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Immunomodulatory receptors are differentially expressed in B and T cell subsets relevant to autoimmune disease

Katherine A. Murphy[a], Kartik Bhamidipati[a], Samuel J.S. Rubin[a], Lucas Kipp[b], William H. Robinson[a,*], Tobias V. Lanz[a,c,*]

[a] Division of Immunology and Rheumatology, Department of Medicine, Stanford University School of Medicine, Stanford, CA, United States
[b] Division of Neuroimmunology, Department of Neurology and Neurological Sciences, Stanford University School of Medicine, Stanford, CA, United States
[c] Department of Neurology, Medical Faculty Mannheim, University of Heidelberg, Mannheim, Germany

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ABSTRACT

Inhibitory cell-surface receptors on lymphocytes, often called immune checkpoints, are powerful targets for cancer therapy. Despite their direct involvement in autoimmune pathology, they are currently not exploited therapeutically for autoimmune diseases. Understanding the expression pattern of these receptors in health and disease is essential for targeted drug design. Here, we designed three 23-colour flow cytometry panels for peripheral-blood T cells, including 15 lineage-defining markers and 21 immunomodulatory cell-surface receptors, and a 22-marker panel for B cells. Blood samples from healthy individuals, multiple sclerosis (MS), and lupus (SLE) patients were included in the study. Several receptors show differential expression on regulatory T cells (Treg) compared to T helper (Th) 1 and Th17 cells, and functional relevance of this difference could be shown for BTLA and CD5. Unbiased multiparametric analysis revealed a subset of activated $CD8^+$ T cells and a subset of unswitched memory B cells that are diminished in MS and SLE, respectively.

1. Introduction

T cells are a major component of the adaptive immune system and the basis for immune memory. Cytotoxic $CD8^+$ T cells recognize intracellular pathogens and tumor antigens presented on major histocompatibility complex (MHC) class I and attack target cells with perforins, granzymes, and Fas ligand [1]. T helper (Th) cells express CD4, recognize extracellular antigens on MHC class II, and guide the immune response via cell-cell interactions and cytokines. Some Th cells drive the immune response by activating and attracting innate immune cells and inducing immunoglobulin class switching in B cells [2], while other Th subsets such as regulatory T cells (Treg) suppress excessive immune responses [3].

T cells mature in the thymus and then circulate in the periphery, where they encounter their cognate antigen and differentiate into effector and memory T cells. Naïve (T_{NAIVE}) and effector T cells (T_{EFF}) can be distinguished from memory T cells by the differential expression of CD45RA or CD45RO and CCR7. Memory T cells can be further subdivided into $CCR7^-$ effector memory T cells (T_{EM}), which traffic to inflamed tissue and elicit immediate effector function, and $CCR7^+$ central memory T cells (T_{CM}), which primarily reside in secondary lymphatic organs, do not display effector function per se, but proliferate and differentiate quickly into effector T cells upon antigenic stimulation [4,5].

$CD4^+$ effector T cells differentiate into well-defined lineages depending on the antigen recognized and co-stimulatory factors present during T cell receptor (TCR) activation. Th1 cells are involved in defense against intracellular pathogens (key cytokine: IFNγ, key transcription factor: T-bet, surface markers: $CXCR3^+$, $CCR4^-$) [6,7]. Th2 cells drive immune responses against extracellular parasites and allergic reactions (key cytokines: IL-4, IL-13, key transcription factor: GATA3, surface markers: $CXCR3^-$, $CCR4^+$) [6–8]. Th17 cells defend against

Abbreviations: MS, Multiple sclerosis; RRMS, Relapsing-remitting MS; CIS, Clinically isolated syndrome; SLE, Systemic lupus erythematosus; Th, T helper cell; Treg, Regulatory T cell; Tfh, T follicular helper cell; Tph, T peripheral helper cell; T_{NAIVE}, Naïve T cell; T_{EFF}, Effector T cells; T_{CM}, Central memory T cell; T_{EM}, T effector memory cell; MHC, Major histocompatibility complex; CSF, Cerebrospinal fluid; TCR, T cell receptor; APC, Antigen presenting cell; ITAM, Immunoreceptor tyrosine-based activation motif; ITIM, Immunoreceptor tyrosine-based inhibitory motif; BCR, B cell receptor; Citrus, Cluster identification, characterization, and regression; B_{SM}, Switched memory B cells; PB, Plasmablasts; PC, Plasma cell; DN, Double negative B cell; CNS, Central nervous system

