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Butyrophilin 3A1 Plays an Essential Role in Prenyl Pyrophosphate Stimulation of Human Vγ2Vδ2 T Cells

Hong Wang,* Olivier Henry,† Mark D. Distefano,‡ Yen-Chih Wang,† Johanna Räikkönen,‡ Jukka Mönkkönen,‡ Yoshimasa Tanaka,§ and Craig T. Morita*§

Most human γδ T cells express Vγ2Vδ2 TCRs and play important roles in microbial and tumor immunity. Vγ2Vδ2 T cells are stimulated by self- and foreign prenyl pyrophosphate intermediates in isoprenoid synthesis. However, little is known about the molecular basis for this stimulation. We find that a mAb specific for butyrophilin 3 (BTN3A1) superfamily proteins mimics prenyl pyrophosphates. The 20.1 mAb stimulated Vγ2Vδ2 T cell clones regardless of their functional phenotype or developmental origin and selectively expanded blood Vγ2Vδ2 T cells. The γδ TCR mediates 20.1 mAb stimulation because IL-2 is released by βγ Jurkat cells transfected with Vγ2Vδ2 TCRs. 20.1 stimulation was not due to isopentenyl pyrophosphate (IPP) accumulation because 20.1 treatment of APC did not increase IPP levels. In addition, stimulation was not inhibited by statin treatment, which blocks IPP production. Importantly, small interfering RNA knockdown of BTN3A1 abolished stimulation by IPP that could be restored by re-expression of BTN3A1 but not by BTN3A2 or BTN3A3. Rhesus monkey and baboon APC presented HMBPP and 20.1 to human Vγ2Vδ2 T cells despite amino acid differences in BTN3A1 that localize to its outer surface. This suggests that the conserved inner and/or top surfaces of BTN3A1 interact with its counterreceptor. Although no binding site exists on the BTN3A1 extracellular domains, a model of the intracellular B30.2 domain predicts a basic pocket on its binding surface. However, BTN3A1 did not preferentially bind a photoaffinity prenyl pyrophosphate. Thus, BTN3A1 is required for stimulation by prenyl pyrophosphates but does not bind the intermediates with high affinity.

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Abbreviations used in this article: AppP, triphosphoronic acid 1-adenosin-5′-y1 ester 3-(3-methylbut-2-enyl) ester; BPHL, bisphosphonate; BRHPP, bromohydrydin pyrophosphate (3-bromo-3-hydroxybutyl pyrophosphate); BTN, butyrophilin; HMBPP, (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate; IDL, isopentenyl diphosphate isomerase; IPP, isopentenyl pyrophosphate; LC/MS, liquid chromatography/mass spectrometry; βM, β2-microglobulin; MFI, mean fluorescence intensity; PHA, phytohemagglutinin; RMSD, root mean square deviation; sRNA, small interfering RNA; TRIM, tripartite motif.

MHC complex is generally very low such that expansion of reactive cells is required to mount effective T cell immunity. In contrast, γδ TCRs recognize nonpeptide compounds or self-cell surface molecules. Humans and mice have limited numbers of Vγ and Vδ gene segments, and many γδ TCRs exhibit limited or no junctional diversity. Thus, although γδ T cells constitute a minor subpopulation among human and murine T cells, the actual precursor frequencies for a given Ag can be very high compared with the very low frequency of CD4 and CD8 T cells specific for particular peptide/MHC complexes.

The major subset of human γδ T cells express Vγ2Vδ2 (also termed Vγ9Vδ2) TCRs and play important roles in immunity to microbes and tumors (1). Vγ2Vδ2 T cells expand to very high levels during many bacterial and protozoan infections, secrete inflammatory cytokines, chemokines, and growth factors, and kill infected cells and tumor cells. Vγ2Vδ2 T cells perform these functions by using their Vγ2Vδ2 TCRs to sense foreign and self-prenyl pyrophosphates and by using their NK receptors to recognize ligands expressed by tumor and virally infected cells. Prenyl pyrophosphates (also termed prenyl diphosphates) are essential intermediates in isoprenoid biosynthesis required by both microbes and humans. We and others (2, 3) have identified one bacterial Ag as (E)-4-hydroxy-3-methylbut-2-enyl pyrophosphate (HMBPP), an intermediate in the methyl-erythritol-4-phosphate isoprenoid pathway used by some bacteria and all apicomplexan parasites. Two other classes of compounds, aminobisphosphonates (4–8) and alkylamines (8–10), indirectly stimulate γδ T cell recognition of peptides or lipids, cell–cell contact is required for prenyl pyrophosphate stimulation (11, 12). However, Ag processing is not required (11) likely explaining why presentation can be by nonprofessional
Biological processes involving prenyl pyrophosphate (PPP) have been widely studied in various contexts. However, the participation of a surface protein distinct from known Ag-presenting molecules for the PPP activation of T cells is still under investigation. Here, we report that BTN3A1, a member of the butyrophilin-like (BTNL) proteins, is involved in the PPP activation of T cells.

Materials and Methods

To assess dimer formation by recombinant BTN3A1, the extracellular domains were expressed as described previously (34). Moreover, small interfering RNA (siRNA) inhibition of BTN3A1 was performed as described previously (14). Assays were combined and had a molecular mass of 25 kDa on SDS-PAGE under reducing conditions.

In vitro expansion of blood VγVδ2 T cells

For in vitro expansion of blood VγVδ2 T cells, PBMCs were prepared from the blood or leukopaks of normal donors by Ficoll–Hypaque density centrifugation. PBMCs (1 × 10^6) in 0.2 ml media in 96-well round bottom wells were pulsed with the compounds for 2–6 h, washed twice, or cultured continuously with the compounds. IL-2 was added to 1 nM on day 3. The cells were harvested on day 9, stained with FITC-anti-CD3 (HIT3a) or PE-conjugated goat–anti-mouse IgG (H+L) Abs, and analyzed using flow cytometry. Blood was drawn from healthy adult donors who were enrolled with written informed consent in accordance with the requirements of the University of Iowa Institutional Review Board.

Stimulation of the DBS43 VγVδ2 TCR transfectant

Derivation of the DBS43 VγVδ2 TCR transfectant has been described previously (17). Stimulation of TCR transfectants for IL-2 release was performed as previously described in the presence of 1 × 10^7 glutaraldehyde-fixed Va2 cells and 10 ng/ml PMA (8, 17, 33). For IL-2 assays, the supernatants were thawed and used at a 1:8 dilution to stimulate the proliferation of the IL-2–dependent cell line HT-2.

Measurement of intracellular IPP levels

MCF-7 breast cancer cells were treated with zoledronate or the 20.1 mAb for 16 h, harvested, washed twice with PBS, counted, and spun down. Cell extracts were prepared as described previously (34). Levels of IPP were determined by separation of metabolites on high-performance liquid chromatography using a Gemini C18 column (Phenomenex, Torrance, CA) with N,N-dimethylethylamine formate as the ion pairing agent and analysis by negative ion electrospray ionization mass spectrometry as described previously (34).

