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in situ differentiation of B cells cross-reactive to Epstein-Barr virus and autoantigens in hidradenitis suppurativa

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An abstract of a dissertation submitted to the faculty of
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Abstract

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By Karla Navarrete

Hidradenitis suppurativa (HS) is a chronic inflammatory skin disease characterized by painful nodules, abscesses, and tunnels involving intertriginous skin. HS negatively impacts the mental wellbeing and economic and educational standing of sufferers. HS is not uncommon with an estimated prevalence of 0.1% in the United States. Prevalence is highest among Black women. While the etiology of HS remains unknown, emerging evidence suggests an autoimmune component. B cells are known to infiltrate affected skin and the presence of autoantibodies correlates with disease severity. To understand the B cell contribution to pathogenesis, we conducted single cell transcriptomics and repertoire analysis on B cells and plasma cells from HS surgical excisions and peripheral blood. We found that infiltrating B cells exhibit a chronic activation phenotype and contribute to the inflammatory milieu of the skin by producing TNFα. A large subset of infiltrating B cells has immunosuppressive potential, producing anti-inflammatory TGBβ. Infiltrating B cells undergo antigen-driven *in situ* clonal expansion and plasma cell differentiation, which is associated with inflammatory cytokine production. A serological screen revealed increased reactivity to Epstein-Barr virus (EBV) epitopes in HS patients compared to healthy controls. Subsequently, we identified clonally expanded and differentiated autoreactive B cells that cross-reacted with EBV protein EBNA1, and autoantigens DNA topoisomerase I (SCL-70) and P21 activated kinase 4 (PAK4). Our findings suggest that chronic activation, differentiation, and clonal expansion of autoreactive B cells contribute to HS pathogenesis and that EBV reactivation may be a factor in HS etiology.

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Preface: A History Lesson

In 1954, during the centennial celebration of Paul Ehrlich's birthday, Ernest Witebsky, a second-generation mentee of Ehrlich's, said in a speech, "The validity of the law of *horror autotoxicus* certainly should be evident to everyone interested in the field of blood transfusion and blood disease. Autoantibodies––namely, antibodies directed against the receptors of the same individual––are not formed".[1] What Witebsky referred to as a law was the concept introduced by Ehrlich that organisms are unable to form toxic autoantibodies. Today, we know that the development of autoantibodies is not only possible, but also the cause of significant morbidity in humans.

In the year 1900, 8 years before winning the Nobel Prize for Medicine, Ehrlich reached his conclusion of *horror autotoxicus* after a series of experiments he conducted with Julius Morgenroth. In these experiments, Ehrlich and Morgenroth immunized animals with blood from a different species, the same species, and from the individual. These experiments resulted in xenohemolysins and isohemolysins, but failed to elicit autohemolysins. Ehrlich concluded, "It would be dysteleologic in the highest degree, if under these circumstances self-poisons of the parenchyma––autotoxins––were formed," and coined the term *horror autotoxicus,* the unwillingness of organisms to endanger themselves by mounting immunity against their own antigens.

Between the time of Ehrlich's observation and Witebsky's speech, evidence of autoimmunity abounded. Serge Metalnikoff observed antibody formation in animals injected with their own spermatozoa.[2] This challenge to *horror autotoxicus* was dismissed by Ehrlich's camp because the sperm antibodies were not shown to cause disease *in vivo*. Stronger evidence would come in 1904, when Julius Donath and Karl

Landsteiner demonstrated that paroxysmal cold hemoglobinuria (PCH) involved autoantibodies to erythrocytes. PCH is a rare disease characterized by hemolysis upon patient exposure to cold temperatures. In a series of experiments, Donath and Landsteiner demonstrated that a factor found only in the serum of PCH patients bound to healthy human erythrocytes at cold temperatures, which caused erythrocyte lysis upon introduction of healthy human serum at body temperature. The factors in PCH patient serum were antibodies to the P antigen on erythrocytes.

Here was evidence of an autoantibody causing disease and clear rationale for revision of the "law" of *horror autotoxicus*. When referring to the findings of Donath and Landsteiner, Ehrlich expressed his interest in this "amboreceptor" acting upon red blood cells. However, Ehrlich's camp ostensibly saw this as an exception to the rule and the law of *horror autotoxicus* still reigned 50 years later. So much so that even as Witebsky was making his speech at Ehrlich's centennial, he was sitting on his and Noel Rose's discovery of autoantibodies to thyroid proteins and their role in thyroiditis, which was published three years later.[3] Today, Ehrlich is credited with coining the term synonymous with autoimmunity, but one is left to wonder whether progress was hindered by deference to such a bright star. Autoimmunity finally emerged as an accepted concept in the 1960s and immunologists have made tremendous strides in its identification and treatment since.

Immunologists hold core tenets that are necessary to ground findings and separate the plausible from the absurd. However, adopting pillars of immunology as if they are irrefutable laws can be an impediment to progress. During my training I have participated in research that, while carefully designed and methodically executed, was too challenging to immunological dogma to be taken seriously. But I am optimistic. The

existence of autoimmunity was a fact waiting to be discovered by new generations of immunologists who did not equate challenging dogma to sacrilege. It has been an honor to be among a new generation of immunologists preparing for long careers of pushing the boundaries of human knowledge. May we remain pliant to new ideas, and may there be many more generations to challenge us in case we do not.

Chapter 1: Introduction

Self-tolerance

A healthy immune system exists along a spectrum of tolerance to the body's own proteins and activity against pathogens. Extreme tolerance means immunodeficiency and a lack of tolerance means autoimmunity. To strike a balance, the immune system is regulated by layers of tolerance mechanisms that counteract its ability to build a response against an infinite variety of proteins. Tolerance mechanisms can be categorized into central and peripheral tolerance. Central tolerance is the process that occurs during development of B and T cells to ensure that autoreactive cells do not mature and enter circulation. This process is not infallible, as the balance between maximizing repertoire diversity and preventing self-antigen (autoantigen) specificity is delicate. A second layer of protection against autoimmunity is peripheral tolerance, a combination of mechanisms to deter activity of circulating autoimmune B and T cells that escape central tolerance. In a healthy individual, these tolerance mechanisms prevent autoimmune disease. However, individuals with genetic susceptibility or exposure to certain environmental triggers experience a breakdown of tolerance, leading to the development of autoimmune disease. In this chapter, I discuss central and

peripheral tolerance mechanisms, factors leading to tolerance breakdown, and the development of autoimmune disease.

The diversification of B and T cell receptors is vital to the function of the adaptive immune system. However, the same mechanisms that maximize receptor diversity can lead to the development of receptors that bind the body's own proteins. The TCR is comprised of an α and a β chain that develop through a process known as rearrangement. The TCR locus in the genome is comprised of several redundant gene segments that must be narrowed down to a genomic sequence containing one of each. This process involves selecting among these redundant gene segments and joining them together while splicing out the extras. Immature thymocytes developing in the thymus commence TCR development by rearranging their β chains. First, one of two D_β gene segments is joined to one of 12-13 J_β segements, followed by the joining of one of 40-48 V_β segments to the selected $D_\beta J_\beta$ segments. If the resulting β chain is functional, rearrangement of the α chain begins. Unlike the β chain, the α chain does not have a D gene segment, and its rearrangement simply involves the joining of one of 43-45 V_α segments to one of 50 J_α segments. The process of selecting among redundant V, D, and J segments during rearrangement is a significant source of diversity in the T cell repertoire. A second source of diversity is junctional diversity, which arises as a result of the deletion and addition of nucleotides during the imperfect joining of gene segments.

Once thymocytes express rearranged α and β chains, they undergo positive and negative selection based on the binding properties of their TCR. TCRs are major histocompatibility complex (MHC)-restricted, meaning that they will only bind antigen-derived peptides when they are being presented to them on MHC by antigen presenting

cells (APC). TCR+ thymocytes are programmed for cell death unless they receive survival signals from thymic epithelial cells (TEC) and other APC in the thymus. Positive selection requires the TCR to be adequately responsive to peptide/MHC complexes during a specific window of time during their development. The vast majority (90-95%) of TCR+ thymocytes fail to receive these survival signals and die, ensuring that mature T cells that enter circulation can recognize their cognate antigen when complexed with MHC. Before MHC-restricted thymocytes can enter circulation however, they are subjected to negative selection—the essential mechanism of central tolerance. Thymocytes that bind and react too strongly to autoantigens during development are deleted from the immature repertoire. In order to test thymocyte reactivity to autoantigen while confined to the thymus, TEC express proteins found in peripheral tissues, a process known as ectopic antigen expression. Expression of these ectopic antigens, or tissue restricted autoantigens (TRA), by TECs is mediated by the transcription factor, autoimmune regulator (AIRE). AIRE regulation allows TECs to present TRA:MHC complexes to lymphocytes. Strong binding at this stage of lymphocyte development leads to deletion from the repertoire, preventing T cells specific for autoantigens from entering circulation. The balance between positively selecting thymocytes with self-MHC reactivity and eliminating thymocytes with autoantigen reactivity is believed to depend on the strength of TCR signaling and binding strength, a theory called the affinity hypothesis. Of course, central tolerance is not infallible, and autoreactive T cells are known to regularly enter the circulation of healthy individuals, necessitating peripheral tolerance mechanisms.

B cell receptors (BCR) develop to be highly specific to antigen at multiple stages throughout the life cycle of the B cell. Immature B cells in the bone marrow undergo

rearrangement of their BCR similarly to thymocytes. BCR are comprised of a light chain and a heavy chain, encoded by genes at the immunoglobulin (Ig) locus of the genome. The Ig locus contains redundant V, D, and J gene segments, and their combinatorial joining is mediated by RAG recombinases. BCR rearrangement begins with heavy chain joining of one of 25 D_H segments and one of 6 J_H segments. This is followed by the joining of one of 40 V_H genes to the D_HJ_H segments. The BCR heavy chain also includes a constant region, or C gene, that confers function to the BCR and determines its isotype. All immature B cells in the bone marrow develop a BCR with an IgM isotype, conferred by the C_μ constant region. The rearranged heavy chain is expressed in conjunction with a surrogate light chain composed of *VpreB* and λ5. The associated surrogate light chain and heavy chain are the pre-BCR, which is expressed to test proper heavy chain folding and association with Igα and Igβ, a surface bound signaling complex that transduces signal for the BCR. If pre-BCR expression and folding is successful, it is downregulated, and light chain rearrangement is triggered. Humans have two light chain alleles, κ and λ. Light chain rearrangement begins on the κ allele, where one of 40 V_κ segments is joined to one of 5 J_κ segments. If the κ chain is unsuccessful, a λ is chain is attempted by joining one of 30 V_κ segments to one of 4 J_κ genes. A successful light chain rearrangement will be expressed, and associate with the heavy chain as a complete IgM BCR on the surface of the B cell.

A diverse B cell repertoire is necessary for a functional immune system. B cell diversity is built by layers. The availability of multiple alleles, combinatorial joining of gene segments, and junctional diversity all contribute to BCR specificity and consequently, the breadth of antigens that can be recognized. However, B cells

developing in the bone marrow can rearrange BCR that are specific for autoantigens and must therefore be subjected to central tolerance mechanisms. B cells specific for autoantigens in the bone marrow have multiple potential fates based on the strength of their binding. These fates include clonal deletion by apoptosis, adjustment of specificity via receptor editing, functional suppression, or release into the periphery as clonally ignorant mature B cells. B cells that respond strongly to autoantigen undergo death by apoptosis, which is known as clonal deletion. Alternatively, autoreactive B cells can be rescued from apoptosis by re-entering the rearrangement phase of development. Their autoreactive light chain can be replaced by a newly rearranged version, which will also be subjected to a tolerance test. This receptor editing process will continue until a non-autoreactive BCR is produced or until the cell exhausts its available genes for rearrangement. If the latter occurs, the cell will apoptose. Autoreactive B cells can exit the bone marrow anergic, or functionally unresponsive to their cognate antigen. If anergic B cells are at a disadvantage in the periphery and will eventually die in competition with functionally competent B cells for survival signals. Finally, autoreactive B cells that are not exposed to their cognate autoantigen in the bone marrow or are weakly reactive enough to escape deletion can enter the periphery as mature B cells. These immunologically ignorant cells can become activated if they encounter large volumes of their cognate antigen or under inflammatory conditions. Some weakly autoreactive B cells can cross-react to non-self pathogens, making their inclusion in the peripheral repertoire beneficial. Therefore, the fact that self-reactive B cells are culled, but not entirely eliminated by central tolerance mechanisms is reflective of the balance between self-tolerance and non self-reactivity that the immune system must strike.

Autoreactive B and T cells that escape central tolerance and are able to mature and enter the periphery are subject to peripheral tolerance mechanisms. Autoreactive B and T cells that encounter and are activated by their cognate antigen are either deleted or rendered anergic. Autoreactive T cells have also been shown to alter their phenotype into that of a regulatory T cell with immunosuppressive effector functions, effectively eliminating their potential for harm, in a process called functional deviation. One final peripheral tolerance mechanism is autoantigen sequestration. B and T cells are prevented from entering certain tissues of immune privilege to prevent potentially autoreactive cells from encountering their cognate antigen and causing tissue damage. Immune privileged tissues tend to be those evolutionarily necessary for survival such as the eyes and the brain. Despite the host of peripheral tolerance mechanisms, they are not effective unless employed. How do autoreactive B and T cells encountering cognate autoantigen know that they have not encountered a pathogen and that they should become anergic or apoptotic? The answer is evidently context. Under inflammatory conditions, which would arise during infection or injury, B and T cells receive signals from surrounding cells that prime them for antigen-dependent activation. In the absence of inflammatory conditions and danger signals, cognate antigen activation leads to apoptosis, anergy, or functional deviation.

Autoimmune Disease Etiology

The most severe consequence of failures in tolerance mechanisms is autoimmune disease. Autoimmune diseases are diverse, complex, and involve many facets of the immune system in both their origin and progression. Often, autoimmune diseases do not have a single direct cause, but a combination of contributing factors that alone are

insufficient to cause disease. Additionally, patients with the same disease presentations may have had different triggers and patients with the same triggers may have different disease presentations. Despite its complexity, tremendous strides have been made in the field of autoimmunity, which have led to the discovery of genetic and external factors leading to disease, mechanisms of disease progression, and treatments.

Autoimmunity can result from genetic factors, particularly those that cause defects in tolerance mechanisms. People with autoimmune polyglandular syndrome type 1 (APS1) have a mutation in *AIRE*, the regulatory factor that mediates ectopic TRA expression in the thymus.[4] The functional defect in *AIRE* affects negative selection of developing T cells, leading to autoreactive T cells entering the periphery and causing damage to multiple organs. An X-linked mutation in the regulatory T cell transcription factor *FOXP3*, leads to a decreased numbers of peripheral regulatory T cells.[5] Without the ability to functionally deviate autoreactive T cells or to mitigate their effects, people with this mutation develop immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX). Male infants with this inherited mutation present with a wide array of systemic autoimmune symptoms and usually do not survive their second year of life. Type 1 diabetes and Grave's disease, an autoimmune disease affecting the thyroid, are both associated with inherited alterations to the *CTLA4* gene.[6] *CTLA4* is an inhibitory receptor expressed by T cells. The mechanism disrupted by *CTLA4* defects is not well understood but is believed to contribute to the induction of anergy in autoreactive T cells. Autoimmune lymphoproliferative syndrome (ALPS) is caused by a defect in the FAS, a death receptor on B and T cells.[7] As they proliferate in response to stimulatory signals and antigen binding, B and T cells become increasingly susceptible to FAS-mediated apoptosis. This mechanism becomes an especially important

constraint on chronically stimulated autoreactive B and T cells. Certain human leukocyte antigen (HLA) haplotypes are also associated with autoimmunity. An individual's haplotype determines the range of peptides that can be presented on MHC and therefore affects T cell activation. Finally, certain epigenetic factors are associated with autoimmunity. Changes in DNA methylation, histone modification, and microRNA expression are associated with systemic lupus erythromatosus (SLE), rheumatoid arthritis (RA), and primary biliary cholangitis (PBC).[8]

Most autoimmune diseases are not known to be invariably caused by a genetic mutation. Instead, they arise when a combination of external triggers exacerbates the effects of minor genetic factors or the naturally occurring autoreactive B and T cells in a healthy repertoire. One example of such a trigger is injury. When tissue damage occurs due to injury, large amounts of autoantigens are released into the lymphatic system. This exposes B and T cells to autoantigens that they were not tolerized against during development. Injury also causes inflammation that relays danger signals to B and T cells. Naturally occurring autoreactive B and T cells, that may not have otherwise encountered their cognate autoantigen and died of neglect, can then mount a destructive immune response to the body's own tissues. This is the case in sympathetic ophthalmia, a dangerous autoimmune response to the eyes that is triggered by an eye injury.[9] Eye antigens are normally sequestered from the immune system as a site of immune privilege but this changes with severe eye injury. Activated eye-reactive effector T cells can then enter both eyes and cause further damage and blindness. During an autoimmune response, the immune system itself becomes the source of tissue damage, autoantigen exposure, and inflammation, leading to a positive feedback loop and

amplification of autoimmune responses. As a result, many autoimmune diseases become chronic and must be managed for a lifetime.

Pathogens have co-evolved with humans for as long as humans have existed. The human immune system has evolved to recognize and eliminate pathogens while pathogens have evolved to evade the immune system of their host. This interplay has previously been thought of as an arms race, evoking a war spanning centuries. However, many now subscribe to the more pacifistic idea that the immune system has evolved to maintain homeostasis with our commensal microbiome in the gut and on the skin. Regardless, the very strategies that pathogens have evolved to evade the immune system tend to have the capacity to cause autoimmune responses. The mechanisms by which pathogens cause or exacerbate autoimmune disease are not well understood and are largely theoretical. The general theories include bystander activation, molecular mimicry, and epitope spreading.

The immune system can cause bystander activation and non-specific tissue damage in its efforts to rid the body of bacterial or viral infection. This type of non-specific autoimmunity may resolve once the pathogen is cleared. However, as with injury, tissue damage due to infection may flood the lymphatic system with inflammatory signals and autoantigens at volumes that B and T cells would not normally encounter. This creates opportunity for autoreactive lymphocytes to establish a specific autoimmune response that may not have occurred in the absence of infection.

Some bacteria and viruses evade the immune system by mimicking self-antigens. In theory, molecular mimicry would protect pathogens from an immune system that is tolerized against the self-antigens they mimic. However, cross-reactive B cells that can bind both the pathogenic antigen and the self-antigen it resembles can still become

activated. This potential for cross-reactivity with pathogens can be an advantage of naturally occurring autoreactive lymphocyte “leak” into the periphery. Cross-reactive B cells can engage similarly autoreactive T cells and together mount an immune response that eliminates the pathogen. However, while a normal immune response would resolve once a pathogen is eliminated and its antigens are no longer in circulation, the cognate autoantigen that activates cross-reactive cells will remain. Thus, cross-reactive responses fail to resolve, inflammation and tissue damage continue, and autoimmune disease is established.

Some viruses have evolved the ability to remain latent in host cells. Latency allows the virus to “hide” from the immune system until it reactivates an active, lytic, infection. This strategy allows the virus to remain seeded in the host without ever being entirely cleared from the body. Depending on viral, host, and external factors, a latent virus may never reactivate or do so infrequently. However, in some cases a latent virus can reactivate frequently and become the source of cyclical immune activation and pathology. Given that the adaptive immune system is designed to hone the specificity of responses over time, persistent viruses can shape the immune repertoire throughout the lifetime of their hosts. In a process known as epitope spreading, B cells begin to recognize a wider array of antigen motifs, or epitopes, the longer the antigen persists. As a result, a B cell that may not have originally been autoreactive can gain the ability to bind autoantigens and B cells with reactivity to an autoantigen can gain the ability to bind new autoantigens.

While establishing causation between infection and autoimmune pathogenesis in humans is extremely difficult, several have been implicated and exhibit the aforementioned mechanisms in humans and murine models. Molecular mimicry by

herpes simplex-1 (HSV-1) is believed to lead to autoimmunity in the eye in a condition called autoimmune herpes stromal keratitis.[10] A capsid protein found in HSV-1 can be recognized by autoreactive T cells specific for corneal antigens. Bacterial infection with *Streptococcus pyogenes* causes autoimmunity to the heart due to its M protein mimicking cardiac myosin.[11] This was demonstrated experimentally in a murine model when mice immunized with human cardiac myosin cross-reacted with *S. pyogenes* M protein.[12] Enteroviruses, a genus named for its intestinal transmission route, have been associated with autoimmune diseases including type 1 diabetes and autoimmune myocarditis. Coxsackie virus is an enterovirus that is the most common cause of infectious myocarditis (before the emergence of SARS-CoV2). Coxsackie virus can infect and lyse cardiac myocytes in vitro and viral RNA can be detected in the heart of mice up until day 14, but myocarditis symptoms persist in the absence of viral detection, suggesting an autoimmune component.[13] After infection, autoantibodies to myosin, tropomyosin, and actin arise in mice.[14] Finally, coxsackie virus has been implicated in the development of type 1 diabetes. Beta cells can be infected by coxsackie virus, leading to innate cell effector responses, inflammation, tissue damage, and the opportunity for lymphocyte exposure to autoantigens.[15] Later in this chapter, I will discuss Epstein-Barr virus, which has been implicated in several autoimmune diseases including MS, RA, and SLE.

