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Regulation of Immunity by Butyrophilins

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BTN1A, BTN2A, BTN3A, B30.2, PRYSPRY

Abstract

Butyrophilin molecules (commonly contracted to BTN), collectively take their name from the eponymous protein in cow's milk. They are considered to be members of the B7 family of costimulatory receptors, which includes B7.1 (CD80), B7.2 (CD86), and related molecules, such as PD-L1 (B7-H1, CD274), ICOS-L (CD275), and B7-H3 (CD276). These coreceptors modulate T cell responses upon antigen presentation by major histocompatibility complex and cognate αβ T cell receptor engagement. Molecules such as BTN3A1 (CD277), myelin oligodendrocyte glycoprotein, and mouse Skint1 and Btnl2, all members of the butyrophilin family, show greater structural and functional diversity than the canonical B7 receptors. Some butyrophilins mediate complex interactions between antigen-presenting cells and conventional αβ T cells, and others regulate the immune responses of specific γδ T cell subsets by mechanisms that have characteristics of both innate and adaptive immunity.

INTRODUCTION

Dysregulation of the immune response underlies the pathology of many infections, autoimmunity, and cancer, and there is considerable interest in exploiting the immune system to combat these diseases. B7 costimulatory receptors and their T cell ligands, such as CD28, CTLA4, ICOS, and PD-1, are emerging as key targets for influencing immune responses. Specific antibody, or soluble Fc fusion proteins, targeting B7 molecules and their ligands have shown promise, particularly in cancer treatment, by reversing the inhibitory signals to activated T cells that are generated by cancer cells themselves and their tissue microenvironment (1, 2). Checkpoint intervention therapies to inhibit inappropriate T cell activation have also been proposed for autoimmune conditions (3, 4).

Human immunoglobulin (Ig) superfamily receptor proteins of the butyrophilin and butyrophilin-like families, termed BTN and BTNL, have also been recognized as potentially important immune modulators (5–8). The Ig domains from butyrophilins exhibit some structural features of the B7 family of coreceptors, such as CD80 and CD86, ICOS-L, and PD-L1 (9–11). So far, counter-receptors for BTNs have not been identified (12–14). Molecules in the CD28/CTLA4 group, the classic receptors for B7 family members, have been largely ruled out as ligands. The immunogenetics and structural diversity of butyrophilins point toward complex functions for these molecules in regulating immune responses. As such, functional characterization and potential therapeutic exploitation has lagged behind that of the canonical B7 receptors.

Some of the insight into understanding the immunomodulatory role of butyrophilins has come from their influence on γδ T cells. These cells represent an important effector subset of innate-like T lymphocytes that express variant T cell receptors (TCRs) consisting of a γ and δ chain heterodimer (γδ TCR) (15, 16). Understanding of the molecular basis of γδ T cell activation and maturation, particularly in humans, has not kept pace with that of αβ T cell biology and represents a gap in our knowledge of T lymphocyte function (17, 18). Identification of activating antigens, selection of the γδ TCR repertoire, and the elements controlling tissue trafficking of γδ T cell subsets remain largely unresolved. Potential counterparts of a system of B7 costimulatory interactions, which are so important in αβ TCR biology, also remain poorly understood for γδ T cells (19–21). Research linking Skint1 (22) and BTN3A1 (23) proteins to the regulation of specific γδ T cell subsets has suggested that butyrophilins may fulfill at least some of these functions in γδ T cell biology. However, the butyrophilin proteins involved are structurally distinct and the genes and γδ T cell subsets are not conserved between species, thus making extrapolation difficult.

IMMUNOGENETICS

Butyrophilin genes are encoded in clusters that have evolved by gene duplication, deletion, diversification, and pseudogene formation (**Figure 1**). In comparisons of human and mouse sequence data, gene orthologs are not always found. Although not rigorously analyzed to date, butyrophilin genes do not appear to be particularly polymorphic within populations, and in only a few cases have disease associations been assigned genetically. Nevertheless, clusters of duplicated genes and differences in gene number between species are both consistent with rapid evolution by selection. Tripartite motif (TRIM) genes are often found in proximity to butyrophilin loci, suggesting a common evolutionary link. Several published reviews cover these general aspects of the structure, genomic organization, and expression of the butyrophilins (6–8).

The two butyrophilin gene clusters on human chromosome 6—the major *BTN* locus telomeric to classical major histocompatibility complex (MHC) class I genes, and the *BTNL* locus near MHC class II loci—illustrate the effects of evolution by gene duplication. The major cluster of *BTN* genes on human chromosome 6p22.1 (**Figure 1*a***) was identified, before the era of large-scale genome