Photoaffinity Ag labeling of BTN3 proteins and Western blotting

To assess dimer formation by recombinant BTN3A1, the extracellular domain of human BTN3A1, residues A29 to R247, was expressed in Escherichia coli as inclusion bodies, solubilized in 6 M guanidine, and refolded in 0.1 M Tris-HCl buffer (pH 8), containing 1 M arginine, 0.25 mM reduced glutathione, and 0.25 mM oxidized glutathione. The refolded protein was concentrated using DE52 anion–exchange resin, isolated by Q Sepharose HP anion–exchange column chromatography, followed by size separation by Superdex 200 gel filtration. Molecular mass standards used were bovine thyroglobulin 670 kDa, bovine gammaglobulin 158 kDa, chicken OVA 44 kDa, and vitamin B12 3.15 kDa. The major peak fractions were combined and had a molecular mass of 25 kDa on SDS-PAGE under reducing conditions, whereas the calculated molecular mass is 23.5 kDa.

Purified full-length recombinant BTN3A1 and BTN3A2 proteins were purchased from OriGene and dialyzed against PBS with 0.05% Tween 20 and 0.5% sucrose in 50 μl buffer added per round-bottom well of a 96-well plate. OVA (Sigma-Aldrich, St. Louis, MO) was used as a control protein. Recombinant protein molecular weights and protein concentrations were confirmed by Coomassie blue staining of SDS-PAGE–separated proteins.

**Materials and Methods**

**Abs and flow cytometry**

mAbs used included anti-Class-III (anti-T8R31 [5A6E9]), anti-Vγ1 (23D12), anti-Vγ2 (7A5), anti-Vδ1 (6TCS1) (all from Pierce Abs, Thermo Scientific, Rockford, IL), anti-Vδ2 (BB3), anti-Vδ3 (P11.5B), anti-BTN3 (1A6) (OriGene, Rockville, MD), FITC–anti-CD3 (HT3a) (eBiocience, San Diego, CA), PE–anti-Vδ2 (B6) (BD Biosciences, San Jose, CA), unconjugated or PE-conjugated mouse IgG1 isotype control mAb (P3) (eBiocience), and unconjugated or PE-conjugated anti-BTN3/CD277 (20.1, 12G12, and the weakly cytotoxic CD4+ γδ T cell clones, HF2, JN.23, and JN.24, have been described previously (30–32). T cell proliferation assays were performed as described previously (14). Assays were done in duplicate or triplicate in round-bottom 96-well plates with 1 × 10^5 T cells/well in the presence of non-fixed (mitomycin C treated) or glutaraldehyde-fixed APC at 1 × 10^5 cells/well for mAb, Ag, and PHA stimulation. The cultures were pulsed with 1 μCi [3H]thymidine (2 Ci/mmol) on day 1 and harvested 16–18 h later. Mean proliferation and SEM of duplicate or triplicate cultures are shown. Staining compounds and inhibitors were used as indicated in the figure legends. The 20.1 and 1A6 mAbs were diazoylated prior to use in functional assays. The P3 IgG1 mAb was used as an isotype control mAb. For cytokine release, culture supernatants were harvested after 16 h and assayed for TNF-α or IFN-γ levels by DuoSet sandwich ELISA (R&D Systems, Minneapolis, MN). The primate B cell lines GAB-LCL (derived from baboons [Papio anubis] B lymphoblastoid cell line) and V038 AGM BLCL (derived from Sabaean monkeys [Chlorocebus sabaeus] B lymphoblastoid cell line) were obtained from the National Institutes of Health Nonhuman Primate Reagent Resource.

**In vitro expansion of VγVδ2 T cells**

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To assess binding of the photoaffinity Ags, the biotin-\(p\)-benzophenone-C-
HMBPP ester was added to wells containing BTN3A1, BTN3A2, or OVA at final concentrations of 0, 0.2, or 10 \(\mu\)M. The plate was then placed on ice and exposed to a 350 nm UV light. After 30 min, 30 \(\mu\)L of 2\% Laemmli buffer (Bio-Rad Laboratories, Hercules, CA) containing 5\% 2-ME was added to each well and mixed, and the samples were moved to microtubes. Samples were then boiled for 5 min, placed on ice for 5 min, and briefly spun, and 20 \(\mu\)L was loaded to a well of a Ready Gel Tris-HCl gel (10–20\% linear gradient, Bio-Rad Laboratories) for separation by SDS-PAGE. Proteins were then transferred overnight at 30 V, 90 mA to an Immun-Blot poly-
vinyldene difluoride membrane (Bio-Rad Laboratories). The polyvinyl-
dene difluoride membrane containing the transferred proteins was washed twice with TBS with 0.05\% Tween 20 (TBS-T) and then blocked with 1\% BSA in TBS-T for 2 h. The membrane was then washed with TBS-T three times and probed with 1\%0,000 diluted streptavidin-HRP (GE Healthcare, Pittsburgh, PA) for 1 h. After washing with TBS-T five times, the mem-
brane was developed using the Visualizer Western blot Detection kit (Millipore, Temecula, CA) with Kodak BioMax Light film.

### Transfection of siRNAs and cDNAs

Silencer-27 siRNAs for BTN3 and control duplexes were purchased from OriGene and reconstituted in the provided duplex buffer to obtain a 20 \(\mu\)M stock solution. The stock solution was further diluted to 5 \(\mu\)M with the same buffer for transfection and cotransfection with cDNA. Expression plasmid containing BTN3A1 and BTN3A2 cDNA (True ORF Gold) were purchased from Origene. An expression plasmid containing BTN3A3 cDNA (OmicLink) was purchased from GeneCopoeia (Rockville, MD). The vector pcMV6-XL5 was purchased from Origene. For trans-
fections, HeLa cells were plated at 17 \times 10^3 cells/well in a 24-well plate 1 d prior to transfection. To transfet 1 well of HeLa cells with siRNA, 4 \(\mu\)L of either a control siRNA or a BTN3 siRNA was added to 100 \(\mu\)L Opti-
MEM I medium and then briefly mixed by vortexing. To transfect cDNA, 1 \(\mu\)g of either the control plasmid or a BTN3-expressing plasmid was added to 100 \(\mu\)L Opti-
MEM I medium and then briefly mixed by vortexing. For cotransfection of siRNA and cDNA, 1 \(\mu\)g cDNA was added to 100 \(\mu\)L Opti-MEM I medium, followed by 4 \(\mu\)L siRNA. After mixing, 4.5 \(\mu\)L Attractene transfection reagent (QiaGen, Valencia, CA) was added to the siRNA and cDNA pre-
parations. They were then vortexed for 5 s, incubated at room tem-
perature for 15 min, and added dropwise to a well of HeLa cells. After 72 h, the transfecants were trypsinized and harvested for flow cytometric
analyses or use in proliferation assays.

### BTN3 sequence alignments and comparisons

BTN3A1 sequences from human (Homo sapiens), bonobo (Pan paniscus), chimpanzee (Pan troglodytes), gorilla (Gorilla gorilla), Sumatran orang-
 utan (Pongo abelii), olive baboon (Papio anubis), and thessen monkey (Macaca mulatta) were analyzed. Most BTN3A2 sequences were obtained from the
PubMed DNA database or from the University of California Santa Cruz
Genome Bioinformatics Web site. Because the full-length baboon BTN3A1
sequence was not available, the BTN3A1 sequence was determined by using a combination of expressed sequence tag sequences from the “Ba-
boon Seeksequence” site (http://www.baboon.washington.edu/) and genome
aboon sequences from the Human Genome Sequencing Center, Baylor
College of Medicine (Houston, TX) (http://www.hgsc.bcm.tmc.edu/ content/baboon-genome-project). The BTN3A1 sequence was determined
using contig 6004 for exon 1, contig 705213 for exon 2, and contig
507608 for exons 4, 5, 6, 7, 8, 9, and 10. For exon 3 (residues 29–144),
residues 29–65 were from contig 58218, and residues 64–144 were from
the expressed sequence tag clone 5974.1, all from \(P.\) anubis. The DNA
sequence of exons 3 and 4 of \(P.\) anubis is identical to \(P.\) papio hamadryas
(trace 1910189173 from the National Center for Biotechnology Information
Trace Archive). Sequences were aligned using the Clustal W method in the
MegAlign program (Lasergene, DNAStar). Phylogenetic trees and se-
quence differences were determined using the MegAlign program.