Delineating the etiology of autoimmune disease is complex, as it is nearly always multifactorial. Despite its challenges, it is a necessary endeavor to undertake because many current therapeutic strategies are not disease specific and fail to adequately manage symptoms in all patients. Therefore, understanding autoimmune disease triggers is vital for identification of disease-specific treatment strategies and prevention.

Pathogenic Mechanisms and Treatment Strategies

Autoimmune diseases can share an etiology but vary in their manifestation. The pathology and clinical presentation of the disease is determined by the group of antigens targeted and the immune mechanisms that dominate. The same immune mechanisms that work in concert to clear foreign pathogens from the body are at play in an autoimmune response. B and T cells are the crux of this response due to the binding properties of their receptors and their consequent ability to direct mechanisms towards self. However, B and T cells do not operate alone, and instead coordinate with the innate arm of the immune system to cause tissue damage and perpetuate disease.

There are two main type of T cells involved in immune and autoimmune responses: CD4+ and CD8+ T cells. CD4+ T cells, also known as helper cells, coordinate the immune response by producing signaling proteins called cytokines, which have a variety of functions and target cells. The cytokines produced by the activated CD4+ T cells depend on the circumstances under which it was activated, namely the co-stimulatory signals it received from the antigen presenting cell (APC) in the form of cytokine and surface receptor binding. In the context of autoimmunity, an autoantigen may be taken up by an APC such as a dendritic cell or B cell because it has been exposed to circulation by infection or injury. That APC is subjected to danger signals due to local tissue damage, which causes it to phagocytose antigens and enter the lymphatic system to find a CD4+ T cell to activate. An autoreactive CD4+ T cell, which may be in circulation due to failures in central or peripheral tolerance due to genetic factors, is presented its cognate autoantigen in complex with MHC class II, which is only expressed by APC. The APC communicates via cytokines and co-stimulatory molecules

on its surface that the T cell should not become anergic because alarm is warranted. The APC can also communicate the location where it obtained the autoantigen, which will allow the CD4+ T cell to home to that location via expression of integrins, surface molecules that bind to adhesion molecules expressed by endothelial cells under inflammatory conditions. Upon stimulation, the CD4+ T cell will begin to secrete inflammatory cytokines such as IFNγ, TNFα, IL-17, IL-1β, IL-6, and GM-CSF. CD4+ T cell cytokines in turn activate innate immune cells with effector functions that result in direct tissue damage, additional immune cell recruitment, and overall amplification of inflammation.

Autoreactive CD8+ T cells are also activated by APC, but their cognate autoantigen is presented on MHC class I, which is expressed by all nucleated cells. Under homeostasis, nucleated cells express MHC class I complexed with their own endogenous autoantigens. This communicates to non-autoreactive T cells and NK cells that they are not infected by an intracellular bacteria or virus. However, an activated autoreactive CD8+ T cell can bind its cognate autoantigen presented by a cell that normally expresses that antigen, and directly kill that cell via toxic molecule release or death receptor binding.

B cell generation of autoreactive antibodies is a major factor in autoimmune disease. Autoreactive naïve B cells encounter their cognate autoantigen in secondary lymphoid organs with the help of follicular dendritic cells that function as antigen traps. Naïve B cells internalize their BCR:antigen complex and present the antigen to a subset of CD4+ T cells call follicular helper cells, which provide an activation signal to the B cell. At this point, the activated B cell undergoes class-switch recombination (CSR), which changes the isotype of their BCR from IgM/D to IgG, IgE, or IgA. What follows is

a germinal center reaction in which the B cell's affinity for its cognate antigen increases in a process called affinity maturation. A B cell undergoing a germinal center reaction mutates its previously rearranged Ig locus, which alters its BCR. Mutation of the Ig locus within the germinal center is called somatic hypermutation (SHM) and is mediated by the protein activation-induced cytidine deaminase (*AID*). Germinal center B cells compete over a limited amount of antigen to present to T follicular helper cells, which provide survival and proliferation signals. B cells that do not improve their affinity for antigen, lose the competition and die of neglect. Through iterative cycles of mutation, proliferation, and competition for antigen, the germinal center results in a set of B cells with the same high affinity for cognate antigen. Affinity matured B cells can exit the germinal center reaction as memory B cells, which have the potential to be activated by their cognate antigen in the periphery, or plasma cells, which produce large amounts of secreted antibody.

In the context of autoimmunity, affinity maturation of autoreactive B cells to autoantigens can occur in germinal centers, leading to autoreactive memory B cells, plasma cells, and secreted autoantibodies. Late stage, chronic autoimmunity can repeatedly flood secondary lymphoid organs with autoantigen, leading to long-lived germinal centers that yield highly mutated, potentially epitope-spread, autoreactive B cells. Autoreactive memory B cells, like T cells, can home to sites of inflammation via integrin expression and become activated when they encounter their cognate autoantigen. Memory B cells have a lower threshold for activation than naïve B cells, so cognate antigen encounters lead to rapid proliferation, cytokine production, and differentiation into plasma cells.

Autoreactive plasma cells secrete autoantibodies, which are significant mediators of autoimmune pathology. Depending on their isotype, which confers function to an antibody, autoantibodies can cause tissue damage in a variety of ways. Autoantibodies binding soluble autoantigen form immune complexes that are a major activator of innate immune mechanisms like complement and cellular action. Complement is an inflammatory pathway comprised of proteins that can individually cause inflammation and together cause direct lysis of cells. Autoantibodies can lead to autoantigen phagocytosis by innate immune cells in a process called opsonization. Most innate cells, all with their own tissue-damaging effector functions, have Fc receptors on their surface, which bind the Fc domain of autoantibodies. Innate cell effector functions are triggered when they bind autoantibodies bound to autoantigen. Finally, autoantibodies to cell surface receptors can cause disease by agonizing or antagonizing those receptors. The receptor being bound is either over-stimulating the cell or being blocked from stimulating the cell.

Understanding how the normal effector functions of the immune system become dysregulated and erroneously targeted in an autoimmune disease is important for developing therapeutic strategies and treatments. The most common therapeutic strategies for autoimmune diseases target cytokines, co-stimulatory molecules, integrins, and B cells.[16] The most used therapeutics for autoimmunity are janus kinase (JAK) inhibitors. JAK are a group of signal transducing molecules that mediate the expression of receptors for more than 50 cytokines. JAK inhibitors essentially blocking cells from responding to cytokine signaling.[17] The cytokines affected by JAK inhibitors do not include TNF, IL-1, and IL-17. JAK inhibitors, tofacitinib and baricitinib, have been successfully used to treat RA, psoriatic arthritis, psoriasis, inflammatory bowel

disease, and alopecia areata. However, the non-specific nature of these inhibitors leads to the side effect of increased susceptibility to infection.

Direct binding and blocking of cytokine receptors by biologics, a class of drug comprised of engineered monoclonal (single target, identical) antibodies, have also been useful in treating autoimmune disease. Biologics to inflammatory TNFα, IL-17, IL-12/23, and IL-6 receptor have all been successfully used to treat Crohn's, RA, psoriasis, and ulcerative colitis. In theory, blocking a cytokine that contributes to the exacerbation of inflammation would reduce inflammation and ameliorate symptoms. While this is sometimes the case, many anti-cytokine biologics fail in some patients or work initially and stop working over time. This is likely due to the complexity of inflammatory microenvironments, as well as cytokine functional redundancy. Efficacy also varies widely from patient to patient and disease to disease, highlighting the diversity of pathogenic mechanisms.

Blocking co-stimulation between APC and T cells is another therapeutic target for autoimmune diseases. CD40 expressed by APC, including B cells, associates with CD40L on T cells resulting in T and B cell activation and B cell isotype switching. A CD40L binding protein without an Fc domain is currently under clinical testing and show promise in patients with RA.[18] Another set of co-stimulatory molecules, CD80/86 expressed by APC and CD28 on T cells, have been targeted with therapeutics. Interaction between CD80/86 and CD28 activates effector T cells but can be blocked with a fusion protein (abatacept) that binds to CD80/86 with higher affinity than CD28, effectively outcompeting it for binding. Abatacept has been used to successfully treat psoriatic arthritis, juvenile idiopathic arthritis, and RA but is ineffective in irritable

bowel disease, multiples sclerosis (MS), and lupus nephritis, highlighting the different mediators of pathology among autoimmune diseases.

Integrins are expressed by lymphocytes homing to areas of inflammation. The endothelial cells of local lymphatic and blood vessels receive inflammatory signals and recruit immune cells by expressing adhesion molecules that bind to integrins. Chemokines, a subset of cytokines involved in homing, also play a role in this process. Integrins are an attractive target for therapeutics as they prevent immune cells from infiltrating inflamed tissue and causing further damage. Monoclonal antibodies to the α4 subunit of integrins α4β7 and α4β1 have shown efficacy in multiple sclerosis and inflammatory bowel disease. α4β7 binds to Mad-CAM-1 cell adhesion molecule, which is specifically expressed by endothelial cells in the gut and α4β1 binds VCAM-1 adhesion molecule expressed more widely by inflamed endothelial cells. A monoclonal antibody to the α4β7 complex has also been developed and shown efficacy in ulcerative colitis.

Autoantibody production and other B cell effector functions, including cytokine production and T cell co-stimulation, play a significant role in autoimmune disease. Therefore, B cell targeting has emerged as a therapeutic strategy. A monoclonal antibody to CD20, a surface molecule expressed by B cells, but not plasma cells, depletes B cells to reduce their numbers in circulation. was initially developed to treat B cell proliferative diseases. Anti-CD20 was initially developed to treat proliferative B cell diseases, but has now been tested and shown efficacy in autoimmune diseases with demonstrable B cell contributions, including MS and RA.[19] The downside of indiscriminate B cell depletion is that any benefits conferred by regulatory B cells, an anti-inflammatory B cell subset, are lost. Another method by which to target B cells is by

preventing their survival and differentiation. This has been attempted via BAFF/APRIL inhibitors and BTK inhibitors. BAFF and APRIL are cytokines that mediate B cell maturation and activation. Biologics and fusion proteins targeting BAFF and APRIL have had mixed results, showing efficacy in SLE patients, and MS mouse models, but exacerbating MS during clinical trials.[20,21] BTK is an intracellular molecule involved in transducing BCR signals that mediate activation, differentiation, and proliferation of B cells.[22] BTK inhibitors for MS are currently being evaluated in clinical trials and several, differing in selectivity and potency, have shown promise in mouse models.

Epstein-Barr Virus and Autoimmune Disease

Epstein-Barr virus (EBV) is a human herpesvirus that causes infectious mononucleosis, also known as "kissing disease". EBV is associated with several autoimmune diseases and cancers. However, establishing a causal link between EBV and disease is complicated by the fact that greater than 95% of people will acquire EBV in their lifetimes, but the majority of them will never develop EBV-associated malignancies. EBV may therefore not be the single causative agent of autoimmune disease, but rather a trigger compounding with other host factors to cause autoimmune pathology that would not otherwise arise. A study published in Science in 2022 by Bjornevik et al. established the strongest link between EBV and an autoimmune disease, namely MS.[23] In this longitudinal study, the department of defense serum repository was leveraged to test the EBV seropositivity of military personnel over time. 800 out of 801 people who developed MS were seropositive for EBV at least 1 year prior, with an estimated average of 7.5 years between EBV seroconversion and MS onset. It was found that the risk of MS was increased 32-fold after EBV infection, but not cytomegalovirus (CMV) infection, a

similarly transmitted virus. This may be the strongest and most recent evidence, but an association between EBV and MS, RA, SLE, and several other autoimmune diseases has been suspected for decades.

EBV is a complex DNA virus transmitted via saliva exchange. It is a B cell tropic virus, meaning that it predominantly infects B cells via their complement receptor, CD21. EBV can infect epithelial cells, which is likely the mechanism by which it crosses the mucosal epithelium to establish a B cell infection. EBV has two distinct replication strategies: latent and lytic.[24] Latent replication is characterized by the tethering of a circularized EBV genome, called an episome, to the DNA of its host cell. When an EBV-transformed host cell divides, it replicates the EBV episome along with its own DNA. During latency, EBV does not express its full range of proteins, reducing the potential for viral protein presentation to the immune system. There are 4 latency types characterized by the set of proteins expressed and the differentiation stage of its host B cell. There is a strong link between EBV and B cell lymphomas due to its ability to influence B cell proliferation. In fact, one proposed model for EBV persistence is the germinal center model, wherein naïve B cells are primarily infected (latency III) and subsequently stimulated to enter a germinal center reaction (latency II). Germinal center B cells undergo proliferative bursts under normal conditions, but the survival and proliferation of EBV-transformed GCB cells are influenced by pro-proliferation, pro-survival, and anti-apoptotic EBV proteins. In this manner, EBV ensures that its host cell will survive the germinal center reaction and exit as a memory B cell (latency 0, I), wherein it ceases expression of the majority of its proteins and is most hidden from immune detection. An alternative model of EBV persistence suggests that latency 0 and I can be established without a preceding latency II and III. This model suggests that a

germinal center reaction can be circumvented and that either clonal expansion or EBV-induced proliferation are sufficient for EBV replication within the memory B cell compartment.

In a healthy individual, cytotoxic cellular immunity (NK cells and CD8+ T cells) is capable of managing the latent replication mechanisms of EBV and preventing its reactivation into lytic replication. The trigger for EBV reactivation is unclear, but likely involves a variety of factors such as immune deficiencies, inflammation, genetics, and even acute psychological stress. Lytic replication occurs when memory B cells carrying latent EBV are stimulated to differentiate into plasma cells via their BCR. Plasma cell transcription factors, *XBP1* and *BLIMP1* induce expression of EBV lytic protein, BZLF1.[25,26] EBV further stimulates plasma cell differentiation by downregulating the BCR. Upon differentiation, EBV expresses all of its 80 proteins and assembles virions that burst from the plasma cell, causing its death. Virions are then able to establish infection of oral epithelial cells, priming its host to shed and transmit the virus.

There are several suspected mechanisms by which EBV may trigger and exacerbate autoimmunity. Molecular mimicry between EBV proteins and disease-relevant autoantigens has been observed in multiple disease contexts.[27,28] Symptoms could arise as a result of bystander tissue damage perpetrated by local cytotoxic immune responses to EBV reactivation. Latently infected, naturally occurring autoreactive B cells could be rescued from deletion via EBV-induced stimulation and differentiation. Chronic EBV reactivation could also lead to autoantigen exposure to naturally occurring autoreactive lymphocytes. Finally, EBV infection could lead to the accidental presentation of self-antigen on MHC class II, which has been observed *in vitro*.[29] Serological and histological methods have helped determine that these diseases are

associated with chronic reactivation of EBV. It may therefore not be coincidental that these diseases are characterized by cyclical symptom remission and relapse. Whether EBV reactivation triggers autoimmune responses in the chronic phase of disease or vice versa, is unclear.

EBV-associated autoimmune diseases include RA, SLE, Sjogren's syndrome (SS), systemic scleroderma (SSc), and MS. MS is characterized by autoimmunity to the central nervous system (CNS). Destruction of myelin sheaths surrounding nerve fibers causes an array of symptoms including muscle weakness, impaired coordination, vision loss, fatigue, and tremors. The link between EBV and MS is arguably the most well studied. In addition to the longitudinal study that established that EBV infection increased the risk of MS by 32-fold, a very strong case has been made for molecular mimicry between EBV protein, EBNA1, and glialCAM, a cell adhesion molecule of the CNS.[30] Clonally expanded B cells in the CSF of MS patients were cross-reactive to EBNA1 and glialCAM, suggesting that this cross-reactivity is linked to pathology.

RA is characterized by inflammation of the synovium of the joints, causing connective tissue deposition and bone erosion. RA patients have autoantibodies to rheumatoid factors, citrullinated proteins, and nuclear antigens, which form inflammatory immune complexes in the join synovium.[31] Investigations into the etiology of RA have revealed a genetic component including defects in self-tolerance and an HLA polymorphism association, namely in the HLA-DRB1 gene. However, EBV emerged as a potential trigger of RA due to the high EBV seropositivity of RA patients. EBV has been found in affected synovium and the quantity of EBV DNA in the blood correlates with disease activity. Characteristic antibodies to citrullinated proteins cross-react with the EBV protein, EBNA, when it becomes citrullinated by a host cell enzyme.[32]

SLE is a complex disease with heterogenous clinical presentation of skin rashes, fever, fatigue, mucosal ulcers, alopecia, vasculitis, nephritis, and stroke. SLE patients have increased titers of autoantibodies to DNA and nuclear antigens and genetic factors such as defects in cellular waste removal mechanisms and an HLA-DRB1 polymorphism have been found to correlate with disease development.[27] SLE patients have a higher seropositivity to EBV, increased Ab titers, and higher viral DNA load in the blood, compared to controls. Molecular mimicry between EBV protein, EBNA1, and nuclear protein Ro60 has been observed. SLE patients also have a 10-fold increase in frequency of latently infected B cells and more latent and lytic EBV protein expression in their blood compared to controls. Increased expression of EBV protein, LMP, in the blood implicates LMP-induced B cell activation in SLE.[33] Increased viral DNA in the blood of SLE patients has also led to the suggestion that SLE patients have dysregulated cellular immunity that fails to control EBV reactivation.

SS is characterized by progressive damage to salivary and lacrimal glands. Patients with SS present with dry mouth and eyes, as well systemic symptoms like fever and fatigue. While SS etiology is less studied than other systemic autoimmune diseases, it shares similarities including a *HLA-DBR1* polymorphism association, autoantibodies to nuclear antigens, and an association with EBV.[34] EBV DNA has been detected in the lacrimal glands of SS patients.[35]

SSc is affects the skin and internal organs. Clinical presentation is heterogenous, but generally involves skin abnormalities like thickening and tightening coupled with more systemic effects on endothelial cells and microvasculature.[36] The association between EBV and SSc was established upon observation of elevated EBV antibody titers and EBV antigens in SSc patients. Viral DNA load is higher in the blood and plasma,

confirming lytic replication and not excluding latent replication. It has been found that SSc patients with high EBV DNA loads in the blood have an increased number of ulcers in their fingers and reduced skin perfusion compared to SSc patients with undetectable EBV DNA loads. Lytic EBV proteins have been found in the monocytes (innate immune cells) of SSc patients, while EBER RNA was detected in endothelial cells in the skin.[37] Elevated autoantibodies to DNA topoisomerase (*SCL-70*) is a hallmark of SSc. Interestingly, *SCL-70* reactive B cells derived from healthy controls are stimulated to secrete antibody when exposed to EBV *in vitro*.[38] This suggests that *SCL-70* specificity is naturally occurring in healthy B cell repertoires but is not sufficient to cause disease.

Hidradenitis Suppurativa

"*As I have it under my armpit, the pain disturbs absolutely in EVERYTHING during the day, because I just cannot move my limb. When I move, it hurts me so much that I feel my brain is going to be ripped out. So it is in everything, from cooking to writing"*

"Home, sweet home. I can walk here, I can ventilate myself. I will create some conditions to survive it somehow. I can go to the backstage, wash up myself."

"I have a depression. It means…, I had. I really do not want to get out of my bed. Man wonders — why should I get out of bed? It hurts me all the same. When I get up, sit down, it is going to rupture. There is black despair, yes, there is. There are black thoughts that nobody shakes hands with you… It is easy to fall down, easy to get lost"

Statements from patients on living with HS[39]

Hidradenitis suppurativa (HS) is a highly debilitating inflammatory skin disease characterized by painful nodules and abscesses, which rupture and cause the formation of sinus tracts, fistulas, and scarring. HS lesions are often chronic and patients report recurrent periods of worsening symptoms, or flares.[40] Prevalence estimates vary, but it is estimated that 0.1% of the United States population suffers from HS, with prevalence being highest among women and Black or biracial people.[40,41] Treatment options are limited to one FDA approved therapeutic, anti-TNFα, which has a 50% response rate in patients. Non-responders must often have their chronic lesions surgically excised to control symptoms.[42]

While severity of cases varies, the distressing nature of this disease must be emphasized. HS affects every facet of life. People with HS experience extreme chronic pain, social stigma, economic challenges due to disruptions in education and employment, and mental distress and illness. [43] HS is a relatively understudied disease and very little is known about its etiology and immunopathogenesis. Fortunately, awareness is spreading due to the tireless work of advocates and HS has become an emerging field of study over the past 10 years.