* Corresponding authors at: Division of Immunology and Rheumatology, Department of Medicine, Stanford University School of Medicine, 269 Campus Drive, CCSR, Room 2240, Stanford, CA 94305, United States.
E-mail addresses: w.robinson@stanford.edu (W.H. Robinson), tlanz@stanford.edu (T.V. Lanz).

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extracellular pathogens by attracting neutrophils and macrophages and have ambiguous roles in tumor immunology (key cytokines: IL-17, key transcription factor: RORγt, surface markers: $CCR6^+$, $CD161^+$) [7,9,10]. T follicular helper cells (Tfh) aid in B cell germinal center formation, and promote B cell proliferation, class switching, and survival in the germinal center (key cytokines: IL-21, IL-6, key transcription factor: Bcl6, surface marker: $CXCR5^+$) [11]. T peripheral helper cells (Tph) were recently described in rheumatoid arthritis patients and are believed to provide similar help to B cells in chronically inflamed tissue [12]. Regulatory T cells (Treg) attenuate immune responses and prevent autoimmunity (key cytokines: IL-10, TGFβ, key transcription factor: FOXP3, surface markers: $CD25^+$, $CD127^-$) [3,7,13].

Th1, Th17, Tfh, and Treg as well as B cells have been implicated in the pathogenesis of autoimmune diseases, including MS and SLE. Elevated Th17 cell counts as well as Th17 cytokine levels have been reported in peripheral blood of MS and SLE patients and correlated with disease severity [14–19]. In MS patients there is an abundance of the Th17 population in the cerebrospinal fluid (CSF) [20]. Th17 cells can be detected in active MS lesions and kidney biopsies of patients with lupus nephritis [21,22]. While some earlier reports found elevated Th1 cytokines in SLE, more recent studies have emphasized the role of Th17 cells over Th1 [18,19,23]. Both Th1 and Th17 responses seem to drive MS progression, and the Th immune response in an individual patient can be dominated by either lineage. A skewed Th1 or Th17 response has been correlated with disease progression and response to IFN-β treatment [24,25]. In mouse models of both diseases, Th17 cells accumulate in inflamed tissue and Th17 cytokines aggravate the disease [26]. Adoptive transfer of autoreactive Th1 or Th17 cells can induce the mouse model of MS in naïve mice, but with differing respective pathological characteristics [25,27].

Treg deficiency and dysfunction have been implicated in multiple autoimmune diseases. While there is some controversy about decreased Treg counts in SLE and MS patients [16,28–30], multiple reports suggest that Tregs are dysfunctional in both diseases [31–33]. Increased levels of IL-10, a key cytokine produced by Tregs, are frequently detected in SLE patients and likely contribute to pathogenesis by promoting B cell differentiation [34,35].

Tfh are crucial for germinal center formation and B cell activation, differentiation, and proliferation [11]. Elevated Tfh frequencies have been reported in the blood and in inflammatory lesions in the central nervous system (CNS) of MS patients [36,37]. B cells contribute to autoimmune pathology by secreting autoantibodies but also by presenting antigens and stimulating Th cells via cell-cell interactions and cytokines. In particular, class-switched memory B cells and plasmablasts (PB) have been shown to traffic to sites of inflammation [38]. PB and plasma cells (PC) are also the main sources of autoantibodies in SLE. The downregulation of the inhibitory Fcγ-receptor CD32b has been detected on B cells of SLE patients and is thought to enable the generation of autoreactive PC from the germinal center [39]. Treatment with B cell depleting anti-CD20 antibodies has been shown to be successful in MS patients [40], whereas clinical trials in SLE failed despite the undeniable involvement of B cells in the pathologic process [41]. PB/PC subsets do not express CD20 and are therefore not directly targeted by anti-CD20 antibody therapies. A more selective approach targeting PB/PC directly might be more promising for treatment of SLE patients.