### Human BTN3A1 and other structural models

BTN3 extracellular domain structures used in this study include BTN3A1
(1F8Q), BTN3A2 (1FQG), BTN3A3 (1FS7), and BTN3A1 complexed with
the 20.1 mAb (1FPL) (26). IgVlC and IgClC dimer structures of
BTN3A1, BTN3A2, and BTN3A3 were provided by Dr. E. Adams. Modeling of other butyrophilin IgV domains and B30.2 domains was done at the Swiss-Model Web site (http://swissmodel.expasy.org/) using stan-
dard settings. The BTN3A1 BTN3B2 model was based on the structure of
pyrin/tripartite motif (TRIM) 20 (2W1l) and was similar to other B30.2
domains differing in backbone Ca from TRIM21 by 1.35 Å root mean
square deviation (RMSD), from pyrin/TRIM20 by 0.68 Å RMSD, and
from TRIM72 by 1.65 Å RMSD. All figures were made using PyMOL
X1 Hybrid (Schrödinger LLC). The B30.2 domains are identically scaled
using a PyMOL script provided by Dr. W.L. DeLano. Electrostatic surface
potential was calculated with the APBS PyMOL plugin (35) with, in some
cases, externally generated PQR files from the PDB2PQR Web site (http://
nbr222.ucsd.edu/pdb2pqr_1.8/) (36) and are colored from red (negative
potential, ~10 kT) to blue (positive potential, +10 kT).

### Results

#### Anti-BTN3 mAb 20.1 stimulates Vy2V62 T cells to proliferate and secrete IFN-\(\gamma\) and TNF-\(\alpha\)

The anti-BTN3 mAb 20.1 stimulated Vy2V62 T cells to prolifer-
ate, whereas a second BTN3 specific mAb 1A6 and a control
mAb had no effect (Fig. 1A). The epitopes of the 20.1 and 1A6
Abs differ because binding of 1A6 does not block 20.1 binding
(data not shown). Neither mAb inhibited the 12G12 response to
HMBPP (data not shown). The 20.1 mAb also stimulated IFN-\(\gamma\)
and TNF-\(\alpha\) release in a dose-dependent manner (Fig. 1B). Cyto-

cine responses to the 20.1 mAb were less than those seen with
HMBPP but more than noted with stimulation with the aminobi-
osphosphonate, zoledronate (Fig. 1B). The 20.1 mAb could be
“pulsed” onto APC more efficiently than HMBPP with the 20.1
mAb dose-response curve shifting ~3- to 5-fold higher compared
with the HMBPP dose-response curve that shifted 54-fold higher
(Fig. 1C). Similar to responses to prenyl pyrophosphates (11, 16),
Vy2V62 T cell clones varied in their requirement for APC to enable
them to respond to the 20.1 mAb, presumably because of
dughter–daughter presentation. The 12G12 Vy2V62 clone re-
quired APC for responsiveness to the 20.1 mAb whereas the
HF2 clone did not (Fig. 1D). Thus, stimulation of Vy2V62 T
cells by the 20.1 mAb results in proliferation and cytokine
production, the mAb can be “pulsed” on APC, and, like prenyl
pyrophosphate stimulation, 20.1 mAb stimulation does not al-
ways require APC.

#### Stimulation of T cells by the 20.1 mAb is restricted to Vy2V62 TCR-expressing cells regardless of functional phenotype or developmental origin and mediated by the Vy2V62 TCR

To assess the specificity of stimulation by the 20.1 mAb, we
stimulated human PBMC with either the 20.1 mAb or HMBPP for
9 d and assessed Vy2V62 T cells by flow cytometry. The 20.1
mAb stimulated expansion of Vy2V62 T cells in all donors (mean
Vy2V62 T cells = 32.7 ± 14.9\%), albeit at slightly lower levels
than HMBPP (mean Vy2V62 T cells = 50.0 ± 18.9\%) (Fig. 2A).
Thus, the 20.1 mAb expanded only Vy2V62 T cells from the
variety of \(\alpha\) and \(\gamma\) T cells present in PBMC.

BTN3 family members are Ig superfamily proteins that are
similar to ligands for costimulatory and inhibitory receptors. If
BTN3 molecules were playing similar roles in \(\gamma\) T cell stimu-
lation, Vy2V62 T cell clones with different functional capabilities
and surface phenotypes or those derived from different stages in
development or different anatomic locations might have different
requirements for either costimulation or inhibition and, thus,
might have different responses to the 20.1 mAb. We compared
cytotoxic Vy2V62 T cell clones that were CD8\(\alpha\)ex positive or CD4
and CD8 negative (Fig. 2B, 12G12 and HD.108, solid bars) with
noncytolytic clones expressing CD4 (Fig. 2B, HF.2, JN.23, JN.24,
open bars) (30) or with clones from cord blood (Fig. 2B, CB.32.26,
solid bar) (29) or fetal liver (Fig. 2B, AC.8, open bar) (30). De-
spite these differences, all clones expressing Vy2V62 TCRs pro-
liferated when cultured with the 20.1 mAb (Fig. 2B), suggesting
that BTN3 does not stimulate Vy2V62 T cells through mechanism
involving alterations in costimulation or inhibition.
To further assess the specificity of 20.1 mAb stimulation, human γδ and αβ T cell clones expressing a variety of V gene pairs (29, 30, 32, 37) were cultured with the 20.1 mAb. Only γδ T cells expressing Vγ2Vδ2 TCRs were stimulated (Fig. 2B). Clones expressing Vγ2 paired with Vδ1 did not respond, including one clone, JR.2.28 (37), that uses the Jγ1.2 gene segment normally used by Vγ2Vδ2 TCRs. Clones expressing Vγ1Vδ6 TCR also did not respond nor did clones expressing Vγ1Vδ1 or αβ TCRs. Note that γδ clones showed identical 20.1 mAb staining whether they expressed Vγ2Vδ2 TCRs or not, making it unlikely that the 20.1 mAb cross-reacts with the Vγ2Vδ2 TCR (data not shown).

To determine whether the Vγ2Vδ2 TCR mediates 20.1 mAb stimulation, we incubated a Vγ2Vδ2 TCR Jurkat transfectant with the 20.1 mAb in the presence of Va2 APC. The 20.1 mAb stimulated IL-2 release by the DBS43 Vγ2Vδ2 TCR transfectant in a dose-dependent fashion (Fig. 2C, right panel) although at somewhat lower levels than HMBPP (Fig. 2C, left panel). Thus, transfer of the Vγ2Vδ2 TCR to β− Jurkat thymoma cells (from the αβ T cell lineage) conferred the ability to respond to the 20.1 mAb.