The etiology of HS is suspected to involve genetic, microbial, and autoimmune components. 30% of HS cases have a family history, indicating a genetic factor in some cases.[44] A mutation of the gene that encodes γ-secretase, an intramembrane protease, was identified in several members of Chinese families with HS. The proposed mechanism by which this mutation affects the skin is that a defective γ-secretase impacts notch signaling, which plays a role in keratinocyte proliferation and

differentiation. Deficiencies in notch signaling may prevent stem cells in hair follicles from differentiating into keratinocytes, leading to consequential disruption of the hair follicle cycle, buildup of cellular debris, and inflammation.[39] However, the majority of HS patients with a positive family history do not have γ-secretase mutations and the genetic factor leading to these cases is unknown. There is also no know genetic association with sporadic HS, the most common type of HS. Observations have been made regarding the locations on the body where HS lesions tend to present and the commensal bacteria therein. HS lesions usually arise in skin folds like the armpits, groin, and under the breasts, which are regions of higher temperature, moisture, and friction. Increased bacterial colonization of chronic HS lesions compared to corresponding location in healthy controls has been reported.[45] The most compelling hypothesis is that HS is an autoimmune disease of the skin. HS is associated with autoimmune diseases including ulcerative colitis, RA, SLE, and MS. Antibodies to a variety of autoantigens have been detected in the blood and skin of HS patients and they correlate with disease severity.[46] Autoantibodies to carboxyethyl lysine, a ubiquitous protein modification, has been observed across patients.[47]

Mounting evidence suggests that B cells are significant contributors to HS pathology. Beyond autoantibody production, B cell infiltration in the skin is a hallmark of HS.[48] B cells in the skin express activation markers and gene expression profiles indicative of stimulation and proliferation. Furthermore, B cell structures reminiscent of germinal center reactions have been observed within affected skin.[49] A recent study has found that anti-TNFα therapy reduces attenuates B cell activity by reducing the plasma cell compartment in the skin, suggesting that this may contribute to its efficacy.[50]

It was against this backdrop that we set out to clarify the B cell role in HS pathogenesis 3 years ago. Our work outlines a model of B cell infiltration and differentiation within HS skin. We identified skin B cells are differentially regulated than their peripheral counterparts. We found that B cells play both inflammatory and protective roles within the skin and proposed an explanation as to why many patients fail to respond to anti-TNFα. Finally, we discovered that expanded clones within the skin of HS patients are cross-reactive to EBV and autoantigens. To our knowledge, this is the first instance of EBV being linked to HS pathology.

It has been a privilege to be part of this important research on a team of passionate and talented scientists and clinicians. I hope that there are many more HS studies to come and that we have provided them a solid foundation.

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Chapter 2: *in situ* differentiation of B cells autoreactive to Epstein-Barr virus and autoantigens in hidradenitis suppurativa

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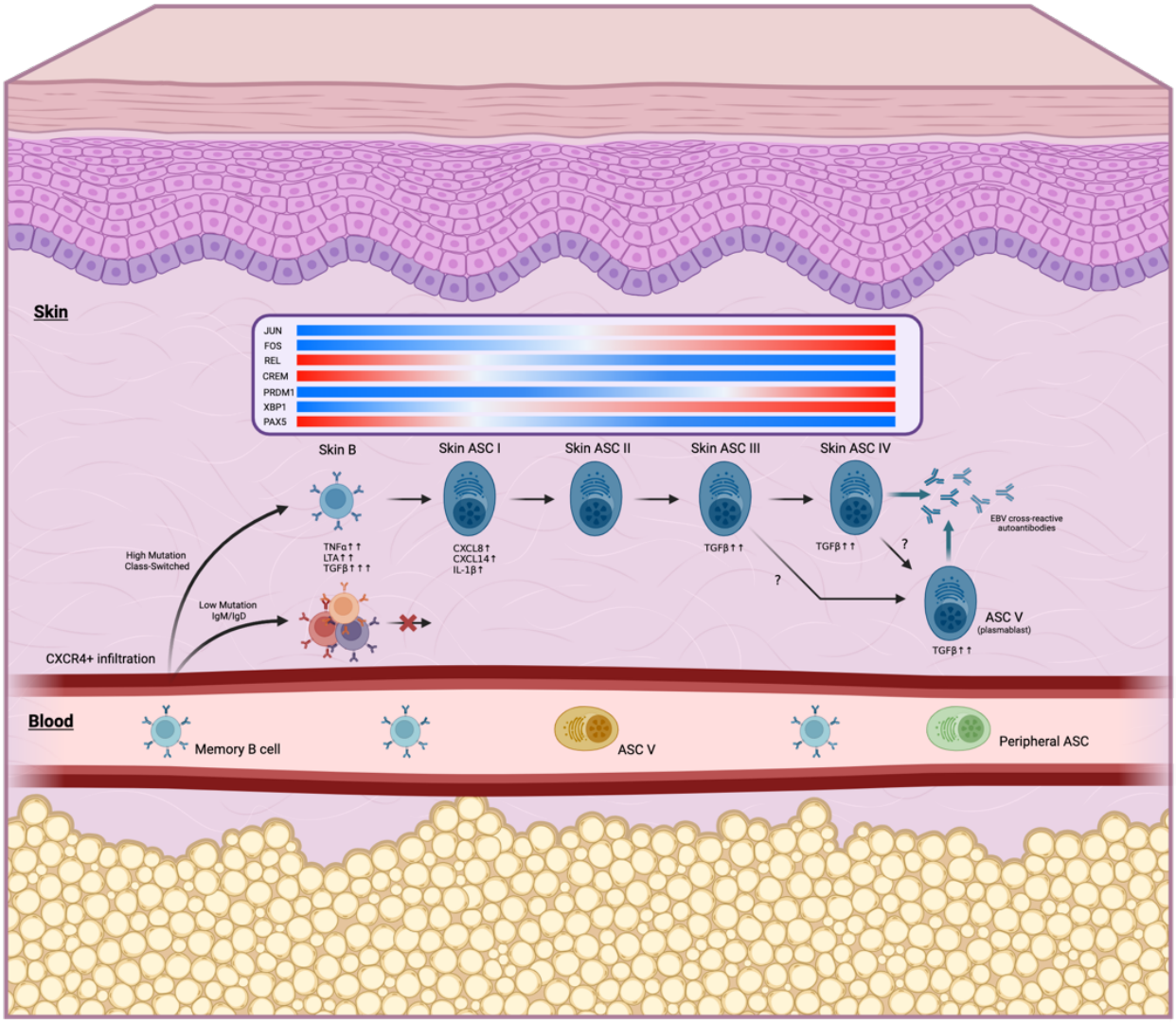
Abstract

Hidradenitis suppurativa (HS) is a chronic inflammatory skin disease characterized by painful nodules, abscesses, and tunnels involving intertriginous skin. While much remains unknown about the immunopathogenesis of HS, B cell infiltration and autoantibodies in the skin have emerged as characteristics of disease. To understand the B cell contribution to pathogenesis, we conducted single cell transcriptomics and repertoire analysis on B cells and plasma cells from HS surgical excisions and peripheral blood. We found that infiltrating B cells take on a chronic activation phenotype and have immunomodulatory potential via production of inflammatory TNFα or immunosuppressive TGFβ. Infiltrating B cells also undergo antigen-driven *in situ* plasma cell differentiation and clonal expansion. A serological screen revealed increased reactivity to EBV epitopes in HS patients compared to healthy controls and we identified clonally expanded autoreactive B cells that cross-reacted with Epstein-barr virus protein, EBNA1, and autoantigens, DNA topoisomerase I (SCL-70) and P21 activated kinase 4 (PAK4). Our findings suggest that chronic activation, differentiation, and clonal expansion of autoreactive B cells contribute to HS pathogenesis and that EBV reactivation may be a factor in HS etiology.

Graphical Abstract



Introduction

Hidradenitis suppurativa (HS) is a highly debilitating inflammatory skin disease characterized by painful nodules, abscesses, and tunnels. HS tunnels are chronic, and patients report recurrent, episodic worsening symptoms, or flares.[1] The distressing nature of this disease cannot be understated. HS patients experience increased rates of depression and suicidal ideation, social stigma, and disruptions in education and employment due to poorly controlled disease.[2–6] The overall prevalence of HS in the United States is estimated to be 0.1%.[7,8] However, prevalence is highest among women (137 in 100,000) and Black (296 in 100,000) or biracial patients (218 in 100,000).[7]

The etiology of HS has yet to be firmly established, but mounting evidence supports an autoimmune component. HS is epidemiologically associated with autoimmune diseases including inflammatory bowel disease (IBD), inflammatory arthritis and spondyloarthritis, and may also be linked to systemic lupus erythematosus (SLE), and multiple sclerosis (MS).[9–11] The presence of autoantibodies in HS skin correlates with disease severity and autoantibodies specific for carboxyethyl lysine (CEL), a ubiquitous protein modification, have been observed across patients.[12,13] It is unknown whether these autoantibodies contribute to disease pathogenesis or emerge as a result of underlying dysregulation.

Emerging data suggests that B cells may play an important role in HS pathogenesis. Beyond driving autoantibody production, B cell activity in the skin has emerged as a characteristic of HS. B cells are the dominant infiltrating leukocytes and express activation signatures *in situ*.[14,15] Another characteristic of HS is increased levels of inflammatory cytokines, but the contribution of B cells to the cytokine milieu in the skin

is unknown.[16–18] B cells have also been observed forming "germinal center-like" lymphoid structures adjacent to HS lesions, though activity therein remains unclear.[15]

Elucidating whether B cells contribute to HS pathogenesis could have important implications for therapeutic strategies. Adalimumab, a fully humanized anti-tumor necrosis factor alpha (TNFα) antibody, is the only FDA approved therapeutic for treating moderate-to-severe HS. However, half of patients fail to adequately respond and predicting non-responders is a major clinical challenge.[19] Previous studies suggest that anti-TNFα agents may function, at least in part, by reducing the B cell numbers and activation.[20] This, along with mixed reports of B cell depletion therapy (anti-CD20) ameliorating symptoms, suggests that examining the role of B cells in HS pathogenesis could advance therapeutic improvements and discovery.[21,22]

In this study, we explore the role of B cells in HS pathogenesis by conducting single cell transcriptomic and repertoire analyses on B cells and plasma cells from patient surgical excision specimens and peripheral blood. We found that infiltrating B cells exhibit a chronic activation phenotype and immunomodulatory potential. Antigen-driven plasma cell differentiation occurs in the skin, and is associated with a transient inflammatory cytokine production. Serological phage immunoprecipitation sequencing (Phip-seq) screen against viral families demonstrated significant reactivity to Epstein-Barr virus (EBV) epitopes. We subsequently found that expanded clones in the skin were cross-reactive to EBV protein, EBNA1, and autoantigens, DNA topoisomerase I (SCL-70) and P21 activated kinase 4 (PAK4). These findings implicate chronic autoreactive B cell activation, differentiation, and clonal expansion in HS pathogenesis and suggest that EBV reactivation may contribute to HS etiology.

Results

Transitional B cell populations are present in HS skin

Seeking to understand the B cell dynamics of HS on a single cell level, we characterized the single cell transcriptional profiles (scRNA-seq) and V(D)J repertoires (scVDJ-seq) of B cells derived from whole blood (WB) (n=3) and surgical skin excisions (n=4) from 5 patients with moderate-to-severe HS (Table 1). 2 patients provided matched samples of WB and excisions. This was accomplished using the 10x Genomics Chromium platform on peripheral blood mononuclear cells (PBMC) isolated from WB and positively selected $CD19^+$ or $BCMA^+$ B lineage cells from surgical excisions. Following quality control steps (see methods, Fig. S1) and subsetting, 11,352 B cells remained for downstream analysis.

We conducted unsupervised clustering using the Seurat pipeline, which divided cells into clusters A-I (Fig. 1A). Generally, peripheral B cells clustered in A, B, and I, and B cells from the skin clustered in C-G. (Fig. 1B). Cluster H contained B cells from both the periphery and skin. Clustering patterns were consistent across patients, with no cluster being exclusively comprised of cells from a single patient sample (Fig. 1B, S1E).

We employed canonical B cell subset expression profiles to classify each cluster. These classifications are summarized in Table 1. Cluster A expressed CD20 (MS4A1), IgD, and lacked CD27, an expression pattern consistent with naïve B cells (Fig. 1C).[23,24] Cluster B expressed CD20 and CD27 but lacked IgD and was therefore classified as a memory B cell cluster (Fig. 1C).[23,24] Clusters A and B also expressed the B cell master regulator PAX5, IRF8 and HLA type II genes (Fig. 1C, D). Cluster C was classified as skin B cells (Skin B), given their source and expression of CD20, CD19, HLA type II, IRF8 and PAX5 (Fig. 1C, D).[25–27] These cells expressed activation markers CD86 and

CD83, consistent with previous studies demonstrating that infiltrating B cells in HS skin have activated phenotypes (Fig. 1C, S2).[14,20] Cluster C lacked IgD and CD27, a phenotype associated with double negative (DN) B cells.[23]

We determined that clusters D-G contained antibody secreting cells (ASC) from the skin at various stages of plasma cell differentiation based on their immunoglobulin (Ig)-specific transcript counts and lack of CD20 and PAX5 (Fig. 1D-F).[23,26] Clusters D and E appear to contain ASC in the early stages of differentiation from B cell to plasma cell. These clusters were classified as skin ASCI and ASCII based on their intermediate expression of XBP1, SDC1, and Ig-specific transcripts.[28] Clusters F and G contained more mature plasma cells in late stages of differentiation. These clusters were classified as skin ASCIII and ASCIV based on their level of XBP1, SDC1, and PRDM1expression and high Ig-specific transcript counts.[29]

Cluster H and I also had characteristic ASC gene expression and high Ig-specific transcript counts. Cluster H expressed MKI67 and HLA type II, fitting the canonical definition of plasmablasts (Fig. 1C, S2).[23] Cluster H and I were classified as ASCV (contains cells from skin and periphery) and Peripheral ASC, respectively.

To further explore the transcriptional states of these clusters, we conducted a gene set enrichment analysis (GSEA) using gene sets associated with biological processes. In this analysis, each cluster had a distinct expression pattern (Fig. 1G). Notably, Skin ASCI-IV clusters were less active than Skin B, suggesting that a slowing in some biological processes occurs as B cells shift to the plasma cell transcriptional program and that Skin ASCII, the most quiescent cluster, could represent an inflection point in plasma cell fate decision.

B cells and ASC populations in HS skin are transcriptionally distinct from peripheral counterparts

We conducted differential gene expression analyses to further characterize B cells in HS-affected skin and to identify phenotypic differences between them and their peripheral counterparts (Fig. 2A-F).

When compared to peripheral Memory B (cluster B), Skin B (cluster C) differentially expressed genes associated with B cell activation including CD83, CD69, and NR4A1/2 (Fig. 2A).[30–32] Skin B also differentially expressed CXCR4 compared to Memory B and Skin ASCI (Fig 2A, S2, S3). CXCR4 is a chemokine receptor that responds to CXCL12, which is produced in the skin. [33–35] Skin B had higher average expression of CXCR4 than Skin ASCI-IV, which expressed no other chemokine receptor, suggesting B cells rather than ASC infiltrate HS skin (Fig. S4). This is further supported by previous HS studies, which identified an increased number of memory B cells, rather than plasma cells, in acutely inflamed skin.[20] Overall, $NR4A^+$ $CD83^+$ $CXCR4^+$ B cells from HS skin represent an activated, infiltrating population with the potential for plasma cell differentiation.

To delineate transcription factors that influence activity and cell state, we conducted a single-cell regulatory network inference and clustering (SCENIC) analysis. SCENIC utilizes scRNA-seq data to identify groups of transcription factors and their co-expressed direct targets (regulons) with significant activity in each cluster.[36] As expected, naïve, memory, and skin B cell clusters showed high activity of characteristic regulons BCL11A, PAX5, SPIB, and IRF8 (Fig. 2G).[27,37,38] In the skin compartment, activity of these regulons was highest in Skin B, greatly reduced in Skin ASCI, and extinguished in Skin ASCII-IV (Fig. 2G). Conversely, ASC clusters had higher XBP1 and

CREB3L activity than B cell clusters (Fig. 2G). Regulon activity levels in Skin ASCI and ASCII were intermediate between Skin B and ASCIII and ASCIV, further underscoring their transitional nature.

Skin B had the highest activity of CREM and REL regulons across all clusters (Fig. 2A, G, H). CREM expression was positively correlated with NR4A1/2 and REL, and REL expression was positively correlated with MS4A1 and HLA type II genes (Fig. 2I). Both were negatively correlated with plasma cell transcription factors, XBP1 and MZB1, implying that non-ASC B cells were more likely to express CREM and REL in the skin compartment.

Skin ASCIII and ASCIV had high activity of FOSB, JUN, and JUND, which encode AP-1 signaling complex subunits (Fig. 2G, H). Genes included in these regulons were also seen differentially expressed in Skin ASCIII and ASCIV compared to peripheral ASC (Fig. 2D, E). Expression of FOSB and JUN correlated positively with NR4A1, and FOSB expression correlated positively with CREM (Fig. 2I). Although FOSB and JUN expression were negatively correlated with CD79b, suggesting that they were more likely to be expressed by ASC than B cells in the skin, Skin B showed some FOSB and JUND activity.

Taken together, these findings suggest that CREM and REL are key regulators of undifferentiated B cells in HS skin. Upon differentiation, plasma cells in the skin become regulated by the AP-1 signaling complex.

Infiltrating B cells undergo in situ plasma cell differentiation and have immunomodulatory potential

To confirm the direction of differentiation from Skin B to Skin ASCIV, we conducted an RNA velocity analysis using the scVelo workflow. Briefly, scVelo predicts the future transcriptional state of a cell using measurements of RNA velocity, which are derived from the ratio of unspliced (immature) to spliced (mature) mRNA within a cell.[39] Velocity can be applied in a gene-specific manner or globally by cell. A higher ratio of unspliced mRNA for a specific gene (relative to an inferred steady-state ratio) yields positive velocity and implies upregulation, while a lower ratio of unspliced mRNA yields negative velocity and implies downregulation. Global velocity measurements can be used to assign direction to a set of static transcriptional states by distinguishing progenitor cells from cells that have reached steady state expression.[39,40]

Velocity analysis suggested that Skin ASCII represents progenitors of Skin ASCIII, and that Skin ASCIII represents progenitors of Skin ASCIV (Fig. 3A, S5, S6). This supports the hypothesis that Skin ASCI-IV represent transitional states from early to late plasma cell. Skin ASCII and Skin ASCI were erroneously identified as progenitors of Skin B. The scVelo methodology identified ASCII as the bifurcation point for differentiation likely due to its low percentage of unspliced mRNA and apparent transcriptional quiescence (Fig. 3B). Plasma cell differentiation is considered a unidirectional process, rendering this outcome an artifact.[41] Nevertheless, Skin ASCII likely represents a transition point along the axis of differentiation from B cell to plasma cell in the skin compartment, characterized by transcriptional quiescence, loss of PAX5, and upregulation of XBP1 and Ig. In other words, it is at this point that PAX5

enforcement of B cell lineage ceases and XBP1 mediated commitment to plasma cell fate ensues (Fig. 3A).

We examined the transcriptional dynamics of genes of interest by comparing their velocity, a measurement of upregulation, and expression, a measurement of total transcript (Fig. 3C). We observed expected PAX5 upregulation and expression in peripheral clusters, Naïve B and Memory B. Skin B and Skin ASCI showed a reduction in PAX5 upregulation. REL and CREM upregulation and expression were highest in Skin B. Skin B and Skin ASCII showed upregulation of REL, but Skin ASCII had no expression, while Skin B had high expression. All skin clusters showed upregulation of CREM, but Skin B and Skin ASCI had highest CREM expression.

Previously, we observed that XBP1 expression increased incrementally from Skin B to Skin ASCIII and ASCIV (Fig. 1D), which we saw recapitulated here. XBP1 upregulation begins to occur in Skin ASCI and peaks in Skin ASCII (Fig. 3C). JUND was previously identified as a key regulator of B cells in the skin (Fig. 2G). Here, we observed that while Skin B had the highest JUND expression, the most JUND upregulation occurred in Skin ASCIII and ASCIV (Fig. 3C). Similarly, Skin B had the highest FOSB expression, but Skin ASC populations demonstrated more FOSB upregulation.

Next, we determined the contribution of each cluster to the *in situ* cytokine milieu. We found that Skin B expressed inflammatory IL6, TNFβ, and TNFα (Fig. 3D, S7). 11% of Skin B highly expressed TNFα, which contrasted with the lack of TNFα expression in Skin ASCI-IV. We observed a lack of TNFα upregulation in transitional populations Skin ASCI and ASCII, suggesting that differentiation is associated with a halt in TNFα production (Fig. 3E). This is underscored by the positive correlation of

TNFα expression with Skin B marker CD20 (MS4A1) and negative correlation with plasma cell marker XBP1 (Fig. 3F).

Skin ASCI expressed inflammatory IL-1β (7.9%), CXCL8 (IL-8) (9.6%), and CXCL14 (9.6%) (Fig. 3D). This contrasted with the lower level of expression of all three of these cytokines in Skin B and Skin ASCII-IV. Given that Skin ASCI represents a transitional state of early plasma cell differentiation, this observation suggests that early differentiation in the skin is associated with transient production of inflammatory cytokines.

B cells and ASC in the skin compartment expressed anti-inflammatory TGFβ, but not IL-10 (Fig. 3G, S7). 74% of Skin B had high expression of TGFβ. We observed similar expression, but less upregulation in peripheral Naïve B and Memory B compared to Skin B (Fig. 3H). 76% of Skin ASCIII and 65% of Skin ASCIV had low expression, as did peripheral ASC. Skin ASCI and II had dramatically low TGFβ expression. TGFβ upregulation occurred in every cluster, but the correlation between the expression of TGFβ and MS4A1supports that B cells, rather than ASC, are the dominant producers in the skin (Fig. 3H, I). Of note, $TNF\alpha^+$ and $TGF\beta^+$ Skin B are distinct populations (Fig. S7A). TGFβ expression is positively correlated with expression of MHC type II and $TGF\beta^+$ Skin B differentially express CREM and CD86 when compared to $TNF\alpha^+$ Skin B (Fig. S7C, E). Undifferentiated B cells may, therefore, have immunomodulatory potential in HS skin, taking on a pro-inflammatory role by producing TNFα or an anti-inflammatory role by producing TGFβ.