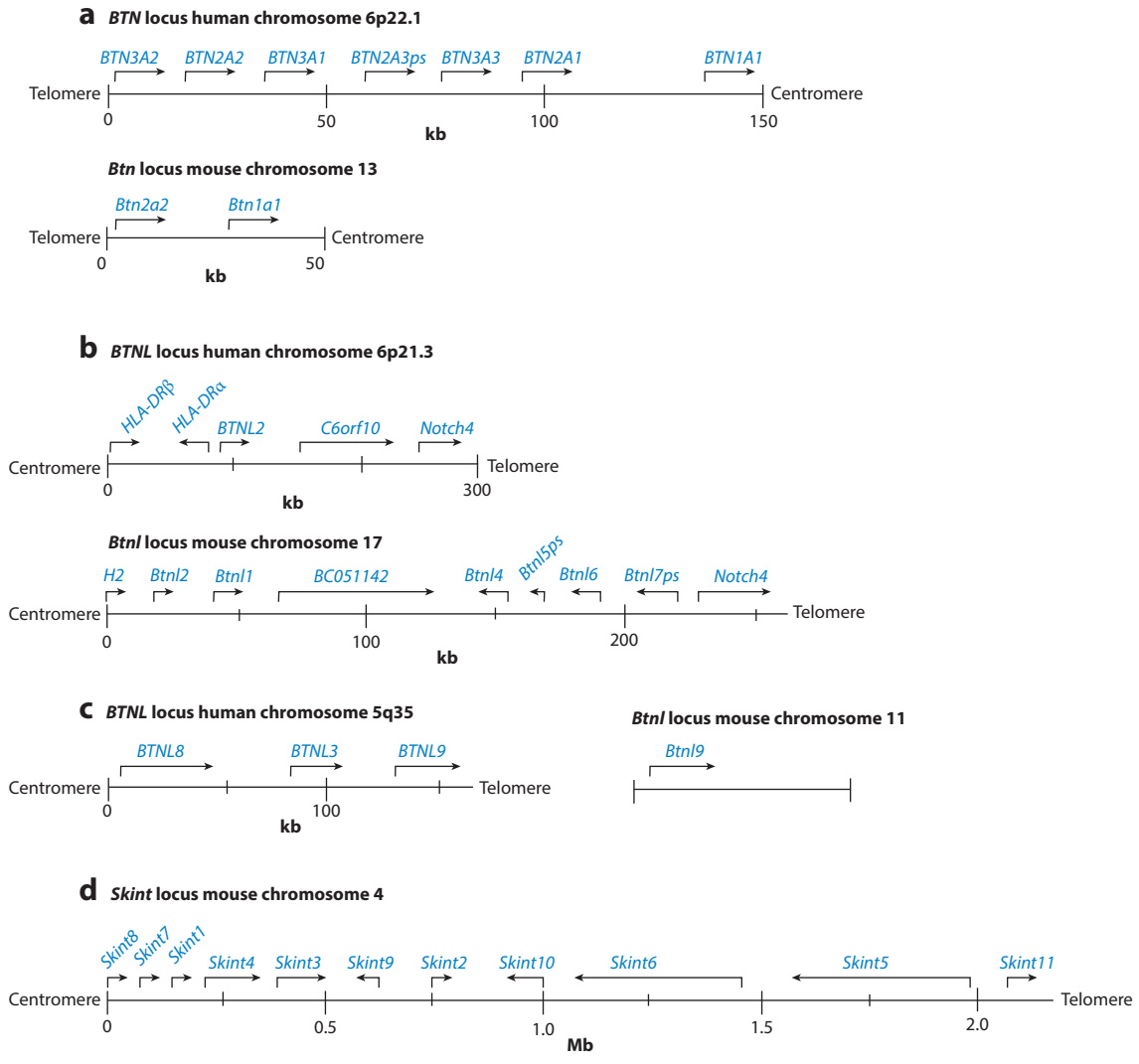

Figure 1

Arrangement of butyrophilin genes in the human and mouse genomes. Butyrophilins have evolved by duplication to form gene clusters subject to expansion and contraction. The absence of gene orthologs between species is a feature of some butyrophilins. (*a*) The *BTN* cluster on human chromosome 6 is composed of 7 genes from 3 subfamilies, *BTN1A*, *BTN2A*, and *BTN3A*, in a pair-wise arrangement. The syntenic region of mouse chromosome 13 is composed of two genes, *Btn1a1* and *Btn2a2*. (*b*) The *BTNL/Btnl* locus is adjacent to the human MHC class II and mouse H2 regions. The mouse *Btnl* locus has expanded to include six related loci, but the human locus contains only a single gene, *BTNL2*. (*c*) The *BTNL* locus on human chromosome 5q35 is composed of three genes, *BTNL8*, *BTNL3*, and *BTNL9*. The syntenic locus in mouse harbors a single gene, *Btnl9*. (*d*) The *Skint* locus on mouse chromosome 4 is composed of 11 genes covering >2 Mb of genomic DNA. The human *SKINTL* locus is represented by a single pseudogene (not shown). Additional conserved butyrophilin loci, represented by single genes, are *MOG* (*BTNL11*), *BTNL10* (*BTN4*), and *ERMAP* (*BTN5*) (not shown). The human genome contains 15 butyrophilin loci. Gene maps were assembled from genome sequence data using the Ensembl genome browser at **http://www.ensembl.org** and **http://www.ncbi.nlm.nih.gov/gene**. Abbreviations: kb, kilobase pairs; Mb, megabase pairs; MHC, major histocompatibility complex.

sequencing, as part of the effort to clone the hemochromatosis gene, *HFE* (24, 25). Several genes showing similarity to *BTN1A1* were identified using complementary DNA selection strategies in the hemochromatosis candidate region. The characterization of the genomic sequence revealed a cluster of six *BTN1A1*-related human genes that have clearly evolved by *cis* duplication (10). The seven *BTN* genes together encompass approximately 150 kb of genomic DNA. *BTN1A1* is a single gene, but each of the *BTN2A* and *BTN3A* families comprises three members—*BTN2A1*, *BTN2A2*, and *BTN2A3* and *BTN3A1*, *BTN3A2*, and *BTN3A3*, respectively—which are present in a pair-wise arrangement. The sequences show approximately 90% amino acid identity within each family and approximately 50% between families (26). The extended MHC region encompassing the human *BTN* gene cluster has been linked to type 1 diabetes by genetic analysis (27). The conserved *MOG* (myelin oligodendrocyte glycoprotein) gene, positioned nearer to MHC class I genes, shares sequence homology with *BTN* and is also considered to be part of the butyrophilin gene family.

In contrast to the human *BTN* gene cluster on chromosome 6, the orthologous genomic region of mouse chromosome 13 contains only 2 genes, the *BTN1A1* ortholog, *Btn1a1*, and a single representative of the *Btn2* family, *Btn2a2* (**Figure 1*a***). A similar arrangement is found in the rat genome. Functional orthologs of *BTN1A1/Btn1a1* and *BTN2A2/Btn2a2* are present in all vertebrate species that have been examined. The *BTN3A* gene lineage is absent from rodent species. Therefore, of the members of the butyrophilin gene family in the extended human MHC region, *BTN1A1*, *BTN2A2*, and *MOG* are framework genes conserved across species, whereas *BTN3A* sequences are less widely conserved. In higher primates, such as *Pan troglodytes* (chimpanzee) and *Macaca mulata* (rhesus monkey), the genomic organization of the *BTN* cluster appears to be conserved, with variation perhaps only in which loci are represented by nonexpressed pseudogenes, for example, human *BTN2A3*.

The *Btnl* cluster in the *H2* region of mouse chromosome 17 includes four complete genes, namely *Btnl2*, *Btnl1*, *Btnl4*, and *Btnl6*, and two pseudogenes, *Btnl5* and *Btnl7*, but the orthologous human MHC class II region contains only *BTNL2* (**Figure 1*b***). A polymorphic variant affecting transcript splicing at the human *BTNL2* locus has been linked by genetic analyses to inflammatory autoimmune conditions, including sarcoidosis, ulcerative colitis, and Crohn's disease (28–30). *BTNL2* lies close to the highly polymorphic MHC class II *HLA-DRB* locus, a genomic region characterized by extensive and variable linkage disequilibrium, making it difficult to resolve genetic associations independently from those documented for MHC class II alleles (31, 32).

The human *BTNL* gene cluster on chromosome 5q35 is composed of three genes, *BTNL8*, *BTNL3*, and *BTNL9*, but the orthologous mouse chromosome 11 locus harbors only *Btnl9* (**Figure 1*c***).

In a striking example of species-specific gene expansion, the *Skint* locus (selection and upkeep of intraepithelial T cells) on mouse chromosome 4 has expanded to include 11 genes, whereas the human *SKINTL* locus is represented by a single pseudogene (**Figure 1*d***). *Skint* loci exhibit polymorphism between mouse strains, both in gene sequence and genomic structural organization (22). *Skint* molecules have two butyrophilin-related Ig domains and three transmembrane regions. They were linked initially to γδ T cell biology by genetic mapping in the FVB/Tac mouse strain (22). The absence of the predominant intraepithelial lymphocyte (IEL) lineage carrying the clonal Vγ5Vδ1 TCR observed in the FVB/Tac mouse was linked to a mutation introducing a premature stop codon in the open reading frame of *Skint1*. Therefore, *Skint1* has been shown to determine thymic selection, maturation, and skin-tissue homing of murine Vγ5Vδ1 dendritic epithelial T cells (DETCs) (22).