Stimulation by the 20.1 mAb is not due to the accumulation of IPP in APC

Aminobisphosphonates and alkylamines indirectly stimulate Vγ2Vδ2 T cells by blocking farnesyl diphosphate synthase causing IPP and its ATP derivative (ApppI) to accumulate and stimulate Vγ2Vδ2 T cells (5, 7, 10). Because this process is dependent on the flow of metabolites down the mevalonate pathway, inhibiting the upstream 3-hydroxy-3-methylglutaryl-CoA reductase enzyme with a statin blocks stimulation by these compounds even at low statin concentrations (8, 16, 38). To determine whether the 20.1 mAb uses a similar indirect mechanism, we determined the sensitivity of 20.1 mAb stimulation to statin treatment (Fig. 3A). The 20.1 mAb response required high mevastatin concentrations for inhibition (IC50 = 45 μM) that were identical to those needed to block the HMBPP response (Fig. 3A). In contrast, the response to the aminobisphosphonate risedronate was highly sensitive to statin inhibition with complete inhibition even at the lowest mevastatin concentration (IC50 = <0.1 μM) (Fig. 3A). Similar findings were noted with the less potent statin pravastatin. Confirming the statin inhibition results, IPP and ApppI levels remained below detectable levels after 20.1 treatment of MCF-7 cells (< 1 pmol/1 × 106 cells) (Fig. 3B), whereas zoledronate treatment increased IPP to 912 pmol/1 × 106 cells and ApppI to 44 pmol/1 × 106 cells. Thus, the 20.1 mAb does not stimulate Vγ2Vδ2 T cells through IPP or ApppI accumulation.

siRNA inhibition of BTN3A1 abolishes stimulation of Vγ2Vδ2 T cells by IPP

The BTN3 family is composed of three members, BTN3A1, BTN3A2, and BTN3A3, that have highly similar IgV domains (99%) but less conserved IgC domains (90–91%) and different intracellular tails (Supplemental Fig. 1). The 20.1 mAb binds all three members (26). To assess the role of the different BTN3 family members in prenyl pyrophosphate stimulation, siRNAs specific for each of the BTN3 proteins were used to decrease their expression. The HeLa cell line was used for these experiments because its low BTN3 expression allowed near total inhibition of BTN3 expression. Cells were transfected with siRNA and then
tested for BTN3 surface expression and for their ability to function as APCs for IPP. After siRNA treatment, HeLa cells downregulated their low expression of BTN3 proteins to near background levels (Fig. 4A). HeLa cells treated with siRNAs specific for BTN3A1 lost their ability to efficiently support IPP responses (Fig. 4B, upper center panel). In contrast, HeLa cells treated with siRNA specific for either BTN3A2 or BTN3A3 still presented IPP (Fig. 4B, middle and lower center panels). Each of the siRNAs partially inhibited the 20.1 mAb response (Fig. 4B, left panels). None of the siRNAs inhibited responses to the PHA mitogen (Fig. 4B, right panels). These results suggest that all three family members can support 20.1 mAb responses, but only BTN3A1 supports IPP responses.

Re-expression of BTN3A1 but not BTN3A2 or BTN3A3 in siRNA-treated HeLa cells restores presentation of IPP to VγVδ2 T cells

To ensure that the loss of BTN3A1 on APC was responsible for their inability to present IPP rather than off-target effects or toxicity from the siRNA treatment, we restored BTN3A1 expression in HeLa cells after siRNA knockdown and assessed the ability of the cells to present IPP and to support 20.1 mAb responses. Consistent with our earlier experiments, BTN3A1 siRNA treatment decreased 20.1 mAb staining (Fig. 5A) and greatly decreased IPP presentation (Fig. 5B, middle lower panel, open circles). siRNA for BTN3A1 also partially decreased 20.1 mAb responses (Fig. 5B, left lower panel). Cotransfection of the BTN3A1 siRNA with BTN3A1 cDNA in an expression plasmid restored BTN3 expression on HeLa to levels higher than normal (Fig. 5A). Importantly, expression of BTN3A1 in siRNA-treated cells restored their ability to present IPP such that they were even more efficient than untreated cells (compare closed circles with open squares in Fig. 5B, middle lower panel). Similarly, expression of BTN3A1 in siRNA-treated cells restored their ability to support 20.1 mAb responses (Fig. 5B, left lower panel). All treated cells were able to support control PHA mitogen responses (Fig. 5B, right panels).

Thus, the effect of siRNA to BTN3A1 is due to the downregulation of the BTN3A1 protein.

To further assess the specificity of presentation of prenyl pyrophosphates by BTN3A1, we compared the ability of the different BTN3 family members to restore presentation by HeLa cells treated with siRNA. Despite restoring BTN3 expression in siRNA-treated cells to levels higher than those found on the untreated cells (Fig. 5C), HeLa cells expressing either BTN3A2 or BTN3A3 were unable to present IPP (Fig. 5D, middle and right panels), whereas HeLa cells expressing BTN3A1 presented IPP similar to cells treated with a control siRNA (Fig. 5D, left panel). Thus, despite strong similarities in the extracellular domains among BTN3A1, BTN3A2, and BTN3A3 (Supplemental Fig. 1), only BTN3A1 is required for the presentation of prenyl pyrophosphates.

BTN3A1 does not bind prenyl pyrophosphates with high affinity

The requirement for BTN3A1 surface expression for the presentation of prenyl pyrophosphates suggests that it could function as a presenting molecule similar to MHC class I and II or CD1 molecules. Proteins binding prenyl pyrophosphates and other phosphorylated compounds commonly use two mechanisms to accommodate their negative charges. In some cases, positively charged binding pockets are formed by the presence of lysine and arginine basic amino acids with additional polar residues (39). These can form ionic and hydrogen bonds to the pyrophosphate moiety. A second mechanism used by many enzymes in isoprenoid synthesis is the presence of precisely positioned acidic residues (the DDXXD motif) that coordinate the binding of three positively charged divalent magnesium cations that then form ionic bonds to the negative charges on the pyrophosphate moiety (40, 41). Hydrophobic pockets accommodate the isoprenoid acyl tails (41). To determine whether there were potential binding pockets in BTN3A1, the electrostatic surface potential of the molecule was calculated. Only a shallow, mildly basic area was noted on the extracellular portion of BTN3A1 (Fig. 6A), whereas
Baboon and rhesus monkey APCs present prenyl pyrophosphates to human \( \gamma \delta T \) cells

Responsiveness to prenyl pyrophosphates is conserved within the primate lineage. Both Old World and New World monkeys have \( \gamma \delta T \) cells that recognized IPP and other prenyl pyrophosphates (33, 42–44) and that can expand during bacterial infections (45, 46). However, xenogeneic presentation of prenyl pyrophosphates to human \( \gamma \delta T \) cells has not been studied extensively. Therefore, we tested APCs from different primate species for their ability to present prenyl pyrophosphates to human \( \gamma \delta T \) cell clones and for BTN3 cross-reactivity with the 20.1 mAb. Both baboon and rhesus monkey cells reacted with the 20.1 mAb (Fig. 7A, 7B). Baboon B cell APC stimulated the CD8\(^{+} \) 12G12 \( \gamma \delta T \) cell clone after culture with HMBPP or risedronate (Fig. 7C). Unexpectedly, despite apparent mAb reactivity, baboon B cells support very minimal 20.1 mAb responses (Fig. 7C), although baboon and other primate B cells did support PHA responses by 12G12 \( \gamma \delta T \) cells (Fig. 7C). The CD4\(^{+} \) HF:2 \( \gamma \delta T \) cell clone gave similar results (Fig. 7D, 7E). In this case, baboon B cells augmented the self-presentation of HMBPP to HF:2 with identical dose response curves to human B cells (EC\(_{50}\) of 0.20 nM for baboon versus 0.13 nM for human APC). However, baboon B cells supported only weak 20.1 mAb responses (Fig. 7C). Rhesus monkey PBMC also presented HMBPP as efficiently as human PBMC (EC\(_{50}\) of 1.3 nM for rhesus versus 1.0 nM for human APC), but again, 20.1 mAb stimulation was inefficient requiring 20-fold higher concentrations to stimulate half maximal responses (EC\(_{50}\) of 0.60 \( \mu \)g/ml for rhesus PBMC versus 0.03 \( \mu \)g/ml for human PBMC). Thus, the ability to present prenyl pyrophosphates to human \( \gamma \delta T \) cells is highly conserved between humans and baboons/rhesus monkeys but less conserved for stimulation by the 20.1 mAb.