B cells and ASC in the skin are class-switched and clonally expanded

We conducted a repertoire analysis of 7210 full-length, paired scVDJ sequences to gain insights on clonal dynamics of infiltrating B cells in HS. We observed that both B cells and ASC in the skin contained class-switched populations. Skin B included 51% class-switched B cells with dominant IgA1 (19.3%) and IgG1(15.2%) (Fig. 4A). This isotype profile was comparable to peripheral Memory B, which were 45.2% class switched with dominant IgG1 (18.1%) and IgA1 (12.4%). Both Skin B and Memory B clusters contained IgG2, IgG3, and IgA2, but only Skin B contained IgG4. In fact, IgG4 was only found in the skin compartment (Skin B, Skin ASCI-III, and skin fraction of ASCV). Notably, autoantibodies involved in the pathogenesis of the autoimmune skin disease, pemphigus vulgaris, are predominantly IgG4.[42]

In contrast to Skin B, Skin ASCI-IV were almost entirely class-switched. Skin ASCI-IV were IgG dominant in contrast to peripheral ASC, which was IgA dominant. Generally, Skin ASC clusters had large fractions of IgA1 (13.7-36.7%) and IgG1 (33.9-51.9%) and had large fractions of IgG2 (9.2-25.3%) and IgG3 (7-16.1%) compared to Skin B. These findings suggest that class-switched infiltrating B cells are more likely to undergo plasma cell differentiation than unswitched infiltrating B cells.

We examined the V_H gene repertoire in the skin and periphery of two patients with matched samples and observed distinct patterns of usage in each compartment. Specifically, there was a skewing of V_H gene usage whereby lower frequency genes in the periphery became high frequency genes in the skin (Fig. 4B). Additionally, 6 of the 10 most frequent V_H genes in the skin of each patient were shared: IGHV3-23, IGHV3-30, IGHV5-51, IGHV3-21, IGHV4-39, IGHV1-18.

Finally, we found that B cell and ASC populations in HS skin were clonally expanded and had high connectivity across clusters. Unlike peripheral clusters, which only contained single-member and small clones (2-5 members), Skin B and ASC clusters contained medium (6-20 members), large (21-100 members), and hyperexpanded (101-500 members) clones (Fig. 4C, D). We utilized complementarity-determining region 3 (CDR3) sequence matching to trace clones across clusters and found that skin clusters and ASCV had high proportions of shared clones (Fig. 4E). While there appeared to be shared clones between peripheral and skin clusters, these were skin cells that clustered with peripheral cells. Therefore, we did not observe clones with members in both the periphery and the skin. The expanded and highly connected nature of skin clusters lends further support to the hypothesis of *in situ* activation, expansion, and differentiation of infiltrating B cells. Skewed V_H gene usage and preferential differentiation of class-switched clones further implies specificity-dependent selection.

Class-switched and mutate infiltrating B cells are preferentially selected for differentiation

Repertoire analysis revealed that B cells and ASC in the skin are mutated. IgH mutation loads tended to be higher among expanded clones in Skin B compared to clones in peripheral Memory B (Fig. 5A). Skin ASCI-IV and ASCV were significantly more mutated than peripheral Memory B and their IgH mutation loads were generally consistent regardless of clone size. IgM in Skin ASC clusters was more highly mutated than IgM found in Skin B, suggesting that infiltrating B cells with higher mutation loads are preferentially selected for differentiation (Fig. 5B). Further characterization of mutations showed higher replacement to silent mutation (R/S) ratios in complementarity-determining regions (CDR) than in framework regions (FR) in Skin B

and Skin ASC clusters, indicating that these cells have undergone antigen-driven mutation (Fig. 5C).

We used the number of IgH mutation difference between the most and least mutated member of each clone (Δmt) as a measurement of intra-clonal diversity to evaluate clones in the periphery and skin compartment (Fig. 5D). By this measure, we found that clones in the skin had low intra-clonal diversity. Of clones with 2 or more members (n=617), 53% had an Δmt of 0 nucleotides (nt) and 24% had a Δmt of less than 5 nt. To further examine a particularly large and diverse clone, we generated a lineage tree based on IgH mutations (Fig. 5E). We observed that even the least mutated member of this clone was highly mutated with 40 IgH mutations, while its most mutated member had 55 IgH mutations. This clone was exclusively IgG1 and highly connected, with its members present in every skin cluster. We generated three additional lineage trees for representatively sized clones, all of which were found in multiple skin clusters (Fig. 5F). The least mutated members of these clones were substantially mutated and had an intra-clonal range of 30-42, 12-32, and 22-40 IgH mutations, respectively (Fig. 5F). Finally, we compared the isotypes of disconnected, single cluster clones (1661 clones) and connected multi-cluster clones (415 clones) (Fig. 5G). We found that disconnected clones were exclusively of a single isotype and that multi-isotype clones, though uncommon, were all connected clones (67 clones). The most frequently observed combination of isotypes was IgG1 and IgA1(32 clones), followed by IgG1and IgG3 (24 clones).

Taken together, these mutation analyses suggest that clones found in HS skin have undergone antigen-dependent somatic hypermutation (SHM) and class-switch recombination (CSR), which are hallmarks of affinity maturation within a germinal

center. We observed a lack of activation-induced cytidine deaminase (AID) expression in the skin, suggests that infiltrating B cells do not undergo *in situ* SHM and CSR, as AID is a prerequisite for these processes. Without *in situ* AID expression, bona fide germinal center reactions likely do not occur, implying an alternate function for previously observed ectopic lymphoid structures in advanced HS.[13,15] Our findings indicate that during advanced HS, class-switched and mutated memory B cells traffic to the skin and are preferentially selected for differentiation over their unswitched low mutation counterparts, likely based on their specificity to antigens present in the skin.

Expanded and differentiated clones from HS skin are cross-reactive to EBV EBNA1, human SCL-70 and PAK4

To characterize peripheral B cell repertoires, we conducted phage immunoprecipitation sequencing (PhIP-Seq) using phage display libraries of human (HuScan) and viral proteins (VirScan) with serum from HS patient (n=10) and healthy controls (n=14) (Fig. 6A). Compared to healthy controls, HS patients had significantly higher reactivity to collagen 1 alpha chain (COL1A1), nik-related protein kinase (NRK), matrix extracellular phosphoglycoprotein (MEPE), elastin microfibril interfacer-1 (EMILIN-1), and protocadherin gamma subfamily A11 (PCDHGA11), which are ubiquitous proteins found in all major tissues, including the skin (Fig. 6B). HS patients also had significantly higher reactivity to common viruses including rhinovirus, respiratory syncytial virus, and cytomegalovirus (CMV) (Fig. 6C). The most dramatic difference in reactivity between controls and HS patients was to Epstein-Barr virus (EBV). HS patients had reactivity to EBV epitopes that were not immunogenic in healthy controls and generally demonstrated reactivity to a wider diversity of epitopes (Fig. 6D). We tested the

seropositivity of HS patients (n=23) to EBV and found that 22 (95.7%) were IgG seropositive and IgM seronegative for EBV viral capsid antigen (VCA) (Table 2). One patient was both IgG and IgM seropositive for EBV VCA. HS patient seroprevalence was comparable to EBV seroprevalence among healthy adults, which is estimated to be approximately 95%.[43]

Using punch biopsies of HS-affected skin from patients (n=21), we conducted RT-PCR to investigate whether EBV was detectable in the skin (Fig. 6A). We tested for amplification of EBV DNA and RNA encoding proteins associated with latency and lytic replication. Raji cells, an EBV-transformed B cell line, and Ramos cells, an EBV-negative B cell line, were used as controls. 12 samples (57.1%) had detectable EBV DNA or RNA (Fig. 6E). 10 (47.6%) samples showed amplification of Epstein-Barr nuclear antigen 1 (EBNA1) DNA, but none showed amplification of EBNA1 RNA. 11 samples (52.3%) showed amplification of EBV non-coding RNA, EBER1, EBER2, or both. EBER1/2 expression is associated with EBV latency type 0 and dual expression of EBNA1 and EBER1/2 is associated with EBV latency type I.[44] No patients showed amplification for latent membrane protein 1 (LMP1) RNA, associated with EBV latency types II and III, or BamHI Z Epstein-Barr virus replication activator (BZLF1), associated with EBV lytic reactivation.[45] While HS patients had significant serum reactivity to CMV, no skin biopsy samples showed amplification of CMV DNA.

Heightened reactivity to EBV epitopes and *in situ* detection of viral DNA are characteristic of patients with EBV-associated autoimmune diseases, particularly multiple sclerosis (MS).[46] We compared our HS VirScan reactivity data to previously published MS VirScan reactivity data and found that of all viral families, reactivity to EBV was most significant for both cohorts (Fig. S8). These findings suggest that HS

pathogenesis may have an autoimmune component and an etiology similar to that of EBV-associated MS.

To determine which antigens drive expansion and differentiation of skin-infiltrating B cells, we screened the specificity of expanded clones against >21,000 full-length human proteins and EBV EBNA1 and capsid protein, p23, in their native conformations (Fig. 6F). These clones (6 clones, 2 from each of 3 patients) had IgH mutations, were highly expanded, and had members in multiple skin clusters (Fig. 6G). Six monoclonal antibodies (mAb) were generated and screened separately, along with 3 unrelated mAb. Five of 6 HS mAb cross-reacted to EBNA1, DNA topoisomerase I (SCL-70), and P21 activated kinase 4 (PAK4) (Fig. 6H, S9). One mAb reacted most significantly to N-terminal Xaa-pro-lys N-methyltransferase 1 (NTMT1) and did not cross-react with EBNA1. None of the screened mAbs reacted to EBV p23. Compared to control mAb, HS mAb demonstrated significantly higher reactivity to EBNA1 ($p<0.05$) and SCL-70 ($p<0.05$) (Fig. 6I). These findings suggest that autoreactive clones, some of which cross-react with EBV EBNA1, are selected for *in situ* differentiation and may drive chronic inflammation in HS.

Discussion

We report that autoreactive B cells infiltrate HS skin and undergo chronic antigen-driven activation, clonal expansion, and plasma cell differentiation. Infiltrating B cells modulate immune responses in the skin by producing pro-inflammatory TNFα or anti-inflammatory TGFβ. Additionally, we provide evidence supporting an association between HS and EBV reactivation by demonstrating epitope spreading and cross-

reactivity of expanded clones with EBV EBNA1and autoantigens SCL-70 and PAK4. These findings hold implications for HS pathogenesis, as well as for therapeutic improvements.

In HS, B cells infiltrate via CXCR4 expression, ostensibly responding to CXCL12 produced by skin fibroblasts, which is a skin trafficking pattern observed in autoimmune, viral infection, and wound healing contexts.[33–35] Once in the skin, B cells adopt an activation phenotype, expressing CD83, CD69, CD86, and NR4A1/2. This phenotype may indicate chronic antigen stimulation, as NR4A1 expression is known to aggregate in response to B cell receptor (BCR) stimulation.[32] NR4A1 expression has also been identified as a distinguishing characteristic of activated B cells in the autoimmune context of rheumatoid arthritis (RA).[47] In the skin, B cells employ transcriptional programs regulated by CREM and REL. These transcription factors are also associated with chronic B cell stimulation and autoimmunity, as expression has been observed in germinal centers, RA synovium, and SLE.[47–50] Upon differentiation, plasma cells in the skin become regulated by the AP-1 signaling complex. B cell AP-1 expression has been observed in response to BCR stimulation and is known to play a role in plasma cell differentiation.[51,52] The difference in significance of AP-1 regulation between skin and peripheral plasma cells may be due to recency of differentiation in the skin compared to periphery. Infiltrating B cells are largely IgD- and CD27-, defining them as double negative (DN) B cells.[23] DN B cells have been extensively characterized in the context of SLE, and have been identified as activated effector ASC precursors associated with active disease and poor response to B cell depletion therapy.[53]

We posit that infiltrating B cells have immunomodulatory potential, taking on inflammatory or immunosuppressive roles in the skin. Undifferentiated B cells are the

main contributors of TNFα within the B cell compartment of the skin. This is in opposition to a previous histological study that observed colocalization between TNFα and SDC1$^+$ (CD138$^+$) plasma cells, not B cells, in the skin of HS patients.[14] We observed widespread SDC1 expression by epithelial cells (data not shown), which may have confounded that study.[54] Differentiation is associated with a transient burst of inflammatory IL-1β, CXCL8, and CXCL14 production, which peaks in early stages. Undifferentiated B cells are also major contributors of TGFβ in the skin. TGFβ-producing B cells represent a subset of regulatory B cells that suppress immune responses in inflammatory environments by inducing regulatory T cell differentiation and reducing the number of IL-17-producing Th17 T cells.[55–57]

Our findings regarding B cell cytokine contributions in the skin have implications for current and future therapeutic strategies. Anti-TNFα therapy (adalimumab) inadequately controls disease in approximately half of HS patients. We echo previous studies in hypothesizing that non-responders have large infiltrating TNFα-producing B cell populations that overcome current dosage recommendations.[20] Adalimumab also fails to target the effects of inflammatory cytokines associated with differentiation. We posit that a potential therapeutic strategy is to control the chronic activation and differentiation of infiltrating B cells while promoting the effects of TGFβ-producing regulatory B cells. This may be achieved with BTK inhibitors, which are under investigation for MS treatment, or anti-BAFF (belimumab), which has been approved for SLE treatment.[58,59] In one case report, pan-B cell depletion with anti-CD20 (rituximab) improved HS symptoms.[21] Based on our findings, rituximab treatment may have inflammatory effects via depletion of TGFβ-producing regulatory B cells.

Additionally, rituximab cannot deplete plasma cells and may fail to prevent the inflammatory effects of autoantibody production in the skin. Fingolimod is an approved therapeutic for MS that has been shown to promote TGFβ-dependent regulatory B cell activity and reduce $CXCR4^+$ B cell trafficking, which could also be beneficial in HS based on our findings.[60,61]

We provide evidence that preferential selection and differentiation of mutated class-switched clones occurs in HS skin. Both switched, high mutation and unswitched, low mutation clones infiltrate as undifferentiated B cells, but plasma cells are predominantly switched and highly mutated. This is indicative of specificity-dependent selection by cognate antigen found in the skin. This process may be occurring in previously described ectopic lymphoid structures.[13,15] However, we echo others in observing a lack of AID expression in the skin.[13] Without evidence of AID expression or germinal center organization of light and dark zones, it is unlikely that SHM and CSR occur in the skin during the chronic phase of advanced HS. Our findings instead suggest that antigen-experienced memory B cells traffic to the skin and are subsequently activated without additional affinity maturation.

EBV is a ubiquitous gamma herpesvirus associated with several autoimmune diseases including RA, MS, and SLE.[46,62,63] A recent longitudinal study with a large cohort provided strong evidence that EBV is a trigger for autoimmunity by observing that EBV infection increased the risk of future MS onset by 32-fold.[64] While the mechanisms by which EBV may trigger autoimmunity are unclear, there is compelling evidence that chronic EBV reactivation and molecular mimicry between EBV and human proteins are factors.[65–67]

We observed similarities between HS and MS patients including significant serologic reactivity to diverse EBV epitopes, a high rate of EBV seropositivity, detectable EBV in affected tissue, and local expanded clones with cross-reactivity to EBNA1 and a disease-relevant autoantigen.[66] EBV establishes latency type 0 in memory B cells, where it limits expression to EBER1/2 RNA.[68] Latency type I, also established in memory B cells, is mediated by EBNA1. EBV can exit latency and initiate lytic replication, mediated by BZLF1, when its host cell undergoes plasma cell differentiation. HS patients had detectable EBER1/2 RNA, but not BZLF1 RNA, suggesting that EBV is latent in the skin. However, HS patients demonstrated epitope spreading to EBV proteins, including lytic proteins BZLF1 and BRLF1, which is indicative of continual affinity maturation facilitated by antigen persistence due to chronic reactivation and intractable latency. Finally, we identified expanded clones in affected skin of multiple HS patients with cross-reactivity between EBNA1, and autoantigens SCL-70 and PAK4. SCL-70 is a nuclear protein that is not specific to the skin, however, anti-SCL-70 autoantibodies are a known clinical feature of scleroderma, an inflammatory autoimmune disease that affects the skin, as well as other tissues.[69] P21 activated kinase 4 (PAK4) is serine-threonine kinase with a role in melanogenesis in skin melanocytes.[70] The presence of these proteins in the skin suggests that they may be driving chronic B cell activation, differentiation, clonal expansion, and autoantibody production, thus contributing to HS pathology.

This study is not without limitations. Our patient cohort was small and skin excisions used for scRNA-seq were exclusively from a cohort composed of a single race and sex, Black women. However, this demographic has the highest prevalence in the US, so the importance of their inclusion in HS studies cannot be understated. Additionally,

we elected to limit our scope to B cells, which surely excluded significant disease factors and required enrichment methods with unknown effects on transcription.

Additionally, all patients used in scRNA analyses were of a limited range of Hurley stages (II-III), and thus the results presented here should be interpreted in the context of established disease. Consequently, we cannot conclude that the results presented here are independent of medication regimen and history. Subsequent studies should be directed to understanding if the axis of differentiation and proportion of class-switched and mutated B cells is linked with early disease progression.

Despite these limitations, this study provides insights into B cell and EBV contributions to autoimmune pathology in HS, as well as avenues for future HS study. The link between a lymphotropic virus and HS is novel and future studies should aim to confirm EBV associations in a separate cohort. In addition, given the periodic worsening of disease (flares) that HS patients experience, it would be of significant interest to identify if either local EBV reactivation or B cell differentiation is linked to the phenomenon. Further work on the associations presented here could provide critical insights, leading to novel therapeutic strategies for HS patients.

Methods

Single cell suspension preparation

Large surgical excisions (>10cm^2) from HS patients with severe symptoms (Hurley stage II/III) were obtained from the Emory Department of Dermatology (Table 2). Tissue was processed into a single cell suspension immediately upon receipt as follows: first, subcutaneous fat was removed from the dermis. The remaining tissue was minced and placed in gentleMACS C tubes (Miltenyi Biotec) containing 5ml PBS with 0.4%

collagenase II (Gibco) and 10 ul/ml DNase I (Thermo Fisher). Approximately 1cm^2 of tissue was included in each tube. Tubes were run on a gentleMACS dissociator (Miltenyi Biotec) for 53s using the "spleen_m_01" program. Tubes were placed in a shaking incubator at 37°C for 1h and run on a gentleMACS dissociator once more. Cells were strained through a 70um strainer and washed three times with PBS containing 2% FBS. B cells were positively selected from the single cell suspension according to the MagniSort Human CD19 Positive Selection Kit (Thermo Fisher) protocol. Biotinylated CD19 and BCMA antibodies were used for selection in equal parts, as well as an Fc receptor blocking solution (Bio Legend).

HS patient blood samples were obtained from the Emory Department of Dermatology. PBMCs were isolated using SepMate tubes (Stemcell) containing Lymphoprep density gradient medium (Stemcell).

Single cell RNA and VDJ sequencing

Single cell suspensions were prepared for capture using the 10X Genomics Chromium Controller with the Chromium NextGEM Single Cell 5' Library & Gel Bead v2 kit.[71] Gene expression and B cell immunoglobulin libraries were prepared according to manufacturer instructions and sequenced on an Illumina NovaSeq 6000 with a paired-end 26x91 configuration targeting a depth of 50,000 reads per cell for the gene expression libraries and 5,000 reads per cell for the immunoglobulin libraries. The data was processed using Cell Ranger v7.0.1 software with Human (GRCh38) v7.0.0 and Human (GRCh38) 2020-A reference genome files provided by 10X Genomics.

Computational analysis

Seurat

Following Cell Ranger annotation, scRNA samples were processed with Seurat v4.3.0. In brief, all gene expression samples were concatenated into a single Seurat object for processing. To control for low quality cells and doublets we removed cells with the number of genes observed <200 and >7000. To further ensure that cells were high quality we removed cells who had a mitochondrial transcript percent in excess of 10% of the total transcripts recorded for those cells. To ensure that cells from separate samples were comparable, raw counts per cell were normalized across total counts for all genes per cell, multiplied by a standard scale factor (10000) and natural log transformed. Normalized counts were then scaled across cells using a linear transformation that adjusts expression such that the mean across cells per gene is 0 and has a resulting variance of 1. Clustering was performed using Seurat v4.3.0 "FindNeighbors" and "FindClusters" functions that utilize an unsupervised graph-based approach. Resolution was set to 0.5. Cells were then projected into a UMAP embedding for visualization.

Separately, VDJ libraries were analyzed with scRepertoire v1.7.0. As with gene expression libraries, these were concatenated into a single object and merged with the gene expression Seurat object. Cells were then filtered again with clusters that did not contain VDJ library information being excluded from downstream analysis. These clusters were then reclustered as above.

GSEA

Gene set enrichment analysis was performed using singleseqgset v0.1.2.9000. Gene sets were downloaded from the Human Molecular Signatures Database, C5 collection, Biological Process subset using msigdbr v7.5.1. Log normalized scaled data was used to

derive log fold change values between clusters for all genes. Enrichment scores were then calculated across all gene sets. Statistical tests were corrected for multiple comparisons. Significant gene sets were reported using Z-scores

SCENIC

Transcription factor analysis was performed using SCENIC v1.1.2. Using normalized log transformed counts, genetic regulatory network (regulon) activity was inferred using co-expression modules with the GENIE3 algorithm (https://github.com/vahuynh/GENIE3). Regulons were then filtered such that only those with target genes containing the transcription factor binding motif in the regulatory region were kept. Reference regulatory regions were obtained from cisTarget databases “Homo sapiens - hg38 - refseq_r80 - v9” (https://resources.aertslab.org/cistarget/databases/homo_sapiens/hg38/refseq_r80/mc9nr/gene_based/). Regulon activity scores were then calculated for each cell using AUCell.