Other conserved butyrophilin loci are the aforementioned *MOG* gene (*BTNL11*) on human chromosome 6p21.3, *ERMAP* (*BTN5*, chromosome 1), and *BTNL10* (*BTN4*, chromosome 1). The

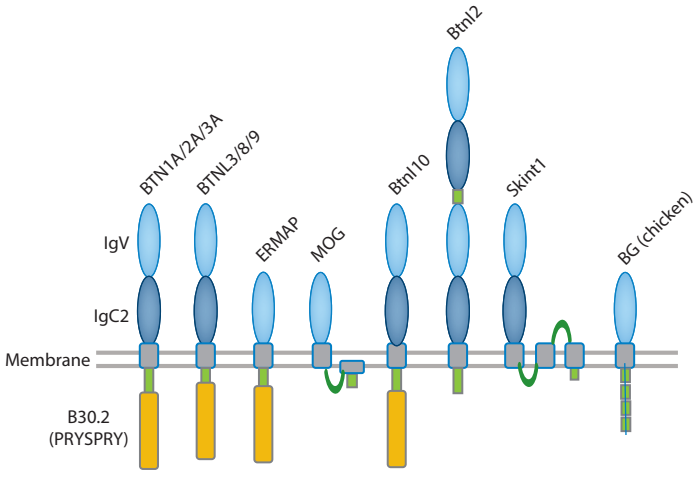

Figure 2

Protein domain structure of butyrophilins. The defining feature of the butyrophilin family is IgV and IgC2 immunoglobulin (Ig) domains, related to the B7 costimulatory receptors, CD80 and CD86. Most BTN molecules contain a cytosolic B30.2 (PRYSPRY) domain. The B30.2 (PRYSPRY) domain is encoded by a single exon, which can be subject to alternative splicing. The three BTN protein families BTN1A, BTN2A, and BTN3A (BTN1A/2A/3A) have identical structures, apart from BTN3A2, which lacks the B30.2 domain. The BTNL molecules BTNL3, BTNL8, and BTNL9 have the canonical butyrophilin protein structure of two Ig domains and a B30.2 domain. Mouse Btnl2 has an unusual arrangement of duplicated Ig domains without a B30.2 domain. It is unclear whether the human *BTNL2* locus produces a complete transcript. Also, human *BTNL10* may not be expressed. The other molecules in the Btnl cluster in the mouse H2 region—Btnl1, Btnl4, and Btnl6—have the conventional butyrophilin structure, showing sequence similarity to human BTN proteins. The other molecules, including Skint1, MOG, ERMAP, and chicken BG, show considerable structural variation in their Ig and cytosolic domains.

human genome, therefore, encodes 15 butyrophilin-related loci, of which *BTN2A3* and *SKINTL* are considered to be nonexpressed pseudogenes. It is not clear whether human *BTNL10* produces a full-length transcript, and *BTNL2* transcripts have been reported only rarely.

STRUCTURE OF BUTYROPHILINS

Figure 2 shows the structure of all the major classes of butyrophilin proteins encoded in the human genome and other important examples in mouse and chicken. Butyrophilins are type 1 receptor glycoproteins of the Ig family, containing one or two extracellular Ig domains that have as a defining feature amino acid sequence similarity to the B7 costimulatory receptors B7.1 and B7.2 (CD80 and CD86). In fact, sequence similarity between B7 and butyrophilin receptors is quite distant, precluding identification of the conserved amino acid motifs required for binding to candidate T cell ligands, such as CD28 or CTLA4 (33, 34). In addition, potentially complex trafficking and membrane topology is suggested by the presence of multiple, predicted transmembrane regions, particularly in Skint and MOG proteins.

In contrast to most other immune receptors of the Ig family, butyrophilins have large cytosolic domains that vary among different members and that do not generally harbor conventional motifs involved in intracellular signaling or trafficking. Instead, the majority of butyrophilins have a B30.2 domain (also termed PRYSPRY) (35). The importance of the B30.2 domain, particularly with regard to the function of TRIM proteins, such as TRIM5α, TRIM21 (Ro52), and TRIM20 (pyrin/MEFV), which share this cytosolic protein domain, has shifted attention to the role that

TRIPARTITE MOTIF MOLECULES IN CELL-AUTONOMOUS IMMUNITY

The tripartite motif molecules (TRIMs) TRIM5 and TRIM21 exemplify the complex functions of TRIM proteins that are dependent on B30.2 (PRYSPRY) domain interactions. The rhesus TRIM5α molecule has been shown to act as a restriction factor against the human retrovirus HIV, the causative agent of AIDS (36). In an example of cross-species pathogen restriction, the B30.2 (PRYSPRY) domain of rhTRIM5α protein binds to the HIV capsid, targeting the virus for proteasome-dependent degradation and initiating innate immune signaling (48). Amino acid changes in the B30.2 (PRYSPRY) domain render human TRIM5α incapable of binding to HIV and, thereby, unable to restrict HIV replication. TRIM21 has been shown to be a high affinity Fc receptor, binding to the CH2–CH3 domain interface of IgG, overlapping the binding site of several other Fc receptors, notably those encoded by some bacteria and viruses (37, 44). In the context of infection, virus with bound antibody, upon entering the cell cytoplasm, recruits TRIM21 via B30.2 (PRYSPRY) domain interaction. TRIM21–IgG binding coordinates complex immune responses, including pathogen restriction and innate immune signaling, in a mechanism termed antibody-dependent intracellular neutralization (ADIN) (45, 46).

the domain plays in the function of butyrophilins (36–39). In these TRIM molecules, the B30.2 domain acts in a manner analogous to pattern-recognition receptors [such as Toll-like receptors (TLRs) or nucleotide-binding oligomerization domain (NOD)-like receptor proteins], which bind molecules associated with infection [such as pathogen-associated molecular patterns (PAMPs)] or cell damage [such as damage-associated molecular patterns (DAMPs)] (40). TRIM family members have a variety of functions, but have been studied extensively as immune signaling molecules and viral restriction factors (41–45) (see sidebar Tripartite Motif Molecules in Cell-Autonomous Immunity). Each B30.2 domain appears to have evolved to bind with high affinity to a specific ligand, which may not necessarily be an endogenous molecule present normally in the cell's cytoplasm. Such binding, in turn, induces signaling and effector function to initiate immune responses (46–48). The presence of a large cytosolic protein domain at the C terminus of two protein families that are otherwise unrelated appears to be unique in the human protein catalog (35).

BUTYROPHILIN AND T CELL MODULATION

Btnl2 and Btn2a2

In in vitro assays, several BTN proteins have inhibited T cell activation. Typically, these assays used immobilized Fc fusion proteins to interact with T cells activated by anti-CD3 antibody. The best-studied examples are mouse Btnl2 and Btn2a2. Btnl2 has been detected predominantly in intestine, and in a mouse model of inflammatory bowel disease, expression was increased by inflammatory immune pathology at this site (12, 49). Btnl2-Fc negatively modulated B7-dependent T cell activation upon anti-CD3 antibody cross-linking, manifested as reduced TCR-dependent signaling, and cytokine production and proliferation, accompanied by induction of Foxp3, a signature of regulatory T cells (Tregs) (14). A potential Btnl2 ligand has been detected on activated T and B cells, but the known T cell–expressed B7 ligands did not bind Btnl2 (12, 49).