T cell activation requires stimulation via the TCR by antigen-MHC class I or II complex on the surface of antigen presenting cells (APC). Concomitantly, the APC provides co-stimulatory signals to the T cell such as CD80/86, which binds to CD28 and is essential for T cell activation. Ligation of both receptors triggers a tyrosine phosphorylation cascade, which starts with the phosphorylation of ZAP70 at immunoreceptor tyrosine-based activation motifs (ITAM) of the intracellular domain of CD3ζ in the TCR complex. Ultimately, nuclear translocations of key transcription factors such as AP1, NFAT, and NF-KB are triggered and result in activation, proliferation, migration, and effector function of T cells (for a comprehensive review see [42]). The signaling cascade of the B cell receptor (BCR) follows pathways similar to the TCR signaling pathway.

T cells and B cells express several co-receptors that can enhance or suppress the cellular response upon activation. Several inhibitory co-receptors share immunoreceptor tyrosine-based inhibitory motifs (ITIM) motifs (e.g. PD-1, TIGIT, BTLA, CD32b, and CD22), which attract the tyrosine phosphatases SHP1 and SHP2 that interrupt the TCR or BCR signaling cascade. Antibodies that block inhibitory receptors with and without ITIM motifs, e.g. PD-1 and CTLA-4 have been used successfully in cancer therapy [43]. In contrast, there is currently no approved drug for autoimmune or autoinflammatory disorders that exploits the suppressive function of these receptors, despite their direct involvement in autoimmune pathology. Expression of inhibitory receptors is tightly regulated and restricted to certain lymphocyte subsets. Targeting of specific pathogenic subsets might be achieved by selecting the right set of receptors. Here, we describe the expression of functional activating and inhibitory receptors on T and B cells in detail and compare healthy individuals to MS and SLE patients.

2. Material and methods

2.1. Patient characteristics

Female SLE and MS patients consented and participated voluntarily in the study. All SLE patients were diagnosed according to the diagnostic criteria established by the American College of Rheumatology, tested positive for anti-nuclear antibodies (ANA) and presented with SLEDAI scores ≥ 4. All MS patients met the 2017 McDonald criteria for relapsing-remitting MS (RRMS) [44] and blood draws were performed at clinical onset of disease before starting treatment. All MS patients were followed up for at least 6 months to confirm RRMS diagnosis (see Table 3 for patient characteristics). Blood samples from healthy female individuals were provided by the Stanford Blood Center. The use of human samples was approved by the Stanford Institutional Review Board.

2.2. Isolation and immunostaining of human PBMCs

Blood was collected into venous blood collection tubes with sodium heparin (BD). PMBCs were isolated by Ficoll-Paque (GE Healthcare) density gradient centrifugation. Cells were cryopreserved in stocks of 10 million cells per milliliter in Recovery™ Cell Culture Freezing Medium (Invitrogen) and stored in liquid nitrogen. Prior to immunostaining, cells were thawed in a 37 °C water bath and washed once in complete media consisting of RPMI supplemented with 10% FBS. Samples were treated with DNAse I and 2.5 mM MgCl for 10 min at room temperature. Cells were washed once with staining buffer (PBS containing 2% FBS). Staining was performed on ice in a 96-well round bottom plate (Corning). PBMCs from each subject were stained with four antibody panels (Supplementary Table 1). Monoclonal antibodies are listed in Supplementary Table 2. 500,000 cells were incubated with 100 µL antibody cocktail for 30 min. Cells were washed twice in staining buffer and resuspended in staining buffer with 1:10,000 LIVE/DEAD Fixable Violet (ThermoFisher).

2.3. In vitro ADCC cell depletion assay

Freshly isolated PMBCs from healthy donors were resuspended in RPMI supplemented with 10% FBS at 2 million cells per mL. Azide and endotoxin free anti-BTLA (Clone MIH26, BioLegend), anti-CD5 (Clone L17F12, BioLegend), and mouse IgG2a isotype (Clone MG2a-53, BioLegend) antibodies were added at 0.1 µg/mL and incubated at 37 °C for 24 h.