The BTN3A1 protein is highly conserved across evolutionary time in the primate lineage (Fig. 8A, Supplemental Fig. 3A, 3B) but not present in other mammalian species. Human BTN3A1 has 92.4% sequence identity with baboon BTN3A1 and 91.6% identity with rhesus monkey BTN3A1 (Supplemental Fig. 3B). The IgV domains are the most highly conserved regions (93.9% for baboon and 94.8% for rhesus), followed by the B30.2 (94.3% for baboon and 94.3% for rhesus) and IgC domains (92.8% for baboon and 89.2% for rhesus) (Supplemental Fig. 3A, 3B). Regions of dimer contacts (yellow and green shaded sequences) are also highly conserved (Fig. 8A). Similarly, the 20.1 mAb binding site is either completely conserved (five primates) or has a single proline to alanine difference at residue 92 in baboons and rhesus monkey (Fig. 8A). Signal peptide sequences and the intracellular regions before the B30.2 domains are the least conserved.

Given that baboon and rhesus monkey APC can present prenyl pyrophosphates to human \( \gamma \delta T \) cells, the sequence differences between human and monkey BTN3A1 do not disrupt presentation. Baboon and rhesus monkey BTN3A1 are highly homologous (96.3% sequence identity) with only three amino acid differences in their extracellular domains (Fig. 8A). When the amino acid differences between humans and monkeys are mapped onto the structure of the extracellular domains of BTN3A1, the differences are concentrated on the outer face of the molecule opposite from the IgC:IgC dimer contact region leaving a completely conserved top and inner face that partially wraps around the middle of the molecule (boxed regions in Fig. 8B and shown in more detail in Supplemental Fig. 4A, 4B). When differences between human and baboon or human and gorilla B30.2 domains are located on a model of the BTN3A1 B30.2 domain, they clustered in regions away from the binding face (where the basic pocket is...
FIGURE 4. siRNA inhibition of BTN3A1 in APC abolishes IPP stimulation of Vγ2Vδ2 T cells. (A) siRNA inhibition of BTN3A1, BTN3A2, and BTN3A3 expression. HeLa cells were transfected with either control siRNA or with siRNA specific for each of the members of the BTN3 family. After 72 h, transfected HeLa cells were stained with the 20.1 mAb and BTN3 surface expression determined by flow cytometry. (B) siRNA inhibition of BTN3A1 greatly reduces IPP stimulation of Vγ2Vδ2 T cells. siRNA-transfected HeLa cells were used as APC for 20.1 mAb, IPP, and PHA stimulation of the 12G12 Vγ2Vδ2 T cell clone. Note that for each of the siRNA shown, there was at least one additional siRNA with a similar effect (i.e., oligo A for BTN3A1, oligo C for BTN3A2, and oligo A and C for BTN3A3).

Discussion
Stimulation by prenyl pyrophosphates is a unique feature of human Vγ2Vδ2 T cells that allows them to detect microbes producing isoprenoid metabolites such as HMBPP as well as human cells with alterations in their isoprenoid metabolism resulting in elevated levels of IPP. Although stimulation is mediated by the Vγ2Vδ2 TCR (17) and dependent on residues in all CDRs of the Vγ2 and Vδ2 regions (19, 23), the molecular basis for this stimulation has not been determined. In this paper, we have studied the role of the BTN3 family of Ig superfamily proteins in prenyl pyrophosphate stimulation. We find that BTN3A1 plays a major role in this stimulation. Binding of the 20.1 mAb to BTN3 proteins activates Vγ2Vδ2 T cells, regardless of their functional phenotype or developmental origin, in an identical manner to HMBPP, IPP, and other prenyl pyrophosphates. Importantly, siRNA inhibition of the expression of one family member, BTN3A1, abrogates stimulation of Vγ2Vδ2 T cells by IPP that can be restored by re-expression of BTN3A1 but not by BTN3A2 or BTN3A3. Moreover, baboon and rhesus monkey APC stimulate human Vγ2Vδ2 T cells in the presence of either HMBPP or the 20.1 mAb demonstrating the conservation of BTN3 function despite their divergence ~23 million years ago (47). All of the results point to a primary role for BTN3A1 in Vγ2Vδ2 TCR recognition of prenyl pyrophosphates.

Most previous studies on the function of BTN3 family members examined their effects on αβ T cells and APCs and did not distinguish between the different BTN3 family members. These studies pointed to BTN3 functioning as ligands binding to inhibitory receptors on αβ T cells (48, 49) or as stimulatory signaling molecules expressed by αβ T cells (50) and APC (51). Overexpression of BTN3 on tumor cells or the addition of anti-BTN3 mAbs moderately inhibits CD4 and CD8 αβ T cell responses to anti-CD3/anti-CD28 mAbs (48, 49). Similar effects are observed with other butyrophilin (52, 53) and butyrophilin-like molecules (54–56) and likely reflect BTN/BTN-L binding to an inhibitory receptor(s) expressed by T cells. BTN3 proteins also may function as signaling molecules. Thus, enhanced stimulation of CD4 αβ T cells by the addition of plate bound 20.1 anti-BTN3 mAbs to anti-CD3/anti-CD28 mAbs suggests that ligation of BTN3 provides a costimulatory signal to T cells, especially for IFN-γ secretion (50). Ligation of BTN3 on monocytes and immature dendritic cells provides a survival signal, upregulates CD80/CD86 (B7-1 and B7-2) and MHC class II, and increases secretion of IL-8, IL-1β, and IL-12/ p70 to LPS (51). Thus, markedly different functions are described for BTN3 depending on the cell type, suggesting that BTN3 functions as both a ligand for counterreceptor(s) on αβ T cells and as a signaling receptor on T cells and APCs. The effects of BTN3 on αβ T cells are in general agreement with the role of most B7-related Ig family members as they commonly function as ligands for costimulatory or inhibitory receptors expressed by T cells.