Studies of mouse Btn2a2 have found similar negative regulatory effects in T cell proliferation assays using soluble Btn2a2-Fc protein (13). Binding of Btn2a2-Fc to anti-CD3-stimulated T cells inhibited CD3ε, Zap70, and Erk1/2 phosphorylation, linked to specific signaling events of the prosurvival PI3K and Akt pathways. Foxp3-GFP-transgenic naive T cells have been used to show that Btn2a2-Fc induced de novo expression of Foxp3 GFP (50). In agreement with these functional

EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

Experimental autoimmune encephalomyelitis (EAE) is an inflammatory, demyelinating disease of the central nervous system (CNS), with features that overlap the symptoms of the human CNS demyelinating disease multiple sclerosis. EAE is an animal model of brain inflammation studied most commonly in mice and rats, and is induced by immunization with purified myelin proteins, myelin oligodendrocyte glycoprotein, or myelin basic protein. EAE can also be induced by the transfer of T cells reactive to these myelin antigens.

assays, Btn2a2-Fc has been found to stain activated T cells, suggesting the presence of a receptor on these cells (W. Reith, unpublished data).

The generation and analysis of *Btn2a2* knockout mice has recently provided genetic evidence for a key role of *Btn2a2* as a negative regulator of T cell responses, thereby supporting conclusions drawn from the aforementioned in vitro studies performed with Btn2a2-Fc (51). *Btn2a2*-deficient mice develop enhanced T helper type 1 (Th1) and type 2 (Th2) responses upon immunization under conditions favoring Th1 and Th2. Their response to antitumor (B16-OVA) vaccination is also boosted, resulting in reduced tumor growth. *Btn2a2*-deficient mice exhibit exacerbated Mog-induced experimental autoimmune encephalomyelitis (EAE) (see sidebar Experimental Autoimmune Encephalomyelitis). Increased susceptibility to EAE was a consequence of increased pathogenic effector Th1 and Th17 responses, coupled with reduced protective Treg expansion. T cell transfer experiments, and the generation of bone marrow chimeras, have suggested that enhanced responses to vaccination and exacerbated EAE are a consequence of deficient Btn2a2 expression by antigen-presenting cells (APCs), rather than by T cells or cells of nonhematopoietic origin. Therefore, like Btnl2, Btn2a2 appears to function as a negative costimulatory molecule that modulates T cell responses by inhibiting Th cell development while favoring Treg expansion.

Butyrophilin Expression and T Cell Regulation

How does BTN-dependent regulation of T cells fit with immune regulation in the whole organism? An answer to this question may come from the study of when and where BTN molecules are expressed. Although widespread expression in lymphocytes has been reported for BTN/Btn and some BTNL/Btnl molecules, in both human and mouse studies expression has been associated particularly with tissue epithelium. Human *BTN* molecules did not reveal any specific tissue expression by RNA analysis, but rather low-level widespread expression (10, 52). Widespread expression in multiple tissues has also been reported for mouse Btn2a2 protein (13). In immune cells, Btn2a2 protein expression has been documented on APCs, including B cells, macrophages, and dendritic cells (DCs) (13). In accordance with having a role in T cell regulation, transcription of the human *BTN2A2* and mouse *Btn2a2* genes in APCs and IFN-γ-induced cells has been found to be regulated by the class II transactivator (CIITA) and regulatory factor X (RFX) (51), two transcription factors known to be crucial for the expression of genes implicated in MHC class II–mediated antigen presentation (53). Thus, *BTN2A2* messenger RNA (mRNA) expression is markedly reduced in cell lines derived from patients suffering from MHC class II deficiency, also called bare lymphocyte syndrome, a hereditary immunodeficiency disease resulting from mutations in the genes encoding CIITA or RFX. Similarly, *Btn2a2* mRNA expression is decreased in B cells, DCs, and IFN-γ-treated embryonic fibroblasts from RFX- and CIITA-knockout mice.

Increased levels of BTN2A1 have been reported in tumors, together with variation in high-mannose oligosaccharide modification for BTN2A1, which influenced binding of the lectin-like receptor DC-specific intercellular adhesion molecule-3–grabbing nonintegrin (DC-SIGN) (52). BTN3A (CD277) molecules have shown increased expression in ovarian tumor tissues (54, 55). These reports suggest that the modulation of T cell responses by BTN expression gives the tumors a selective advantage.

Apart from mouse Btnl2, other molecules of the BTNL family, particularly human BTNL proteins, are largely uncharacterized. Murine Btnl1 has been detected in intestine, with a predominantly cytosolic localization (56), as well as in other lymphoid cells (57). Btnl1 suppressed inflammatory mediators in co-culture with murine epidermal IELs (56), and neutralizing anti-Btnl1 antibodies caused enhanced immune pathology in mouse models of autoimmune disease, which, again, is consistent with negative regulatory effects on T cells (57). Evidence for a novel activating ligand on T cells has also been presented for human BTNL8 (58). These data suggest that molecules of the BTNL lineage also modulate T cell responses.

ACTIVATION OF γδ T CELLS

Skint1 Determines the Predominant Vγ5Vδ1 Intraepithelial Lymphocyte Lineage of Murine Skin

A series of publications have reported that *Skint1* guides the thymic selection and organ-specific homing of the Vγ5Vδ1 IEL, which gives rise to murine DETCs (59). The absence of the skin Vγ5Vδ1 DETC lineage, in the FVB-Tac laboratory mouse strain, has been linked to a spontaneous point mutation at the *Skint1* locus (22). The Glu324stop mutation truncates the Skint1 protein at the third predicted transmembrane domain, thereby affecting the complex membrane topology, which appears vital to its normal function (60). Other members of the *Skint* gene family show prominent tissue expression that is restricted similarly to skin and thymic epithelium, implying a potential role for additional *Skint* genes in shaping these tissue epithelia for functional selection of DETC (22). In comparisons of early thymocytes derived from mutant FVB-Tac and wild-type FVB mice, *Skint1* has been shown to influence an expression profile that is dependent upon the transcription factors Egr3, NFAT, and NFκB to support expansion of Vγ5Vδ1$^+$ thymocytes, a regulatory network nominally induced by TCR engagement (61). Similarly, Vγ5Vδ1$^+$ thymocytes could be rescued from FVB-Tac mice using TCR antibody cross-linking to complement the *Skint1* mutation (59). These data support the notion that *Skint1* provides the direct ligand for the Vγ5Vδ1 TCR receptor, although other potential interpretations have not been ruled out (62).

Human BTN3A Molecules and Vγ9Vδ2 T Cells

The human protein BTN3A1 has been linked to phosphoantigen-dependent activation of Vγ9Vδ2 T cells. It has been known for some time that Vγ9Vδ2 T cells, which are present predominantly in the blood of higher primates, including humans, are activated specifically by small molecule intermediates of the mevalonate pathway, collectively termed phosphoantigens (pAgs) (63). Phosphoantigens are ubiquitous environmental stimuli, the presence of which is transmitted to Vγ9Vδ2 T cells by BTN3A1 using a novel antigen-presentation mechanism. The γδ T cells, in turn, are activated to proliferate rapidly, secrete inflammatory cytokines, such as tumor necrosis factor (TNF)-α and IFN-γ, and kill targeted cells. The archetypal pAgs, (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) and isopentenyl pyrophosphate (IPP), are structurally very similar, with a molecular weight of approximately 250 Da, the approximate size of two amino acids (see sidebar Phosphoantigens as Activators of Vγ9Vδ2 T Cells). Nevertheless, the BTN3A1-dependent