2.4. Flow cytometry

Flow cytometry was performed using an Aurora flow cytometer (Cytek, Fremont CA). The instrument was configured with 4 lasers (405 nm, 488 nm, 561 nm, 640 nm) and 48 detectors. The 48 detectors are spread out across the entirety of the emission spectrum, which facilitates deconvolution of fluorescent signals and allows for simultaneous use of dyes that are incompatible on a traditional flow cytometer with a single detector, e.g. APC and Alexa Fluor 647. Dyes were selected with distinct spectral properties to allow for accurate marker detection (Supplementary Tables 1 and 2). A total of 21 cell surface receptors were included in three T cell panels in addition to 15 lineage markers that were the same across all T cell panels. The B cell panel contained 22 lineage and functional markers (Supplementary Table 1). Calibration was performed with SpectroFlo QC beads (Cytek) and compensation was set using antibodies bound to anti-mouse Ig compensation beads (BD). Events were collected at < 5000 events per second and 250,000 events were recorded for each sample. To exclude debris from the event count, the minimum forward scatter threshold was set to 100,000 units. Compensation was calculated using SpectroFlo software (Cytek) and data was exported in .fcs file format.

2.5. Data analysis

Data in .fcs format was imported to the Cytobank web application for analysis (cytobank.org). Live single cells were identified by forward and side scatter parameters and live/dead staining. B and T cells were identified by manual gating of $CD19^+$ and $CD3^+$ populations, respectively (Supplementary Fig. 1). B and T cell subsets were identified by manual gating using markers described in Table 1 (Supplementary Figs. 1 and 2). Gates were applied uniformly across subjects and were not adjusted to individuals. Mean fluorescence intensity (MFI) was calculated for each subset and exported in .csv format. Z scores were calculated by subtracting the MFI of the subset from the average MFI and dividing by the standard deviation. Heat maps were generated using the Morpheus web application (https://software.broadinstitute.org/morpheus).

Cluster analysis was performed using Citrus within the Cytobank web application. B cells were clustered using all markers except CD19 and T cells were clustered using all markers except CD3 with the nearest shrunken centroid (PAMR) association model. Clusters were characterized by abundances using equal event sampling. 10,000 T cells were sampled from each file and the minimum cluster size was set at 5%. 1500 B cells were sampled from each file to allow for equal representation of samples with limited B cell counts and the minimum cluster size was set at 2%.

2.6. Statistics

Statistical analysis was performed with GraphPad Prism version 8.0.2. Bar graphs depict averages and error bars indicate standard deviation. The Mann-Whitney test was used to compare groups without the assumption of a gaussian distribution. $n = 5$ for comparisons between disease groups, and $n = 15$ for comparisons between cell subsets. Bonferroni's correction for multiple comparisons was applied by multiplying the P value by the number of parameters. Statistical significance was defined as $P < .05$. Citrus utilizes linear regression models to report statistically significant differences.

3. Results and discussion

3.1. Selection of candidate receptors

Receptors that influence activation states of lymphocytes were identified in a broad literature search and included in our T and B cell panels (Table 2). Immunomodulatory receptors that signal through tyrosine phosphorylation of activating ITAMs and inhibitory ITIMs were preferentially selected as their signaling pathways are well characterized and therefore could be ideal therapeutic targets. Each receptor was classified as activating or inhibitory in order to facilitate interpretation of the results. While some receptors such as PD-1 are well characterized inhibitory receptors, others such as 2B4 have been reported to have both activating and inhibitory properties [45,46]. In these ambiguous cases, we used Gene Ontology (GO) terms and signaling domains reported in UniProt (UniProt.org) to assign a functional classification to receptors of interest.

3.2. Activating and inhibitory receptors are differentially expressed in Th cell subsets

Several $CD4^+$ T effector cell subsets such as Th1 and Th17 promote inflammation, while others such as Tregs suppress inflammation. Subset-specific differential expression of immunomodulatory receptors could potentially be exploited therapeutically to treat autoimmune conditions. To determine activating and inhibitory receptor expression across $CD4^+$ T cell subsets, each lineage was identified by manually gating on subset-defining surface markers (Table 1, Supplementary Figs. 1A and 2A) [5,7,12,47]. MFI for each marker was calculated and compared across subsets (Fig. 1A). As T cells in MS and SLE patients are chronically activated and dysregulated, we expected differences in

Table 1
Cell surface markers used to identify B and T cell populations.

