MAST CELL ACTIVATION AND SIGNALING IN THE AUTOIMMUNE DISEASE
BULLOUS PEMPHIGOID

Lisa A. Heimbach

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Approved by:

Zhi Liu, PhD
Roland Tisch, PhD
Edward Collins, PhD
Jenny P-Y Ting, PhD
Lishan Su, PhD
ASTRACT

LISA HEIMBACH: Mast cell activation and signaling in the autoimmune disease bullous pemphigoid
(Under the direction of Dr. Zhi Liu)

Bullous pemphigoid (BP) is an autoimmune skin blistering disorder primarily observed in the elderly. Autoantibodies directed against the hemidesmosomal protein BP180 trigger a pathological inflammatory response that causes separation of the epidermis from the underlying dermis. Complement, mast cells (MCs), and polymorphonuclear neutrophils (PMNs) are required for disease in experimental BP, an animal model that closely mimics the clinical, immunological and histological features of human BP. In this work, we investigated MC activation and signaling in experimental BP.

MC degranulation occurs downstream of complement activation and the generation of complement component 5a (C5a) in experimental BP. We determined that C5a binds to the C5a receptor (C5aR) on MCs in neonatal mice given disease-inducing antibodies. C5a-C5aR interaction significantly increases phosphorylation of the intracellular signaling protein p38 mitogen-activated protein kinase (p38MAPK). Pharmacologically blocking p38MAPK activation protected mice from MC degranulation and clinical disease. Taken together, we demonstrated that the binding of C5a to C5aR on MCs activates the p38MAPK signaling pathway and leads to MC degranulation and skin blistering.

Upon degranulation, MCs release bioactive compounds from their secretory granules, including tumor necrosis factor-alpha (TNF-α). Here, we report that MC-derived TNF-α is
required for disease in experimental BP. Mice lacking TNF-α globally or in MCs alone fail to recruit sufficient numbers of PMNs to the skin and do not develop clinical blisters following injection with pathogenic anti-BP180 antibodies. C5aR-deficient mice are protected from blistering and do not exhibit elevated TNF-α levels or MC degranulation. TNF-α receptor 1 (TNFR1) expression on MCs is required for development of experimental BP, suggesting that TNF-α acts in an autocrine fashion on MCs.

The findings described in this dissertation refine our understanding of the mechanisms of MC degranulation in experimental BP. MCs are activated by the binding of C5a to C5aR. C5a-C5aR interaction leads to activation of p38MAPK and MC degranulation. MC degranulation releases TNF-α, and TNF-α acts in an autocrine manner on MC TNFR1 to promote disease development. In addition to providing insight into the pathogenesis of BP, our data also suggests that C5a, p38MAPK, and TNF-α may be promising therapeutic targets for treatment of human disease.
This dissertation is dedicated to my husband, Matthew, and my parents, Mike and Kim.

Thank you for your love and support.
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**LIST OF TABLES**

**Table 2.1.** Role of C5a receptor and p38 MAPK in experimental BP. ............................... 61
LIST OF FIGURES

Figure 1.1  Organization of BP180 in human, mouse, and the NC16A+/+ humanized mouse…………………………………………………………………….5
Figure 1.2  Mechanism of subepidermal blistering in experimental BP………………7
Figure 2.1  Level of C5a in the skin is significantly reduced in pathogenic IgG-injected mice lacking the classical pathway of complement………………………….55
Figure 2.2  Mice lacking C5a receptor are resistant to experimental BP……………..56
Figure 2.3  Mice lacking C5aR show impaired p38MAPK activation induced by pathogenic antibodies………………………………………………………57
Figure 2.4  Blocking p38MAPK activation abolishes experimental BP………………58
Figure 2.5  C5a activates p38MAPK of MCs in vivo and in vitro………………………59
Figure 2.6  MC reconstitution restores BP in MC-deficient mice.……………………60
Figure 3.1  Pathogenic anti-mBP180 IgG induces BP blisters associated with an elevated level of TNF-α in mouse skin.……………………………………..84
Figure 3.2  TNF-α-deficient mice are resistant to experimental BP.………………….85
Figure 3.3  Time-course of TNF-α expression during BP disease development in mice…………………………………………………………………………………86
Figure 3.4  MC reconstitution restores BP in TNF-α-/- and MC-deficient mice………87
Figure 3.5  C5aR on MCs are required for TNF-α release and skin blistering………88
Figure 3.6  TNFR1 and not TNFR2 is required for experimental BP…………………..89
Figure 3.7  Local reconstitution of MCs bearing TNFR1 and not TNFR2 restores BP disease in TNFR-deficient mice…………………………………………….90
Figure 3.8  Experimental BP induced by patient anti-BP180 autoantibodies depends on TNF-α……………………………………………………………………..91
Figure 3.9  Proposed model for MC regulation of experimental BP…………………92
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>BMZ</td>
<td>Basement membrane zone</td>
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<tr>
<td>BP</td>
<td>Bullous pemphigoid</td>
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<tr>
<td>BPAG</td>
<td>Bullous pemphigoid antigen</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>C3a</td>
<td>Complement component 3a</td>
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<tr>
<td>C3aR</td>
<td>Complement component 3a receptor</td>
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<td>C5a</td>
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<tr>
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<td>Complement component 5a receptor</td>
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<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonic acid</td>
</tr>
<tr>
<td>CTMC</td>
<td>Connective tissue mast cell</td>
</tr>
<tr>
<td>COLXVII</td>
<td>Collagen seventeen</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DEJ</td>
<td>Dermal-epidermal junction</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>F(ab)</td>
<td>Fragment (antigen binding)</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas associated death domain</td>
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<tr>
<td>Fb</td>
<td>Factor B</td>
</tr>
<tr>
<td>Fc</td>
<td>Fragment, crystallizable</td>
</tr>
<tr>
<td>FcγIIIR</td>
<td>Fragment, crystallizable gamma three receptor</td>
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<tr>
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<td>Description</td>
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<tr>
<td>g</td>
<td>Gravity</td>
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<tr>
<td>GPCR</td>
<td>G protein coupled receptor</td>
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<tr>
<td>GB</td>
<td>Gelatinase B</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony stimulating factor</td>
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<td>GST</td>
<td>Glutathione S-Transferase</td>
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<td>IVIG</td>
<td>Intravenous immunoglobulin</td>
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<td>Hematoxylin and eosin</td>
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<tr>
<td>LC</td>
<td>Langerhans cell</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<td>MAPKKK</td>
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<tr>
<td>mBP180</td>
<td>Murine BP180</td>
</tr>
<tr>
<td>MC</td>
<td>Mast cell</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>MIP-2</td>
<td>Macrophage inflammatory protein 2</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
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<tr>
<td>Symbol</td>
<td>Term</td>
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<td>----------------------------------</td>
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<tr>
<td>μl</td>
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<tr>
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<td>Micromolar</td>
</tr>
<tr>
<td>mMCP4</td>
<td>Mouse mast cell protease 4</td>
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<tr>
<td>MΦ</td>
<td>Macrophage</td>
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<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>MTMC</td>
<td>Mucosal tissue mast cell</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
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<tr>
<td>NC</td>
<td>Non-collagen</td>
</tr>
<tr>
<td>NE</td>
<td>Neutrophil elastase</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T cells</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Nuclear factor-kappa B</td>
</tr>
<tr>
<td>Ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear neutrophil</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>rC5a</td>
<td>Recombinant complement component 5a</td>
</tr>
<tr>
<td>RIP</td>
<td>Receptor-interacting protein</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>SODD</td>
<td>Silencer of death domain</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumor necrosis factor receptor</td>
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<tr>
<td>TRADD</td>
<td>TNF-receptor associated death domain</td>
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<tr>
<td>TRAF</td>
<td>TNF-receptor associated factor</td>
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<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
</tr>
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<td>WT</td>
<td>Wild type</td>
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CHAPTER 1

BACKGROUND AND SIGNIFICANCE

1.1 BULLOUS PEMPHIGOID

Bullous pemphigoid (BP) is a human autoimmune and inflammatory disease first described by the dermatologist Walter Lever in 1953 (1). BP is the most prevalent autoimmune skin blistering disease, with 10 new cases per million population reported annually in the United States (2). Unlike many other autoimmune diseases, BP primarily afflicts men and the elderly, with an average age of onset in the 80s (3). BP is associated with significant morbidity and mortality, with between 10% and 40% of patients dying within three years of disease onset (4, 5). The global disease burden of BP is expected to increase over time as the population ages. Although it is a relatively uncommon disease, BP is particularly useful as a paradigm for an organ-specific autoimmune disease based on the well understood mechanism of pathogenesis.

1.1.1 Clinical Features of Bullous Pemphigoid in Humans

BP is characterized by the presence of tense, fluid filled blisters on the skin (1). Blister formation occurs in areas where the basal keratinocytes of the epidermis detach from the underlying dermis (6). This dermal-epidermal junction (DEJ) separation is associated

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with a cellular inflammatory infiltrate in the upper dermis and the bullous cavity. Eosinophils are the predominant cell type present, along with polymorphonuclear neutrophils (PMNs), lymphocytes, mast cells (MCs), and monocytes/macrophages (7-12). Eosinophils, PMNs, and MCs are thought to be locally activated in the dermis by the multiple inflammatory mediators present in the lesional skin and blister fluid. These mediators include granular proteins derived from degranulated leukocytes, such as eosinophil cationic protein, eosinophil major basic protein, and PMN-derived myeloperoxidase (MPO) (13-15), and chemoattractants and cytokines, such as C5a fragments, histamine, leukotriene B4, interleukin (IL)-1, -2, -4, -5, -6, -8, -15, tumor necrosis factor (TNF)-α, interferon-γ, RANTES, and eotaxin (16-22).

A hallmark of BP is the deposition of autoantibodies and complement components along the basement membrane zone (BMZ) of the skin (23, 24). The humoral autoimmune reaction in BP is directed against components of the hemidesmosome, a cellular adhesion complex that anchors the basal keratinocytes of the epidermis to the underlying extracellular matrix (ECM) (24, 25). A polyclonal repertoire of both circulating and tissue-bound autoantibodies targets two major hemidesmosomal antigens, the 230 kilodalton (kDa) protein termed BP230 or bullous pemphigoid antigen (BPAG) 1, and the 180 kDa protein termed BP180, BPAG2, or type XVII collagen (26-29). BP230 localizes to the hemidesmosomal plaque within the intracellular domain of the keratinocyte (30, 31). BP180 is a type II transmembrane protein, and the amino-terminal region also localizes to the intracellular hemidesmosomal plaque (30, 32, 33). The carboxyl-terminal portion of BP180 extends into the extracellular milieu of the basement membrane zone (BMZ) and interacts with laminin-332 and alpha-6 integrin to promote keratinocyte stability and adhesion (34, 35).
ectodomain of human BP180 consists of 15 collagen domains (designated C1 through C15) interspersed by 16 non-collagen sequences (NC1 through NC16A) (Figure 1.1) (32). NC16A is the largest of the non-collagen regions and is adjacent to the transmembrane domain of BP180. Epitope mapping studies demonstrated that the NC16A domain of BP180 harbors multiple epitopes recognized by autoreactive immunoglobulins (Ig) of IgE and IgG isotypes and IgG1 and IgG4 subclasses (36-40). Serum titers of anti-NC16A autoantibodies are correlated with disease severity (37, 41, 42). Together, these data demonstrate that BP180 is the key autoantigen in BP.

In addition to the humoral response directed against BP180, most BP patients also exhibit T cell mediated autoimmunity as well. Autoreactive CD4+ T lymphocytes expressing memory cell surface markers recognize epitopes within the extracellular region of BP180, mostly clustering within the NC16A domain (43, 44). The autoreactive T cells exhibit a Th1/Th2 mixed cytokine profile. CD4+CD25+Foxp3+ regulatory T cells (Tregs), a subpopulation of T lymphocytes associated with downregulation of autoimmunity (45), do not appear to play a major role in BP pathogenesis. Tregs are present in normal numbers in the circulation of BP patients, and numerous Tregs are found in the lesional and healthy skin of BP patients (46, 47). Tregs isolated from BP patients are also functionally intact and are able to suppress T cell proliferation (47). A recent study found that elevated levels of Th17 cells, a subset of T lymphocytes associated with inflammation and tissue damage in autoimmunity (48), are found in the lesional skin of BP patients; however, no correlation between Th17 cell levels and disease severity were observed (46). Further research into Th17 cell involvement in the pathogenesis of BP is needed.
1.1.2 Animal Models of Bullous Pemphigoid

Efforts to develop an animal model of BP date back to the early 1970s (49). Based on the strong correlation between BP disease severity and serum anti-BP180 and anti-BP230 antibody titers (24), researchers suspected that autoantibodies triggered blister formation. In the first attempt to demonstrate human patient autoantibody pathogenicity in vivo, researchers transfused Rhesus monkeys with plasma from human BP patients (50). Only limited autoantibody deposition was observed at the BMZ, and all three animals failed to develop blisters. Attempts to elicit disease in mice by passively transferring purified BP patient autoantibodies were also unsuccessful (49). Researchers were puzzled by the failure of the experiments, as two related autoimmune intraepidermal blistering diseases, pemphigus vulgaris and pemphigus foliaceus, were successfully modeled in mice using this same passive transfer strategy (51-53).

A breakthrough came in 1993, when Zhi Liu and colleagues determined that the amino acid sequence of NC16A, the human BP180 protein recognized by pathogenic patient autoantibodies, is very poorly conserved in murine BP180 (mBP180) (54). Human patient autoantibodies fail to cross react with the homologous murine antigen, termed NC14A (Figure 1.1), and thus do not trigger disease. Liu et. al. then cloned the NC14A portion of mBP180 and expressed the peptide in DH5α cells as a glutathione S-transferase (GST) fusion protein. New Zealand White rabbits were immunized with the purified mBP180 fusion protein and the polyclonal IgG fraction from the serum was collected.

Neonatal Balb/c mice injected intradermally with purified rabbit anti-mBP180 IgG developed the key hallmarks of human disease, including clinical skin lesions, \textit{in vivo} deposition of rabbit IgG and mouse C3 at the basement membrane by direct
immunofluorescence (IF), dermal-epidermal separation and an extensive inflammatory cell infiltration by H&E staining, with PMNs being the predominant cells (54).

Figure 1.1 Organization of BP180 in human, mouse, and the NC16a+/+ humanized mouse. The human form of BP180 (top) consists of 15 collagen domains (yellow) interspersed by 16 non-collagen domains. The non-collagen domain closest to the cell membrane, NC16A (red), harbors the epitopes recognized by pathogenic autoantibodies. In mouse (middle), BP180 consists of 13 collagen domains and 14 non-collagen domains. NC14A (black) is the murine homolog of NC16A, but the sequence is sufficiently divergent such that antibodies do not cross-react between the two regions. In the humanized NC16A+/+ mouse, the NC16A region replaces the NC14A sequence in murine BP180.