In contrast to the costimulatory/inhibitory role for αβ T cells, BTN3 plays a central role in the activation of Vγ2Vδ2 T cells. As reported in this study and in other recent studies (25, 26), stimulation by the 20.1 mAb precisely mimics stimulation of human Vγ2Vδ2 T cells by prenyl pyrophosphates. Unlike most Abs specific for CD28 or other costimulatory receptors, 20.1 mAb ligation of BTN3 stimulates full Vγ2Vδ2 T cell activation in vitro with proliferation and IFN-γ release. Moreover, although costimulation receptors vary between CD24 and CD8 αβ T cells, the 20.1 mAb stimulated both CD4+ and CD8αε+CD4−8− Vγ2Vδ2 T cells regardless of their functional phenotype or de-
Proliferative responses were assessed as in Fig. 1A. luted IPP, followed by addition of 12G12 T cells. transfectants were then cultured with half-log diluted, and treated for 1 h with mitomycin C. The cDNA. After 72 h, the transfectants were harvested, BTN3A1 siRNA oligo B and the indicated BTN3 were transfected with the indicated siRNA or cotransfected with cDNA. After 72 h, the transfectants were transfected with the indicated siRNA or cotransfected with the indicated siRNA and cDNA. After 72 h, the transfectants were washed, and treated for 1 h with mitomycin C. The transfectants were then cultured with half-log dilutions of the 20.1 mAb, IPP, or PHA, followed by addition of 12G12 T cells. Proliferative responses were assessed as in Fig. 1A. (C) Transfection of BTN3A1, BTN3A2, or BTN3A3 cDNA restores BTN3 expression on HeLa cells treated with BTN3 siRNA. HeLa cells were transfected with BTN3A1 siRNA oligo B or cotransfected with BTN3A1 siRNA oligo B and the indicated BTN3 cDNA as BTN3A1 but not BTN3A2 or BTN3A3 cDNA in described in (A). After 72 h, BTN3 expression was BTN3A1 cDNA in APC restores BTN3 siRNA-assessed by flow cytometry. (D) Transfection of BTN3A1 but not BTN3A2 or BTN3A3 cDNA in BTN3 siRNA-treated APC restores IPP stimulation of VγVδ2 T cells. HeLa cells were transfected with BTN3A1 siRNA oligo B or cotransfected with BTN3A1 siRNA oligo B and the indicated BTN3 cDNA. After 72 h, the transfectants were harvested, washed, and treated for 1 h with mitomycin C. The transfectants were then cultured with half-log diluted IPP, followed by addition of 12G12 T cells. Proliferative responses were assessed as in Fig. 1A.

FIGURE 5. Re-expression of BTN3A1 but not BTN3A2 or BTN3A3 in siRNA-treated APC restores IPP stimulation of VγVδ2 T cells. (A) Transfection of BTN3A1 cDNA restores siRNA-inhibited BTN3 expression on HeLa cells. HeLa cells were transfected with the indicated BTN3 siRNA or cotransfected with BTN3 siRNA and cDNA. After 72 h, the transfectants were stained with either PE-conjugated isotype control mAb or the 20.1 mAb and analyzed by flow cytometry. The relative mean fluorescence intensity (MFI) was calculated as 20.1 mAb MFI minus isotype control MFI. (B) Transfection of BTN3A1 cDNA in APC restores BTN3 siRNA-inhibited IPP stimulation of VγVδ2 T cells. HeLa cells were transfected with the indicated siRNA or cotransfected with the indicated siRNA and cDNA. After 72 h, the transfectants were transfected, washed, and treated for 1 h with mitomycin C. The transfectants were then cultured with half-log dilutions of the 20.1 mAb, IPP, or PHA, followed by addition of 12G12 T cells. Proliferative responses were assessed as in Fig. 1A. (C) Transfection of BTN3A1, BTN3A2, or BTN3A3 cDNA restores BTN3 expression on HeLa cells treated with BTN3 siRNA. HeLa cells were transfected with BTN3A1 siRNA oligo B or cotransfected with BTN3A1 siRNA oligo B and the indicated BTN3 cDNA as detailed in (A). After 72 h, BTN3 expression was assessed by flow cytometry. (D) Transfection of BTN3A1 but not BTN3A2 or BTN3A3 cDNA in BTN3 siRNA-treated APC restores IPP stimulation of VγVδ2 T cells. HeLa cells were transfected with BTN3A1 siRNA oligo B or cotransfected with BTN3A1 siRNA oligo B and the indicated BTN3 cDNA. After 72 h, the transfectants were harvested, washed, and treated for 1 h with mitomycin C. The transfectants were then cultured with half-log diluted IPP, followed by addition of 12G12 T cells. Proliferative responses were assessed as in Fig. 1A.

velopmental origin. Costimulatory receptors are generally shared between αβ T cells will similar functions or differentiation states. In contrast, we find that 20.1 mAb stimulates only VγVδ2 clones and not other γδ clones expressing other TCRs, even though they have similar functional capabilities and were derived in the same cloning. Finally, both human β− Jurkat (this paper and Ref. 25) as well as murine 58α− Jurkat cells (25) transfected with VγVδ2 TCRs responded to the 20.1 mAb, despite the αβ T cell origins of the parent cells line and despite the fact that mice do not express BTN3 orthologs, making it unlikely that they express BTN3-specific costimulatory receptors. Also, because murine cells do not express BTN3 and show no 20.1 reactivity on flow cytometric analysis, 20.1 mAb stimulation of a murine VγVδ2 model 4 (Fig. 9) are unlikely to explain BTN3A1 function in prenyl pyrophosphate stimulation.

If BTN3A1 does not stimulate VγVδ2 T cells by binding to costimulatory receptors or blocking inhibitory receptors or by signaling into APC, how might it function in prenyl pyrophosphate recognition? One possibility is that it could function like an MHC class I/II or CD1 presenting molecule. In this case, BTN3A1 would bind to prenyl pyrophosphates in its extracellular structure. Moreover, all three members serve to stimulate VγVδ2 T cells when bound to the 20.1 mAb and are highly homologous in their extracellular domains, yet only BTN3A1 is required for prenyl pyrophosphate stimulation.

In this respect, it would differ from the superagonistic anti-CD28 mAb TGN1412 (57), which does not show this specificity. Although the TGN1412 anti-CD28 mAb stimulates T cells to proliferate and produce cytokines, it activates most effector CD4 αβ T cells without V-region specificity (57, 58). Thus, model 3 and model 4 (Fig. 9) are unlikely to explain BTN3A1 function in prenyl pyrophosphate stimulation.

Although it is possible that the 20.1 mAb functions as a superagonist mAb, it would be required to be highly specific only for VγVδ2 T cells and to function in prenyl pyrophosphate stimulation but not in stimulation by mitogens or anti-CD3 mAbs. In
Although there is no evidence for direct prenyl pyrophosphate binding to BTN3A1, the ability of monkey APC to present prenyl pyrophosphates to human V\_γ\_2V\_δ\_2 T cells suggests that there may be conserved binding regions in the BTN3A1 extracellular domain. Consistent with this hypothesis, amino acid differences between monkey and human BTN3 family members all localize to the outer face of the BTN3A1 dimer (where the 20.1 binding site is located (26)), with the inner, outer middle, and top faces totally conserved. We speculate that these areas are potential binding regions for the human V\_γ\_2V\_δ\_2 TCR or for other proteins involved in prenyl pyrophosphate recognition. The ability of another anti-BTN3 Ab, 103.2, to specifically block prenyl pyrophosphate responses is consistent with direct binding to BTN3 for activation (25, 26).

Primate APC showed similar efficiency as human APC in HMBPP stimulation of V\_γ\_2V\_δ\_2 T cells but only supported weak 20.1 mAb responses (Fig. 7C–E), despite similar levels of BTN3 expression by 20.1 mAb staining (Fig. 7A, 7B). This result may reflect differences in the amino acid sequences of primate and human BTN3 family members. We speculate that these areas are potential binding regions for the human V\_γ\_2V\_δ\_2 TCR or for other proteins involved in prenyl pyrophosphate recognition. The ability of another anti-BTN3 Ab, 103.2, to specifically block prenyl pyrophosphate responses is consistent with direct binding to BTN3 for activation (25, 26).