RNA Velocity

RNA velocity analyses were performed with either ScVelo v0.2.5 or UniTVelo v0.2.4 with Python v3.7. Using Cell Ranger output, spliced and unspliced matrices were calculated for each sample using ScVelo and saved as .loom files. Loom files were merged into a single loom object and were merged again into the exported Seurat object saved as h5ad file with Seurat Disk v0.0.0.9011. RNA velocity pipelines for either ScVelo or UniTVelo were then performed.

For ScVelo, genes were selected by detection of >20 counts and high variability between cells. Spliced and unspliced count matrices were then normalized and log transformed as above. Principal components are then calculated for all count matrices. RNA velocity is then estimated using the dynamical model which uses a likelihood-based expectation-maximization framework to learn the unspliced/spliced phase trajectory for each gene.

For UniTVelo, data processing proceeds using the pipeline of ScVelo. RNA velocity, however, is estimated using either a unified latent time model which is leveraged over the entire transcriptome to incorporate stably and monotonically changed genes, or an independent model which retains gene-specific time matrices. In both models, the root cluster was assigned to be “Skin B”.

Phage immunoprecipitation sequencing

Serum from HS patients (n=10) and healthy controls (n=14) was provided to CDI Labs for antibody profiling using their HuScan and VirScan PhIP-seq services. Patient samples were tested against phage display libraries of 48,921 human proteins (49-mer, 25-AA overlap) and proteins from >1,000 viral families (56-mer, 28-AA overlap). Detailed descriptions of the immunoprecipitation, sequencing, and analytics pipeline for HuScan and VirScan have been previously published.[72,73]

Serology

HS patient serum (n=23) was tested for EBV viral capsid antigen reactivity using Gold Standard Diagnostics EBV VCA-IgG and -IgM ELISA kits (Fisher).

Real-time PCR

4mm punch biopsies of lesional skin from HS patients were obtained from the Emory Department of Dermatology and stored at -80°C in RNAlater solution (Thermo Fisher). DNA and RNA were extracted from the same biopsy using the RNeasy fibrous tissue mini kit (Qiagen) and the Wizard genomic DNA purification kit (Promega) in tandem. cDNA was generated from the RNA fraction using Superscript III first strand synthesis (Invitrogen) with gene-specific primers found in the table below. Real-time PCR was carried out using Taqman master mix and accompanying protocol (Thermo Fisher). Real-time PCR was performed on the LightCycler 480 system (sw.v. 1.2.9.11, Roche). A second derivative maximum analysis was performed and the reaction was deemed "positive" if the crossing point (CP) was reached within 45 cycles.

Gene Target	Primer/Probe	Sequence 5'-3'	Fluorescence	Quencher	Reference
EBNA1	Probe	AGGGAGACACATCTGGACCAGAAGGC	FAM	ZEN–Iowa Black FQ	74
	Forward	TACAGGACCTGGAAATGGCC			
	Reverse	TCTTTGAGGTCCACTGCCG			
EBER1	Probe	AGACAACCACAGACACCGTCCTCACCA	FAM	ZEN–Iowa Black FQ	74
	Forward	TGCTAGGGAGGAGACGTGTGT			
	Reverse	TGACCGAAGACGGCAGAAAG			
EBER2	Probe	CGACCCGAGGTCAAGTCCCGG	FAM	ZEN–Iowa Black FQ	74
	Forward	AACGCTCAGTGCGGTGCTA			
	Reverse	GAATCCTGACTTGCAAATGCTCTA			
LMP1	Probe	TCCAGATACCTAAGACAAGTAAGCACCCGAAGAT	FAM	ZEN–Iowa Black FQ	74
	Forward	AATTTGCACGGACAGGCATT			
	Reverse	AAGGCCAAAAGCTGCCAGAT			
BZLF1	Probe	ATAATGGAGTCAACATCCAGGCTTGGGC	FAM	ZEN–Iowa Black FQ	75
	Forward	AAATTTAAGAGATCCTCGTGTAAAACATC			

	Reverse	CGCCTCCTGTTGAAGCAGAT			
GAPDH	Probe	CAAGCTTCCCGTTCTCAGCC	FAM	ZEN–Iowa Black FQ	
	Forward	GAAGGTGAAGGTCGGAGTA			
	Reverse	GAAGATGGTGATGGGATTTC			
CMV	Taqman assay	ID: Vi06439643_s1 (Thermo Fisher)			

HuProt monoclonal antibody screen

Six monoclonal antibodies (mAb) were generated from the observed sequences of 3 HS patients (2 mAb per patient). Full-length VDJ sequences were selected based on their clone size and mutation load. Heavy chains were cloned into pFUSE-CHIg-hG1 and light chains were cloned into pFUSE-CHIg-hK or -hL2. These plasmids were used to transfect CHO cells using the ExpiCHO Expression System (Gibco) according to the manufacturer's instructions. mAbs were isolated from culture supernatants via purification on columns containing Pierce protein A agarose (Thermo Fisher). 2 patient-derived influenza mAbs with validated reactivity to H3 protein and a SARS-CoV2 mAb with validated reactivity to S2 protein (1A9, Invitrogen) were included as controls.

mAb samples were provided to CDI Labs for antibody profiling using their Human Proteome Microarray (HuProt) protocol. The HuProt microarray includes >21,000 full-length proteins in their native conformation. Recombinant full-length EBNA1(abcam), p23 (abcam), H3, and S2 were provided for inclusion on the microarray. HuProt development, pipeline, and validation have been previously published.[76]

Tables

Table 1: Cluster classification

Compartment	Lineage	Cluster	Classification	Expression
Periphery	B cell (CD19+, CD20+, PAX5+)	A	Naive B	IgD↑, IgM↑↑, CD27-, CD38-, HLA-DRA↑
		B	Memory B	IgD-, IgM↑, IgG/IgA↑, CD27↑, CD38-, HLA-DRA↑
Skin		C	Skin B	IgD-, IgM↑, IgG/IgA↑↑, CD27-, CD38-, HLA-DRA↑, CXCR4↑, CD83↑
	Plasma cell (CD20-, PAX5-, IgM-,high Ig-specific transcripts)	D	Skin ASC I	CD19-, IgG/IgA↑↑↑, CD27-, CD38-, HLA-DRA^{low}, XBP1↑, SDC1-, Ig-specific transcripts↑
		E	Skin ASC II	CD19-, IgG/IgA↑↑↑, CD27-, CD38-, HLA-DRA-, XBP1↑↑, SDC1^{low}, MZB1↑, Ig-specific transcripts↑↑
		F	Skin ASC III	CD19^{low}, IgG/IgA↑↑↑, CD27↑, CD38↑, HLA-DRA-, XBP1↑↑↑, SDC1↑↑, MZB1↑, PRDM1↑, Ig-specific transcripts↑↑↑
		G	Skin ASC IV	CD19^{low}, IgG/IgA↑↑↑, CD27-, CD38↑, HLA-DRA-, XBP1↑↑↑, SDC1↑, MZB1↑, PRDM1↑, Ig-specific transcripts↑↑
Skin/Periphery	Plasmablast	H	ASC V	CD19^{low}, IgG/IgA↑↑↑, CD27↑, CD38↑, HLA-DRA^{low}, XBP1↑↑↑, SDC1^{low}, MZB1↑, PRDM1↑, MKI67↑, Ig-specific transcripts↑↑
Periphery	Plasma cell (CD20-, PAX5-, IgM-, high Ig-specific transcripts)	I	Peripheral ASC	CD19^{low}, IgG/IgA↑↑↑, CD27↑↑, CD38↑, HLA-DRA^{low}, XBP1↑↑↑, SDC1-, MZB1↑, PRDM1↑, Ig-specific transcripts↑↑

Table 2: Clinical characteristics of study participants

	Demographic			EBV			Hidradenitis Suppurativa				Surgically Excised Lesion		PBMC
Participant ID	Age	Sex	Race	VCA-IgG	VCA-IgM	PCR	Age of Diagnosis	Hurley Stage	History with anti-TNFα	Concomitant autoimmune condition	Type	Location	Provided PBMC?
HS-1002	20s	F	White	+	-		10s	II	N/A				
HS-1003	30s	F	White	+	-		20s	II	Responder	Crohn's disease			
HS-1004	50s	M	African American	+	-		10s	III	Non-responder				
HS-1005	40s	F	African American	+	-		40s	III	Non-responder				
HS-1007	30s	F	Asian	+	-		20s	III	N/A				
HS-1009	60s	F	African American	+	-		50s	II	Non-responder	Crohn's disease			
HS-1010	40s	F	African American	+	-		40s	II	N/A				
HS-1013	30s	F	White	+	-		20s	I	N/A				
HS-1014	50s	F	African American	+	-								
HS-1017	60s	F	White	+	-		50s	II	N/A				
HS-1019	30s	M	African American	+	-		20s	III	Responder				
HS-1020	30s	M	African American	-	-		20s	III	N/A				
HS-1027	40s	F	African American	+	-		20s	II	N/A				
HS-1031	40s	F	African American	+	-								
HS-1039	40s	F	African American	+	-		30s	III		Neuromyelitis optica			
HS-1041	30s	F		+	-								
HS-1006	20s	F	African American	+	-	+	10s	II	Responder				
HS-1021	20s	M	African American	+	+	-	10s	III	Non-responder				
HS-1023	50s	M	African American	+	-	+							
HS-1026	30s	F	White	+	-	-	10s	III	Responder				
HS-1029	30s	M	Asian	+	-	+	20s	III	Non-responder				
HS-1040	50s	F	African American	+	-	-	40s	II	Non-responder	Crohn's disease-luminal and cutaneous	Tunnel	Axilla	No
HS-1044	40s	F	African American	+	-	+							
HS-1001	30s	F	White			+	30s	II					
HS-1012	30s	F	African American			+	30s	II	N/A				
HS-1018	30s	M	White			-	30s	II	Responder	Crohn's disease, rheumatoid arthritis			
HS-1022	30s	F	African American			+	30s	II					
HS-1030	30s	F	African American			-							
HS-1036	20s	M	African American			-		II	N/A				
HS-1032	40s	F	African American			-	20s	III	Responder		Tunnel	Axilla	Yes
HS-1038	40s	F	White			-	20s	II	Responder				
HS-1043	40s	M				+	30s	III	Non-responder				
HS-1045	30s	F				+	20s	III	N/A				
HS-1046	20s	F	White			+	10s	II	Non-responder				
HS-1047						-							
HS-1052	40s	F	African American			+	40s	II	Non-responder		Tunnel	Breast	Yes
HS-1054	40s	F	African American			-	40s	II	N/A				
HS-1055	30s	F					20s	II	Responder				Yes
HS-1056	20s	F	African American				10s	III	Responder		Tunnel	Axilla	No

Unavailable data left blank; PCR: amplification of EBV nucleic acid from skin biopsies; N/A: not applicable; Non-responder: history of discontinued use due to inadequate response

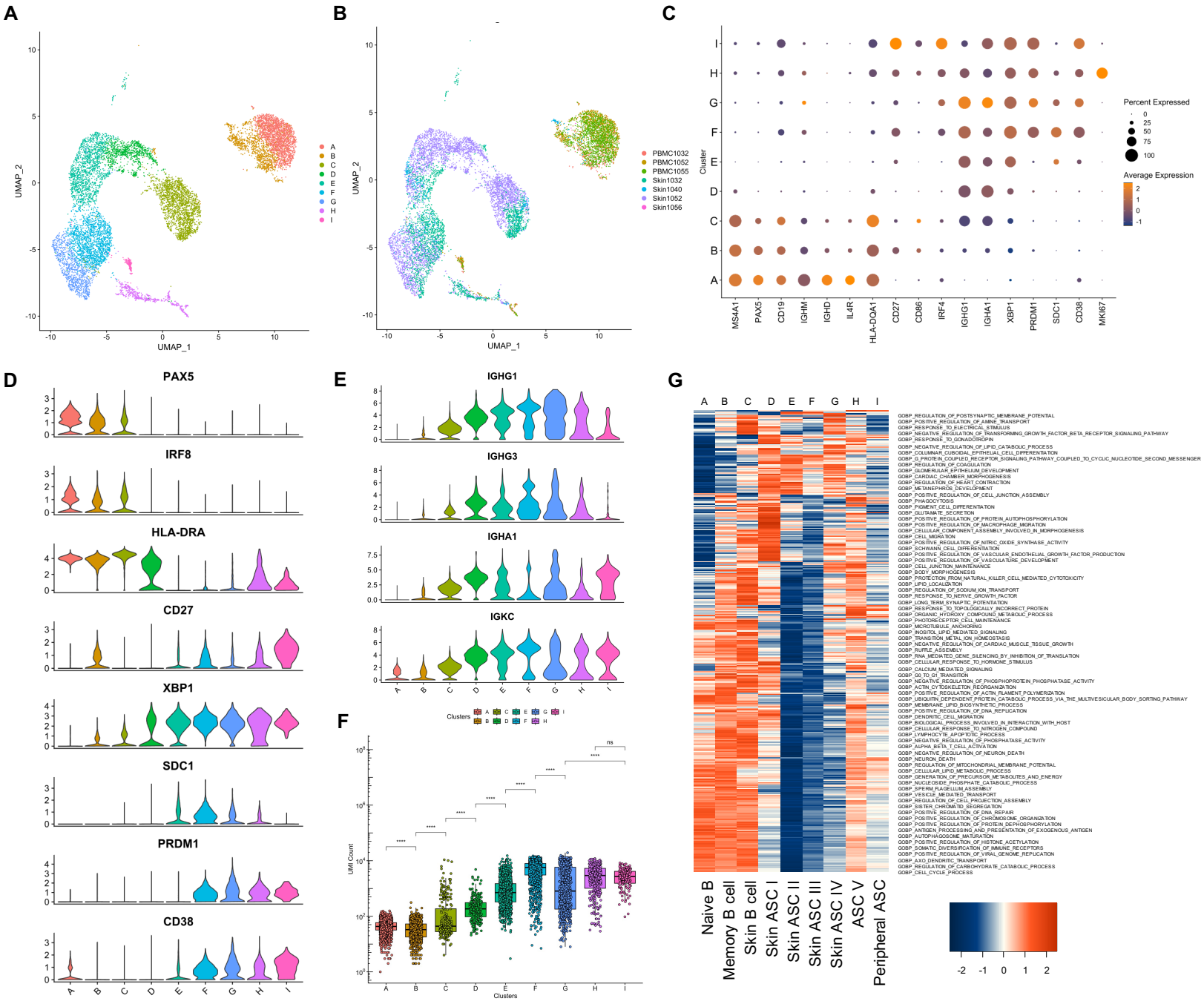


Figure 1. HS skin and peripheral B cells form clusters based on differential gene expression. (A) UMAP of B cells from on PBMCs (n=3) and skin B cells from the surgical excisions (n=4) of 5 patients with moderate-to-severe HS. Colored according to sample **(B)** and cluster. **(C)** Dot plot depicting relevant gene expression. Dot size represents percent of cells in cluster expressing the gene. **(D)** Violin plots depicting expression level of relevant genes by cluster. **(E)** Violin plots depicting expression level of immunoglobulin genes by cluster. **(F)** Immunoglobulin-specific transcripts by cluster (t-test with Bonferroni correction for multiple comparisons, ****p<0.0001; ns, nonsignificant). **(G)** Gene set enrichment analysis using the biological processes subset of gene ontology gene sets (GOBP). These gene sets are compiled in the molecular signatures database (MSigDB v2023.1.Hs) and sourced from ontology resources. Heat map depicts enrichment level for each gene set from low (blue) to high (orange) across clusters. Clusters are labeled according to classification described in Table 1. ASC, antibody secreting cell.

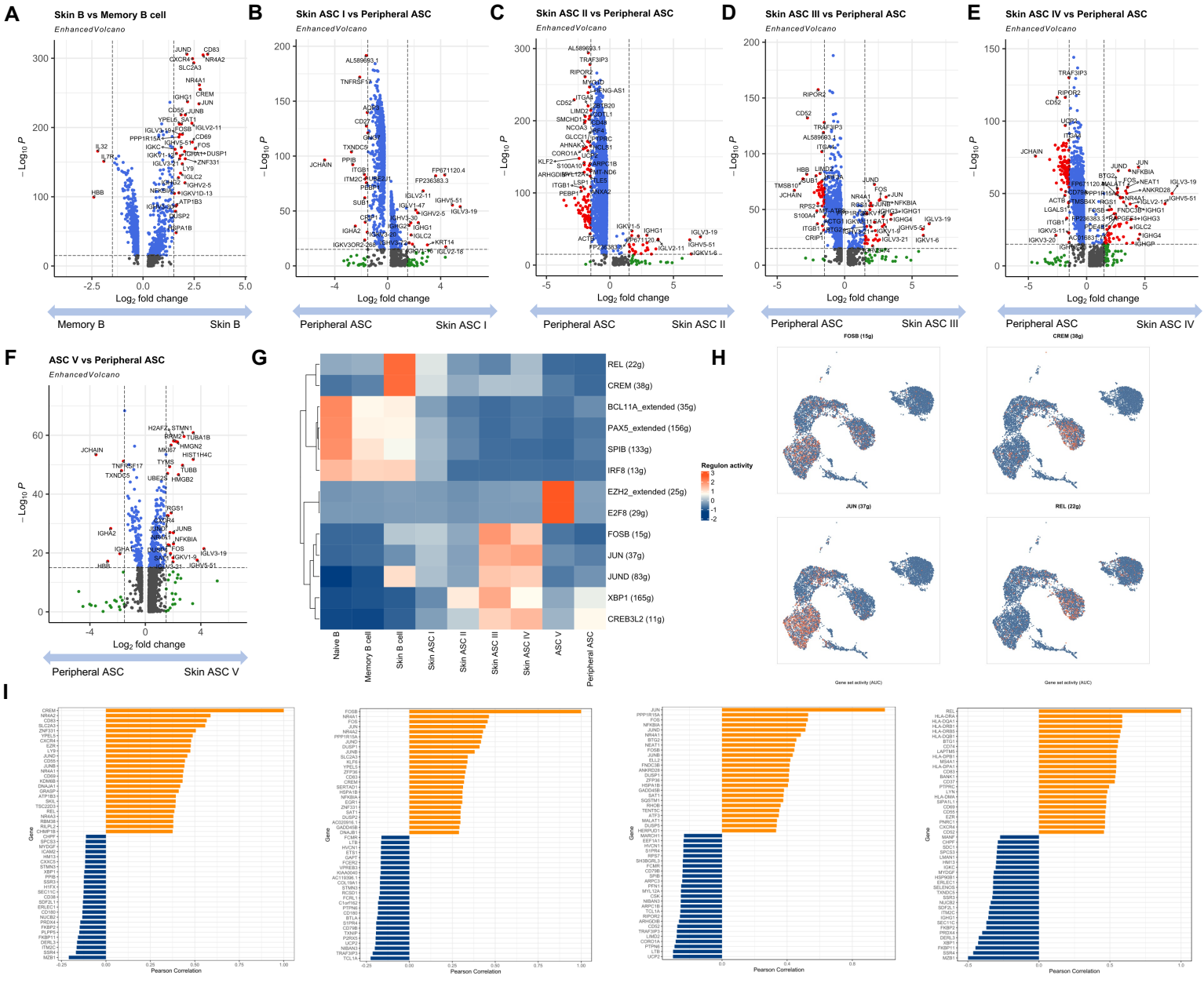


Figure 2. B cells in HS skin express markers and transcription factors associated with chronic activation. (A-F) Volcano plots depicting differentially expressed genes between two populations. Genes are plotted according to p-value and fold change on a log scale. Genes represented by red dots are above significance thresholds. **(G)** SCENIC heatmap depicting top active regulons comprised of transcription factors and their direct target genes. Labels include number of genes in regulon. Color corresponds to regulon activity from low (blue) to high (orange). **(H)** UMAP colored according to positive regulon activity (orange). **(I)** Histograms depicting genes with positive correlation (orange) or negative correlation (blue) with CREM, FOSB, JUN, and REL expression.