The murine model of experimental bullous pemphigoid developed in 1993 has proven to be an invaluable tool for understanding the etiopathogenesis of disease. The research describing the cellular and inflammatory events that contribute to experimental BP development is reviewed in section 1.3 of this document. However, the rabbit anti-mBP180 IgG passive transfer model does not allow for experimentation with BP autoantibodies isolated from human clinical samples. To assess the pathogenicity of human anti-BP180
autoantibodies, Liu et. al generated a novel mouse strain in which the murine BP180NC14A was replaced with the homologous human BP180NC16A epitope cluster region (Figure 1.1) (55). The humanized NC16A (NC16+/+) mice injected with anti-BP180NC16A autoantibodies purified from human BP patients exhibit in vivo deposition of human IgG and mouse C3 at the basement membrane, dermal-epidermal separation, clinical blistering, and inflammatory cell infiltration. A second humanized animal model was developed by Nishie et. al. that replaces the entire mBP180 protein with the human BP180 homolog (56). Upon injection of human anti-BP180 autoantibodies, these mice (termed COLXVII-humanized) reproduce the DEJ separation at the lamina lucida, the deposition of human IgG along the DEJ, and the inflammatory cell infiltrate consisting of PMNs and eosinophils seen in human disease.

1.1.3 Immunopathogenesis of Experimental BP in the Murine Model

Development of in vivo systems to study an experimental BP model has allowed for great progress in defining the etiopathogenesis of disease. The rabbit anti-mBP180 IgG passive transfer model is easily adapted from use with Balb/c mice to numerous genetically modified mice to explore the requirement for a wide swath of inflammatory mediators in the development of experimental BP. Specifically, the roles of pathogenic antibodies, the complement system, MCs, PMNs, and proteolytic enzymes have been elucidated in the context of the rabbit anti-mBP180 IgG passive transfer model. Figure 1.2 summarizes the key steps identified in the mechanism of subepidermal blistering in experimental BP.
**Figure 1.2 Mechanism of subepidermal blistering in experimental BP.** Anti-BP180 antibodies bind to the NC14A domain of BP180 on basal keratinocytes. Antibody binding triggers complement activation and the generation of C5a. C5a induces MC degranulation and the release of proinflammatory mediators. MΦ are activated. PMNs are recruited to the site of inflammation, and the FcRs on PMNs interact with tissue-bound antibodies. PMNs release proteolytic enzymes, which degrade extracellular matrix proteins and cause blistering.

-Pathogenic Antibodies-

Injection of anti-mBP180 IgG into a normal neonatal mouse is sufficient to initiate subepidermal blister formation, and the levels of circulating anti-mBP180 antibodies tightly control disease onset and severity (54, 57), indicating that IgG is the primary class of antibody that mediates experimental BP. The rabbit immunization protocol used to raise anti-murine BP180 NC14A generates a polyclonal repertoire of antibodies, and not all anti-BP180 NC14A antibodies are pathogenic. The pathogenic epitope of experimental BP was
mapped using a panel of deletion mutants of the mBP180 ectodomain in concert with immunoadsorption and immunoblotting assays (58). These mapping studies demonstrated that pathogenic anti-BP180 antibodies recognize a 9-12 amino acid stretch within the murine BP180 NC14A region of the antigen (58). This epitope overlaps the region of the human BP180 NC16A containing the immunodominant epitopes recognized by human BP autoantibodies, supporting the relevance of the murine system as a model for human disease. Epitope mapping studies in the humanized mouse are not yet published.

---Complement---

Complement activation occurs via three pathways—the classical, lectin, and alternative pathways (59). The classical pathway initiates when antigen-antibody binding induces a conformational change in the Fc portion of the antibody, enabling the antibody to bind C1. The C1r subunit is converted to a serine protease that cleaves C4 and C2 to C4a + C4b and C2a + C2b. C4b associates with C2a to form the C3 convertase, while C4a and C2b diffuse and promote inflammation. The alternative pathway does not involve antibody, but instead initiates when the labile thioester bond in the C3 molecule spontaneously hydrolyses to produce C3a and C3b. C3b associates with factor B, which is cleaved by factor D to form C3bBb, the C3 convertase. The mannose binding lectin pathway is activated when mannose binding lectin binds sugars on the surface of a bacteria. This binding attracts MASP-1 and MASP-2 to interact with the lectin, and leads to the cleavage of C2 and C4 and generation of the C3 convertase akin to that of the classical pathway. All three pathways of complement activation converge in function at the formation of the C3 convertase capable of cleaving C3 to C3a and C3b. C3b associates with the C3 convertase to form the active C5 convertase; this C5 convertase cleaves C5 into C5a and C5b. The anaphylotoxin C5a is a potent
chemoattractant and inflammatory mediator, and acts on a wide range of myeloid and lymphoid lineage cells, including skin MCs (60, 61). C5b binds to the surface of a target cell and initiates the assembly of the membrane attack complex. Subsequent binding of additional serum proteins (C6, C7, C8, C9, sequentially and with many C9s binding) generates an amphiphilic helix that inserts into the lipid bilayer and creates a membrane spanning channel. Disruption of the cell membrane integrity leads to lysis of the cell. C3b is also involved in the innate immune response by directly coating particulate antigen and immune complexes, leading to their enhanced opsonization by macrophages.

Autoantibodies bind to basement membrane antigens and activate complement in human BP. Lesional skin from BP patients exhibits deposition of complement components along the DEJ, and the murine models of experimental BP recapitulates this feature of human disease (23, 54-56). In the rabbit anti-mBP180 IgG passive transfer model, complement activation is required for disease development. Both BALB/c mice depleted of complement by pretreatment with cobra venom and C5-deficient mice are resistant to experimental BP. In turn, reconstitution of the C5-deficient mice with recombinant C5a restores disease susceptibility (62).

F(ab’2) fragments generated from the pathogenic anti-mBP180 IgG cannot induce subepidermal blisters in C5-sufficient mice (62), indicating a key role for the classical pathway of complement activation. Similarly, mice deficient in complement component C4 (specific for the classical and lectin pathways), but not in factor B (specific for the alternative pathway), are resistant to BP (63). Wild-type mice depleted of complement component C1q (also specific for the classical pathway) fail to activate MCs or develop BP skin lesions when injected with pathogenic IgG (63). These data indicate that the classical pathway of the
complement activation plays a major role in development of BP disease through the generation of C5a (64).

-Mast Cells-

MCs are key effector cells involved in innate and adaptive immunity, as well as allergic and anaphylactic reactions (65, 66). MCs are found in particularly high concentrations in the connective tissues just below the epithelial surfaces of the body, such as the submucosal tissues of the gastrointestinal and respiratory tracts and the dermis of the skin, positioning them as key sentinels against external challenges. Upon activation, MCs degranulate and release preformed bioactive compounds from their secretory granules, including histamine, cytokines, proteoglycans, and proteases (67). Together, these inflammatory mediators increase the permeability of local blood vessels and recruit effector cells to the site. In addition to their role in protection against environmental stimuli, MCs are also implicated in chronic inflammatory and autoimmune diseases. Mice lacking MCs are partially or completely protected from disease in animal models of multiple sclerosis, collagen-induced arthritis, and BP (64, 68, 69).

Intact and degranulated MCs are commonly observed in the dermis of BP patients, and histamine and other MC-derived factors are present in high concentrations in the affected skin (12, 13, 18, 70). Mice injected with pathogenic anti-mBP180 antibodies exhibit extensive MC degranulation in the lesional skin, similar to that observed in human BP (12, 64). MC-deficient mice are resistant to experimental BP, but reconstitution of these mice with MCs restores susceptibility to disease. MC activation precedes PMN infiltration, and either the absence of MCs or the inhibition of MC degranulation prevents PMN infiltration and blister formation. However, MC-deficient mice reconstituted locally with PMNs, or
injected locally with PMN chemoattractants IL-8 or C5a form blisters in response to anti-mBP180 IgG. These results suggest that MCs release proinflammatory cytokines critical for PMN recruitment (64).

Skin-resident MCs function in both an IgE dependent and IgE independent manner. The IgE independent activation of MCs can be mediated by products of complement activation, proinflammatory cytokines, Toll-like receptors (TLRs), or by Fc receptors (71). Skin MCs express C5a receptor (C5aR, or CD88) (72), and interaction of C5a and C5aR induces MC degranulation and the release of proinflammatory cytokines.

-Neutrophils-

PMNs, produced in vast numbers in the bone marrow, circulate in the blood and act as the front line of defense against microorganisms that breach skin and mucous membrane barriers. PMNs extravasate into tissue under the direction of local endothelial cells, which are activated by inflammatory signals such as TNF-α, IL-1β, or IL-17. (73). The activated local endothelial cells express the adhesion molecules P-selectin, E-selectin, intracellular adhesion molecule (ICAM)-1, ICAM-2, and vascular adhesion molecule (VCAM)-1, which engage their ligands on the PMNs to capture rolling PMNs from the circulation. The endothelial cells also produce the PMN chemoattractant and activators IL-8 and macrophage inflammatory protein 2 (MIP-2) (73). PMNs have a wide range of effector capabilities, including phagocytosis of microbes, production of reactive oxygen species, release of lytic enzymes, synthesis of both proinflammatory and anti-inflammatory mediators, and capture of microbes via fibrillar networks termed neutrophil extracellular traps (NETs) (74-76). PMNs are involved in the pathology of numerous chronic inflammatory and autoimmune disorders,
including chronic obstructive pulmonary disease, systemic lupus erythematosus, small vessel vasculitis, inflammatory arthritis, and BP (77-82).

PMN infiltration is required for the development of experimental BP, and there is a strong correlation between severity of disease and number of infiltrating PMNs (80). Depletion of PMNs with anti-PMN antibodies, or disruption of the events upstream of PMN infiltration (namely complement activation and MC degranulation), renders mice resistant to BP blister formation. However, complement system impairments and MC deficiencies do not block the pathological effects of BP180 autoantibodies unless PMN infiltration is prevented as a consequence of the deficiency. Mice deficient in complement or MCs are still susceptible to experimental BP when PMNs are recruited by injection with IL-8 or rC5a (63, 64). Thus, complement activation and MC degranulation mediate disease progression by promoting PMN recruitment to the skin. These data demonstrate that infiltrating PMNs are the critical cells that directly mediate tissue injury at the DEJ, leading to BP skin blisters.

**-Proteolytic Enzymes-

PMNs promote skin injury by release of proteolytic enzymes. The FcγIIIR on the cell surface of infiltrating PMNs interacts with the Fc portion of the tissue-bound pathogenic anti-mBP180 IgG (83). PMNs activated by the Fc-FcγIIIR molecular interaction secrete proteolytic enzymes known to degrade the ECM, including neutrophil elastase (NE) and gelatinase B (GB, also called matrix metalloproteinase-9) (83). A lack of either of these two enzymes blocks blister formation in mice (84, 85). *In vitro*, although both GB and NE are capable of degrading the recombinant BP180 protein, only NE produces DEJ separation when incubated with skin sections (84, 86). *In vivo*, the degradation of BP180 depends on NE activity (87). These findings suggest that GB is the upstream actor that proteolytically
inactivates the physiological inhibitor of NE (α1-proteinase inhibitor), which allows for unmitigated NE cleavage of ECM proteins (including BP180), resulting in DEJ separation (88). MCs also release proteolytic enzymes upon degranulation (67). Mouse mast cell protease-4, the likely murine homolog of human MC chymase, directly degrades BP180, and also activates GB (89).

-Other Inflammatory Cells-

Anti-mBP180 autoantibodies trigger experimental BP with equivalent severity in WT mice and mice deficient in T and B lymphocytes (90), suggesting that B and T cells do not play a role in this animal model. The limitations of the passive transfer injection model prevent investigation of the events that precipitate loss of tolerance and the generation of autoreactive B cells. Indeed, little is understood about the etiology of BP or lymphocyte regulation. Macrophages (MΦ) play an accessory role to MCs in PMN recruitment upon pathogenic antibody injection (90). MΦs are activated downstream of MCs, and they serve to amplify the PMN recruitment mediated by MCs.

1.1.4 Current Therapeutic Strategies For Treatment of Human BP

The mortality rate for BP is high, reported to be between 12% and 40% in the first three years after diagnosis (4, 5). The conventional therapy for BP over the past several decades has been high-dose, long-term systemic oral corticosteroids and immunosuppressive agents (91). However, long term use of corticosteroids is associated with deleterious health effects, including severe infection, diabetes mellitus, osteoporosis, hypertension, renal failure, lymphoma, and squamous cell carcinoma (92). The negative side effects of immunosuppression therapy may account for a significant portion of the morbidity and
mortality associated with BP. Even when disease is successfully treated, up to 45% of patients suffer a relapse within six months of cessation of therapy (93), exacerbating the need for novel therapeutic interventions.

In the past ten years, clinical management of BP has shifted away from oral corticosteroids in favor of topical corticosteroids (94). However, topical treatments are not always feasible for cases of extensive blistering, particularly in elderly patients who may be bedridden or without the mobility to apply medications regularly.

Patients who fail both oral and topical corticosteroid treatment may be treated with biologicals such as intravenous immunoglobulin (IVIG) or rituximab (95). Patients treated with IVIG receive huge amounts of pooled IgG isolated from thousands of healthy human donors (96). The exact mechanism of IVIG is debated, however, there is compelling evidence that IVIG decreases the prevalent of pathogenic antibodies in circulation by increasing the rate of catabolism of IgG (97). Rituximab targets the CD20 molecule on the surface of B cells, depleting the antibody producing cells from circulation (98). No randomized controlled trials have been performed for either of these therapies yet, but anecdotal evidence suggests that these biologic approaches to treatment of BP offer treatment alternatives for patients (99, 100).

1.2 MAST CELL BIOLOGY

MCs are innate immune cells primarily distributed in tissues that contact the external environment, such as the skin, airways, and the intestinal mucosa (101). While widely known as the culprits in type I hypersensitivity responses, MCs are now known to participate in host defense, modulation of innate and adaptive immune responses, angiogenesis, wound
healing, and tissue homeostasis and remodeling (102). Better understanding the mechanisms of MC activation, signaling, and cross-talk with other members of the immune system presents new opportunities for treatment of human disease.

1.2.1 MC Development and Function in the Skin

MCs develop from hematopoietic stem cells (HSC), the common precursor to lymphocytes, granulocytes, and monocytes. The HSC precursors develop into CD34+/CD117 (or cKit+)/CD13+ multipotent hematopoietic progenitors (103, 104). Committed MC progenitors exit the bone marrow, circulate in the peripheral blood, and undergo final maturation under the direction of microenvironmental cues from the tissue where the MCs stake long-term residence (105-109). Final MC maturation in the peripheral tissues is regulated by the MC growth factors stem cell factor (SCF, or c-kit ligand) and IL-3 (110, 111), as well as tissue specific factors. MC populations exhibit considerable heterogeneity between tissue sites, based on tissue-specific final maturation.