PHOSPHOANTIGENS AS ACTIVATORS OF Vγ9Vδ2 T CELLS

Vγ9Vδ2 T cells are an innate-like T cell subset carrying an unconventional T cell receptor of restricted diversity. Vγ9Vδ2 T cells are activated by biochemical intermediates of isoprenoid biosynthesis, called phosphoantigens. Isoprenoids are synthesized from the precursors isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate and are essential components in all cells. Eukaryotes, fungi, and some bacteria produce IPP through the mevalonate pathway, whereas some parasites, gram-negative and some gram-positive bacteria, including both pathogenic and commensal species, utilize the nonmevalonate or 2-C-methyl-D-erythritol-4-phosphate pathway. HMB-PP [(*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate] is an intermediate in the nonmevalonate pathway and is the most potent activator of Vγ9Vδ2 T cells that has been identified. The endogenous phosphoantigen IPP is a less potent activator.

mechanism is capable of differentiating between the two compounds by direct interaction (64–66). How such binding is then transmitted to the γδ TCR remains poorly understood and is an active area of research. Most evidence now points toward a mechanism of indirect pAg presentation, which is critically dependent upon the cytosolic B30.2 domain of BTN3A1. The question of most pressing concern is whether BTN3A1 is a ligand for the γδ TCR (67). In addition, because the selection of Vγ9Vδ2 T cells by BTN3A1 shows similarity to the selection of mouse Vγ5Vδ1-positive DETC by *Skint1*, these findings imply that other butyrophilin molecules may regulate the maturation and functional potential of T cells carrying distinct γδ TCR specificities.

The metabolite IPP is an intermediate of the mevalonate, or cholesterol biosynthesis, pathway (also called the HMG-CoA reductase pathway). The importance of this pathway is highlighted by the fact that it is widespread in prokaryotic and eukaryotic cells, being essential for both host cells and infectious microorganisms. The pathway provides isoprenoid precursors for essential biochemical processes, such as cholesterol biosynthesis, which is required for the structural integrity of the cell membrane, and for posttranslational modification of membrane-anchored proteins, such as N-glycosylation, prenylation, farnesylation, and geranylgeranylation. Importantly, the pathway is targeted by two classes of commonly prescribed drugs, namely statins (HMG-CoA reductase inhibitors), used to lower blood cholesterol, and aminobisphosphonates (such as zoledronate), used to treat bone metastases of tumors, as well as osteoporosis. Treating human cells with these agents modulates the intracellular IPP concentration and the response of Vγ9Vδ2 T cells, acting through the BTN3A–pAg sensing mechanism (68). The widespread use of these drugs, particularly with regard to the efficacy and mechanism of action of statins, could have implications for human health, which at present are not clear (69).

The microbial isoprenoid precursor HMB-PP is produced by many gram-negative and gram-positive bacteria and is a potent activator of Vγ9Vδ2 T cells (70). HMB-PP is an intermediate of the nonmevalonate pathway of isoprenoid biosynthesis. We speculate that microbes may have evolved an alternative pathway for isoprenoid biosynthesis because the endogenous pathway is subject to regulatory control as a result of infection, possibly representing a host response to controlling pathogen replication (71, 72). Therefore, the nonmevalonate pathway may be regarded as a pathogen countermeasure to host cell regulation. Other mechanisms of host–pathogen interaction impinge on the classical mevalonate pathway; for instance, the protein viperin, an antiviral protein that interacts with farnesyl pyrophosphate synthase, acts in part to influence cellular lipid metabolism (73, 74).

Competing regulatory signals centered on the mevalonate pathway are also likely to occur as a result of adaptation to cell transformation (75). BTN2A and BTN3A molecules have been shown

MR1-RESTRICTED PRESENTATION OF BACTERIAL METABOLITES TO MAIT CELLS

MAIT cells (mucosal associated invariant T cells) are a conserved innate-like T cell population carrying a T cell receptor of restricted diversity (85). MAIT cells are associated with tissue mucosa, including the intestine, lung, and genitourinary tract of humans and mice, where they act in immune recognition of bacterial infection (84). MAIT cells respond to bacterial metabolic intermediates of vitamin B metabolism, which are presented to the MAIT receptor by the monomorphic major histocompatibility complex class I–related molecule MR1 (83).

to be regulated efficiently during viral infection by a mechanism analogous to viral downregulation of MHC class I or NKG2D ligands, implying that immune evasion of BTN-mediated activation of γδ T cells is a requirement for viral infection (76–78).

The BTN3A–pAg sensing mechanism results in recruitment of the killing potential of activated Vγ9Vδ2 T cells to sites of infection. The response may be used to identify and kill transformed cells where the intracellular IPP concentration is increased (68). Inappropriate Vγ9Vδ2 T cell activation in the absence of regulatory checkpoints may predispose to autoimmunity. The BTN3A–pAg sensing mechanism, therefore, impacts on important areas of human health, and further study of the molecular basis of the pathway may allow for its manipulation using specific drugs (65).

Phosphoantigen Binds to the B30.2 Domain of BTN3A1

Some progress has been made in elucidating the molecular control of the BTN3A–pAg sensing mechanism. The structure of the B30.2 domain of BTN3A1 has revealed it to be composed of layered, antiparallel β sheets with variable loops, homologous to the Ig fold. Binding studies have shown specific interaction of the pAg molecules IPP and HMB-PP with the BTN3A1 B30.2 domain. Physiologically relevant consensus affinities of <1 μM for HMB-PP binding to the B30.2 domain have been reported, and IPP bound with a lower affinity, in the millimolar range (0.5–0.6 mM) (64–66).

Structural analyses of a B30.2–pAg complex have proven problematic due to the small size of the ligand, but a binding pocket required for pAg binding has been identified (64). The mutation of charged residues within the pocket renders cells carrying the variant BTN3A1 molecules unresponsive in T cell assays, confirming a critical role for pAg binding to the B30.2 domain (64, 66).

These data support a model of indirect pAg presentation that differs from a proposed direct-interaction model, whereby pAg binds to the external IgV domain of BTN3A1 (67). The direct-interaction model, reviewed extensively in the literature (79–82), mimics other mechanisms of bacterial metabolite presentation to specialized cells of the immune system. MR1-dependent presentation of vitamin B derivatives to MAIT cells (83, 84) (see sidebar MR1-Restricted Presentation of Bacterial Metabolites to MAIT Cells; 85) and CD1d-restricted presentation of lipid to the invariant TCR on natural killer T (NKT) cells (86) both rely upon MHC class I–like structures for antigen presentation to their respective TCRs. At present, these issues of pAg presentation to the Vγ9Vδ2 TCR remain unresolved and it is unclear whether activated BTN3A1, with bound pAg in the B30.2 domain or the external IgV domain, is the ligand for the γδ TCR itself or whether BTN3A-dependent recruitment of other molecules is required for TCR engagement (64, 67, 87).

Interaction of BTN3A1 with the Plakin Protein Periplakin

The cytoskeletal adaptor protein periplakin has been identified as a BTN3A1 binding partner (66). Periplakin is a member of the plakin family of cytolinker proteins, which have been characterized mainly as structural elements in epithelial cell integrity, forming part of a large protein complex contributing to the formation of the cornified envelope of keratinocytes (88–90). A number of additional protein interactions have been demonstrated for periplakin, including with other plakin family members—for example envoplakin, involucrin, and plectin (91, 92)—with kazrin (93), and also with F-actin (94). Periplakin interaction with Fc receptor CD64 (FcγR1) increased receptor avidity and antibody uptake in monocytes (95). Periplakin also performed a scaffolding function to localize the serine–threonine protein kinase activity of the signaling molecule PKB (AKT) (96).