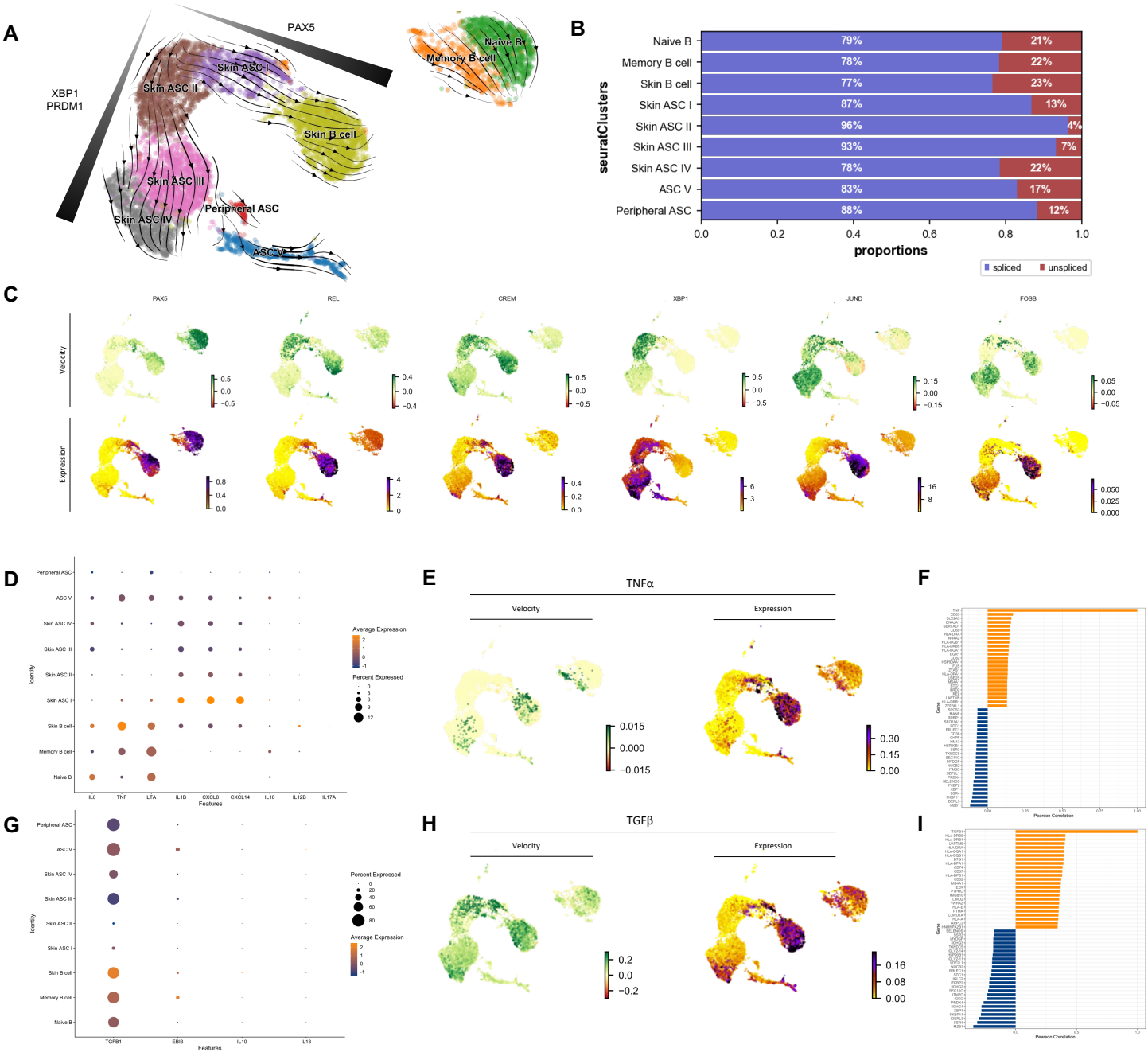


Figure 3. RNA velocity analysis demonstrates B cell to plasma cell differentiation and cytokine production in the skin. (A) scVelo velocity vector field embedded in UMAP. Velocity analysis predicts the future transcriptional state of a cell based on its ratio of immature (unspliced) to mature (spliced) mRNA. **(B)** Proportion of spliced to unspliced mRNA by cluster. **(C)** Top: UMAP colored according to velocity. Positive velocity (green) implies upregulation of a gene from steady state (yellow), while negative velocity (red) implies downregulation of a gene from steady state. Bottom: UMAP colored according to expression from low (yellow) to high (black). **(D)** Dot plot of pro-inflammatory cytokine expression by cluster. Size of dot represents percent of cells in cluster with expression, while color corresponds to average expression level. **(E)** Velocity and expression UMAPs for TNFα. **(F)** Histogram depicting genes with positive correlation (orange) and negative correlation (blue) with TNFα expression. **(G)** Dot plot of anti-inflammatory cytokine expression by cluster. **(H)** Velocity and expression UMAPs for TGFβ. **(I)** Histogram depicting genes with positive correlation (orange) and negative correlation (blue) with TGFβ expression.

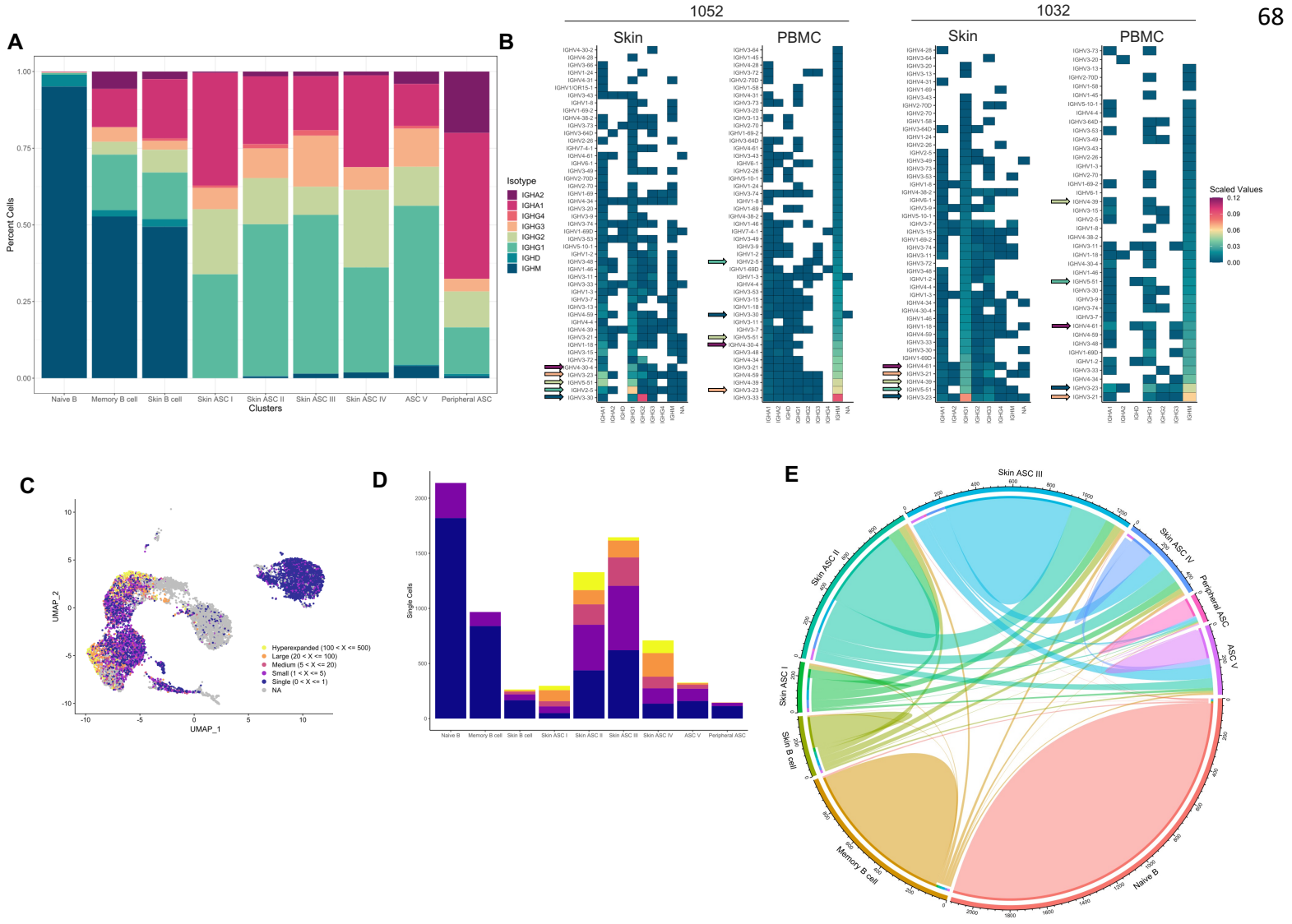
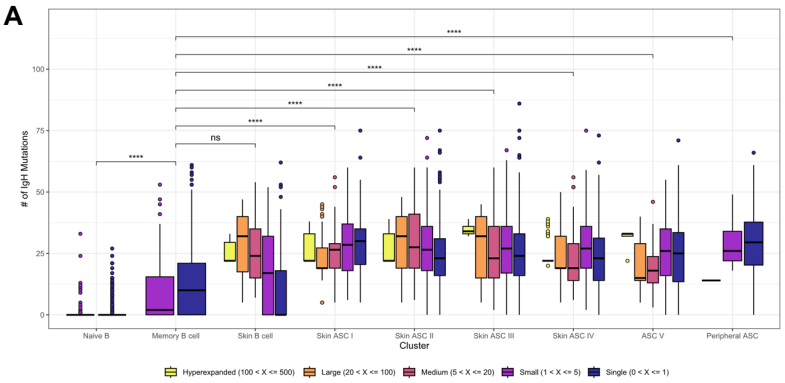
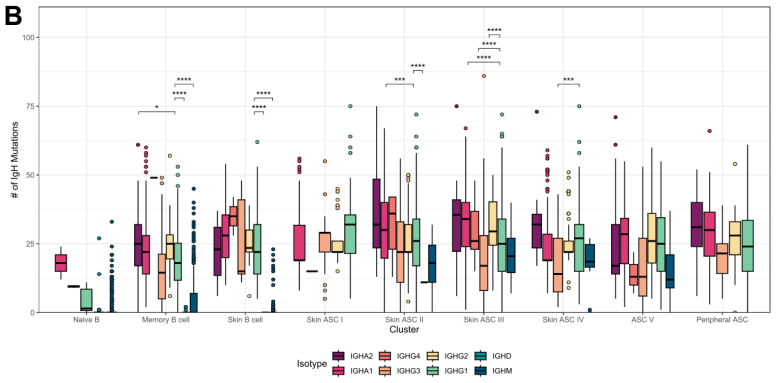
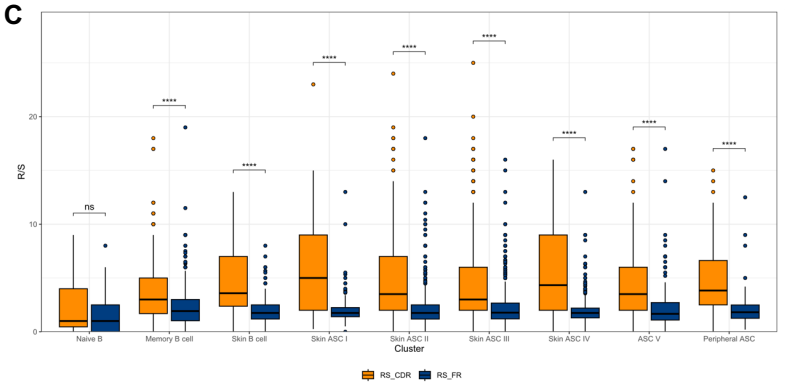
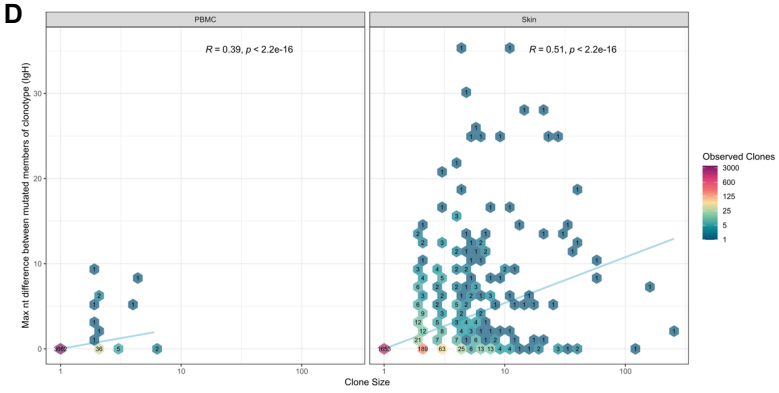


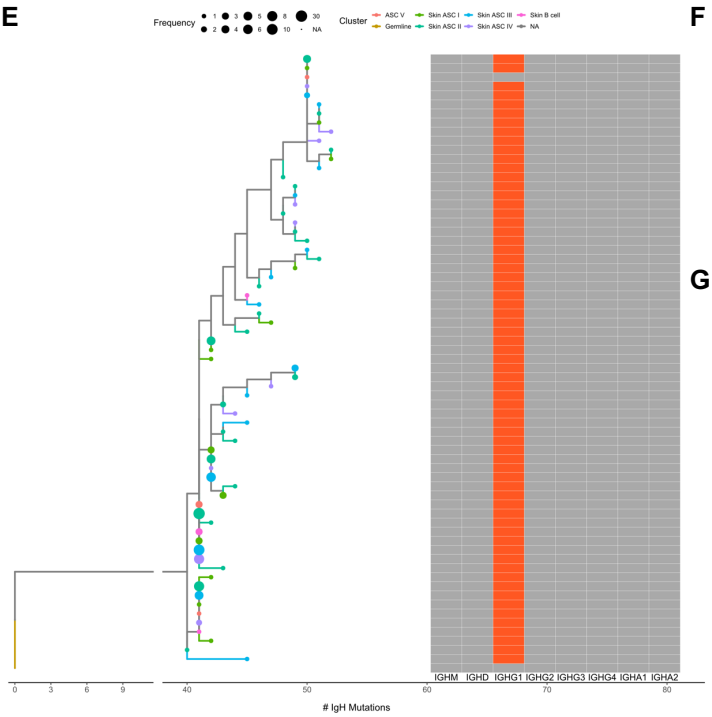
Figure 4. HS skin B cells and ASC are class-switched and clonally expanded. (A) Isotype expression by cluster shown as percent of total cells. **(B)** IGHV gene usage by isotype in the skin and periphery of participants 1052 and 1032. Color corresponds to proportion of total sequences. **(C)** UMAP colored according to clonotype expansion level. **(D)** Number of cells at each clonotype expansion level by cluster. **(E)** Circos diagram depicting connectivity between clusters based on shared CDR3 sequence. Connecting ribbons represent clones found in both clusters, weight of ribbon indicates number of shared clones.

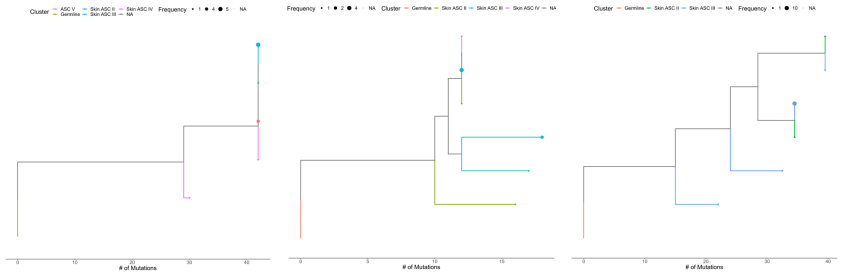

A
# of IgH Mutations
Naive B
Memory B cell
Skin B cell
Skin ASC I
Skin ASC II
Skin ASC III
Skin ASC IV
ASC V
Peripheral ASC
Cluster
ns
****

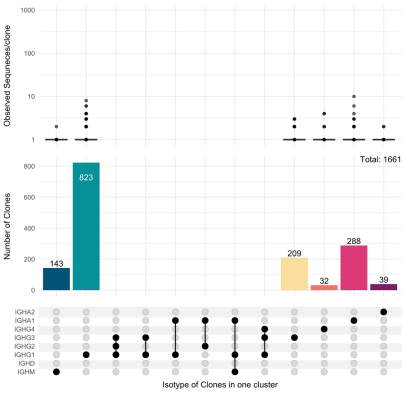

B
# of IgH Mutations
Cluster
Isotype
IGHA2
IGHG4
IGHG2
IGHD
IGHA1
IGHG3
IGHG1
IGHM


C
Hyperexpanded (100 < X <= 500)
Large (20 < X <= 100)
Medium (5 < X <= 20)
Small (1 < X <= 5)
Single (0 < X <= 1)
RS
Cluster
RS_CDR
RS_FR


D
PBMC
Skin
R = 0.39, p < 2.2e-16
R = 0.51, p < 2.2e-16
Max nt difference between mutated members of clonotype (IgH)
Clone Size
Observed Clones


E
Frequency
Cluster
IgH Mutations
IGHM
IGHD
IGHG1
IGHG2
IGHG3
IGHG4
IGHA1
IGHA2


F
# of Mutations


G
Observed Sequnces/clone
Number of Clones
Total: 1661
143
823
209
32
288
39
Isotype of Clones in one cluster

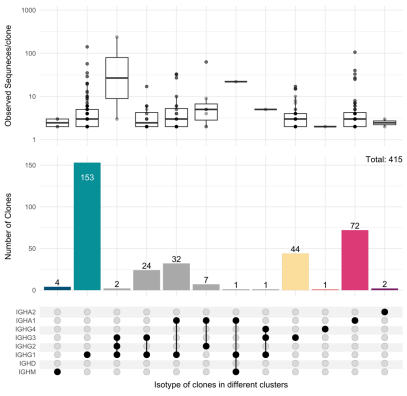

H
Observed Sequnces/clone
Number of Clones
Total: 415
4
153
2
24
32
7
1
1
44
1
72
2
Isotype of clones in different clusters

Figure 5. Expanded clones in the skin are highly mutated, intra-clonally homogenous, and are primarily of a single isotype. (A) Box plot depicting number of heavy chain (IgH) mutations found in clones at each expansion level by cluster. Lower and upper bars represent the first and third quartiles, middle bar is the median, whiskers extend to 1.5 times the interquartile range. **(B)** Number of heavy chain mutations found per isotype by cluster. Statistical significance measured in comparison to IgG1. **(C)** Characterization of mutations in terms of replacement to silent (R/S) ratio in CDR vs. framework regions by cluster.**(D)** Clonal diversity in terms of maximum mutation difference between members by clone size (Δmt). Color and number correspond to total observed clones of equivalent size and diversity (linear regression in blue, Pearson correlation). **(E)** Lineage tree of large clones from skin of patient 1052. Tree origin represents germline sequence, with branch length corresponding to number of IgH mutations. Dots represent observed members, with size corresponding to frequency and color corresponding to cluster. Member isotype is indicated to the right in orange. **(F)** Representative lineage trees of average-sized clones from skin of patient 1032. **(G)** Characterization of class switching among undifferentiated clones in the skin. Histogram depicts number of clones, while black dots below indicate observed isotype(s) in clones above. Box plots above depict size of clones below. Lower and upper bars represent the first and third quartiles, middle bar is the median, whiskers extend to 1.5 times the interquartile range. **(H)** Characterization of class switching among differentiated clones in the skin. Histogram depicts number of clones, while black dots below indicate observed isotype(s) in clones above. Box plots above depict size of clones below. Lower and upper bars represent the first and third quartiles, middle bar is the median, whiskers extend to 1.5 times the interquartile range. (t-test with Bonferroni correction for multiple comparisons, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$; ns, nonsignificant).