The identities of the factors that promote homeostatic MC progenitor recruitment to the skin and regulate development are not known (112). In mice, nearly all skin MCs are connective tissue MCs (CTMCs) and have granules rich with histamine, tryptase, chymase, carboxypeptidase and cathepsin G-like proteinase, while MCs in the bowel mucosa and airways are predominantly mucosal tissue MC (MTMCs) and have granules that contain either tryptase and histamine or chymase, carboxypeptidase, and histamine but not tryptase (113-115). This same pattern of tissue-based protease distinction is observed in human MCs. Skin and peritoneal cavity MCs are categorized as MC<sub>CT</sub>, indicating that the granules contain both tryptase and chymase (116).
MCs contribute to host defense by acting as sentinels against pathogens that breach the epidermal barrier. MCs are distributed throughout the dermis, but are greatly enriched in the area immediately below the DEJ, a site dense with small blood vessels (117). This proximity to the microvasculature enables MCs to act rapidly both locally and systemically. Localization to the DEJ also enables MCs to readily communicate with the two main sentinels of the epidermis, the keratinocytes (KCs) and Langerhans cells (LC), a class of specialized dendritic cell (DC) (118). KCs and LCs produce a battery of antimicrobial peptides, chemotactic proteins, and cytokines, including IL-1 (α and β) and TNF-α (119-121). IL-1 stimulated MCs secrete IL-3, IL-6, IL-9, and TNF-α, and TNF-α stimulated MCs secrete IL-1 and upregulate FcγR expression (122). Together, these proinflammatory factors can act on endothelial cells to promote local inflammation and recruit leukocytes to the skin, including perhaps additional MC progenitors (112, 123).

TLRs enable MCs to directly respond to microbial agents than penetrate beyond the 4 to 5 cell layers of the epidermis and into the dermis (71). Extensive experimental evidence demonstrates that MCs respond to bacterial membrane components such as lipopolysaccharide (LPS) and peptidoglycan (PGN) via TLR-4 and TLR-2. Mouse MCs stimulated with LPS or PGN secrete TNF-α and IL-6 without degranulation in a TLR-dependent manner, and mice lacking TLR-4 fail to recruit sufficient PMNs to clear bacterial infections (124-129). In vitro evidence suggests roles for TLR-9, TLR-3, TLR-1, and TLR-6 in MC recognition of pathogen associated molecular patterns (130-134).

MCs are able to indirectly respond to pathogens via their complement receptors (discussed in Section 1.2.2) and their Fc receptors. FcγRs on MCs bind the Fc portion of
pathogen-specific antibodies, and cross-linking of the FcγRs by the polyvalent antigen on the pathogen results in MC degranulation and release of proinflammatory mediators (135, 136). FcR signaling can also activate MCs in response to bacterial superantigens, such as *Streptococcus aureus* protein A (137).

### 1.2.2 C5aR mediated MC Activation

The anaphylatoxin C5a interacts with two C5a receptors, C5aR (also called CD88) and the C5a receptor-like 2 (C5L2) (61). Both C5aR and C5L2 are broadly expressed by cells of both myeloid and non-myeloid lineages, including MCs (61, 138-142). C5aR is the classically described ligand for C5a, while C5L2 was considered until very recently to be a non-signaling decoy receptor that served to dampen C5a-mediated inflammation (141). Recent studies have uncovered a functional signaling role for C5L2 (143, 144), however, the vast majority of the understood C5a-mediated signaling is facilitated by C5aR.

C5a is a G protein coupled receptors (GPCRs) (145, 146). Members of the GPCR family are characterized by their seven transmembrane-spanning domains, and they function by coupling to intracellular heterotrimeric G proteins (147). The G protein is comprised of an α, β, and γ subunit. When inactive, the G-α subunit is bound to GDP, and is associated with the β and γ subunits. GPCR ligation causes a conformational change to the intracellular portion of the receptor, enabling it to act as a guanine nucleotide exchange factor. The G-α releases GDP and binds GTP, which causes the β and γ subunits to dissociate from G-α. G-α and G-βγ activate second messengers, including cyclic AMP, phospholipase C, and phosphoinositide 3-kinase. Ultimately, the intracellular signaling cascade leads to production
of the transcription factors nuclear factor κB (NFκB), nuclear factor of activated T cells (NFAT), and activation protein-1 (AP-1), which modulate gene expression (148).

In the case of pathogen assault, complement activation may occur by the classical or mannose binding lectin pathways. While many inflammatory cells (notably PMNs) express complement receptors, MCs are optimally positioned at the DEJ to respond to C5a. MCs express other complement-component receptors, including C3aR (the ligand for C3a) and CR3 (the ligand for C3b), however, degranulation occurs primarily as a result of activation of C5aR (71). Degranulation results in release of TNF-α, histamine, proteases, GM-CSF, IL-6, IL-1, and leukotriene C (71).

1.2.3 p38MAPK Signaling in MCs

p38MAPK is a key signaling molecule involved in translating extracellular environmental conditions, particularly inflammatory conditions, into cellular responses (149). There are four isoforms of p38MAPK, designated –α, -β, -γ, and –δ; MCs express the –α form exclusively (150). p38MAPK is a serine/threonine kinase, thus, it exerts biological effect by phosphorylating proteins.

p38MAPK can be activated by numerous extracellular stimuli, including endotoxins, reactive oxygen species, ultraviolet radiation, TNF-α, IL-1, and TGF-β (151). Upstream of p38MAPK, interaction, mitogen-activated protein kinase kinase kinases (MAPKKKs) are activated in a GTPase-dependent manner. The MAPKKKs phosphorylate the MAPK kinases MKK3, MKK4, and MKK6, which in turn activate p38 via phosphorylation of the Thr180 and Try 182 residues. Activated p38MAPK facilitates the stabilization and increased
transcription of mRNAs for proinflammatory cytokines and receptors associated with inflammation (151).

The p38MAPK pathway plays a key role in numerous immune and inflammatory diseases, including rheumatoid arthritis and the skin autoimmune disease pemphigus vulgaris (152-154). In PMNs and monocytes, the interaction of C5a and C5aR leads to activation of p38MAPK (155-157). MC activation via antigen crosslinking of surface IgE molecules leads to p38MAPK activation and cytokine release (158), however, p38MAPK activation upon engagement of the C5aR on MCs has not yet been reported.

1.2.4 TNF-α mediated MC Activation

TNF-α has been exhaustively described as a product released by MCs (159). Less is known, however, about the effect of TNF-α on MCs themselves. MCs stimulated by KC and LC-derived TNF-α secrete IL-1 and upregulate FcγR expression (122, 160). In cultured human lung MCs stimulated with SCF and anti-IgE, TNF-α was shown to feed back on the MCs to promote enhanced NF-kB activation (161). NF-kB promotes transcription of a wide array of pro-inflammatory cytokines, suggesting that a positive autocrine feedback loop may enable MCs to drive inflammation.

TNF-α signals through the TNF-α receptors (TNFRs) TNFR1 and TNFR2, though TNFR1 mediates the majority of the biological activities of TNF-α (162). Ligation of TNFR1 releases the inhibitory protein silencer of death domains (SODD) from TNFR1’s intracellular domain (ICD), which enables ICD to be recognized by the adaptor protein TNF receptor-associated death domain (TRADD) (163). TRADD serves as a dock for additional adaptor proteins that recruit signaling enzymes to the signaling complex. These adaptors
include TNFR-associated factor 2 (TRAF2), which can activate MAPKKS, including MEKK1 and ASK1. The adaptor Fas-associated death domain (FADD) recruits caspase-8 to the signaling complex, where it promotes progression of apoptosis. Receptor-interacting protein (RIP) is yet another adaptor recruited by TRADD, and RIP mediates NF-kB activation.

1.2.5 MCs in Autoimmunity

MCs are potent effector cells in the innate immune response to infection in large part due to the many avenues that lead to MC activation. However, inappropriate MC activation outside of the context of pathogen invasion can result in significant pathology and autoimmunity (164). The role of MCs in BP is described in Section 1.1.3. In multiple sclerosis, MCs accumulate in the central nervous system plaque lesions and at sites of inflammatory demyelination (165-167). MC-deficient mice are protected from experimental autoimmune encephalomyelitis, an animal model of MS, further supporting the case of MC involvement in disease (69). Similarly, MC-deficient mice are not susceptible to autoantibody-induced arthritis (68). In human rheumatoid arthritis patients, elevated numbers of MCs are present in the synovial compartments, along with high levels of MC-products including TNF-α, IL-1, IL-17, and tryptase.(168, 169). There is also evidence that MCs contribute to pathology in Type 1 diabetes. Cromolyn, a compound that prevents MC degranulation, significantly delays onset of disease in the spontaneously diabetic BioBreeding mouse (170). Furthermore, activated MCs are found in the pancreatic lymph nodes of diseased mice (170).
In the case of experimental BP, the data are clear: MCs attract PMNs to the skin, and PMNs do the damage (see section 1.1.3). However, MCs interact with many types of immune cells, making the mechanisms by which MCs regulate autoimmunity far more complex than the experimental BP model predicts. MC-derived products are known to influence the migration, maturation, and function of DCs, effector T cells and B cells (171, 172). Intriguingly for the consideration of autoimmunity, MCs also interact with Tregs and Th17 cells (164). One well-defined avenue of Treg-MC interaction occurs via OX40, a molecular constitutively expressed on MCs, and OX40L, a protein constitutively expressed by Tregs. When OX40-OX40L interact, MCs can impair Treg suppression of effector T cells, and Tregs are able to downregulate FceR1 expression on MCs and prevent FceR1-mediated degranulation(173-175). MCs can produce Th17/Treg polarizing cytokines, including IL-6, IL-21, IL-23, and TGFβ. In vitro, MCs can drive Treg or Th17 cell differentiation (175). Conceivably, MCs could perform this same polarizing role at sites of secondary activation and T cell priming.

1.3 SUMMARY AND CONCLUDING REMARKS

The murine IgG passive transfer model of BP developed by Liu et al in 1993 has facilitated dissection of the mechanism of disease progression in experimental BP. After nearly 20 years of experimentation, it is clear that BP pathogenesis proceeds through activation of MCs, but the mechanism of MC activation and the consequences of this activation are not clear. MCs express a number of types of receptors capable of responding to proinflammatory ligands, including C5aR. Taken with the evidence that complement
activation occurs upstream of MC degranulation, the C5aR is a promising candidate for facilitating MC signaling.

The primary objective of this dissertation was to elucidate the cellular events that occur immediately upstream and downstream of MC degranulation in experimental BP. Our central hypothesis is that complement components activate MCs, and proinflammatory compounds released from the MCs drive pathology and blistering. In chapters 2 and 3, we described studies that test this hypothesis using a series of knockout mice and MC reconstitution experiments. We found that MCs are activated by the binding of C5a to C5aR. C5a-C5aR interaction leads to activation of p38MAPK and MC degranulation. MC degranulation releases TNF-α, and TNF-α acts in an autocrine manner on MC TNFR1 to promote disease development. Our findings highlight the potential for cross-talk between the complement and TNF-α mediated pathways of MC activation, and also support three potential therapeutic targets for treatment of BP.
1.4 REFERENCES


Bullous pemphigoid (BP) is an autoimmune skin blistering disease characterized by the presence of autoantibodies against the hemidesmosomal proteins BP230 and BP180. In the IgG passive transfer mouse model of BP, subepidermal blistering is triggered by anti-BP180 antibodies and depends on the complement system, mast cell (MC) degranulation, and neutrophil infiltration. In this study, we identify the signaling events that connect the activation of the complement system and MC degranulation. We found that mice deficient in MCs or C5a receptor (C5aR) injected with pathogenic anti-BP180 IgG failed to develop subepidermal blisters and exhibited a drastic reduction in p38MAPK phosphorylation compared to wild-type (WT) mice. Local reconstitution with MCs from WT but not C5aR-deficient mice restored high levels of p38MAPK phosphorylation and subepidermal blistering in MC-deficient mice. Local injection of recombinant C5a (rC5a) induced phosphorylation of p38MAPK in WT, but not MC-deficient mice. Cultured mouse MCs treated with rC5a exhibited a significant increase of p38MAPK phosphorylation and MC degranulation. Taken together, these data demonstrate that C5a interacts with C5aR on MCs,
and that this C5a-C5aR interaction triggers activation of the p38MAPK pathway, subsequent MC degranulation, and ultimately BP blistering.

2.1 INTRODUCTION

Bullous pemphigoid (BP) is an acquired skin autoimmune blistering disease characterized by the presence of circulating and tissue bound autoantibodies against two major hemidesmosomal proteins, BP230 (BPAG1) and BP180 (BPAG2 or type XVII collagen) (1-11). These anti-hemidesmosomal autoantibodies, along with complement components, are deposited along the basement membrane at the dermal-epidermal junction (DEJ) of perilesional skin (12-14). Basal keratinocytes detach from the underlying dermis, leading to blister formation (5). Using an animal model of BP in which neonatal BALB/c mice are injected with anti-murine BP180 (mBP180) antibodies, we previously demonstrated that dermal-epidermal separation is triggered by anti-BP180 IgG and depends on complement activation, mast cells (MCs), and neutrophils (PMNs) (15-17). The binding of pathogenic IgG to BP180 activates the classical pathway of the complement and leads to MC degranulation, PMN recruitment, and subsequent skin blistering (18). However, the cellular processes linking complement activation and MC degranulation are not defined in BP.

Complement activation via the classical, alternative, and lectin pathways generates the anaphylotoxin C5a (19). A potent chemoattractant and inflammatory mediator, C5a acts on a wide range of myeloid and lymphoid lineage cells, including skin MCs (20, 21). Skin MCs express C5a receptor (C5aR, or CD88) (22), and interaction of C5a and C5aR induces MC degranulation and the release of proinflammatory cytokines such as interleukin 1 (IL-1), IL-6, tumor necrosis factor alpha (TNF-α), and granulocyte macrophage colony stimulating...
factor (GM-CSF) (23). In PMNs and monocytes, the interaction of C5a and C5aR leads to activation of p38 mitogen activated protein kinase (p38MAPK) (24-26). p38MAPK is a key signaling molecule involved in translating extracellular environmental conditions, particularly inflammatory conditions, into cellular responses (27, 28). MC activation via antigen crosslinking of surface immunoglobulin E (IgE) molecules leads to p38MAPK activation and cytokine release (29), however, p38MAPK activation upon engagement of the C5aR on MCs has not yet been reported.

In this work, we examined the role of C5a-C5aR interaction in the progression of experimental BP. We found that C5a interacts with C5aR on MCs, and that this C5a-C5aR interaction triggers activation of the p38MAPK pathway and ultimately induces MC degranulation and subsequent BP blistering.

2.2 EXPERIMENTAL PROCEDURES

Materials. The p38MAPK inhibitor SB203580 and the inactive analog SB202474 were purchased from Calbiochem (La Jolla, CA). Rabbit anti-phospho-p38MAPK antibody was purchased from Cell Signaling (Danvers, MA), and rabbit anti-p38MAPK antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The recombinant C5a was purchased from R&D System (Minneapolis, MN). Protein concentration was determined with the RC DC protein assay purchased from Bio-Rad Laboratories (Hercules, CA). The ECL Western blotting analysis kit was purchased from GE Healthcare (Piscataway, NJ).