If the extracellular domain of BTN3A1 does not bind prenyl pyrophosphates, how are these compounds sensed by V\_γ\_2V\_δ\_2 T cells? Harly et al. (25) demonstrated that the intracellular B30.2...
domain of BTN3A1 is required for prenyl pyrophosphate stimulation. Truncation of the BTN3A1 intracellular B30.2 domain abolishes its ability to mediate prenyl pyrophosphate stimulation, whereas swapping the BTN3A3 intracellular domain–BTN3A1 tail protein to restore prenyl pyrophosphate stimulation to cells lacking BTN3A1 (25). Thus, the B30.2 domain is required for BTN3A1 function and may be involved in the sensing of prenyl pyrophosphates.

B30.2 domains are present in most butyrophilin proteins and are homologs of the evolutionarily older PRY–SPRY domains (59). In humans, B30.2/PRY–SPRY domains have been identified in 97 proteins belonging to 15 protein families (60). When their binding partners have been identified, B30.2/PRY-SPRY domains are found to mediate protein interactions (60). Several proteins containing B30.2/PRY–SPRY domains are involved in innate immunity (27, 59, 61). For example, the 68 TRIM family proteins (62), which have E3 ubiquitin ligase activity, include TRIM21 (an intracellular protein that binds to Ig Fc regions (63) as well as to the cytosolic DDX41 dsDNA sensor (64)), TRIM5α (65) (an HIV-1 capsid binding protein that blocks HIV-1 replication in monkeys), and TRIM20/pyrin (66) (a protein binding to caspase-1 blocking IL-1β maturation that is mutated in familial Mediterranean fever), all contain PRY–SPRY domains and are involved in innate immunity.

B30.2/PRY–SPRY domains have a two-layered β-sandwich structure with six hypervariable loops that are similar to Ig CDR regions (63). The variable loops of B30.2 domains form the binding interface for their interacting proteins (63, 65, 67, 68). The v1–v4 variable regions that dictate viral restriction specificity in TRIM5α (65, 69) center over these hypervariable loops. We have identified the variable regions in BTN3A1 by sequence alignment with other B30.2/PRY–SPRY domains and have modeled the BTN3A1 B30.2 domain structure (Fig. 6B). When we compare the B30.2 sequence of rhesus BTN3A1 and human BTN3A3, the v1–v4 regions are highly conserved (one difference in v1, three differences in v3). In contrast, the B30.2 sequence of human BTN3A3 has 4 aa differences in v1, 2 aa differences in v2, and 2 aa differences in v3, along with other framework differences. The strong conservation of V region sequences of the B30.2 domain of BTN3A1 suggests a conserved binding partner between humans and monkeys.
The structural model of the B30.2 domain from BTN3A1 predicts a prominent basic pocket on the binding face of the domain. This basic region is not found in any of the other PRY–SPRY domains whose structures have been determined (Fig. 6B, Supplemental Fig. 2) but is predicted to be present to varying degrees in five of the eight human BTN/BTNL proteins with B30.2.

**FIGURE 8.** Amino acid differences between human and monkey BTN3A1 localize to the outer face of the BTN3A1 dimer. (A) Amino acid alignment for primate BTN3A1. The amino acid sequences for various primate BTN3A1 proteins were aligned using the Clustal W method. Locations of IgV, IgC, and B30.2 structural domains are indicated. Sequences shaded yellow highlight the IgV to IgC contacts, whereas sequences shaded green highlight the IgC to IgC contacts. Red asterisks indicate residues making up the 20.1 mAb epitope. (B) Conservative and nonconservative amino acid differences between humans and baboons/rhesus monkeys localize to the outer face of the BTN3A1 dimer. The BTN3A1 extracellular dimer in complex with the 20.1 mAb is shown with conservative (shaded green) and nonconservative (shaded orange) differences located on the structure. Conserved areas on the top, inner face, and mid-outer face are boxed.

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hypothesize that BTN3A1 molecules detect prenyl pyrophosphates through “inside-out” signaling when cells are activated, causing a change in BTN3A1 conformation or distribution leading to Vγ2Vδ2 T cell stimulation. In model 3, BTN3A1 acts as a ligand binding to a costimulatory or inhibitory receptor on Vγ2Vδ2 T cells. In model 4, BTN3A1 functions as an APC signaling receptor.

FIGURE 9. Models for BTN3A1 involvement in prenyl pyrophosphate stimulation. In model 1, BTN3A1 binds prenyl pyrophosphates extracellularly with or without the contribution of a second protein with direct contract of the prenyl pyrophosphate to the Vγ2Vδ2 TCR. In model 2, the BTN3A1 B30.2 domain associates with a protein that either binds prenyl pyrophosphates directly or stabilized the binding of the prenyl pyrophosphates to the B30.2 domain intracellularly, causing a change in BTN3A1 conformation or distribution leading to Vγ2Vδ2 T cell stimulation. In model 3, BTN3A1 acts as a ligand binding to a costimulatory or inhibitory receptor on Vγ2Vδ2 T cells. In model 4, BTN3A1 functions as an APC signaling receptor.
proteins that function as sensors for intracellular processes would represent a new paradigm for “Ag” recognition by unconventional T cells. Because, if this is the case, there is no direct contact by the γδ TCR to its “Ag,” but instead, the TCR detects changes in the conformation or multimerization of the sensors. The challenge now is to identify the other proteins involved in the sensing process to confirm this hypothesis. Characterizing this sensing pathway will help determine how γVδ2Vδ2 T cells recognize the presence of intracellular pathogens. Elucidating the mechanism of stimulation also will help us understand how tumors such as Daudi and RPMI 8226 are recognized by the γVδ2Vδ2 TCR (1, 17) and allow us to identify tumors that would be highly sensitive to γVδ2Vδ2 T cell immunotherapy.

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Disclosures

C.T.M. is a co-inventor of U.S. Patent 8,012,466 on the development of live bacterial vaccines for activating γδ T cells. The other authors have no financial conflicts of interest.

References


Supplemental Figures

SUPPLEMENTAL FIGURE 1. Characteristics of BTN3A1, BTN3A2, and BTN3A3 family members. A, Schematics of the domain organization of BTN3 family members. All members have IgV and IgC extracellular domains but only BTN3A1 and BTN3A3 have B30.2 intracellular domains. B, Amino acid alignments for BTN3 family members. Amino acid identity is shown as a "." Residues that contribute to the IgC:IgC dimer interface are shaded green whereas those contributing to IgV:IgC dimer interface are shaded yellow. The residues making up the 20.1 mAb epitope are indicated with an "*". C, D, Similarity of the BTN3 IgV domain amino acid sequence to the IgV domains of other immunoglobulin family members. Phylogenetic tree (panel C) and the identity and divergence table (panel D) are shown. Alignment was done by the Clustal W method.

SUPPLEMENTAL FIGURE 2. Surface potential of the B30.2 domains of BTN and other proteins and the synthesis of a biotinylated photoaffinity antigen. A. Surface potential of B30.2 domains of various proteins. Surface potential of the B30.2 domains of BTN3A1, BTN3A3, BTN1A1, BN2A1, BTN2A2, BTNL3, BTNL8, and BTNL9 B30.2 models (top, middle panels) or TRIM72, SPSB-3, pyrin, and Gustavus crystal structures (bottom panels) are shown. The surface potentials were calculated in Pymol using the APBS plugin and are colored from red (-10 kT) to blue (+10 kT). The B30.2 domains are identically scaled using a Pymol script kindly provided by Dr. DeLano. B. Synthesis of biotinylated photoaffinity antigen.