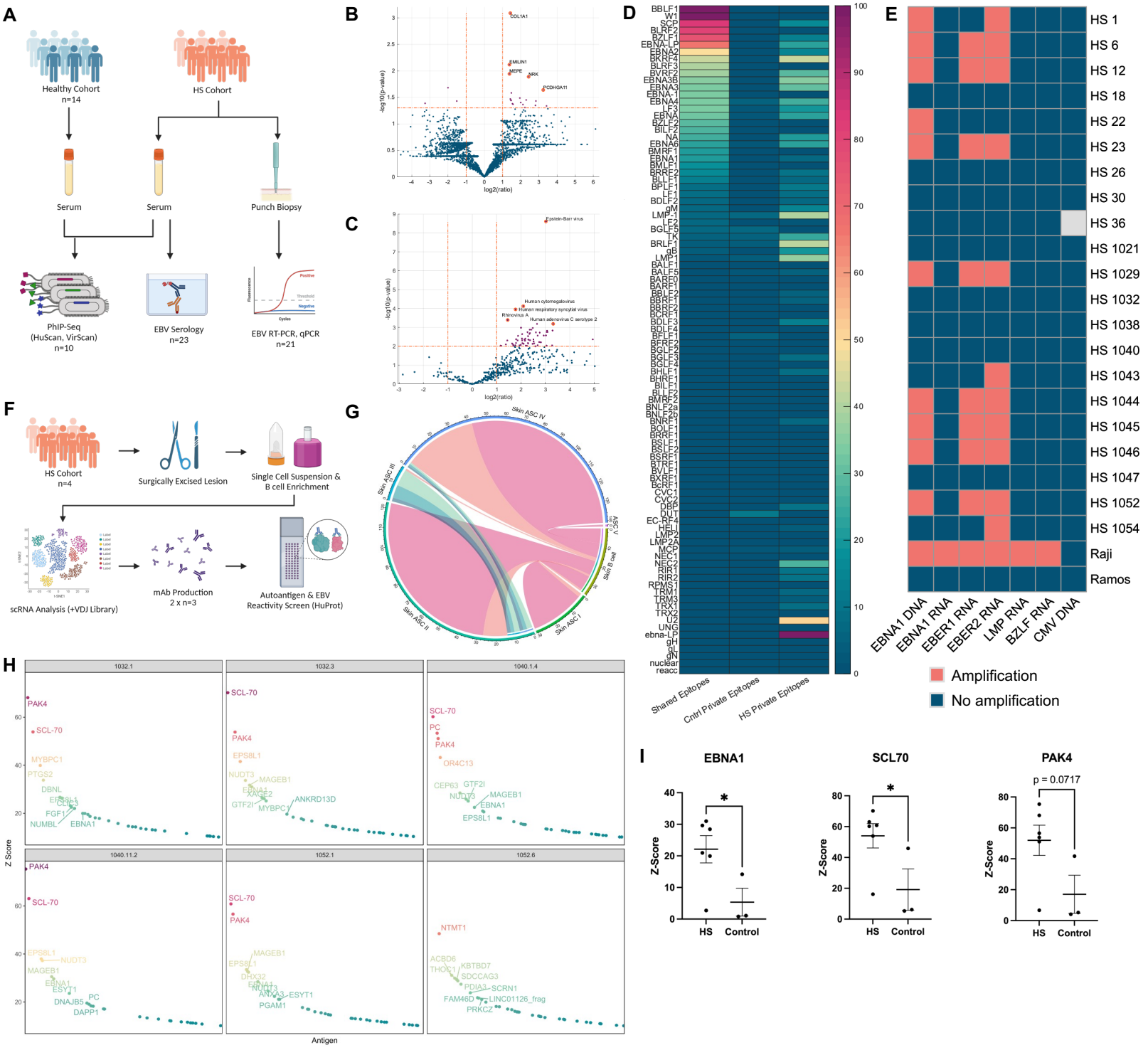
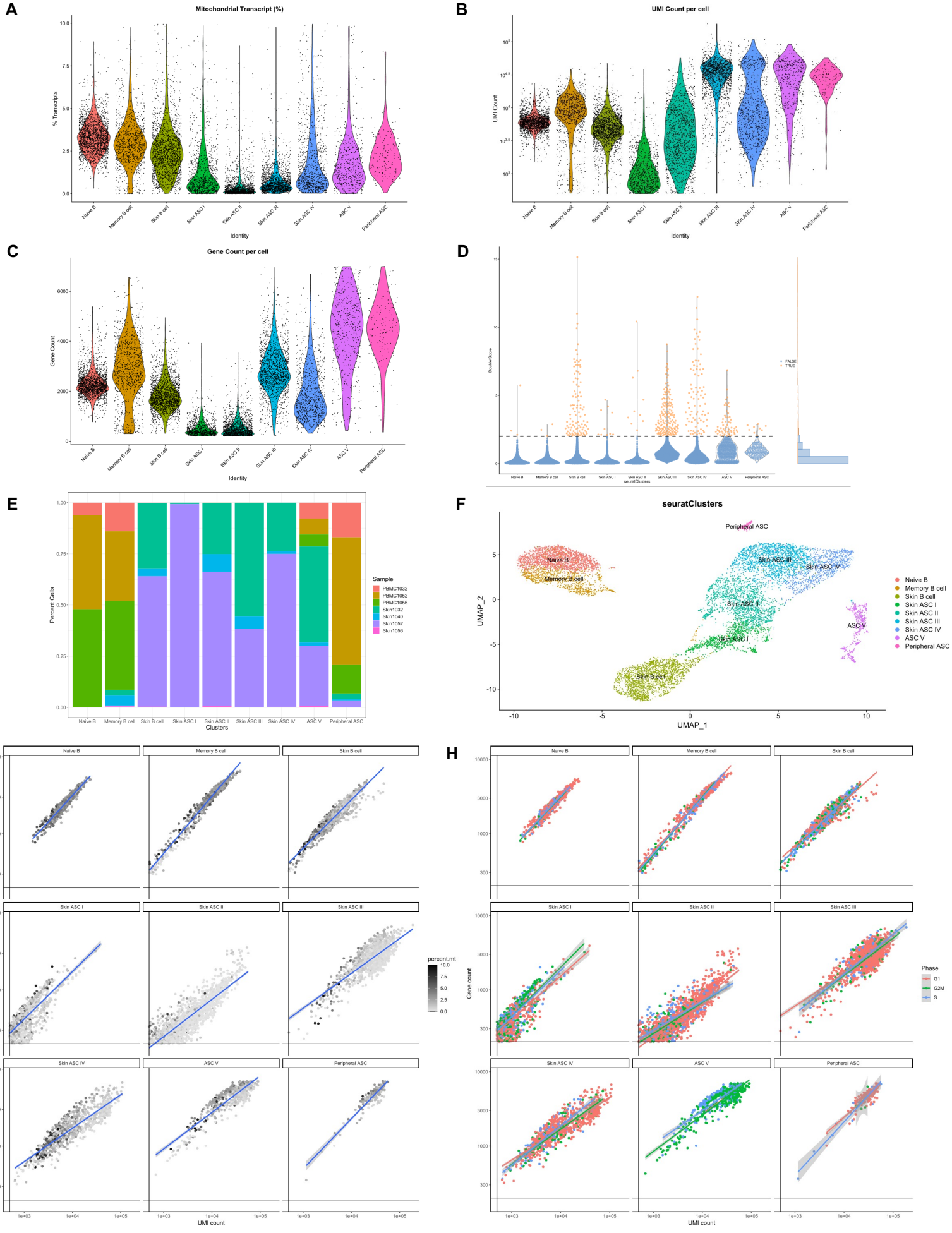
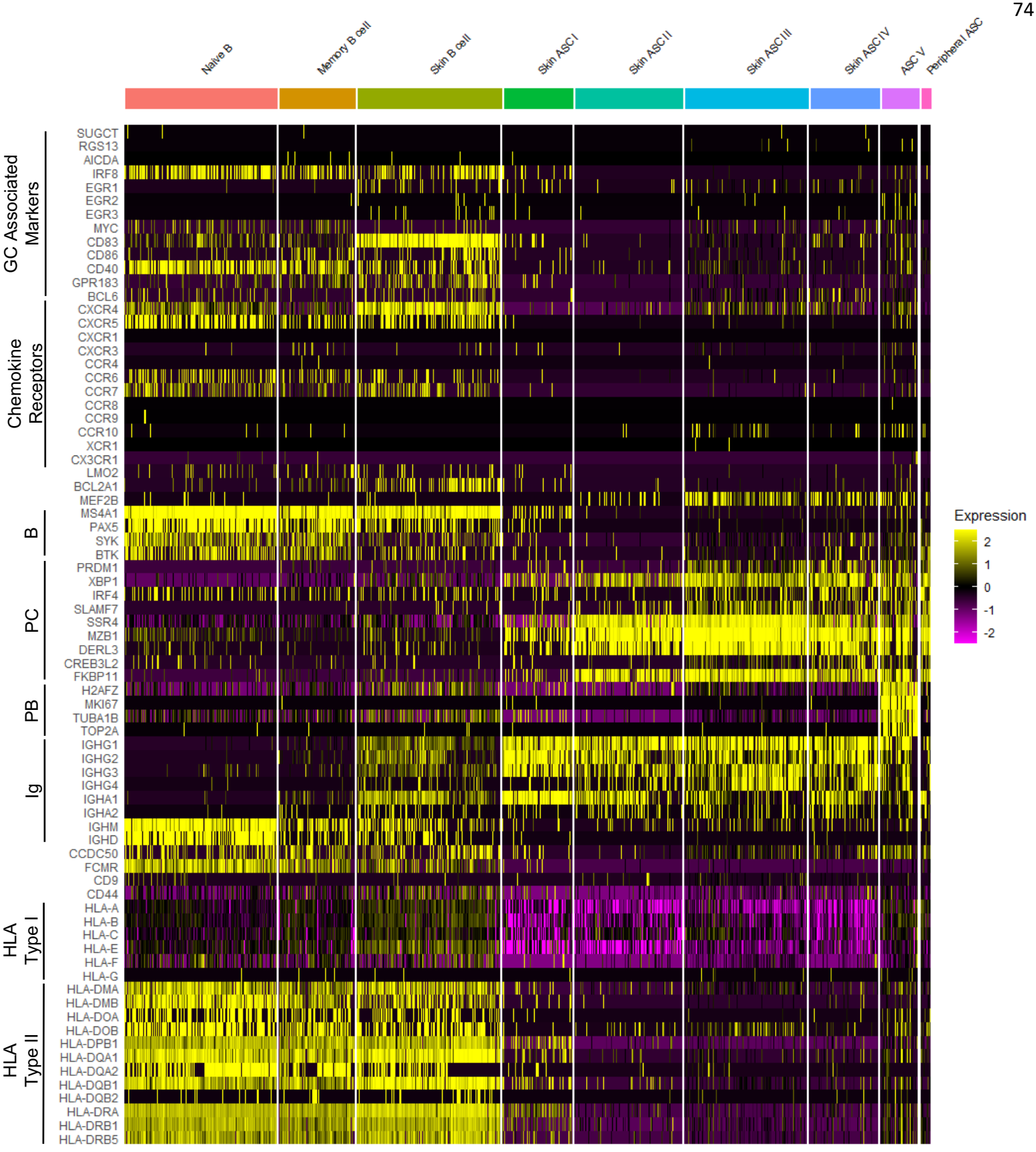


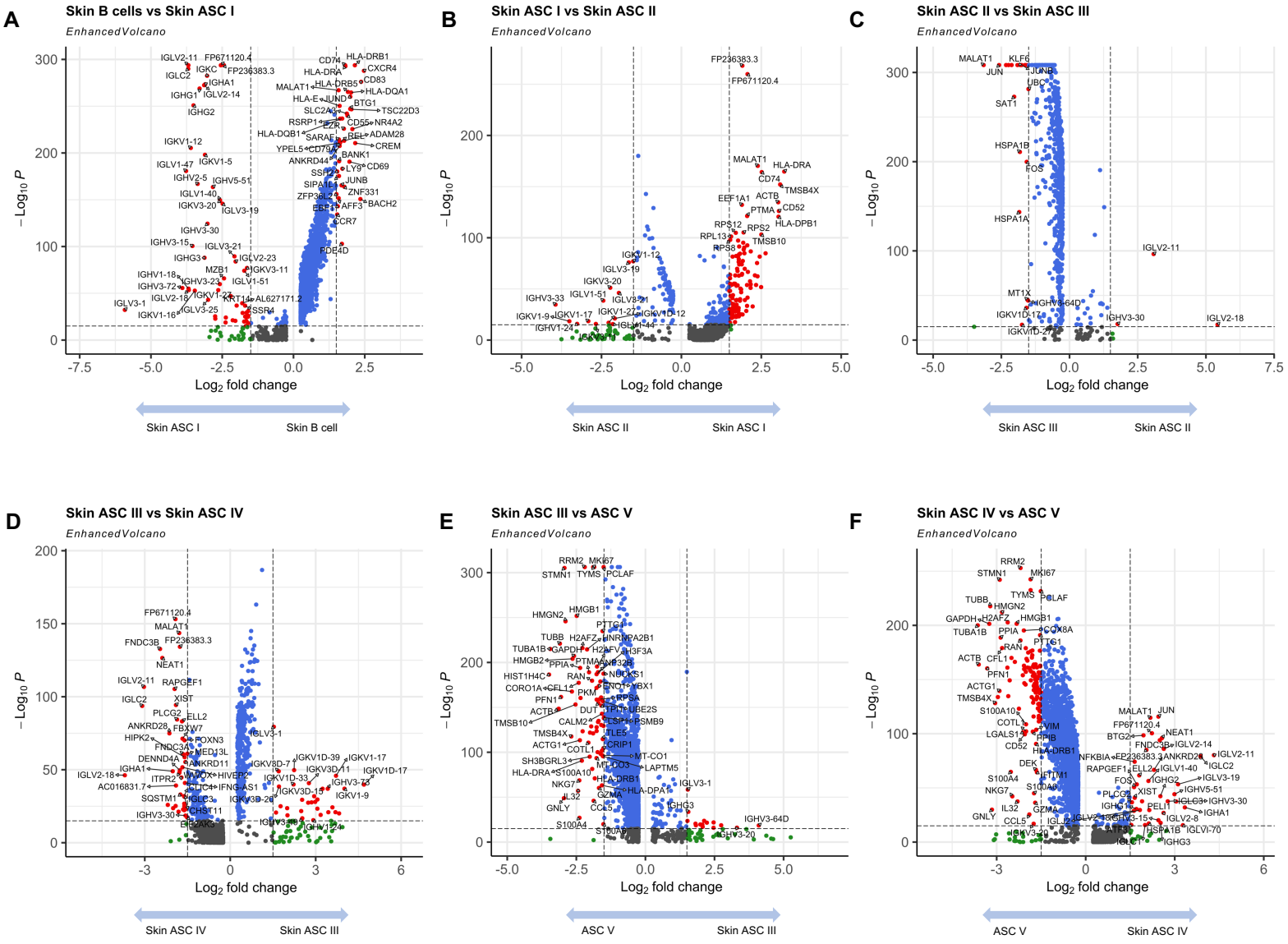
Figure 6. Proteome-wide screening revealed cross reactivity between EBV and autoantigens in clonally expanded HS skin clones. (A) Schematic depicting study design for PhIP-seq, EBV serology and RT-PCR/qPCR analyses. **(B-C)** Volcano plots depicting extent of epitope spread relative to healthy controls (HC) for human antigens (**B**) and viral antigens (**C**) in PhIP-seq analysis. For human antigens, PhIP-seq signal was aggregated at the protein level. For viral antigens, PhIP-seq signal was aggregated at the viral species level (**D**) Heatmap depicting epitope reactivity in PhIP-seq analyses. Each row represents a single EBV protein. For each protein, epitopes were assessed for reactivity in HC and HS sera. The percent of epitopes that had shared reactivity (Shared Epitopes), HC only (Cntrl Private Epitopes), or HS only (HS Private Epitopes) is shown. (**E**) Heatmap depicting RT-PCR/qPCR amplification of EBV transcripts in HS skin biopsies. (**F**) Schematic outlining source of selected monoclonal antibodies for full-length human proteome reactivity screening. (**G**) Circos plot showing that clones from which monoclonal antibodies were generated are found across clusters and thus represent differentiating clones. (**H**) Reactivity of select antibodies against a panel of >21,000 human antigens. Shown are Z-scores of top hits in the panel. (**I**) Z-score comparison of HS derived antibodies versus control antibodies against EBNA1, SCL70, and PAK4. *p<0.05


A
Mitochondrial Transcript (%)
% Transcripts
Identity
B
UMI Count per cell
UMI Count
Identity
C
Gene Count per cell
Gene Count
Identity
D
DoubletScore
FALSE
TRUE
seuratClusters
E
Percent Cells
Clusters
Sample
PBMC1032
PBMC1052
PBMC1055
Skin1032
Skin1040
Skin1052
Skin1056
F
seuratClusters
UMAP_1
UMAP_2
Naive B
Memory B cell
Skin B cell
Skin ASC I
Skin ASC II
Skin ASC III
Skin ASC IV
ASC V
Peripheral ASC
G
Gene count
UMI count
percent.mt
H
Gene count
UMI count
Phase
G1
G2M
S

Supplemental Figure 1. scRNA-seq quality control process. (A) Violin plot of mitochondrial transcript percent by cluster. **(B)** Unique molecular identifier (UMI) count per cell by cluster. **(C)** Number of genes per cell by cluster. **(D)** Predictive doublet scoring per cell by cluster. Dots colored according to likelihood that they are a doublet. Histogram to the right depicts fraction of data under set threshold. **(E)** Sample contribution to each cluster in terms of percent of total cells. **(F)** UMAP projection following reclustering of cells after removal of all heavy/light variable and constant BCR genes from the count matrix. Cells are annotated with their original label prior to reclustering. **(G)** Correlation between gene count and UMI count of cells from each cluster. Color intensity corresponds to mitochondrial transcript percent. Linear regression in blue, bounded in grey by 95% confidence interval. **(H)** Correlation between gene count and UMI count of cells from each cluster. Color corresponds to cell cycle phase. Linear regression shown for each cell phase bounded in grey by 95% confidence interval.

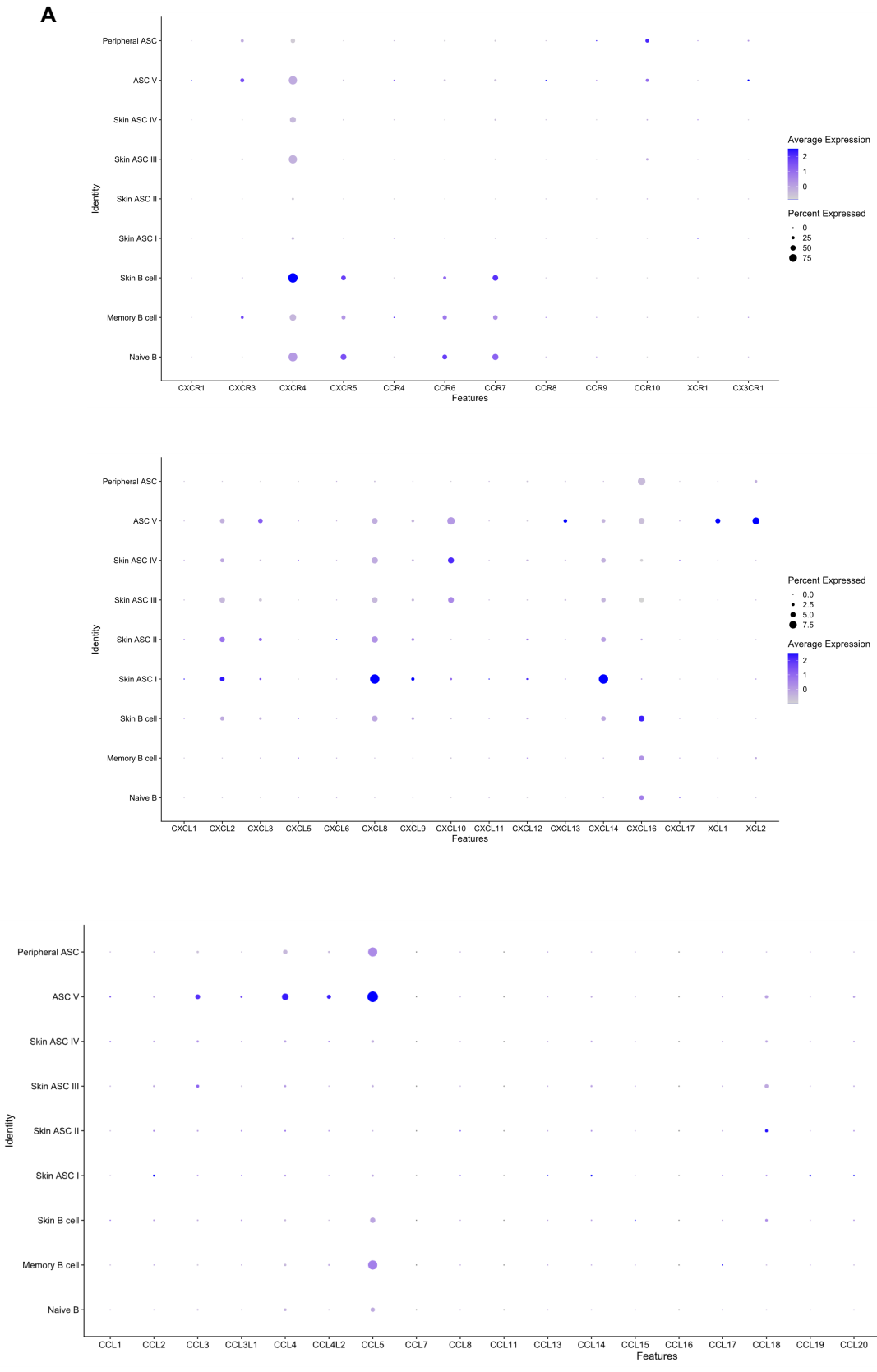


Supplemental Figure 2. Expression of genes of interest. (A) Heat map of gene expression by cluster. Selection includes differentially expressed genes, as well as genes relevant to B cell populations.

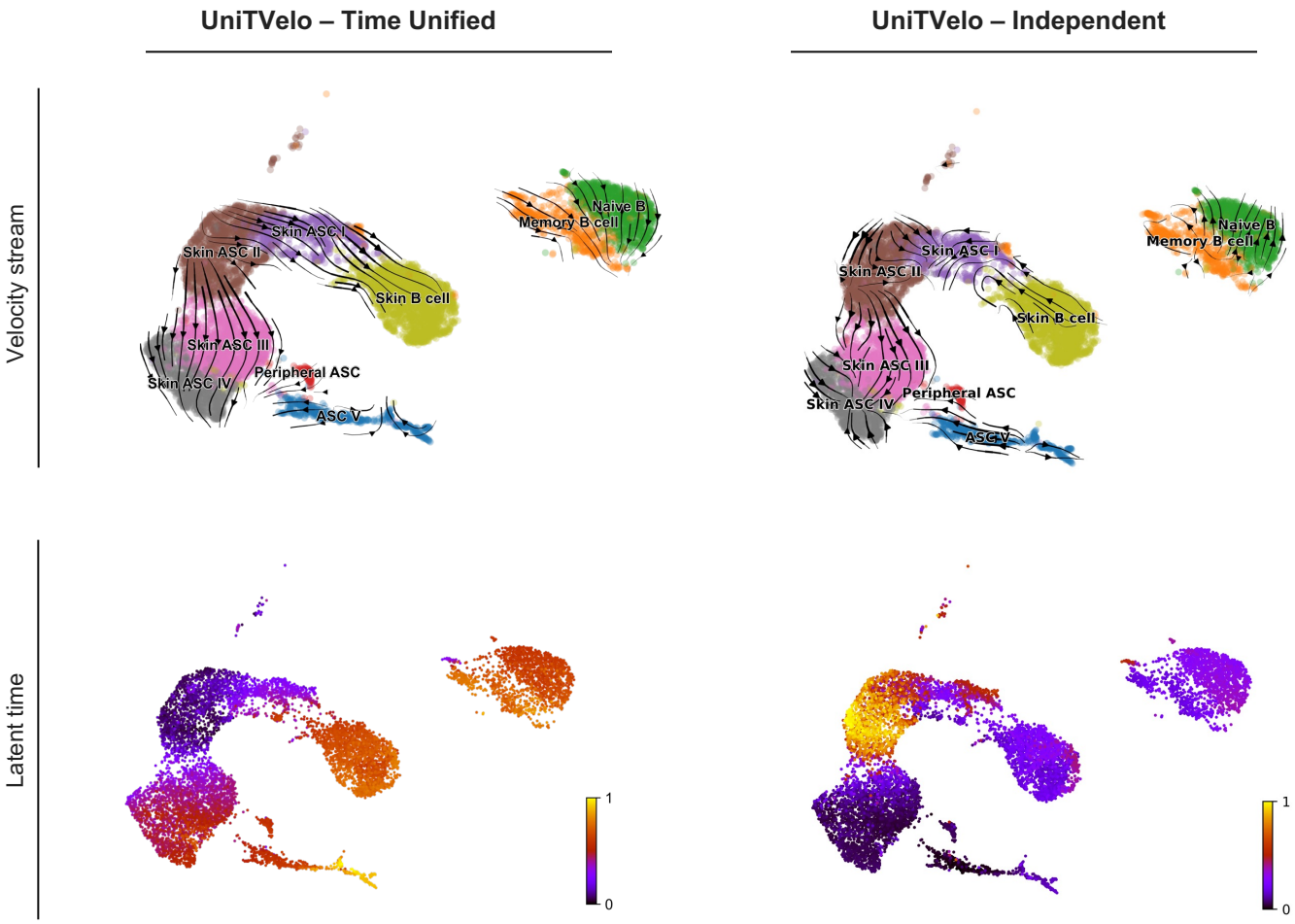


Supplemental Figure 3. Differential gene expression between skin compartment clusters. (A-F) Volcano plots depicting differentially expressed genes between most closely related skin B cell clusters. Genes are plotted according to p-value and fold change on a log scale. Genes represented by red dots are above significance thresholds.