Laboratory animals. Breeding pairs of C57BL/6J, C4-deficient (C4-/-), MC-deficient WBB6F1-Kit+/Kit+/v (MC-/-), and C5a receptor-deficient (C5aR-/-) mice were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained at the University of
North Carolina at Chapel Hill. The MC deficiency in Kit^{w'/Kit^{w''}} mice is caused by distinct mutations in MC c-Kit (30). Neonatal mice (24-36 hours old with body weights between 1.4 and 1.6 grams) were used for passive transfer experiments. All animal care and animal experiments were approved by the UNC Animal Care Committee and were in accordance with the National Institutes of Health guidelines.

**Preparation of pathogenic anti-BP180 IgG.** The preparation of recombinant murine BP180 (mBP180) and the immunization of New Zealand white rabbits were performed as previously described (31-33). Briefly, a segment of the ectodomain of the murine BP180 antigen (31) was expressed as a glutathione S-transferase (GST) fusion protein using the pGEX prokaryotic expression system (Pharmacia LKB Biotechnology, Piscataway, NJ). The murine BP180 fusion protein, designated GST-mBP180ABC containing NC14A and half of C13 domains (**Figure 1.1**), was purified to homogeneity by affinity chromatography using a glutathione-Agarose column (33). New Zealand White rabbits were immunized with the purified mBP180 fusion protein and the IgG fraction from the serum (designated R621) was purified as previously described (31). The IgG fractions were concentrated, sterilized by ultrafiltration and the protein concentrations determined by OD_{280} [E (1%, 1 cm) = 13.6]. The titers of anti-murine BP180 antibodies in both the unfractionated rabbit serum and in the purified IgG fraction were assayed by indirect immunofluorescence (IF) using mouse skin cryosections as substrate. The antibody preparations were also tested by immunoblotting against the GST-mBP180ABC fusion protein. The pathogenicity of these IgG preparations was tested by passive transfer experiment as described below.
**Induction of experimental BP and clinical evaluation of animals.** Neonates were given on the back one i.d. injection of a sterile solution of IgG in PBS (50 μl IgG, 2.5 mg IgG/g body weight) as described elsewhere (31). The skin at the IgG-injection site of the mice from the test and control groups was examined at different time points after the IgG injection. The extent of cutaneous disease was scored as follows: "(−)", no detectable skin disease; "1+", mild erythematous reaction with no evidence of the epidermal detachment sign [this sign was elicited by gentle friction of the mouse skin which, when positive, produced fine, persistent wrinkling of the epidermis]; "2+", intense erythema and epidermal detachment sign involving 10-50% of the epidermis in localized areas; and "3+", intense erythema with frank epidermal detachment sign involving more than 50% of the epidermis in the injection site.

After clinical examination, the animals were sacrificed, and skin and serum specimens were obtained. Skin sections were used for a) routine histological examination by light microscopy (H/E staining) to localize the lesional site and PMN infiltration, b) toluidine blue staining to quantify MCs and MC degranulation, c) direct IF assays to detect rabbit IgG and mouse C3 deposition at the BMZ, and d) myeloperoxidase (MPO) enzymatic assay to quantify the PMN accumulation at the skin injection site described below. The sera of injected animals were tested by indirect IF techniques to determine the titers of rabbit anti-mBP180 antibodies using mouse skin cryosections as the substrate. Direct and indirect IF studies were performed as previously described (31) using commercially available FITC-conjugated goat anti-rabbit IgG (Kirkeggard & Perry Laboratories Inc.). Monospecific goat anti-mouse C3 IgG was purchased from Cappel Laboratories.
**Quantification of PMN accumulation at the skin site.** MPO activity in skin sites of the injected animals was assayed as a measure of PMN infiltration as described (34, 35). A standard reference curve was established using purified MPO (Athens Research and Technology, Inc., Athens, Georgia). The skin samples were extracted by homogenization in an extraction buffer containing 0.1 M Tris-Cl, pH 7.6, 0.15 M NaCl, 0.5% hexadecyl trimethylammonium bromide. MPO activity in the supernatant fraction was measured by the change in optical density at 460 nm resulting from decomposition of H$_2$O$_2$ in the presence of $o$-dianisidine. MPO content was expressed as units of MPO activity/mg protein. Protein concentrations were determined by the Bio-RAD dye-binding assay using bovine serum albumin (BSA) as a standard.

**Determination of C5a levels in skin.** Skin from the IgG injection site of the diseased and control mice was mechanically homogenized in PBS to extract proteins. The level of mouse C5a in the skin was measured by ELISA (R&D Systems, Minneapolis, MN). Microtiter plates were coated with a rat anti-mouse C5a antibody, incubated with skin protein extracts, followed by goat anti-mouse C5a detection antibody, and then developed and read at OD$_{492}$nm. The C5a level was expressed as OD$_{492}$ reading/mg protein.

**Quantification of MCs and MC degranulation.** MCs and MC degranulation in skin samples were quantified according to Wershil, et al. (36), with modification. Briefly, lesional and nonlesional skin sections of IgG-injected mice were fixed in 10% formalin. Paraffin sections (5-μm thick) were prepared and stained with toluidine blue and H&E. The total number of MCs was counted and classified as degranulated (>10% of the granules exhibiting fusion or discharge) or normal in five fields under a light microscope as described previously. The results are expressed as percentage of MC degranulation.
**MC reconstitution.** Kit<sup>w</sup>/Kit<sup>w</sup><sup>-/-</sup> mice were repaired of their MC deficiency selectively and locally by the injection of growth factor-dependent bone marrow-derived cultured MCs into the skin (15, 37, 38). Briefly, femoral bone marrow cells from WT and C5aR<sup>-/-</sup> mice were maintained in vitro for 4 weeks in RPMI 1640 complete medium (Life Technologies, Grant Island, NY) supplemented with 20% WEHI-3-conditioned medium until MCs represented >95% of the total cells as determined by toluidine blue staining and flow cytometry analysis using antibodies specific for the MC cell surface markers FceRI, c-Kit, and CD13 (37). Murine IgE and rat anti-mouse IgE were purchased from Southern Biotechnology Associates (Birmingham, AL). FITC-labeled rat anti-mouse c-Kit and FITC-labeled rat anti-mouse CD13 were obtained from DB Pharmingen (San Diego, CA). MCs (1x10<sup>6</sup> in 20 μl of medium) were injected i.d. into the ears of 8 to 10 week old MC-deficient mice. Medium alone (20 μl) was injected i.d. into the ears of MC<sup>-/-</sup> and MC<sup>+/+</sup> mice to serve as negative controls. This procedure selectively and locally reconstitutes the dermal MC population without systemic effects (38). MC reconstitution was confirmed by staining skin sections from MC-injected sites with toluidine blue. Ten weeks after the adoptive transfer of MCs, when the injected MCs were fully matured into functional cutaneous MCs (15, 37, 38), both ears of the mice were injected i.d. with pathogenic anti-BP180 IgG (2 mg/20 μl/site). 24 hours later, ear skin biopsies were obtained and analyzed by H/E, toluidine blue staining and MPO enzyme assay as described above.

**In vivo and in vitro inhibition of p38MAPK activation.** For the in vivo inhibitor studies, mice were pretreated with 50 μl of the p38MAPK inhibitor SB203580 or inactive analog SB202474 (i.d., 10 μg/g body weight). Two hours later, the mice were injected i.d. with 50 μl of pathogenic IgG or control IgG (2.5 mg/g body weight) and the mice were
examined at different time points for BP skin lesions and p38MAPK activation (as described below). For in vitro inhibitor studies, cultured bone-marrow-derived MCs from C57BL/6J mice were incubated with C5a (25 ng/ml) in the presence of p38MAPK inhibitor SB203580 (20 μM) for 0-60 min and cell extracts were assayed for p38MAPK activation.

**Detection of total and phospho-p38MAPK.** Protein was extracted from skin samples in lysis buffer (8 M urea, 4% 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonic acid (CHAPS), 2.5 mM dithiothreitol, 40 mM Tris, 10 μM pepstatin, 100 μM leupeptin, 10 μM E-64, 1 mM phenylmethylsulfonyl fluoride, 0.1 M sodium orthovanadate, 0.5 mM sodium fluoride, and okadaic acid). For the cell culture studies, bone marrow-derived MCs were incubated with buffer control or C5a (25 ng/ml) in the absence or presence of SB203580 (20 μM) for 0-60 min at 37°C. The cells were harvested and lysed on ice, and 20 μg of each protein extract was loaded on a 10% acrylamide gel and separated by SDS-PAGE. Gels were transferred to PVDF (Millipore) membranes and probed by immunoblot for total p38MAPK and phospho-p38MAPK. Western blots were developed by enhanced chemiluminescence (ECL) reaction (Amersham Biosciences). The signal intensity was quantified by scanning chemiluminescence on a GeneGnome scanner (Syngene Bio Imaging, Frederick, MD) using GeneSnap software.

**Statistical analysis.** The data are expressed as mean ± SEM and were analyzed using the Student's paired t-test. A p value less than 0.05 was considered significant.
2.3 RESULTS

Mice lacking the classical pathway of the complement system are resistant to experimental BP and exhibit a reduced level of skin C5a. We previously reported that the classical pathway of complement activation is critical to the pathogenesis of experimental BP in mice (18). Wild-type (C4+/+) mice and mice deficient in the alternative pathway component factor B (Fb-/-) developed subepidermal blisters following injection with pathogenic anti-mBP180 IgG; however, mice deficient in the classical pathway component C4 (C4-/-) were resistant to blister formation (Fig. 2.1A and Table 2.1). 24 hours following injection, C4-/- mice had significantly lower levels of C5a present in the skin compared to WT and Fb-/- mice (p<0.0001, Fig. 2.1B). Significantly fewer infiltrating PMNs were present in the skin of the C4-/- mice than in the WT or Fb-/- mice, as determined by quantification of the PMN cell marker myeloperoxidase (MPO) enzymatic activity (Fig. 2.1C). These results demonstrate that complement activation via the classical pathway is essential for generation of C5a, recruitment of PMNs to the skin, and blistering in experimental BP.

Mice lacking C5a receptor are resistant to experimental BP and exhibit impairment in MC degranulation and p38MAPK activation. C5a fragments primarily act through binding to the C5a receptor (C5aR, or CD88) (39). Having determined that high levels of C5a are present in the skin of mice susceptible to experimental BP, we next examined the role of the C5aR in disease development. We injected C5aR-deficient mice (C5aR-/-) and WT mice (C5aR+/+) with pathogenic anti-BP180 antibodies and found that C5aR+/+ mice developed blisters, but C5aR-/- mice did not (Figs. 2.2A and Table 2.1). Although C5aR-/- mice failed to develop skin lesions, the amount of C5a present in their skin was comparable
to the WT mice at 4 h post IgG injection (Fig. 2.2B), when the early phase of PMN infiltration begins (17). These data demonstrate that C5aR deficiency does not affect C5a generation. However, MC activation in the skin of the pathogenic IgG-injected C5aR-/- mice was severely impaired as evidenced by toluidine blue staining, a MC-specific histochemical staining. At 2 h post-injection when MC degranulation triggered by pathogenic IgG reaches its peak in experimental BP (15), extensive MC degranulation was observed in the dermis of WT mice (Fig. 2.2C, left panel). In contrast, C5aR-/- mice exhibited a minimal level of MC degranulation in the dermis (Fig. 2.2C, right panel). We quantified MC degranulation and found a significant reduction in the number of degranulating MCs in C5aR-/- mice compared to WT mice (p<0.001, Fig. 2.2D). Because MC degranulation releases cytokines that recruit PMNs to the skin during experimental BP pathogenesis (15), we hypothesized that the C5aR-/- mice would be unable to effectively attract PMNs to the skin. Indeed, the pathogenic IgG-injected C5aR-/- mice showed a significantly reduced PMN infiltration in the skin as compared to the diseased WT mice (Fig. 2.2E).

In addition to the lack of MC activation, C5aR-/- mice injected with pathogenic antibodies also lacked activation of p38MAPK, a critical signaling pathway in inflammation and autoimmunity (28). By immunoblotting, the lesional skin of the pathogenic IgG-injected WT mice exhibited a much more intense phosphorylated p38MAPK band as compared to the skin of the pathogenic IgG-injected C5aR-/- mice (Fig. 2.3A, lane 2 vs. lane 4). Quantitative analysis of intensity of each band on immunoblots confirmed that the phospho-p38 levels in the lesional skin of the C5aR+/- mice were significantly higher than C5aR-/- mice (p<0.0001, Fig. 2.3B, bar 2 vs. bar 4). These results suggest that the molecular interaction
between C5a and C5aR leads to MC activation and p38MAPK signaling, followed subsequently by PMN recruitment and BP blistering.

**p38MAPK activation is required for experimental BP.** To determine whether p38MAPK activation is involved in the disease development or is simply a secondary stress response, neonatal WT mice were pretreated with the p38MAPK inhibitor SB203580 or inactive control SB202474 (i.d., 10 μg/g body weight). Two hours later, the mice were injected i.d. with pathogenic antibodies. The SB202474-treated mice developed clinical and histological subepidermal blisters with IgG deposition at the BMZ (Fig. 2.4A, left panels; also Table 2.1). However, SB203580 treatment completely abolished the BP skin lesions (Fig. 2.4A, right panels). Mice pretreated with SB202474 exhibited significantly higher levels of p38MAPK phosphorylation at 2 and 24 hours post IgG injection (Fig. 2.4B, lanes 2 and 5) than mice pretreated with SB203580 (Fig. 2.4B, lanes 3 and 6). Inhibition of p38MAPK activation also resulted in significantly reduced MC degranulation (Fig. 2.4C, bar 4 vs. bar 3) and PMN infiltration (Fig. 2.4D, bar 4 vs. bar 3). These results demonstrate that p38MAPK is critical for MC activation and subsequent the development of experimental BP.

**C5aR-mediated p38MAPK activation depends on MCs.** The complement system and MC activation are indispensable to the pathogenesis of experimental BP (18, 40). Having demonstrated that p38MAPK activation is also required for disease development, we next investigated the links between complement, MCs, and p38MAPK. MCs express C5aR and can be activated by C5a (41), so we reasoned that the interaction of C5a with the MC C5aR may trigger p38MAPK signaling. To test this hypothesis, we injected neonatal MC-sufficient (MC+/+) and MC-deficient (MC-/-) mice with PBS or recombinant C5a (rC5a)
(i.d., 50 ng in PBS/mouse) and analyzed phosphorylated p38MAPK levels in the skin at the injection sites. We observed a significantly higher level of p38MAPK activation in the rC5a-treated MC+/+ mice (Fig. 2.5A, lane 2) as compared to the rC5a-treated MC-/- mice (Fig. 2.5A, lane 4). In vitro, we found that bone marrow-derived MCs, when incubated with rC5a (25 ng/ml) for as little as 5 minutes, exhibited high levels of p38MAPK activation (Fig. 2.5B). Addition of SB203580 (20 μM) to MC culture prior to rC5a treatment almost completely blocked p38MAPK activation (Fig. 2.5C). These data strongly suggest that p38MAPK activation occurs in MCs following exposure to C5a.

