTIONS**

AF planned, performed, and analyzed experiments, and wrote the manuscript. MK reviewed the manuscript and provided the sequence for *Vicugna pacos* BTN3. LS performed experiments. RT provided samples and reviewed the manuscript. TH conceived the study, planned and analyzed experiments, and wrote the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.00265/full#supplementary-material.
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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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