Schematic for the synthesis is shown. Reagents and conditions: (i)-(ii) see Cole, K. P.; Hsung, R. P. Org. Lett. 2003, 5, 4843-4846. (iii) see Dong, Y. et al. Bioorg. Med. Chem. 2006, 14, 6368-6382. (iv) SOCl₂, reflux, 14 h; then Et₃N, S 1-2, CH₂Cl₂, RT 14 h; then biotin-PEG-amine, DMF, RT, 24 h. (v) TBAF, THF, RT, 14 h. (vi) PPh₃, CBr₄, CH₂Cl₂, RT, 30 min; then [n-Bu₄N]₃[HO₇P₂], CH₃CN, 14 h. Compound 2 was synthesized from 1 as described in Cole, K. P.; Hsung, R. P. Org. Lett. 2003, 5, 4843-4846. Compound 3 was converted to 4 as described in Dong, Y. et al. Bioorg. Med. Chem. 2006, 14, 6368-6382. To produce compound 5, a mixture of
4 (314 mg, 1.16 mmol) and SOCl$_2$ (3 ml, 41.4 mmol) was stirred at reflux for 14 h. Excess SOCl$_2$ was removed by rotary evaporation followed by the addition of CH$_2$Cl$_2$ (4.5 ml) and Et$_3$N (722 μL, 5.18 mmol). The solution was then added to 2 (396 mg, 1.16 mmol) and stirred at RT for 14 h. Biotin-PEG-azide (520 mg, 1.16 mmol) was dissolved in DMF (2 ml), combined with the above CH$_2$Cl$_2$ solution and left stirring at RT for 24 h. The solution was concentrated by rotary evaporation and the resulting mixture was purified by silica gel column chromatography (5% MeOH in EtOAc then 10% MeOH in EtOAc) to yield 5 (460 mg, 450 μmol, 39%, $^1$H NMR (CDCl$_3$): δ 8.13 (d, $J$ = 8, 2H), 7.98 (d, $J$ = 8.5, 2H), 7.82 (m, 4H), 7.66 (m, 4H), 7.39 (m, 6H), 6.67 (t, $J$ = 5, 1H), 6.15 (s, 1H), 5.75 (t, $J$ = 5.3, 1H), 5.46 (s, 1H), 4.70 (s, 2H), 4.46 (t, $J$ = 6, 1H), 4.27 (m, 3H), 3.59 (m, 11H), 3.48 (m, 4H), 3.28 (m, 2H), 2.86 (dd, $J$ = 13, 5, 1H), 2.71 (d, $J$ = 12.5, 1H), 2.16 (t, $J$ = 7.3, 2H), 1.91 (t, $J$ = 6, 2H) 1.70 (m, 3H) 1.62 (m, 3H) 1.54 (s, 3H), 1.23 (m, 2H), 1.02 (s, 9H). ESI-MS [M+Na]$^+$: m/z calc. for C$_{56}$H$_{72}$N$_4$O$_{10}$SSiNa: 1043.4631; found: 1043.5072). To produce compound 6, a solution of 5 (200 mg, 196 μmol) in THF (5 ml) was added ($n$-Bu)$_4$NF (1.0 M solution in THF, 400 μL, 400 μmol). The mixture was stirred at RT for 14 h. The solution was concentrated by rotary evaporation and the resulting mixture was purified by reversed-phase chromatography using a C$_{18}$ Sep-Pak column (solvent A: H$_2$O with 0.1% TFA, solvent B: CH$_3$CN with 0.1% TFA, step gradient: 6 ml with 10%, 20%, 30%, 40% solvent B each) to yield 6 (32 mg, 40.5 μmol, 21%, ESI-MS [M+H]$^+$: m/z calc. for C$_{40}$H$_{55}$N$_4$O$_{10}$S: 783.3639; found: 783.2710). To produce compound 7, to a solution of 6 (23 mg, 29.1 μmol) in CH$_2$Cl$_2$ (7 ml) was added PPh$_3$ on resin (36 mg, 108 μmol) and CBr$_4$ (36 mg, 108 μmol). The mixture was stirred at RT for 30 min. The solution was concentrated by rotary evaporation and the resulting mixture was purified by silica gel column chromatography (20% MeOH in EtOAc). To a solution of the resulting products in CH$_3$CN (2 ml) was added [$n$-Bu$_4$N]$_3$[HO$_2$P$_2$] (39 mg, 43.2 μmol). The mixture was stirred at RT for 14 h. A column was filled with Dowex 50WX8 ion-exchange resin and then converted to its ammonium form by adding three column volumes of H$_2$O/NH$_4$OH (3:1, v/v) followed by equilibration with buffer C (25 mM NH$_4$HCO$_3$, 10 mM 2-mercaptoethanol, 10 mM EDTA). The reaction mixture was loaded onto the column and eluted
with buffer C. The combined fractions were lyophilized and purified by semipreparative reversed-phase HPLC (solvent A: 25 mM NH₄HCO₃, solvent B: CH₃CN, gradient: 0–30% B over 24 min, flow rate: 5 ml/min using a 10 x 250 mm, 10 μm particle size Luna C₁₈ column (Phenomenex). Product-containing fractions were lyophilized to yield compound 7, (E)-4-((hydroxy(phosphonooxy)phosphoryl)oxy)-2-methylbut-2-en-1-yl 4-(4-((15-oxo-19-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-4,7,10-trioxa-14-azanonadecyl) carbamoyl) benzoyl) benzoate. (ESI-MS [M-H]⁺: m/z calc. for C₄₀H₅₅N₄O₁₆P₂S: 941.2814; found: 941.1693).

SUPPLEMENTAL FIGURE 3. Similarity of primate BTN3A1 proteins. Phylogenetic trees (A) and identity and divergence tables (B) are shown for the full length, IgV, IgC, and B30.2 domains of BTN3A1. The amino acid sequences for various primate BTN3A1 proteins were aligned using the Clustal W method and phylogenetic trees and identity and divergence tables calculated using the MegAlign module of the DNAStar/Lasergene 10 suite.

SUPPLEMENTAL FIGURE 4. Location of amino acid differences between human and primates on human BTN3A1 structures. The location of differences between human and baboon or between human/baboon and rhesus monkey are shown on the unbound human BTN3A1 extracellular monomer (A) and dimer (B) structures as they are rotated. Conservative amino acid differences between human and baboon are colored green whereas non-conservative differences are colored light orange. Differences between human/baboon BTN3A1 and rhesus monkey BTN3A1 are colored red. The 20.1 mAb binding site on BTN3A1 is shaded purple. The IgC:IgC dimerization interfaces are colored magenta and cyan. There is a totally conserved area located on the inner face of the dimer that wraps around to the mid-outer face (boxed) whereas most differences are located on the distal- or membrane-proximal outer face of the dimer. C, The location of differences between human and baboon or between human and gorilla on the human BTN3A1 B30.2 domain model as it is rotated. Conservative amino acid differences between
human and baboon are colored green whereas non-conservative differences are colored light orange. Conservative amino acid differences between human and gorilla are colored violet whereas non-conservative differences are colored cyan.
Supplemental Figure 1. Wang et al.
Supplemental Figure 2. Wang et. al.
Supplemental Figure 3. Wang et al.