C5aR is broadly expressed across a number of cell types, and p38MAPK is activated in many immune cells in response to extracellular inflammatory signals (42, 43). To strengthen the evidence that MCs are the key cell type that interact with C5a and promote development of experimental BP, we reconstituted MC-/- mice with MCs from C5aR-/mice, or MCs from C5aR+/+ mice. If p38MAPK activation is dependent on the C5aR on MCs, then MC-/- mice reconstituted with MCs expressing C5aR should be susceptible to experimental BP, but mice reconstituted with C5aR-deficient MCs should be protected from experimental BP. To test this hypothesis, we injected the left ears of MC-/- mice with C5aR-/- MCs and the right ears of the same MC-/- mice with C5aR+/+ MCs (1x10⁶ cells/site). Ten weeks later, both ears of the mice were injected i.d. with pathogenic anti-BP180 IgG (2 mg/20 μl/site) and examined 24 hour post IgG injection. Similar numbers of MCs were present in the C5aR+/+ MC reconstituted ears and the C5aR-/- MC reconstituted ears. MC-/- mice that were not reconstituted with any MCs and mice reconstituted with C5aR-/- MCs developed no skin lesions, while MC-/- mice reconstituted with C5aR+/+ MCs showed subepidermal blistering (Fig. 2.6A). The number of infiltrating PMNs in the C5aR+/+ MC-
reconstituted site was significantly higher than the C5aR-/− MC-reconstituted site (Fig. 2.6B, bar 6 vs. bar 5). Pretreatment with p38MAPK inhibitor SB203580 abolished BP skin lesions (Fig. 2.6A, 4th panel) and markedly reduced PMN infiltration in the MC-/− mice reconstituted with C5aR+/+ MCs (Fig. 2.6B, bar 7). Taken together, these results demonstrate that disease progression is dependent on MCs expressing C5aR and p38MAPK activation.

2.4 DISCUSSION

Activation of the classical pathway of complement generates C5a, a molecule critical for tissue inflammation and the recruitment of PMNs to the cutaneous BMZ. C5a may carry out this role either directly (functioning as a PMN chemoattractant itself) or indirectly (by stimulating local cells to release proinflammatory mediators such as IL-8), or through a combination of these two mechanisms (44). In experimental BP, we found that C5a contributes to disease progression primarily through interaction with C5aR on MCs, as evidenced by the failure of C5aR-/− mice to induce MC degranulation and subsequent PMN accumulation upon passive transfer of pathogenic anti-BP180 antibodies.

Without C5aR, activation of p38MAPK in the pathogenic anti-mBP180 IgG-injected mouse skin is impaired. p38MAPK is an important signaling molecule involved in translating extracellular environmental conditions into cellular responses (27, 28). This signaling pathway plays a key role in numerous immune and inflammatory diseases, including rheumatoid arthritis and skin autoimmune disease pemphigus vulgaris (45-47). Our data show that p38MAPK activation is not a secondary stress response and instead is required for
experimental BP, as mice treated with p38MAPK inhibitors are protected from disease progression.

It has been reported that in PMNs and monocytes, interaction of C5a and C5aR leads to activation of p38MAPK (24-26). In this study, we provide direct evidence that MCs also use the same pathway of C5a-C5aR-mediated p38MAPK activation. WT mice injected with rC5a exhibited significantly greater p38MAPK phosphorylation than MC-/- mice, indicating that MCs are a key source of the p38MAPK signaling required for induction of experimental BP. Compellingly, we found that MC-/- mice reconstituted with MCs expressing C5aR prior to passive transfer of pathogenic antibody develop blisters; however, mice reconstituted with MCs lacking C5aR expression do not exhibit blister formation. Furthermore, rC5a directly activates p38MAPK in C5aR-sufficient but not C5aR-deficient cultured MCs. Taken together, these data demonstrate that C5a interaction with C5aR on MCs and the subsequent MC p38MAPK activation are indispensable for development of experimental BP. In summary, our present studies identify the C5a-C5aR interaction being a critical molecular linker between basal keratinocyte-based complement activation and MC-based p38MAPK activation in experimental BP. Activation of p38MAPK is essential for MC activation, PMN infiltration, and blister formation in experimental BP.

Inhibitors of p38MAPK are currently under investigation for treatment of autoimmune and inflammatory diseases (48-50). We found that BP patients have significantly elevated levels of p38MAPK activation in lesional skin, indicating that p38MAPK is likely an important mediator of inflammation in the human, as well as the mouse. Thus treatment of BP with these p38MAPK inhibitors may prove to be a practical therapy if specific and safe inhibitors can be developed.
Figure 1.1  Level of C5a in the skin is significantly reduced in pathogenic IgG-injected mice lacking the classical pathway of complement. Neonatal wild-type (C4+/+), C4-deficient (C4/-/-), and factor b-deficient (Fb/-/-) mice were injected i.d. with pathogenic rabbit anti-murine BP180 IgG R621 or control rabbit IgG (2.5 mg/g body weight). The injected animals were examined 24 h post-injection. (A) Hematoxylin and Eosin-stained skin sections from these mice showed a subepidermal vesicle with neutrophilic infiltrate in the pathogenic IgG-injected C4+/+ (left panel) and Fb/-/- (right panel) mice. In contrast, R621-injected C4/-/- mice showed no evidence of subepidermal vesiculation at the light microscopic level (middle panel). E, epidermis; D, dermis; V, vesicle. X125 magnifications. (B) A C5a-specific ELISA assay exhibited a significantly reduced levels of C5a in the skin of C4/-/- mice as compared to the diseased mice (p<0.0001, bar 4 vs. bars 2, 6). (C) MPO enzymatic assay of skin protein extracts revealed a significantly lower number of infiltrating PMNs in the C4/-/- mice as compared to the diseased mice (p<0.001, bar 4 vs. bars 2, 6). n=8 for each group. Three independent experiments were done for each group of mice.
Figure 2.2. Mice lacking C5a receptor are resistant to experimental BP. Neonatal wild-type (C5aR+/+) and C5aR-deficient (C5aR−/−) mice were injected i.d. with pathogenic rabbit anti-murine BP180 IgG R621 or control rabbit IgG (2.5 mg/g body weight). The injected animals were examined 2, 4, and 24 h post-injection. (A) Pathogenic R621 IgG induced BP blisters in C5aR+/+ mice (left panel), but not in C5aR−/− mice (right panel) at 24 h post injection. (B) A C5a-specific ELISA assay show no difference in skin C5a levels between C5aR−/− and C5aR+/+ mice at 4 h post IgG injection (p=0.325, bar 2 vs. bar 4). (C) Toluidine blue staining showed an extensive MC degranulation in the C5aR+/+ mice (left panel) at 2 h post IgG injection, while the C5aR−/− mice (right panel) exhibited a minimal level of MC degranulation. (D) Quantification of degranulated MCs on the toluidine blue-stained skin sections showed a drastically increased MC degranulation in the C5aR+/+ mice as compared to C5aR−/− mice (p<0.001, bar 2 vs. bar 4). (E) MPO enzymatic assay of skin protein extracts revealed a significantly lower number of infiltrating PMNs in the C5aR−/− mice as compared to the diseased mice (p<0.001, bar 2 vs. bar 4). n=8 for each group. Three independent experiments were done for each group of mice.
Figure 2.3. Mice lacking C5aR show impaired p38MAPK activation induced by pathogenic antibodies. Neonatal wild-type (C5aR+/+) and C5aR-deficient (C5aR-/−) mice were injected i.d. with pathogenic rabbit anti-murine BP180 IgG R621 or control rabbit IgG (2.5 mg/g body weight). The skin protein extracts of injected animals were examined 24 h post-injection by immunoblotting for p38MAPK activation. (A) Immunoblotting analysis of p38MAPK activation. Skin protein extracts (30 μg/lane) were analyzed by immunoblotting with antibodies to phosphorylated p38MAPK (phospho-p38) and total p38MAPK (total p38). A much more intense band for phospho-p38 was seen in the skin of the diseased C5aR+/+ mice (lane 2) as compared to C5aR-/− mice (lane 4). (B) Quantification of p38MAPK activation. Intensity of each band on immunoblots was quantified with a GeneGnome scanner and GeneSnap software. The phospho-p38 levels in the skin of the C5aR+/+ mice were significantly higher than C5aR-/− mice (p<0.0001, bar 2 vs. bar 4). n=8 for each group. Three independent experiments were done for each group of mice.
Figure 2.4 Blocking p38MAPK activation abolishes experimental BP. Neonatal C57BL/6J mice were administered i.d. with the p38MAPK inhibitor SB203580 or inactive control SB202474 (10 μg/g body weight). Two h later, the mice were injected i.d. with pathogenic rabbit anti-murine BP180 IgG R621 or control rabbit IgG (2.5 mg/g body weight). The injected animals were examined at different time points post IgG injection. (A) Mice pretreated with the inactive control SB202474 prior to injection with pathogenic antibodies developed BP clinically and histologically, with IgG deposition at the BMZ (left panels). In contrast, inhibition of p38MAPK with SB203580 completely abolished the skin lesions clinically and histologically without interference with IgG deposition at the BMZ (right panels). (B) Immunoblotting analysis of p38MAPK activation. Pathogenic antibodies induced high levels of phospho-p38 in SB202427-treated mice at 2 and 24 h (lanes 2 and 5), while SB203580 treatment induced only background levels of p38MAPK phosphorylation (lanes 3 and 6). (C) Quantification of MC degranulation. Pre-treatment with SB203580 (bar 4) significantly reduced MC degranulation at 2 h post pathogenic IgG injection as compared to PBS (bar 2) or SB202474 pre-treatment (bar 3) (p<0.001, bar 4 vs. bars 2, 3). (D) Quantification of MPO activity revealed significantly lower levels of PMN infiltration in the SB203580-treated mice (bar 4) than SB202474-treated mice (bar 3) at 24 h post IgG injection (p<0.01). n=6 for each group. Three independent experiments were done for each group of mice. Arrowheads indicate basal keratinocytes.
Figure 2.5 C5a activates p38MAPK of MCs in vivo and in vitro. (A) C5a activates MC p38MAPK in vivo. MC-sufficient (MC+/+) and MC-deficient (MC-/-) mice were injected i.d. with PBS or C5a (50 ng in 50 μl PBS/mouse) and the skin extracts were examined 4 h later by immunoblotting with antibodies to phospho-p38 and total p38. Significantly higher levels of phospho-p38 were seen in the C5a-injected MC+/+ mice (lane 2) than PBS control (lane 1) and C5a-injected MC-/- mice (lane 4). n=6 for each group. Two independent experiments were done for each group of mice. (B) C5a activates MC p38MAPK in vitro. Bone marrow-derived MCs from C57BL/6 mice were incubated with buffer control (lane 1) or C5a (25 ng/ml, lanes 2-4) for 0-60 min and cell extracts were assayed for p38MAPK activation. All C5a-treated MCs showed high levels of phospho-p38. (C) SB203580 blocks C5a-induced MC p38MAPK phosphorylation in vitro. Bone marrow-derived MCs from C57BL/6 mice were incubated with buffer control (lane 1) or C5a (25 ng/ml, lanes 2-4) in the presence of p38MAPK inhibitor SB203580 (20 μM) for 0-60 min and cell extracts were assayed for p38MAPK activation. SB203580 treatment almost completely blocked p38MAPK activation. A representative of three independent experiments was shown.
Figure 2.6. MC reconstitution restores BP in MC-deficient mice. Pathogenic antibodies were injected into ears of MC+/-, MC-/-, MC-/- reconstituted with 1x10^6 MCs from C5aR/- mice, and MC-/- reconstituted with MCs from C5aR+/- mice with or without pretreatment with SB203580. The ears were examined 24 h post IgG injection. (A) Hematoxylin and Eosin staining showed that MC+/- mice and MC-/- mice reconstituted with wild-type MCs developed DEJ separation following antibody injection. MC-/- mice reconstituted with C5aR/- MCs and MC-/- mice reconstituted with C5aR+/- MCs that were pretreated with SB203580 prior to antibody injection failed to develop DEJ separation. (B) Quantification of PMN infiltration. Pathogenic antibodies induced BP with significantly increased PMN infiltration in MC+/- mice (bar 2), and MC-/- mice reconstituted wild-type MCs (bar 6), but not MC-/- mice reconstituted with C5aR/- MCs (bar 5) and MC-/- mice reconstituted with wild-type MCs plus SB203580 (bar 7). p<0.01, 6 mice per group. Three independent experiments were done for each group of mice.
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<td>R621</td>
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**Table 2.1. Role of C5a receptor and p38 MAPK in experimental BP.** Neonatal wild-type, C4-/-, factor B (Fb)-/-, and C5aR-/- mice were injected intradermally with either control IgG R50 or pathogenic rabbit anti-mBP180 IgG R621 alone (2.5 mg/g body weight) or pretreated with the p38MAPK inhibitor SB203580 or inactive control SB202474 (10 μg/g body weight). All mice were examined 24 h after IgG injection. The extent of cutaneous disease was scored as follows: "(-)", no detectable skin disease; "1+", mild erythematous reaction with no evidence of the "epidermal detachment sign" [this sign was elicited by gentle friction of the mouse skin which, when positive, produced fine, persistent wrinkling of the epidermis]; "2+", intense erythema and "epidermal detachment" sign involving 10-50% of the epidermis in localized areas; and "3+", intense erythema with frank "epidermal detachment" sign involving more than 50% of the epidermis in the injection site. The clinical disease severity is expressed as Mean + SE and analyzed by paired Student t-test.
2.5 REFERENCES


CHAPTER 3

FUNCTIONAL INTERACTION BETWEEN COMPLEMENT AND THE PRO-INFLAMMATORY CYTOKINE TNF-α IN EXPERIMENTAL BULLOUS PEMPHIGOID

Bullous pemphigoid (BP) is a chronic inflammatory skin blistering disease characterized by autoantibodies against two major hemidesmosomal proteins, BP230 and BP180. Existing therapeutic strategies rely heavily on corticosteroids; hence there is urgent need for new treatments. High levels of TNF-α are reported in the sera of BP patients, providing a possible intervention target. However, the role of TNF-α in BP is unknown. In this study, we examined TNF-α’s activity using two IgG passive-transfer mouse models of BP. One of the models is a humanized model, where human anti-BP180 is injected into a knock-in mouse which expresses the human pathogenic portion of BP180. Upon injection with pathogenic anti-BP180 antibodies, mice exhibit sharp mast cell (MC)-dependent increases in TNF-α in the skin and blister fluid. Mice lacking TNF-α or MCs fail to recruit sufficient numbers of neutrophils to the skin and do not develop blisters. TNFR1 but not TNFR2 expression on MCs is required for experimental BP. Additionally, anti-TNF-α therapies abrogated disease pathogenesis. These data indicate that pathogenic antibodies trigger TNF-α release from MCs, and that TNF-α mediates neutrophil infiltration and disease by acting in an autocrine fashion on MCs. Additionally, this study provides rationale for considering anti-TNF therapy for the treatment of BP.
3.1 INTRODUCTION

Bullous pemphigoid (BP) is a chronic, autoimmune skin blistering disease that predominantly occurs in the elderly, but also can affect younger people (1-3). BP is associated with high mortality rates, ranging from 12% and 40% in the first three years after diagnosis (4, 5). The conventional therapy for BP over the past several decades has been high-dose, long-term systemic oral corticosteroids and immunosuppressive agents (6). However, long term use of corticosteroids is associated with deleterious health effects, including severe infection, diabetes mellitus, osteoporosis, hypertension, renal failure, lymphoma, and squamous cell carcinoma (7). The negative side effects of immunosuppression therapy may account for a significant portion of the morbidity and mortality associated with BP. Even when disease is successfully treated, up to 45% of patients suffer a relapse within six months of cessation of therapy (8), underscoring the urgent need for novel therapeutic interventions.