Population	Abbreviation	Defining Surface Markers	References
Th1		$CD3^+$ $CD4^+$ $CXCR3^+$ $CCR4^-$	Kunicki et al. [7]
Th2		$CD3^+$ $CD4^+$ $CXCR3^-$ $CCR4^+$	Kunicki et al. [7]
Th17		$CD3^+$ $CD4^+$ $CD161^+$ $CCR6^+$	Kunicki et al. [7]
T follicular helper	Tfh	$CD3^+$ $CD4^+$ $CXCR5^+$	Rao et al. [12]
T peripheral helper	Tph	$CD3^+$ $CD4^+$ $PD1^{high}$ $CXCR5^-$	Kunicki et al. [7]
T regulatory	Treg	$CD3^+$ $CD4^+$ $CD25^{high}$ $CD127^{low}$	Kunicki et al. [7]
Central memory T cell	T_{CM}	$CD3^+$ CCR7 + $CD45RA^-$	Sallusto et al. [5]
Effector memory T cell	T_{EM}	$CD3^+$ $CCR7^-$ $CD45RA^-$	Sallusto et al. [5]
Naïve T cell	T_{NAIVE}	$CD3^+$ $CCR7^+$ $CD45RA^+$	Sallusto et al. [5]
T effector	T_{EFF}	$CD3^+$ $CCR7^-$ $CD45RA^+$	Sallusto et al. [5]
Naïve B cell		$CD19^+$ $CD27^-$ IgD^+	
Unswitched memory B cell	UM	$CD19^+$ $CD27^+$ IgD^+	
Switched memory B cell	SM	$CD19^+$ $CD27^+$ IgD^-	
Double negative B cell	DN	$CD19^+$ $CD27^-$ IgD^-	
$CD27^-$ IgD^- $CXCR5^{high}$		$CD19^+$ $CD27^-$ IgD^- $CXCR5^{high}$	
$CD27^-$ IgD^- $CXCR5^{low}$		$CD19^+$ $CD27^-$ IgD^- $CXCR5^{low}$	
Plasmablast	PB	$CD19^+$ $CD27^+$ IgD^- $CD38^{high}$	
Plasma cell	PC	$CD19^+$ $CD27^+$ IgD^- $CD38^{high}$ $CD138^{high}$	

Table 2
Protein family and function of T and B cell markers.