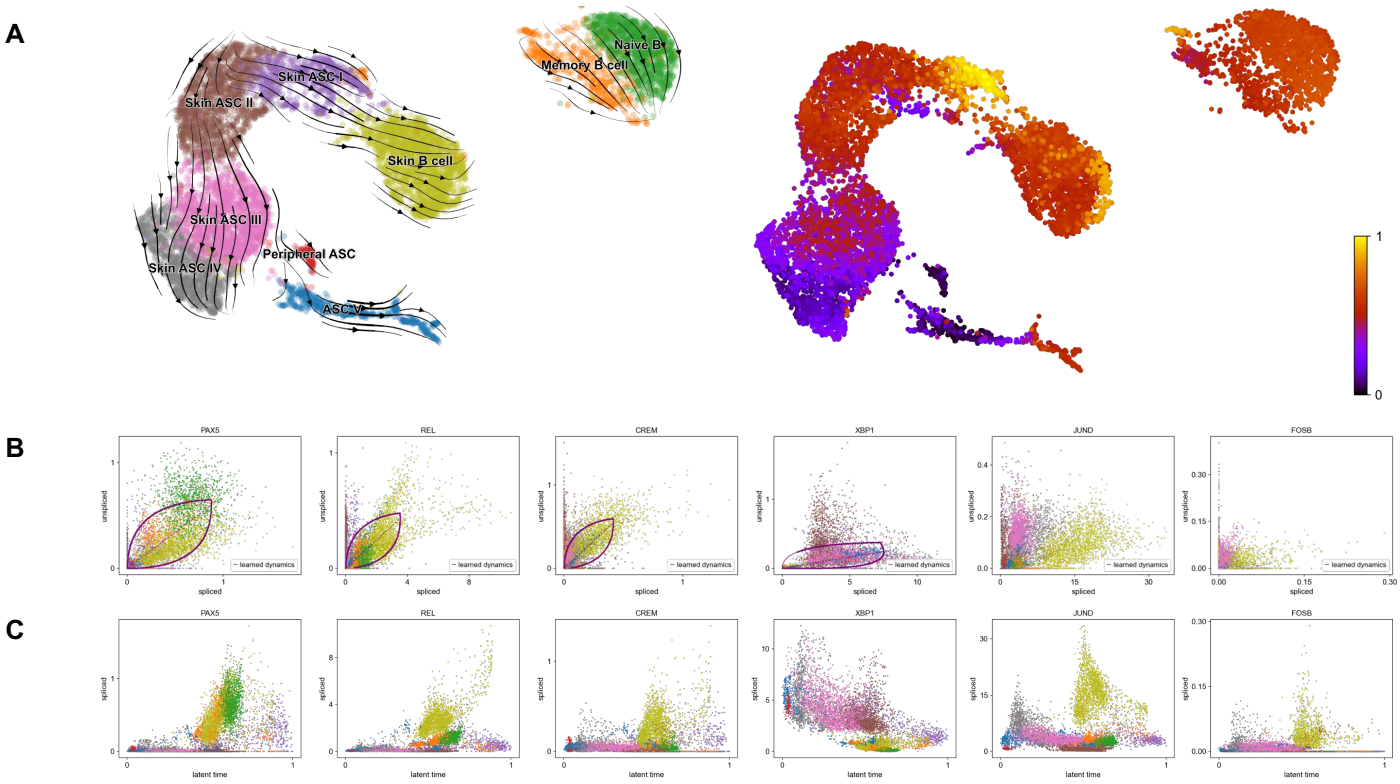
A



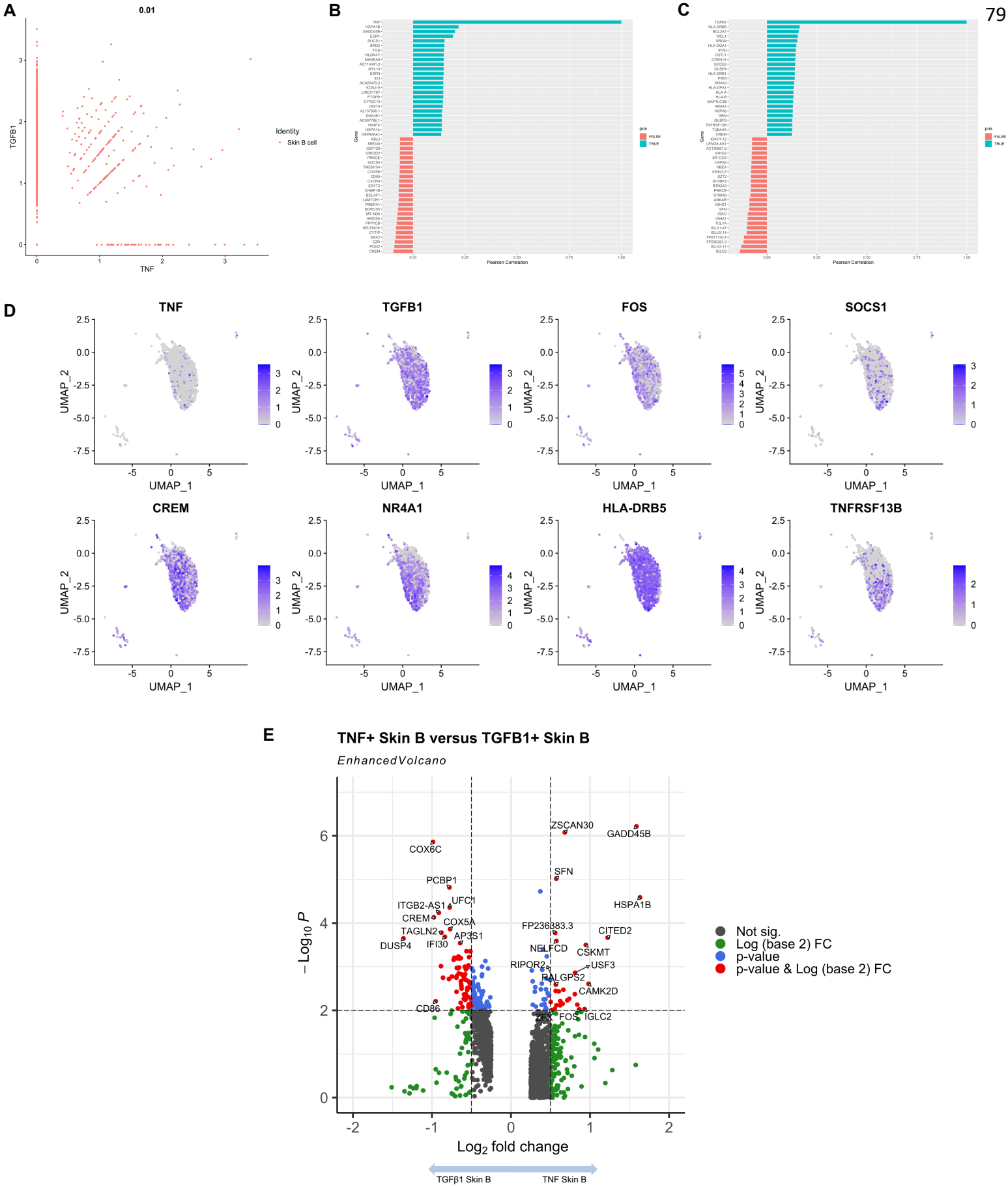
Supplemental Figure 4. Chemokine receptor and ligand expression. (A) Dot plots of chemokine receptor and ligand expression by cluster. Size of dot indicates percent of cells in cluster with expression, color intensity indicates average expression level.



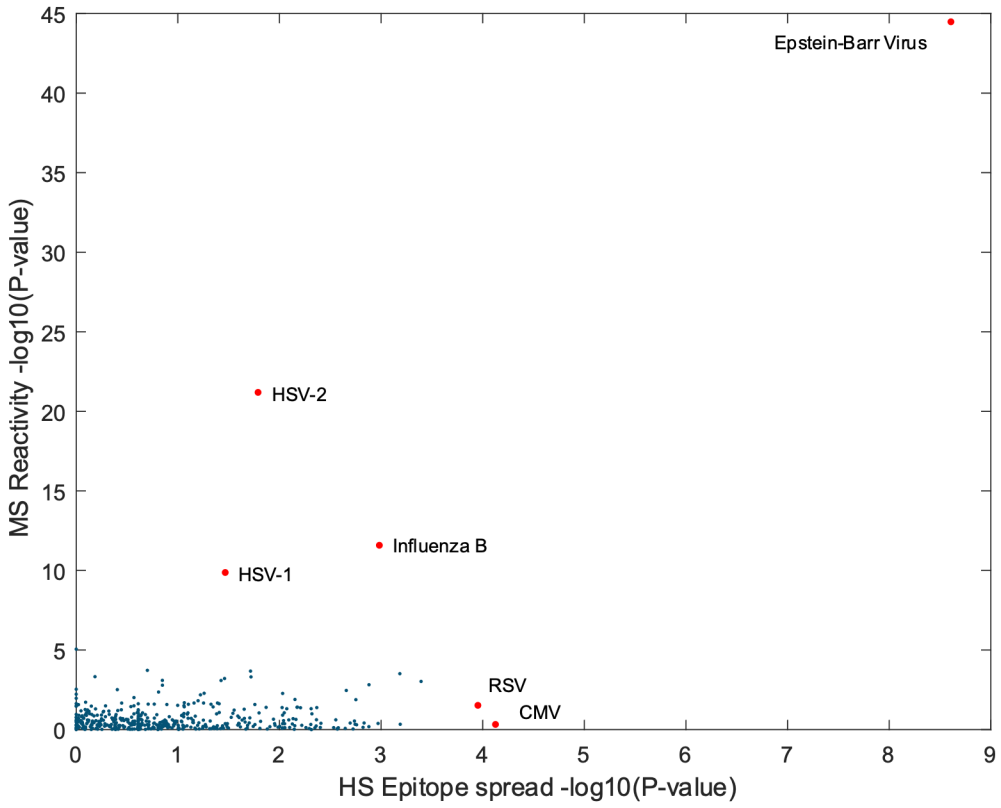
Supplemental Figure 5. RNA velocity estimation with UniTVelo. (A) Velocity stream and latent time analysis conducted with UniTVelo, an alternative to ScVelo, under the time unified or independent model. Each run was conducted with the Skin B population as the root cell cluster.



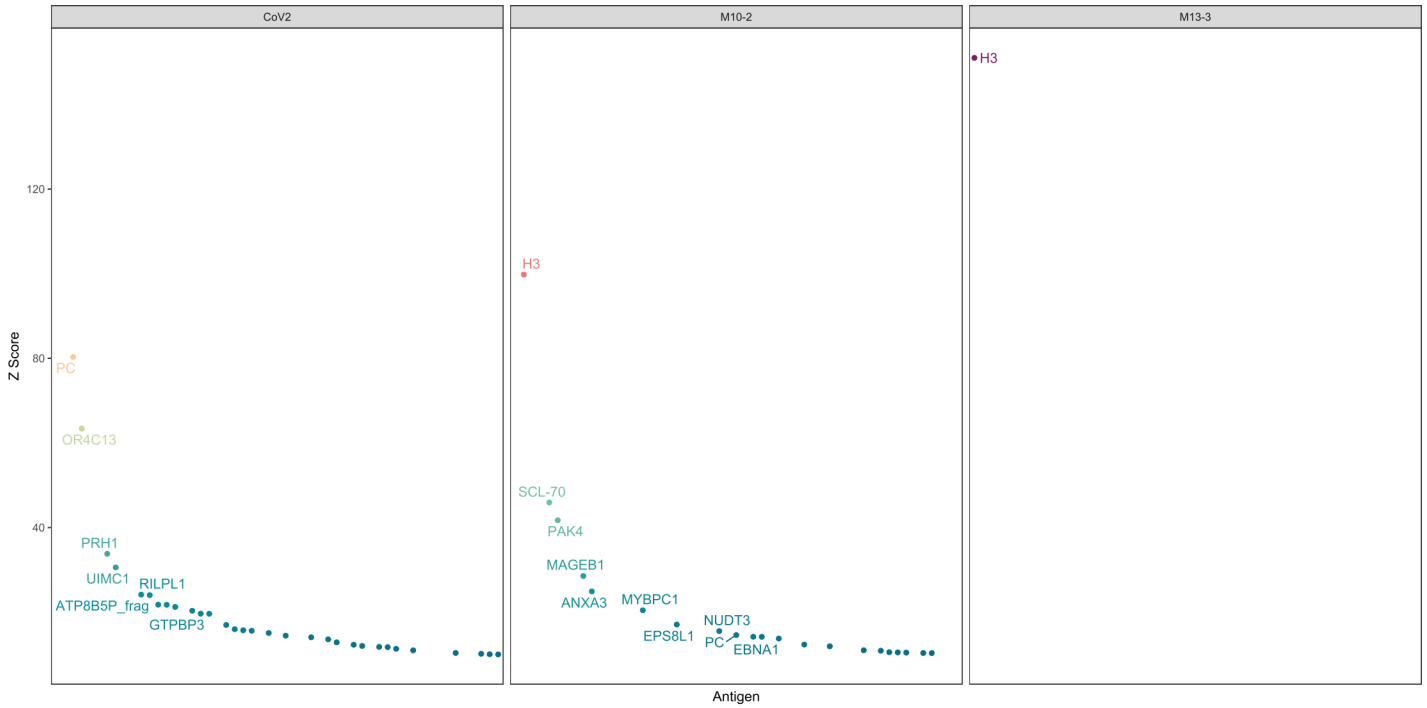
Supplemental Figure 6. Splice dynamics in RNA velocity analysis. (A) scVelo latent time applied to UMAP (left). Color corresponds to pseudotime progression (right). **(B)** Phase portraits of select transcription factors depicting spliced to unspliced mRNA ratios, colored according to clusters in A. Regression represents inferred stead-state ratio, top curve represents induction of gene, while bottom curve represents repression. **(C)** Quantification of spliced mRNA along latent time.



Supplemental Figure 7. Comparison of TNFα+ and TGFβ1+ skin B cells (A) Correlation of TNFα- and TGFβ1-expressing cells. **(B)** Histogram depicting genes with positive correlation (blue) or negative correlation (red) with TNFα and **(C)** TGFβ1expression. **(D)** UMAP of skin B cell cluster colored according to gene expression level. **(E)** Volcano plot depicting differentially expressed genes between TNFα+ and TGFβ1+ skin B cells. Genes are plotted according to p-value and fold change on a log scale. Genes represented in red are above significance thresholds.



Supplemental Figure 8. Comparison of VirScan PhIP-seq reactivity of MS patients and HS epitope spread. HS epitope spread p-values were compared to the MS patient reactivity reported in Bjornveik et al. In both data sets, EBV emerged as highly significant as compared to other viral species.



Supplemental Figure 9. Control antibody reactivity in HuProt antigen screen. Three monoclonal antibodies were chosen to represent affinity-matured antibodies in the HuProt screen against >21,000 full length human antigens. Only data points with Z-scores greater than 10 are shown for each antibody. CoV2 is a SARS-CoV2 spike protein specific antibody, while M10-2 and M13-3 are both influenza H3 hemagglutinin specific antibodies.

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Chapter 3: Discussion

With this project, we endeavored to answer crucial questions regarding the B cell role in HS pathogenesis. We were able to shed light on the type of B cell that infiltrates during the chronic stage of disease, the regulatory factors that control them, their contribution to the cytokine milieu, and why they are stimulated to differentiate in the skin. Furthermore, we added to the mounting evidence of an autoimmune component to HS pathogenesis by evaluating the specificity of expanded clones from HS patients and finding cross-reactivity between EBV and autoantigens. We found that HS patients have high seropositivity to EBV, that many of them have detectable EBV DNA in the skin, and a repertoire exhibiting epitope spreading to EBV antigens. This opens further avenues of investigation regarding the role of EBV in HS and whether HS shares an etiology with other EBV-associated autoimmune diseases. Based on our findings and what is known regarding EBV-associated autoimmune disease etiology, we have developed a working model of the initiation and chronic phases of HS.

Initiation Phase

People who develop HS have a predisposition to EBV reactivation due to genetics, deficits in cellular immunity, or psychological factors. This predisposition is mild enough to remain sub-clinical and not develop into chronic active EBV disease (CAED). These individuals mount an immune response to EBV that might have waned if not for chronic reactivation. Repeated cycles of reactivation, immune response, and return to latency, result in continuous boosting and maturation of memory responses. Long-lived germinal centers become established in secondary lymphoid organs due to a steady

stream of EBV antigens entering circulation. This results in affinity maturation and epitope spreading of EBV-specific B cells. A diverse EBV-specific memory B cell repertoire is established, as is a circulating pool of anti-EBV antibodies. Some of this EBV-specific memory is autoreactive due to molecular mimicry between EBV protein EBNA1, and human proteins SCL-70 and PAK4. These are nuclear antigens that are generally sequestered from the immune system, so they may not become the source of autoimmune pathology until a triggering event occurs.

Skin injury due to friction, bacterial infection, or dysregulation of hair follicle keratinocytes causes localized inflammation that leads to B cell infiltration. EBV-transformed memory B cells infiltrate the skin and the combination of inflammatory microenvironment, TLR activation, and EBV mechanisms, stimulates *in situ* plasma cell differentiation, leading to EBV lytic replication and potential infection of epithelial cells. Previously established, robust anti-EBV memory traffics to the skin in response to active infection. Local activation of anti-EBV memory causes bystander tissue damage that exposes the nuclear antigens of cells in the skin. Cross-reactive memory B cells to EBNA1, SCL-70, and PAK4 infiltrate and the presence of their cognate antigens anchors an autoimmune response to the skin.

Chronic Phase

Reactivation of localized EBV, clonal expansion of cross-reactive B cells, and consequential inflammation, lead to chronic tissue damage at the skin. This floods the lymphatic system with skin autoantigens that would otherwise have remained sequestered, along with inflammatory and localization signals. This leads to germinal centers, affinity maturation, and an autoreactive memory B cell pool. Over time, this

pool will become increasingly diversified to a multitude of skin-specific and ubiquitous antigens, including EMILIN1, MEPE, NRK, and COL1A1. Affinity to autoantigens may outpace affinity to EBV at this stage. The chronic phase of disease can now proceed independently of EBV, but its reactivation may be a driver of symptom remission and relapse.

While much of this model is conjecture, it seems that the optimal conditions for the emergence of HS would be a) chronic EBV reactivation leading to epitope spreading, b) skin damage via injury or local EBV infection, c) emergence of affinity matured cross-reactivity via epitope spreading or EBV-boosted preexisting autoimmunity.

I am optimistic that future research will continue to lead to advancements in therapeutic strategies for HS. Our work indicates that avenues for exploration could be therapeutics already approved for MS, SLE, and RA that target B cell activation and infiltration. These are all well-characterized EBV-associated diseases that have many similarities with HS. BTK inhibitors have shown promise in clinical trials for MS, as have BAFF inhibitors for SLE. Furthermore, our findings indicate that co-treatment with antivirals may reduce instances of symptom flares.

Other helpful avenues for research and development are EBV vaccines and HS animal models. While most people acquire EBV, the majority develop disease. However, enough evidence exists of an association between EBV and several autoimmune diseases and cancers, that efforts to develop vaccine candidates should be increased and well-supported. Careful consideration must be put into candidates so as to not bolster naturally occurring cross-reactive autoimmunity. Finally, the development of animal

models for SLE and MS have been a key factor in characterization and therapeutic development for these diseases. An animal model of HS would be a boon to the field.