Although in vitro studies have confirmed the periplakin–BTN3A1 interaction, with a binding affinity in the low micromolar range (approximately 2 μM), it has generally been difficult to demonstrate interactions between periplakin and BTN3A1 in cells (66). Analyses of periplakin knockdown lines have been consistent with additional interactions having a regulatory role in γδ T cell activation. Other plakin molecules may function in this pathway, and further work will be required to confirm whether these periplakin-interacting molecules are involved (91, 97–99).

Structural studies on the amino-terminal plakin domain of periplakin have not been presented, although study of the related domain in plectin showed a central, noncanonical SH3 domain (92, 100). The position of the SH3 domain in plectin coincided with deletion-mapping studies of the related domain in periplakin and the interaction interface with BTN3A1. A dileucine motif in the juxtamembrane region of BTN3A1, not found in the other BTN3A isoforms (BTN3A2 and BTN3A3), has been shown to bind periplakin (66). This mechanism has similarities to that of E-cadherin–p120-catenin binding, where inside-out signaling by a noncanonical dileucine motif regulates cell–cell adhesion by E-cadherin homotypic interaction (101, 102).

Initially, a conformational change in BTN3A1 was proposed to be induced by pAg binding to the B30.2 domain as a mechanism to account for the differential effect of BTN3A (CD277) monoclonal antibodies on Vγ9Vδ2 T cell activation (23, 103). A conformational change in the B30.2 domain of BTN3A1 has been detected by nuclear magnetic resonance studies (65). Periplakin might represent a structure involved in this switch, serving to anchor BTN3A1 in the cell membrane upon pAg binding by attachment to cytoskeletal structures, such as intermediate filaments or actin (66).

Periplakin has also been shown to bind a membrane-proximal dileucine in BTN1A1 (66). The secretion of lipid into milk requires a specialized secretory mechanism in breast epithelium (104). The requirement for BTN1A1 in this process was confirmed in a study of mice in which the *Btn1a1* gene was genetically ablated (105). Retention of the periplakin interaction motif in BTN1A1 suggests a role for periplakin in this secretory mechanism or in a not-yet-explored immune function of BTN1A1.

Models of Phosphoantigen-Dependent γδ T Cell Activation by BTN3A

Figure 3 outlines potential models for the mechanism controlling pAg-dependent activation of Vγ9Vδ2 T cells by BTN3A molecules. Most evidence is now consistent with pAg binding to the intracellular B30.2 domain of BTN3A1 (64, 65, 67). As an additional component of the pathway, a pAg transporter may enable the movement of externally delivered pAg into the cell cytoplasm in order to allow for binding to the B30.2 domain (79, 80). Although the critical role for BTN3A1 in the mechanism is not disputed, evidence of nonredundant roles for the other BTN3A isoforms, BTN3A2 and BTN3A3, has been presented (66). The significant question then is whether the BTN3A complex is the direct ligand of the γδ TCR (**Figure 3*a***) (64, 67). Both anti-BTN3A

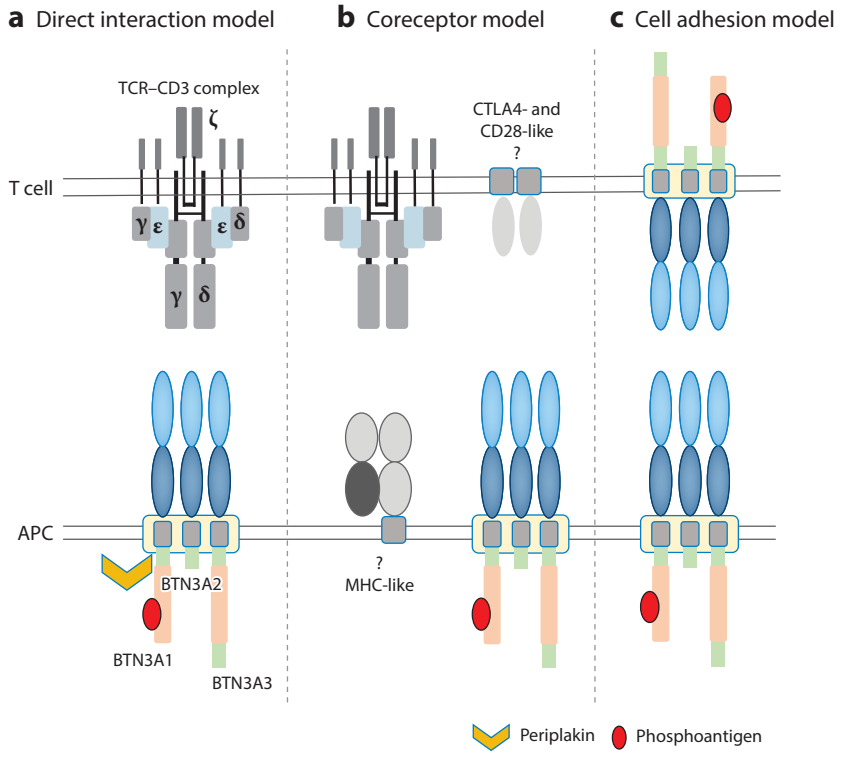

Figure 3

Models of γδ T cell activation by BTN3A. BTN3A1 transmits activation signals to γδ T cells (64, 65, 67). Most data are consistent with the binding of the phosphoantigens (pAgs) HMB-PP and IPP to the cytosolic B30.2 domain of BTN3A1. Some data have suggested there is a role for the BTN3A isoforms BTN3A2 and BTN3A3 and for the recruitment of periplakin to stabilize the complex in the cell membrane (66). The key issue is how the BTN3A–pAg structure conveys activation signals. This could be achieved by (*a*) the direct-interaction model, in which BTN3A1 is the ligand for the Vγ9Vδ2 T cell receptor (TCR); (*b*) the coreceptor model, in which the γδ TCR interacts with a major histocompatibility complex (MHC)-like molecule and BTN3A with a structure related to CTLA4 or CD28 [although this model represents a conventional view of interactions between antigen-presenting cells (APCs) and T cells, such ligands have not been identified]; or (*c*) the cell-adhesion model, in which the BTN3A structure mediates homotypic interactions to promote cell contact among T cells and APCs.

(anti-CD277) antibody and pAg treatment have induced similar signaling in γδ T cells, suggesting a close overlap between BTN3A-dependent pAg sensing and TCR engagement (106). If the BTN3A complex is not the direct γδ TCR ligand, then what could be? In a conventional view of the molecular interactions between APCs and T cells, presenting molecules take the form of an MHC-like structure, with additional B7, CTLA4, and CD28 coreceptor-like molecules providing costimulatory functions, similar to those that control αβ TCR–MHC interactions (**Figure 3*b***). However, no such MHC-related molecule has been linked to the BTN3A–pAg stimulation of Vγ9Vδ2 T cells, and no CTLA4- or CD28-related structure has been identified as the ligand for the B7-related BTN3A molecules. An additional possibility is that BTN3A molecules act simply as adhesion factors promoting cell–cell interaction in a pAg dependent manner (**Figure 3*c***), leaving the nature of the TCR ligand unresolved. Further work will be required to clarify these possibilities.