BP patients develop circulating and tissue bound autoantibodies against two major hemidesmosomal proteins, BP230 (BPAG1) and BP180 (BPAG2, type XVII collagen) (9-14). These anti-hemidesmosomal autoantibodies, along with complement components, are deposited along the basement membrane at the dermal-epidermal junction (DEJ) of perilesional skin (15). Basal keratinocytes detach from the underlying dermis, leading to the formation of tense, fluid filled blisters (1). Blister fluid contains cellular dermal inflammatory infiltrates, including eosinophils, neutrophils (PMNs), lymphocytes, degranulated mast cells (MCs), and monocyte/macrophages (16-21). In addition, various inflammatory mediators that can recruit/activate MCs or leukocytes have been identified in lesional skin and/or blister
fluids of BP patients, including C5a, eosinophilic/neutrophilic chemotactic factors, histamine, leukotrienes, and various cytokines (3, 22-28).

The cytokine TNF-α is a potent and pleiotropic mediator of biological responses and signals through the TNF-α receptors (TNFRs) TNFR1 and TNFR2 (29). TNF-α is implicated in the pathogenesis of a number of inflammatory diseases, including rheumatoid arthritis, inflammatory bowel disease, psoriasis, and pemphigus vulgaris (30). Elevated levels of TNF-α have been reported in the serum and blister fluid of BP patients, and disease severity correlates with TNF-α serum levels (31, 32). In one case study, BP in a patient with severe psoriatic background was successfully treated with a combination therapy of rituximab and etanercept (29), while etanercept alone was used to control psoriasis and BP in another patient (30). These studies are limited in scope but provide the attractive possibility of utilizing established anti-TNF-α therapies to treat BP patients. However, prior to considering the broad application of TNF-α therapies to BP, it is vital to understand the role of this cytokine in the pathogenesis of BP.

To address this issue, we used two IgG passive transfer mouse models of BP that mimic the key features of human BP to investigate the role of TNF-α in the pathogenesis of experimental BP. In the first of these models, neonatal C57BL/6J mice are injected with polyclonal rabbit antibodies that recognize the immunodominant domain of murine BP180, called NC14A (33). NC14A is the murine homolog of NC16A, the domain of human BP180 that harbors multiple epitopes recognized by human BP autoantibodies (34, 35). Animals injected with rabbit anti-BP180NC14A IgG develop skin blistering that depends on complement activation, MC degranulation, and PMN infiltration to the skin (36-38). In the second model, mice “humanized” to express NC16A instead of NC14A are injected with...
BP180-specific IgG isolated from human BP patients. The injected patient antibodies trigger blistering that also requires complement, MC degranulation, and PMN recruitment (39). Using these two mouse models, we describe a key role for TNF-α via TNFRI in the pathogenesis of BP.

3.2 EXPERIMENTAL PROCEDURES

**Laboratory animals.** Breeding pairs of C57BL/6J, MC-deficient WBB6F1-Kit<sup>+/−</sup>/Kit<sup>−/−</sup> (MC<sup>−/−</sup>), C5a receptor-deficient (C5aR<sup>−/−</sup>), TNFR1-deficient (TNFR1<sup>−/−</sup>), TNFR2-deficient (TNFR2<sup>−/−</sup>), and TNFR1 and TNFR2 deficient (TNFR1,2<sup>−/−</sup>) mice were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained at the University of North Carolina at Chapel Hill. The MC deficiency in Kit<sup>+/−</sup>/Kit<sup>−/−</sup> mice are caused by distinct mutations in MC c-Kit (40). Humanized NC16A mice were generated as described (39). In these mice, the mouse BP180NC14A domain was replaced with human BP180NC16A domain. Neonatal mice (24-36 hours old with body weights between 1.4 and 1.6 g) were used for passive transfer experiments. All animal care and animal experiments were approved by the UNC Animal Care Committee and were in accordance with the National Institutes of Health guidelines.

**Preparation of pathogenic anti-BP180 IgG.** The preparation of recombinant murine BP180 (mBP180) and the immunization of New Zealand White rabbits were performed as previously described (33, 41, 42). Briefly, New Zealand White rabbits were immunized with the GST-hBP180NC16A or GST-mNC14A fusion protein. IgG fractions from sera of the immunized rabbits were purified using a protein G Sepharose column (Sigma). The pathogenicity of these IgG preparations was tested by passive transfer
experiment as described below. A pathogenic anti-mBP180 IgG preparation (R530) and a control IgG preparation were used in this study (38). For experiments with human patient IgG, BP180NC16A-specific autoantibodies from BP patients were purified using a BP180NC16A-glutathione Sepharose column (39).

**Induction of experimental BP and clinical evaluation of animals.** Neonates were given on the back one i.d. injection of a sterile solution of IgG in PBS (50 μl IgG, 2.5 mg IgG/g body weight) as described elsewhere (33). The skin at the IgG-injection site of the mice from the test and control groups was examined at different time points after the IgG injection. The extent of cutaneous disease was scored as follows: "(-)", no detectable skin disease; "1+", mild erythematous reaction with no evidence of the epidermal detachment sign [this sign was elicited by gentle friction of the mouse skin which, when positive, produced fine, persistent wrinkling of the epidermis]; "2+", intense erythema and epidermal detachment sign involving 10-50% of the epidermis in localized areas; and "3+", intense erythema with frank epidermal detachment sign involving more than 50% of the epidermis in the injection site.

After clinical examination, the animals were sacrificed, and skin and serum specimens were obtained. Skin sections were used for a) routine histological examination by light microscopy (H&E staining) to localize the lesional site and PMN infiltration, b) toluidine blue staining to quantify MCs and MC degranulation, c) direct IF assays to detect rabbit IgG and mouse C3 deposition at the BMZ, and d) myeloperoxidase (MPO) enzymatic assay to quantify the PMN accumulation at the skin injection site described below. The sera of injected animals were tested by indirect IF techniques to determine the titers of rabbit anti-
mBP180 antibodies using mouse skin cryosections as the substrate. Direct and indirect IF studies were performed as previously described (33) using commercially available FITC-conjugated goat anti-rabbit IgG (Kirkeggard& Perry Laboratories Inc.). Monospecific goat anti-mouse C3 IgG was purchased from Cappel Laboratories.

**Pretreatment with TNF-α neutralizing antibodies and rTNF-α.** Neonatal WT and TNF-α-deficient (TNF-α−/−) mice were injected i.d. with 50 μl of BSA control (1 μg/50 μl PBS), TNF-α neutralizing antibodies (1 μg/50 μl PBS, R&D System), or rTNF-α (50 ng/50 μl PBS, R&D System). Two hours later, the mice were given i.d. with pathogenic anti-mBP180 antibodies and examined 24 h post IgG injection.

**Identification of TNF-α in blister fluid.** One hundred microliters of PBS was injected into the skin blisters (formed 12 hours after pathogenic IgG injection) and nonlesional sites, and withdrawn 1 minute later. The “washout” PBS was centrifuged at low speed (1,000 g) for 5 minutes to remove cells and then at high speed (12,000 g) for 5 minutes to remove cell debris. TNF-α in the supernatant was measured by a mouse TNF-α-specific ELISA kit (R&D System). TNF-α level were expressed as pg/ml.

**RT-PCR.** mRNA was isolated from skin samples using an RNeasy kit (Qiagen). TNF-α, TNFR1, TNFR2 messages were amplified from the cDNA using the following primers: Forward: 5’-TAGTGGTGCCAGCGATGGGT-3’ and Reverse: 5’-GACCTGCCCGGACTCCGCAA-3’ for mouse TNF-α, Forward: 5’-GTGCGTCCCTTGCAGCCACT-3’ and reverse 5’-AGGGTCAGCTCCCTGTTGGTGG-3’ for mouse TNFR1, Forward: 5’- GTTTCAGGAGGCCGCTGAG -3’ and Reverse:5’-
CCATATCCGGCACACCAAGGGGCA -3’ for mouse TNFR2. Sizes of PCR products were 305, 439, and 293 base pairs for TNF-α, TNFR1, and TNFR2, respectively.

**Quantification of PMN accumulation at the skin site.** Myeloperoxidase (MPO) activity in skin sites of the injected animals was assayed as a measure of PMN infiltration as described (43, 44). A standard reference curve was established using purified MPO (Athens Research and Technology, Inc., Athens, Georgia). The skin samples were extracted by homogenization in an extraction buffer containing 0.1 M Tris-Cl, pH 7.6, 0.15 M NaCl, 0.5% hexadecyltrimethylammonium bromide. MPO activity in the supernatant fraction was measured by the change in optical density at 460 nm resulting from decomposition of H₂O₂ in the presence of o-dianisidine. MPO content was expressed as units of MPO activity/mg protein. Protein concentrations were determined by the Bio-RAD dye-binding assay using BSA as a standard.

**Quantification of MCs and MC degranulation.** MCs and MC degranulation in skin samples were quantified according to Wershil, et al. (45) with modification. Briefly, lesional and nonlesional skin sections of IgG-injected mice were fixed in 10% formalin. Paraffin sections (5-μm thick) were prepared and stained with toluidine blue and H&E. Toluidine blue stains histamine in the MC granules (46). Total number of MC was counted and classified as degranulated (>10% of the granules exhibiting fusion or discharge) or normal in five fields under a light microscope as described previously. The results were expressed as percentage of MC degranulating.

**Determination of C5a levels in skin.** Skin from the IgG injection site of the diseased and control mice was mechanically homogenized in PBS to extract proteins. The level of mouse C5a (mC5a) in the skin was measured by ELISA (R&D Systems, Minneapolis, MN).
Microtiter plates were coated with a rat anti-mouse C5a antibody, incubated with skin protein extracts, followed by goat anti-mC5a detection antibody, and then developed and read at OD_{492nm}. The C5a level was expressed as OD_{492} reading/mg protein.

**MC reconstitution.** Kit^{w}/Kit^{w-v} mice were repaired of their MC deficiency selectively and locally by the injection of growth factor-dependent bone marrow-derived cultured MC into the skin (47, 48). Briefly, femoral bone marrow cells from WT and C5aR^{-} mice were maintained in vitro for 4 wk in RPMI 1640 complete medium (Gibco/Life Technologies, Grant Island, NY) supplemented with 20% WEHI-3-conditioned medium until MCs represented >95% of the total cells as determined by toluidine blue staining and flow cytometry analysis using antibodies specific for MC cell surface markers FceRI, c-Kit, and CD13 (47). Murine IgE and rat anti-mouse IgE were purchased from Southern Biotechnology Associates (Birmingham, AL). FITC-labeled rat anti-mouse c-Kit and FITC-labeled rat anti-mouse CD13 were obtained from BD Biosciences (San Diego, CA). MCs (1x10^6 in 20 μl of medium) were injected i.d. into right ears of MC-deficient mice. Medium alone (20 μl) were injected i.d. into left ears of the same mice as negative control. This procedure selectively and locally reconstitute dermal MC population without systemic effects (48). To confirm MC reconstitution, skin sections from MC-injected sites were stained by toluidine blue. Ten weeks after adoptive transfer of MCs, both ears of the mice were injected i.d. with pathogenic anti-BP180 IgG (2 mg/20 μl/site). Twenty four hours later, ear skin biopsies were obtained and analyzed by H&E, toluidine blue staining and MPO enzyme assay as described above.

**Statistical analysis.** The data are expressed as mean ± SEM and were analyzed using the Student's paired t-test. A p value less than 0.05 was considered significant.
3.3 RESULTS

Pathogenic anti-BP180 antibodies induce local expression of TNF-α. As we previously reported (33), wild-type (WT) C57BL/6J mice injected with pathogenic rabbit anti-mBP180 IgG R530 (2.5 mg/g body weight) develop intense clinical blisters after 24 hours (Figure 3.1A, top row, arrow denotes blistered area). A linear pattern of IgG binding along the DEJ is detected by direct immunofluorescence (Figure 1A, middle, arrow marks DEJ), and separation of the dermis from the epidermis is apparent in H&E stained skin sections (Figure 3.1A, bottom). Mice injected with control IgG lack blisters and antibody deposition. Levels of myeloperoxidase (MPO) in the skin are significantly higher in mice given R530 than in mice given control IgG, indicating that PMNs are recruited to the skin in the pathogenic antibody-injected animals (Figure 3.1B). To investigate the role of TNF-α in experimental BP, we collected skin biopsies from the affected area of R530-injected mice and from the equivalent, unblistered areas of control IgG-injected mice and measured TNF-α protein, which was significantly higher (over 25 fold) in the blister fluid from the R530 injected mice than in the wash-out of control IgG injected mice (Figure 3.1C). These results demonstrate that expression of TNF-α is greatly augmented in experimental BP.

TNF-α is required for the development of experimental BP. Having established that TNF-α levels rise dramatically in mice given pathogenic anti-BP180 antibodies, we next investigated whether TNF-α is required for experimental BP disease pathogenesis. TNF-α deficient mice (TNF-α−/−) and WT mice were pretreated locally with PBS, recombinant TNF-α (rTNF-α), or anti-TNF-α neutralizing antibody. Two hours later, the mice were injected i.d. with control IgG or R530. 24 hours post IgG injection, TNF-α−/− mice showed no skin lesions clinically or histologically (Figure 3.2A, left column). However, pretreatment with
rTNF-α restored clinical and histological lesions in TNF-α−/− mice (Figure 3.2A, middle column). WT mice pretreated with TNF-α neutralizing antibody were also resistant to experimental BP (Figure 3.2A, right column). Taken together, these data demonstrate an essential role for TNF-α in experimental BP.

Experimental BP pathogenesis proceeds through the activation of complement, followed by MC degranulation, and then PMN infiltration to the skin (49). We next examined the effect of TNF-α deficiency on each of these processes. Four hours after IgG injection, similar levels of C5a were detected by ELISA in the skin of WT and TNF-α−/− animals injected with control IgG or R530 IgG (Figure 3.2B). Toluidine blue stained skin sections from mice sacrificed two hours after R530 antibody injection, when MC degranulation reaches its peak level (37), revealed equivalent levels of MC degranulation in the dermis of WT and TNF-α−/− animals (Figure 3.2C). While complement activation and MC degranulation were unaffected by the loss of TNF-α, PMN recruitment was significantly impacted (Figure 3.2D). Twenty-four hours after R530 injection, WT mice exhibit a significant increase in skin PMN levels. Pretreatment with the TNF-α neutralizing antibody ablated the PMN recruitment in WT mice. TNF-α−/− animals recruit significantly fewer PMNs to the skin than the WT mice, but PMN recruitment was completely restored in TNF-α−/− animal pretreated with rTNF-α. These experiments demonstrate that TNF-α is required for PMN recruitment in experimental BP.