BTN3A (CD277) Molecules May Have Other Functions

Activating γδ T cells may not be the only function of BTN3A molecules. Some studies have shown that BTN3A proteins may act to inhibit T cell immune responses (11, 107). BTN3A (CD277)-positive cells decreased the proliferation of human T cells in co-culture, with a concomitant reduction of Th1 cytokine profiles, particularly IL-2 and IFN-γ (54). Cross-linking experiments using anti-BTN3A (anti-CD277) antibodies showed regulatory roles for BTN3A expressed on monocytes and DCs, particularly affecting expression of the proinflammatory cytokines IL-8, IL-12/p70, and IL-1β (108). CD277 antibody cross-linking with the simultaneous addition of TLR ligands had synergistic effects on cytokine production. BTN3A molecules showed widespread expression on blood lymphocytes, including CD4, CD8, and NKT cell subsets, possibly with distinct expression patterns for the three BTN3A isoforms (109). Use of the anti-BTN3A (anti-CD277) agonist monoclonal antibody 20.1 increased the production of IL-2 and IFN-γ in proliferation assays using purified $CD4^+$ T cells, consistent with a costimulatory role in this case. These effects were linked to TCR signaling, particularly affecting PI3K, AKT and ERK phosphorylation. No direct effect on primary NK cell activation was detected, although BTN3A isoforms could differentially affect IFN-γ production induced by NKp30 receptor cross-linking (109). Collectively, these data link BTN3A molecules to the complex regulation of T cells. The CD277 monoclonal antibody data hints that T cells may be modulated in different ways, dependent upon relative expression or surface conformation of the three BTN3A isoforms.

Other Butyrophilin Molecules: BTN1A1, MOG, and ERMAP

The butyrophilin protein BTN1A1 is found in milk, and it functions to promote efficient secretion of lipid in mammalian milk. Expression is restricted predominantly to the specialized secretory epithelium of the mammary gland of the pregnant mammal (110). The B30.2 domain of BTN1A1 binds to xanthine oxidoreductase (XO), a housekeeping enzyme involved in producing reactive oxygen species and in purine catabolism (111), although it is also required for milk lipid secretion (112). A role for BTN1A1-coated milk fat globules in promoting apoptosis and involution of breast epithelium has been reported, linked to the disruption of lysosomal membranes and STAT3 signaling (113). BTN1A1 has also been capable of inhibiting T cell responses in in vitro assays (13).

A large body of work has documented the influence of *MOG* (*BTNL11*) on T and B cell interactions associated with EAE (see sidebar Experimental Autoimmune Encephalomyelitis). Immunization protocols in rodents, which include Mog protein, are commonly used to induce demyelination in the central nervous system (CNS) as a model of the human autoimmune condition multiple sclerosis (MS). The MOG protein contains a single IgV domain with two potential transmembrane regions that contribute to a complex membrane topology (114). Strict expression in the oligodendrocytes of the CNS implies a role for MOG in ensuring the structural integrity of the myelin sheath, the outermost layer of nerve fibers. MOG has been linked with the pathogenesis of MS by genetic analysis (115) and by the detection of anti-MOG autoantibodies in serum samples from MS patients. Whether anti-MOG autoantibodies contribute directly to MS pathology is debated (116). MOG protein has been shown to provide immune-dominant peptide epitopes for $CD4^+$ T cell antigen presentation, and the adoptive transfer of MOG-reactive T cells has induced inflammatory responses in the EAE model (117, 118). $Mog^{-/-}$ mice were refractory to Mog-induced EAE, as they exhibited a mild phenotype compared with wild-type controls, confirming that the lack of tolerance is directed specifically toward Mog protein in wild-type mice (119). It has been proposed that BTN1A1, as a common dietary factor, could influence EAE disease progression by molecular mimicry (120). *MOG* transcripts have not been detected in thymic epithelium, leading to the proposal that immune-dominant MOG epitopes result from a failure of

central T cell tolerance (121). Also of relevance, the interaction of modified, fucosylated N-glycans on MOG was shown to support interaction with DC-SIGN, which is expressed on brain microglia and DCs (122). The MOG–DC-SIGN interaction, together with simultaneous activation of TLR4 on DCs, enhanced IL-10 secretion and resulted in decreased T cell proliferation. The MOG–DC-SIGN interaction was sensitive to MOG glycosylation and regulated by inflammatory signals, supporting the notion that variation in the posttranslation modification of MOG influenced T cell responses. These data, which are reminiscent of the previous results describing the interaction of DC-SIGN with BTN2A1 (52), uncover a potentially important regulatory system for the control of immune tolerance in the brain.

ERMAP (erythroid membrane-associated protein, *BTN5*) is highly expressed in erythroid tissues (123, 124). The conserved ERMAP protein, which has an implied function in the development of erythroid cells, shares homology with butyrophilin IgV and particularly with the B30.2 domains. Colocalization on chromosome 1p34.2 of human ERMAP with the serologically described Scianna blood group antigen system led to the demonstration that the human Scianna and Radin blood groups were encoded by allelic variants of ERMAP. The Sc2 allele is a consequence of the ERMAP(Gly57Arg) polymorphism found at a frequency of approximately 1% in Northern Europeans. Similarly, Radin is a consequence of ERMAP(Pro60Ala), a rarer allele (125).

The two Ig domains and B30.2 domain of mouse Btnl10 show approximately 40% amino acid sequence identity to other butyrophilins, but this protein is otherwise uncharacterized. The human *BTNL10* (*BTN4*) gene locus encodes only a single IgV domain with no transmembrane region, so it may represent a truncated transcript.

Chicken *BG* Genes

A large number of butyrophilin-related *BG* genes have been identified in the genome of the chicken, *Gallus gallus*. As a highly populous domesticated species used for food production, the chicken represents a major economic commodity and an important model for studying infection and immunity. *BG* genes were initially characterized as a serological blood group (known as the B locus), determined by polymorphic erythrocyte antigens, and subsequently shown to be encoded by the *BG* multigene family (126, 127). *BG* gene clusters show structural complexity among haplotypes that is indicative of dynamic expansion and contraction with the production of polymorphic hybrid genes. These features are reminiscent of the structural variation observed at the mouse *Skint* locus and are consistent with selection and rapid evolution (128). BG proteins are disulfide-linked dimers composed of a single extracellular butyrophilin-like IgV domain, a single transmembrane region, and a cytoplasmic tail of variable length, with the notable absence of a B30.2 domain. BG molecules showed complex expression in tissues (128) and have been linked to the selection of B cells (129). Preferential antibody responses have been generated toward minor protein fractions in mixed immunization with BG molecules, which is characteristic of an adjuvant effect (130). Resistance to the T cell lymphomas induced in unimmunized chickens by infection with the herpesvirus GaHV-2 (Marek's disease virus) has been linked to the polymorphic *BG1* locus (131). In addition, a separate chicken butyrophilin-like gene, Tvc-1, has been described as the receptor for avian leukosis virus (132). In summary, chicken *BG* genes are an extensive, highly polymorphic gene family with poorly understood functions.

CONCLUSIONS

Butyrophilins are associated with complex immunological phenomena that are only just beginning to be defined at the molecular level. Distinct members of the butyrophilin family regulate the function of T cells by engaging poorly defined receptors on both αβ and γδ T cells. Positive and

negative regulatory effects of butyrophilin molecules acting on T cells have been described, but receptor–ligand interactions have not been confirmed, making the identification of butyrophilin ligands a priority. It is conceivable that butyrophilin molecules are expressed normally in an inactivate conformation, acting to transmit tolerogenic signals to T cells to function primarily in maintaining immune homeostasis in tissue. By mechanisms that overlap innate immune recognition, cell stress or infection may influence cell-surface expression, in which case the molecules may become activatory. The role of the B30.2 domain in the molecules containing this structure may be crucial to controlling this switch. Some butyrophilins also regulate γδ T cells carrying rearranged T cell receptors, which parallels adaptive mechanisms of immune recognition.

The best-studied examples of butyrophilin function, including Skint1, BTN3A1, Btnl2, and BTN2A2/Btn2a2, indicate that there are similarities between B7-dependent costimulatory interactions and the function of butyrophilins. CD80 and CD86 coreceptor interactions can also deliver positive and negative signaling to T cells by engaging their shared ligands, CD28 and CTLA4. Interactions among B7, CD28, and CTLA4 promote initial T cell activation and TCR signaling through the CD3 complex and also regulate self-tolerance by supporting FOXP3$^+$ Treg homeostasis (133). The structural arrangement of B7 receptors at the cell surface, together with variation in expression and differences in affinities for the T cell ligands, accounts for these complex functions (134). The expression of butyrophilins associated with professional APCs, as well as with tissue epithelium, provides new means for regulating T cell activation and tolerance in peripheral tissues. We speculate that butyrophilins normally act to negatively regulate organ-specific cellular immune responses in a manner similar to the homologous PD-L1 molecule (135) but have evolved novel mechanisms to activate T cells in some settings.

SUMMARY POINTS

1. Butyrophilin receptors are B7-related proteins that modulate the function of T cells.
2. Butyrophilins are structurally diverse, and orthologs among mammalian species are in some cases not found, suggesting rapid evolution by selection.
3. Butyrophilins mediate complex interactions between antigen-presenting cells and T cells, acting through unidentified counter-receptors. Butyrophilins have been shown to promote positive and negative T cell receptor–dependent and –independent signaling.
4. Mouse Btnl2 and Btn2a2 molecules downmodulate T cell activation and induce Foxp3$^+$ T cells, a characteristic of T regulatory cells.
5. The BTN3A lineage is involved in Vγ9Vδ2 T cell activation in humans by a mechanism that remains to be clearly defined.
6. Skint1 is required for the selection and tissue homing of skin-associated Vγ5Vδ1$^+$ dendritic epidermal T cells in mice.

FUTURE ISSUES

1. The identification of counter-receptors is critical for filling the gaps in our knowledge of butyrophilin function. Likewise, the identification of molecules that bind to B30.2 domains, which perhaps act as activating antigen, will also be important.

2. For BTN3A1 and Skint1, direct interactions with γδ T cell receptors with restricted receptor diversity are proposed. Validation of these observations is required in order to eliminate alternative interpretations of the data.
3. A major research theme will be to investigate the mechanisms that regulate butyrophilin expression, and particularly the role of interactions with host and viral gene products.
4. Further work will be required to resolve the molecular basis of the BTN3A–pAg pathway of Vγ9Vδ2 T cell activation.
5. Can butyrophilin molecules downmodulate or activate T cells, depending on their configuration at the cell surface, their interaction with antigens, or both?
6. Does BTN1A1 have an immune-modulatory function in addition to being a structural component in milk?
7. Why are there three human BTN2A and BTN3A molecules? Does each work independently or do they act cooperatively?

DISCLOSURE STATEMENT

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Annual Review of Immunology
Volume 34, 2016

Contents

How One Thing Led to Another
Irving Weissman 1

Mechanisms of Pediatric Inflammatory Bowel Disease
Joanna M. Peloquin, Gautam Goel, Eduardo J. Villablanca, and Ramnik J. Xavier 31

T Cell Fate at the Single-Cell Level
Veit R. Buchholz, Ton N.M. Schumacher, and Dirk H. Busch 65

Transcriptional Control of Dendritic Cell Development
Theresa L. Murphy, Gary E. Grajales-Reyes, Xiaodi Wu, Roxane Tussiwand, Carlos G. Briseño, Arifumi Iwata, Nicole M. Kretzer, Vivek Durai, and Kenneth M. Murphy 93

Genomics of Immune Diseases and New Therapies
Michael Lenardo, Bernice Lo, and Carrie L. Lucas 121

Regulation of Immunity by Butyrophilins
David A. Rhodes, Walter Reith, and John Trowsdale 151

Crystal Formation in Inflammation
Bernardo S. Franklin, Matthew S. Mangan, and Eicke Latz 173

Chemokines and Chemokine Receptors in Lymphoid Tissue Dynamics
Olga Schulz, Swantje I. Hammerschmidt, G. Leandros Moschovakis, and Reinhold Förster 203

Galectins and Immune Responses—Just How Do They Do Those Things They Do?
Sandra Thiemann and Linda G. Baum 243

Variations in MHC Class II Antigen Processing and Presentation in Health and Disease
Emil R. Unanue, Vito Turk, and Jacques Neefjes 265

The Innate Lymphoid Cell Precursor
Isabel E. Ishizuka, Michael G. Constantinides, Herman Gudjonson, and Albert Bendelac 299

Heterogeneity of Human $CD4^+$ T Cells Against Microbes
Federica Sallusto 317

Follicular Helper T Cells
Carola G. Vinuesa, Michelle A. Linterman, Di Yu, and Ian C.M. MacLennan 335

Retinoic Acid and Retinoic Acid Receptors as Pleiotropic Modulators of the Immune System
Alexandre Larange and Hilde Cheroutre 369

Autoantigens as Partners in Initiation and Propagation of Autoimmune Rheumatic Diseases
Antony Rosen and Livia Casciola-Rosen 395

Neuroimmunity: Physiology and Pathology
Sébastien Talbot, Simmie L. Foster, and Clifford J. Woolf 421

Fate Mapping and Quantitation of Hematopoiesis In Vivo
Thomas Höfer, Katrin Busch, Kay Klapproth, and Hans-Reimer Rodewald 449

The Immunology of CD1- and MR1-Restricted T Cells
Lucia Mori, Marco Lepore, and Gennaro De Libero 479

Protein Kinase C Enzymes in the Hematopoietic and Immune Systems
Amnon Altman and Kok-Fai Kong 511

Coinhibitory Pathways in Immunotherapy for Cancer
Susanne H. Baumeister, Gordon J. Freeman, Glenn Dranoff, and Arlene H. Sharpe 539

Exploiting Mucosal Immunity for Antiviral Vaccines
Akiko Iwasaki 575

Tissue Tregs
Marisella Panduro, Christophe Benoist, and Diane Mathis 609

Broadly Neutralizing Antibodies to HIV and Their Role in Vaccine Design
Dennis R. Burton and Lars Hangartner 635

Indexes

Cumulative Index of Contributing Authors, Volumes 24–34 661

Cumulative Index of Article Titles, Volumes 24–34 667

Errata

An online log of corrections to *Annual Review of Immunology* articles may be found at http://www.annualreviews.org/errata/immunol