**MCs are the source of TNF-α needed for experimental BP pathogenesis.** Upon degranulation, MCs release large amounts of pre-formed TNF-α (50). However, a number of other skin-resident cell populations can produce TNF-α, including keratinocytes and Langerhans cells (8). To determine if MCs are the critical cellular source of TNF-α in the
development of experimental BP, we first examined the kinetics of TNF-α production in the skin of WT and WBB6F1-Kitw/Kitw-v mice which lacked MC (referred to as MC−/−) post-injection with R530 or control IgG. Mice (n=6 per group) were sacrificed 0, 1, 2, 4, 8, and 24 hours post IgG injection, and blisters or the area of skin where blisters would form were washed with 100 μl of PBS to collect protein. TNF-α levels were measured in the washout fluids by ELISA. Injection with R530 IgG induced an initial peak in TNF-α levels 2 hours post injection, and a second peak 24 hours post-injection in WT mice (Figure 3.3). In contrast, TNF-α levels did not significantly increase in mice injected with control IgG at all time-points. The TNF-α protein level peaked 2 h post injection which coincides with the peak in MC degranulation, suggesting that MCs release of preformed TNF-α may be the key source of the cytokine in the early stages of experimental BP. To further investigate this hypothesis, we tested whether TNF-α was produced in MC−/− mice following R530 injection. TNF-α levels in the washouts from MC−/− mice injected with R530 were not significantly different from the levels observed in mice injected with control IgG (Figure 3.3, closed triangles) indicating that MC is the source of TNF-α in response to R530 injection.

MC reconstitution experiments further confirmed that MCs are the critical source of TNF-α in experimental BP pathogenesis. TNF-α−/− mice did not develop blisters following injection with pathogenic IgG. Furthermore, reconstitution with 1x10^6 MC from TNF-α−/− mice prior to R530 injection does not restore histological blistering. However, TNF-α−/− mice reconstituted with WT MCs prior to R530 injection develop histological blistering (Figure 3.4A). Similarly, MC−/− mice and MC−/− mice reconstituted with TNF-α−/− MCs fail to develop blisters following R530 injection, but MC−/− mice reconstituted with WT MCs develop blisters (Figure 3.4B). Only mice with WT MCs, but not TNF-α−/− MCs are able to recruit
PMNs to the skin and develop blisters following injection with pathogenic antibodies (Figure 3.4C). Taken together, these data demonstrate that TNF-α released by degranulating MCs is required for experimental BP.

**C5a-C5a receptor interaction is required for TNF-α release.** We recently reported that C5a interaction with the C5a receptor (C5aR, or CD88) on MCs triggers activation of p38MAPK in MCs and MC degranulation (51). To assess if TNF-α released by degranulating MCs is also affected by the C5a pathway, we injected WT and C5aR<sup>−/−</sup> neonatal mice with R530 and examined various stages of disease progression. C5aR<sup>−/−</sup> mice fail to develop clinical blisters after 24 hours (Figure 3.5A), despite generating levels of C5a equivalent to the WT mice (Figure 3.5B). MC degranulation is significantly impaired in the C5aR<sup>−/−</sup> mice injected with R530 compared to WT mice injected with R530, and washout fluid from C5aR<sup>−/−</sup> mice contains significantly lower levels of TNF-α than washout from diseased WT mice (Figures 3.5C, 3.5D). C5aR<sup>−/−</sup> mice fail to recruit PMNs to the skin following R530 injection, and reconstitution with C5aR<sup>−/−</sup> MCs or TNF-α<sup>−/−</sup> MCs does not restore PMNs. Only reconstitution with WT MCs restores PMN infiltration to the skin (Figure 3.5E). These experiments place C5a upstream of TNF-α production and PMN recruitment to the skin.

**TNF-α receptor 1 mediates the downstream effects of TNF-α.** TNF-α acts via TNFR1 and TNFR2. To determine which TNF receptors are required for experimental BP pathogenesis, we first looked at receptor expression following pathogenic antibody injection. Over 8 hours post injection, TNFR1 is expressed constitutively, whereas TNFR2 mRNA expression increased over time (Figure 3.6A). We next injected TNFR1<sup>−/−</sup>, TNFR2<sup>−/−</sup>, and TNFR1,2<sup>−/−</sup> mice with R530 and checked for clinical blistering after 24 hours. TNFR2<sup>−/−</sup> mice
developed extensive blisters equivalent to those seen on WT mice, but the TNFR1−/− and TNFR1,2−/− were protected from disease (Figure 3.6B). We found that PMN infiltration is impaired in both TNFR1−/− and TNFR1,2−/− mice, indicating that TNFR1 is required for the transition from MC degranulation to PMN recruitment (Figure 3.6C).

TNFR1 is broadly expressed across cell types, including MCs, keratinocytes, fibroblasts, endothelial cells, and PMNs (52). To investigate the possibility that TNF-α acts on MCs in an autocrine fashion, we performed a series of MC reconstitution experiments. TNFR1,2−/− and MC−/− mice were reconstituted with MCs from TNFR1,2−/−, TNFR1−/−, TNFR2−/−, or WT and then injected with R530 IgG. The TNFR1,2−/− and the MC−/− mice recruited PMNs to the skin and developed BP blisters only when they were reconstituted with TNFR2−/− or WT MCs (Figure 3.7). These data indicate that TNF-α acts on TNFR1 expressed by MCs in an autocrine fashion to promote experimental BP.

**Human BP patient IgG induces TNF-α production in humanized mouse model.**

To extend these findings to the human BP disease, we used a humanized animal model of experimental BP (39). The humanized mice, called NC16A+/+, express the human sequence of the pathogenic portion of BP180 in place of the murine homolog. In these mice, passive transfer of anti-BP180NC16A autoantibodies purified from human patients triggers experimental BP. Similar to the rabbit anti-mouse IgG model, disease development depends on complement activation, MC degranulation and PMN infiltration (39). To test whether human BP autoantibody-induced skin disease also depends on TNF-α, we injected human control IgG or NC16A-specific IgG into NC16A+/+ mice. The anti-NC16A antibodies induced clinical blisters in NC16A mice, and these blisters were prevented by pretreatment with anti-TNF-α neutralizing antibodies (Figure 3.8A). Significantly higher levels of TNF-α
were present in blister washout of the humanized mice given pathogenic antibodies as compared to the humanized mice injected with human control IgG (Figure 3.8B). Pretreatment with anti-TNF-α reduced the total amount of TNF-α present in the washout fluid of the anti-NC16A-injected animals. As expected, increased PMN recruitment was associated with higher levels of TNF-α in the diseased NC16+/+ mice, and blocked by anti-TNF-α neutralizing antibodies (Figure 3.8C). These results suggest that TNF-α also acts to increase skin blistering and granulocyte infiltration in a humanized model of BP.

3.4 DISCUSSION

TNF-α is a pleiotropic cytokine with a broad range of biological effects in regulating cell growth and death, development, oncogenesis, immune function and the inflammatory response (53). Various TNF-α inhibitors are used in the clinic to treat Crohn’s disease, colitis and rheumatoid arthritis. Here, we show evidence for TNF-α’s autocrine effect on MCs in experimental BP. We found that TNF-α is required for development of experimental BP. Mice injected with anti-BP180 antibodies develop a significant increase in serum levels of TNF-α, and TNF-α is detected in the blister fluid from diseased animals. Upon injection with pathogenic antibodies, animals lacking TNF-α fail to recruit PMNs to the skin and are protected from blister formation, but exhibit normal levels of complement activation and MC degranulation. Mice lacking MCs do not exhibit increased serum TNF-α levels upon pathogenic antibody injection and are resistant to experimental BP. Reconstitution with TNF-α sufficient MCs restores disease susceptibility in TNF-α−/− or MC−/− mice, whereas TNF-α+/- MCs do not. Activation of the C5aR on MCs by C5a is required for TNF-α release. We also found that TNF-α acts on MCs in an autocrine fashion through TNFR1. MC−/− mice
develop experimental BP only when they are reconstituted with MCs that express TNFR1. Thus, we conclude that MCs are both the critical source of TNF-α and the key target of TNF-α in experimental BP.

The binding of C5a to C5aR on MCs leads to activation of the p38MAPK signaling pathway and MC degranulation (51), which releases preformed TNF-α from intracellular stores (Figure 3.9). This initial wave of TNF-α binds to MC TNFR1 and initiates an intracellular signaling cascade that leads to activation of the transcription factors NF-kB and c-Jun (54). NF-kB and c-Jun mediate production of pro-inflammatory factors, and our laboratory is currently investigating roles for these compounds in PMN recruitment and BP pathogenesis.

While TNF-α-TNF-R1 interaction on MCs is critical for disease pathogenesis, it is possible that TNF-α has other secondary effects during disease. TNF-α expression in the skin upregulates expression of VCAM-1, ICAM-1, and E-selectin, which promotes extravasation of inflammatory cells to the skin (55-57). PMNs and macrophages also express high levels of TNFR1; the effects of TNF-α on these cell types remains to be investigated. TNF-α also upregulates MC production of matrix metalloproteinase-9 (MMP-9) (58), an enzyme critical for the secondary wave of PMN recruitment in experimental BP (59). While PMNs are the key source of MMP-9 in experimental BP, TNF-α stimulated MCs may augment these levels.

Anti-TNF-α therapies, including anti-TNF-α antibodies and TNF-α blocking peptidesetanercept (Enbrel), infliximab (Remicade), and adalimumab (Humira), are effective in treating a number of inflammatory diseases (30). Limited case reports indicate that
treatment with anti-TNF-α therapies is therapeutically beneficial to BP patients (60, 61). Our work makes a strong case for expansion of anti-TNF-α therapies for the treatment of BP.
Figure 3.1. Pathogenic anti-mBP180 IgG induces BP blisters associated with an elevated level of TNF-α in mouse skin. Neonatal C57BL/6J mice were injected i.d. with normal rabbit IgG (control IgG) or pathogenic rabbit anti-mBP180 IgGR530 (2.5 mg/g body weight) and examined 24 h post injection. (A) Top row: Clinical evaluation of mice 24 hours after antibody injection. Arrow, blister. Middle row: Direct IF for rabbit IgG on skin sections. Bottom row: H&E staining on skin sections. E, epidermis. D, dermis. V, vesicle. Arrow, basal keratinocytes. 100x magnification. (B) MPO assay exhibited a significantly increased number of PMNs in the lesional skin relative to the non-lesional skin of control mice. N=8 for each group. *p<0.01 (bar 1 vs. bar 2). (C) TNF-α ELISA showed a drastic increase in TNF-α protein level in the blister fluid of the diseased mice as compared to that in the “wash-out” of control mice.
Figure 3.2. TNF-α-deficient mice are resistant to experimental BP. Neonatal WT and TNF-α-deficient (TNF-α−/−) mice were pretreated locally with PBS, recombinant TNF-α, or anti-TNF-α neutralizing antibody. Two hours later, the mice were injected i.d. with control IgG or pathogenic rabbit anti-mBP180 IgG R530 (2.5 mg/g body weight) (A) Clinical (top row, arrow) and histological (bottom row) blisters 24 h post IgG injection; (B) Relative levels of C5a in skin extracts from WT and TNF-α−/− mice at 4 h post IgG injection, measured by ELISA. (C) Toluidine blue staining for MC degranulation in the dermis between WT and TNF-α−/− mice at 2 h post IgG injection, when MC degranulation reaches the peak level. (D) Relative MPO activity assay demonstrated that anti-TNF-α antibody treatment resulted in a significant reduction of PMN infiltration, while local reconstitution of TNF-α restored BP disease with an increased level of PMN infiltration similar to the diseased mice at 24 h post IgG injection. n = 8 for each group. *p<0.01 (bar 2 vs. bar 3; bar 5 vs. bar 6).
Figure 3.3. Time-course of TNF-α expression during BP disease development in mice. Neonatal wild-type (WT) and MC-deficient (MC-/-) mice were injected i.d. with control IgG or pathogenic rabbit anti-mBP180 IgG (2.5 mg/g body weight). At 0, 1, 2, 4, 8, and 24 h post injection, mice were given 50 μl of PBS and the levels of TNF-α in the PBS wash-out were quantified by ELISA. N=6. *p<0.05.
Figure 3.4. MC reconstitution restores BP in TNF-α-/− and MC-deficient mice. TNF-α-/− and MC-/− mice were reconstituted at the ear with buffer control (none) or 1x10^6 MCs from TNF-α-/− or WT mice. Ten weeks later, pathogenic abs were injected into ears of the MC-reconstituted mice and the ears were examined after 24 h. (A) H&E staining showed that pathogenic IgG induced blistering in TNF-α-/− mice reconstituted with WT MCs (right panel), but not with buffer control or TNF-α-/− MCs (left, middle panels). (B) H&E staining showed that pathogenic IgG induced blistering in MC-/− mice reconstituted with WT MCs (right panel), but not with buffer control or TNF-α-/− MCs (left, middle panels). (C) Quantification of PMN infiltration. Pathogenic IgG induced blistering and PMN infiltration in WT mice and TNF-α-/− or MC-/− mice reconstituted with WT MCs but not TNF-α-/− MCs. *p<0.01, 6 mice per group. Three independent experiments were done for each group.
Figure 3.5. C5aR on MCs are required for TNF-α release and skin blistering. WT and C5aR-/- mice were injected i.d. with pathogenic rabbit anti-mBP180 IgG and examined at post injection. (A) Pathogenic IgG induced clinical blisters in WT and not C5aR-/- mice. (B) ELISA assay showed compatible skin C5a levels in WT and C5aR-/- mice 4 h post IgG injection. (C) Toluidine blue staining showed a significant reduction of MC degranulation in pathogenic IgG-injected C5aR-/- mice compared to WT mice 2 h post injection. (D) ELISA showed a significantly reduced TNF-α in the “wash-out” of C5aR-/- mice as compared to the blister fluid of the WT mice. *p<0.01 (bar 2 vs. bar 4), 6 mice per group. (E) C5aR-/- mice were reconstituted at the ear with buffer control, 1x10^7 MCs from C5aR-/- mice, TNF-α/-/- mice or WT mice. Ten weeks later, pathogenic abs were injected into ears of the MC-reconstituted mice and the ears were examined 24 h later by H&E and MPO assay. C5aR-/- mice reconstituted with WT MCs and not with C5aR-/- or TNF-α/-/- showed restored PMN infiltration and dermal-epidermal blistering. n=6 per group. *p<0.01 (bar 7 vs. bars 5, 6).
Figure 3.6. TNFR1 and not TNFR2 is required for experimental BP. Neonatal WT and mice lacking TNFR1 (TNFR1-/-), TNFR2 (TNFR2-/-), and both TNFR1 and TNFR2 (TNFR1,2-/-) were injected i.d. with pathogenic rabbit anti-mBP180 IgG (2.5 mg/g body weight) and examined post injection. (A) RT-PCR demonstrated that TNFR-1 was expressed constitutively, while TNFR2 expression was induced in the pathogenic IgG-injected skin of WT mice. (B) Pathogenic IgG induced BP blisters in WT, TNFR2-/-, but not TNFR1-/- and TNFR1,2-/- mice. (C) Pathogenic IgG induced BP with significantly increased PMN infiltration in WT mice and TNFR2-/- mice, but not TNFR1-/- and TNFR1,2-/- mice.
*p<0.01 (bar 2 vs. bars 6, 8), 6 mice per group. Three independent experiments were done for each group of mice.

**Figure 3.7. Local reconstitution of MCs bearing TNFR1 and not TNFR2 restores BP disease in TNFR-deficient mice.** WT, TNFR1,2−/−, and MC−/− mice were reconstituted at the ear with buffer control (none), 1x10^6 MCs from WT, TNFR1−/−, TNFR2−/−, or TNFR1,2−/− mice. Ten weeks later, pathogenic antibodies were injected into ears of the MC-reconstituted mice and the ears were examined 24 h post IgG injection by H&E staining and MPO assay. TNFR1,2−/− and MC−/− mice reconstituted with TNFR2−/− MCs (bars 7, 11) or WT MCs (bar 8) but not with TNFR1,2−/− MCs (bars 5, 9) or TNFR1−/− MCs (bars 6, 10) showed restored PMN infiltration and dermal-epidermal blistering. n= 6 for each group. *p<0.01 (bars 7, 8 vs. bars 5, 6; bar 11 vs. bars 9, 10).
**Figure 3.8.** Experimental BP induced by patient anti-BP180 autoantibodies depends on TNF-α. Neonatal NC16A mice were injected i.d. with normal human IgG (control IgG) or pathogenic anti-BP180NC16A autoantibodies (anti-NC16A IgG) and examined 2 and 24 h post injection. (A) Pathogenic anti-NC16A and not control IgG induced BP blisters in NC16A mice 24 h post injection. Pretreatment with anti-TNF-α antibody abolished experimental BP. (B) ELISA showed a significant increase in TNF-α protein in the blister fluid of diseased mice compared to that in the “wash-out” of control mice. Blocking TNF-α significantly reduced TNF-α in the skin. (C) MPO assay demonstrated a significantly increased number of PMNs in the lesional skin relative to the non-lesional skin of control mice and anti-TNF-α-treated mice. *p<0.01 (bar 1 vs. bar 2), 8 mice for each group.
**Figure 3.9. Proposed model for MC regulation of experimental BP.** (1) C5a binds to C5aR on MCs. (2) p38MAPK is phosphorylated and signals. (3) MCs degranulate, releasing pre-formed TNF-α extracellularly. (4) TNF-α binds to TNFR1 on MCs. (5) TNFR1 signaling leads to transcription of unidentified pro-inflammatory factors. (6) The MC-derived pro-inflammatory mediators lead to recruitment of PMNs to the skin and ultimately blister formation.
### 3.5 REFERENCES


CHAPTER 4
DISCUSSION

4.1 INTRODUCTION

MCs are dynamic mediators of host defense and tissue homeostasis. As is often the case in the immune system, the versatile means of activation and powerful responses that make MCs excellent protectors against microbial invaders also make MCs potent arbiters of inflammatory dysfunction and autoimmunity. Understanding the precise mechanisms that lead to initial MC activation and the continuation of MC signaling provides opportunities to intervene pharmacologically to curb autoimmunity.

MCs are key players in the immunopathophysiology of BP. Intact and degranulated MCs are commonly observed in the dermis of BP patients, and histamine and other MC-derived factors are present in high concentrations in the affected skin (1-4). Mice injected with pathogenic anti-mBP180 antibodies exhibit extensive MC degranulation in the lesional skin, similar to that observed in human BP (3, 5). Importantly, mice lacking MCs do not develop experimental BP, indicating that MCs are required for disease development and are not merely uninvolved or tangentially involved bystanders activated by local inflammation. The critical role of MCs in experimental BP is to recruit PMNs from the circulation to migrate into the skin at the site of inflammation (5). MC degranulation releases a host of
chemoactive compounds capable of acting on local endothelial cells to promote PMN capture and extravasation (6, 7).

Activation of the classical pathway of the complement system and generation of the anaphylatoxin C5a is required for MC activation in experimental BP (8). MCs constitutively express C5a on their surface, making C5a-C5aR interaction the likely mechanism of MC activation. However, C5aR is broadly expressed on a wide range of tissues, so it is possible that C5a acts on another local cell type, such as keratinocytes or Langerhans cells (9, 10).

TNF-α, along with a number of other cytokines, is released from degranulated MCs following FceR crosslinking, FcγR crosslinking, or C5aR ligation (11). A potent and pleiotropic molecule, TNF-α elicits a huge range of cellular effects, from the triggering of apoptosis to the upregulation of proinflammatory cytokines (12). TNF-α released from dermal MCs in experimental BP likely acts on cells in the surrounding microenvironment, but the proximity of the MCs to the microvasculature of the DEJ also makes it possible for TNF-α to act globally (13). MC-derived TNF-α could act on the local endothelial cells to promote PMN recruitment, or could potentially act on circulating PMNs themselves, as PMNs express high levels of TNFR1. Alternatively, TNF-α may regulate BP via its action on KCs, LCs, or dermal fibroblasts.

The primary objective of this dissertation was to elucidate the cellular events that occur immediately upstream and downstream of MC degranulation in experimental BP. Our central hypothesis is that complement components activate MCs, and proinflammatory compounds released from the MCs drive pathology and blistering. We found that MCs are activated by the binding of C5a to C5aR. C5a-C5aR interaction leads to activation of
p38MAPK and MC degranulation. MC degranulation releases TNF-α, and TNF-α acts in an autocrine manner on MC TNFR1 to promote disease development.

4.2 FINDINGS AND IMPLICATIONS

We found that C5a contributes to experimental BP disease progression primarily through interaction with C5aR on MCs. C5aR-/- mice injected with pathogenic anti-BP180 antibodies fail to induce MC degranulation and subsequent PMN accumulation. As discussed, C5aR is broadly expressed, so we performed MC reconstitution experiments to confirm that the C5aR on MCs and not another cell type that links complement activation and MC activation. Mice lacking all MCs due to a mutation in c-Kit were reconstituted with either WT MCs or C5aR-/- MCs prior to anti-BP180 antibody injection. Only the WT MCs restored susceptibility to disease, indicating that if C5aR on MCs directly binds C5a and induces MC degranulation.

The basic signaling mechanism of C5aR is that of a GPCR, where ligation of the extracellular terminus induces G protein-mediated activation of secondary messengers within the cell (14). In the case of C5aR, we found that C5a-C5aR interaction results in activation of p38MAPK, and that the p38MAPK signaling cascade is required for MC degranulation. p38MAPK is significantly less phosphorylated in C5aR-/- mice than WT mice following anti-BP180 injection, indicating that C5a binding is the initiating event in the signaling cascade. WT mice injected with rC5a exhibited significantly greater p38MAPK phosphorylation than MC-/- mice, indicating that MCs are the key site of p38MAPK signaling in experimental BP. Furthermore, rC5a directly activates p38MAPK in C5aR-sufficient but not C5aR-deficient cultured MCs. Taken together, these data demonstrate that
C5a interaction with C5aR on MCs and the subsequent MC p38MAPK activation are indispensable for development of experimental BP. In summary, our present studies identify the C5a-C5aR interaction being a critical molecular linker between basal keratinocyte-based complement activation and MC-based p38MAPK activation in experimental BP. Activation of p38MAPK is essential for MC activation, PMN infiltration, and blister formation in experimental BP.

Having established how MCs are activated in experimental BP, we next looked at the consequences of MC activation. We chose to focus on TNF-α based on BP patient data demonstrating that elevated levels of TNF-α are present in serum and blister fluid, and disease severity correlates with TNF-α serum levels (15, 16).

We found that anti-BP180 antibodies triggered release of TNF-α into the serum and blister fluid of diseased mice. The TNF-α is MC-derived, and MC-/- mice do not exhibit TNF-α increases. Mice that lack either TNF-α or MCs fail to recruit sufficient numbers of PMNs to the skin to mediate blistering. MC-/- mice reconstituted with WT MCs are once again susceptible to disease, but reconstitution with TNF-α-/- MCs does not restore blistering. These MC reconstitution studies strongly demonstrate that MC-derived TNF-α-/- is required for experimental BP. TNF-α deficient mice are able to activate complement and degranulate MCs normally, indicating that the C5a-C5aR mediated activation of MCs is not impaired in the absence of TNF-α. We also noted that C5aR-/- mice do not experience MC degranulation or TNF-α, in keeping with our proposed mechanism of MC activation.

We next asked what key targets of MC-derived TNF-α are involved in disease progression. Cytokines are at their highest concentration at the site of release, so we looked the most local of all environments, the MC itself. We found that MC-/- mice do not develop
disease susceptibility upon reconstitution with TNFR1-/-. Disease susceptibility is restored by reconstitution with TNFR2-/- or WT MCs. We conclude that the autocrine feedback of MC-derived TNFR1-/- on MC themselves is required for experimental BP. Thus, MCs are both the critical source of TNF-α and the key target of TNF-α.

We propose a revised model for the signaling activity of MCs in BP (Figure 3.9). The binding of C5a to C5aR on MCs leads to activation of the p38MAPK signaling pathway and MC degranulation, which releases preformed TNF-α from intracellular stores. Released TNF-α binds to MC TNFR1 and initiates an intracellular signaling cascade that leads to production of pro-inflammatory factors that recruit PMNs to the skin.

The studies detailed in this dissertation focused on three molecules that are targets for FDA-approved drugs. Anti-TNF-α therapies include both anti-TNF-α antibodies and TNF-α blocking peptides. Etanercept (Enbrel), infliximab (Remicade), and adalimumab (Humira), are effective in treating a number of inflammatory diseases (17). Limited case reports indicate that treatment with anti-TNF-α therapies is therapeutically beneficial to BP patients (18, 19). Our work makes a strong case for expansion of anti-TNF-α therapies for the treatment of BP. Inhibitors of p38MAPK have been investigated for treatment of autoimmune and inflammatory diseases (20-22). Based on our findings that p38MAPK modulates MC degranulation and experimental BP pathogenesis, human BP may be effectively treated with these inhibitors. However, p38MAKP inhibitors are associated with negative side effects due to numerous off-target responses (23), Efforts to generate more specific inhibitors are constantly underway, however, the results of randomized control trials are mixed (24, 25). Eculizumab is a humanized monoclonal antibody that prevents cleavage of C5 into C5a and C5b, thus curbing their activities (26). Eculizumab has proven to be very
effective in treatment of paroxysmal nocturnal hemoglobinuria, a complement-mediated blood pathology (27). Side effects are generally mild, but depletion of complement increases susceptibility to encapsulated organisms such as Neisseria meningitides (28). Bacterial infections are particularly dangerous for patients with impaired skin barrier function, and BP patients often have large areas of eroded skin.

Special considerations should be made when evaluating the appropriateness of a clinical intervention in BP. Because elderly patients often have multiple co-morbidities associated with advanced age, physicians must cautiously consider the likelihood of a drug being tolerated. BP affects the skin, making topical application of medications a possibility. The advantage of potentially lower morbidity due to local rather than systemic application must be counterbalanced by mobility constraints that affect many BP patients. In the case of p38MAPK inhibitors, several topical treatments have been tested in the laboratory setting, but no clinical trial data is available yet (29).

All three types of drugs described here have the distinct disadvantage of being extremely costly compared with corticosteroids. These drugs would likely be considered last resorts rather than first-line efforts at disease management.

4.3 REMAINING QUESTIONS AND FUTURE DIRECTIONS

The data described here present an intriguing new look at the signaling events associated with MC activation, function, and the potential for crosstalk between pathways. The binding of C5a to C5aR on MCs activates the p38MAPK signaling pathway and MC degranulation, which releases preformed TNF-α from intracellular stores (30). This initial wave of TNF-α feeds back to MC TNFR1 and likely initiates an intracellular signaling
cascade that leads to activation of the transcription factors NF-kB and c-Jun, though we have not yet demonstrated this. (12). NF-kB and c-Jun mediate production of pro-inflammatory factors, and our laboratory is currently investigating roles for these compounds in PMN recruitment and BP pathogenesis. Cross talk between the NF-kB and c-Jun NH(2)-terminal kinase (JNK) signaling pathways following TNF-R1 stimulation is an active area of research (31). The genes growth arrest and DNA damage-inducing protein (GADD45β) and X-chromosome-linked IAP (XIAP) are induced by NF-kB after TNF-R1 engagement and have been demonstrated to block JNK activation (32, 33). GADD45β activates MTK1/MEKK4, which in turn activates the p38MAPK and JNK pathways (34). The question of whether cross talk exists between the TNF-R1 signaling and the C5aR signaling via is of great interest to the field of MC signal transduction in autoimmunity and has yet to be explored.

The relative contribution of TNFR1 and C5aR signaling to the MC cytokines released is also an interesting question. In the animal model, C5aR signaling releases a large bolus of proinflammatory mediators by causing degranulation, while TNFR1 signaling conceivably activates *de novo* synthesis of inflammatory factors. It seems unlikely that the TNFR1-mediated protein synthesis raises local concentrations of cytokines significantly in the short frame of time between MC degranulation (4 hours post-antibody injection) and PMN recruitment (8 hours post-antibody injection). Do the MCs produce factor(s) not stored in secretory granules upon TNFR1 engagement? If so, what is the identity of the factor(s)? Are these factor(s) directly responsible for PMN recruitment?

Like all animal models, experimental BP has limitations. While the pathogenic events of experimental BP occur within 24 hours, human BP is a chronic condition that can last years, and relapses are common (35). In human BP, is C5a consistently available to
mediate MC degranulation, or are skin complement levels depleted, forcing MCs to release cytokines via signaling cascades other than the C5aR pathway?

MC-deficient WBB6F1-Kit<sup>W</sup>/Kit<sup>W-v</sup> mice have been used to study the role of MCs in physiological processes for many years. Kit<sup>W</sup>/Kit<sup>W-v</sup> mice have mutations in the Kit gene, leading to MC deficiency. However, Kit is involved in development of other cell lineages, so Kit<sup>W</sup>/Kit<sup>W-v</sup> mice are also deficient in erythrocytes, PMNs, melanocytes, germ cells, pacemaker cells, and certain subpopulations of intraepithelial T cells (36). Recently two strains of mice were developed that delete MCs by Cre-lox technology but have intact Kit expression (37, 38). Intriguingly, MC-deficient mice with intact Kit are resistant to experimental autoimmune encephalitis and autoimmune inflammatory arthritis (38), in contrast to the data obtained with Kit<sup>W</sup>/Kit<sup>W-v</sup> animals. In light of this study, it may be pertinent to reexamine the roles of MCs and PMNs in experimental BP using the new MC deficient animal strains.

4.4 CONCLUDING REMARKS

Despite being described over 100 years ago, there is still much to learn about the actions of MCs under homeostatic and pathologic conditions. The findings described here provide a small sliver of insight into the complex network of interactions between MCs and other immune and non-immune cells. In experimental BP, MCs are activated by the binding of C5a to C5aR. C5a-C5aR interaction leads to activation of p38MAPK and MC degranulation. MC degranulation releases TNF-α, and TNF-α acts in an autocrine manner on MC TNFR1 to promote disease development. We hope that these findings may one day lead to alleviation of suffering from BP.
4.5 REFERENCES