Marker	Family	Function	Signaling domains
CD161	C-type lectin	Regulation of immune response	ITIM
CD69	C-type lectin	T cell activation	
CCR4	G-protein coupled receptor 1	Lymphocyte trafficking	
CCR7	G-protein coupled receptor 1	Lymphocyte trafficking	
CCR6	G-protein coupled receptor 1	Lymphocyte trafficking	
CXCR5	G-protein coupled receptor 1	Lymphocyte trafficking	
CXCR3	G-protein coupled receptor 1	Lymphocyte trafficking	
CD127	Type 1 cytokine receptor	IL-7 receptor	
CD25	Type 1 cytokine receptor	IL-2 receptor	
4-1BB (CD137)	TNF receptor superfamily	Co-stimulatory	
CD30	TNF receptor superfamily	Anti-apoptosis	
CD357 (GITR)	TNF receptor superfamily	Co-stimulatory	
OX40	TNF receptor superfamily	Co-stimulatory	
CD40	TNF receptor superfamily	Induction of immunoglobulin secretion	
CD27	TNF receptor superfamily	Anti-apoptosis	
CD40L	Tumor necrosis factor	Co-stimulatory	
IgD	Immunoglobulin superfamily	Antigen binding	
IgM	Immunoglobulin superfamily	Antigen binding	
IgA	Immunoglobulin superfamily	Antigen binding	
CD2	Immunoglobulin superfamily	Co-stimulatory	
CD28	Immunoglobulin superfamily	Co-stimulatory	
TIM-1	Immunoglobulin superfamily	Co-stimulatory	
ICOS	Immunoglobulin superfamily	Co-stimulatory	
CD86	Immunoglobulin superfamily	Co-stimulatory	
ICOSL	Immunoglobulin superfamily	Co-stimulatory	
CD226	Immunoglobulin superfamily	Co-stimulatory	
CD3	Immunoglobulin superfamily	T cell receptor signaling	ITAM
CD4	Immunoglobulin superfamily	TCR Co-receptor, scaffold for LCK	
CD8	Immunoglobulin superfamily	TCR Co-receptor, scaffold for LCK	
PD1	Immunoglobulin superfamily	Negative regulator of T cell activation	ITIM, ITSM
LAG3	Immunoglobulin superfamily	Negative regulator of T cell activation	
TIM3	Immunoglobulin superfamily	Negative regulator of T cell activation	
TIGIT	Immunoglobulin superfamily	Negative regulator of T cell activation	ITIM
2B4 (CD244)	Immunoglobulin superfamily	Negative regulator of T cell activation	ITSM
CTLA-4	Immunoglobulin superfamily	Negative regulator of T cell activation	
VISTA	Immunoglobulin superfamily	Negative regulator of T cell activation	
BTLA	Immunoglobulin superfamily	Negative regulator of T cell activation	ITIM
CD19	Immunoglobulin superfamily	BCR co-receptor	
CD32	Immunoglobulin superfamily	Low affinity Fc receptor	ITIM
CD22	Immunoglobulin, SIGLEC	Regulation of B cell activation	ITIM
CD45RA	Protein-tyrosine phosphatase	Protein tyrosine phosphatase	
CD62L	Selectin/LECAM	Cell-cell adhesion	
CD49b	Integrin alpha chain	Cell adhesion	
CD5	SRCR superfamily	Negative regulator of T cell activation	
CD38	ADP-ribosyl cyclase	Enzymatic, NAD^+ to NADPH	
CD138	Syndecan proteoglycan	Plasma cell survival	
CD20	MS4A	Regulation of calcium flux	
HLA-DR	MHC II	Antigen presentation	
CD43	Leukosialin	Co-stimulatory	

Table 3
Patient characteristics.

Characteristics	Healthy controls	SLE patients	MS patients
Patients, n	5	5	5
Females, n	5/5	5/5	5/5
Median age in years (range)	39 (27–51)	32 (24–69)	32 (18–50)
Median disease duration in years (range)	–	5 (1–36)	0
Treatment at blood draw, n	–	4/5	none
Hydroxychloroquine, n	–	4/5	–
Mycophenolate mofetil, n	–	2/5	–
Median SLEDAI score (range)	–	4 (4–6)	–
Kidney involvement	–	2/5	–
ANA positive, n	–	5/5	–
Anti-dsDNA positive, n	–	3/5	–
CNS symptoms	–	–	5/5
optic neuritis	–	–	3/5
transverse myelitis	–	–	3/5
Oligoclonal bands positive, n	–	–	4/5
Median CSF cell count (range)	–	–	10 (1 − 11)

receptor expression in various T cell subsets across the three diseases. However, while striking differences in expression levels could be detected among T cell subsets, expression levels were surprisingly uniform among the three patient groups (Figs. 1A and 2A). Healthy controls, MS, and SLE subjects were therefore combined into one analysis. Several receptors were identified with higher expression in Th1 and/or Th17 populations compared to Tregs, including the inhibitory receptors PD-1, BTLA, CTLA-4, and CD5 and the activating receptors CD28, CD38, OX40, and CD2 (Fig. 1B). Our expression data is corroborated by publicly available RNA expression data, which shows higher CD5 and BTLA expression in Th1 and Th17 lineages compared to Treg cells (Supplementary Fig. 2C) [48]. To determine if differential surface marker expression on Th1, Th17 and Treg cells was biologically relevant, we tested whether anti-CD5 or anti-BTLA antibodies could selectively deplete Th1 or Th17 cells through antibody-dependent cell-mediated cytotoxicity (ADCC). We used mouse IgG2a antibodies, which have been shown to be potent inducers of ADCC by human PBMCs [49]. Freshly isolated PBMCs were incubated for 24 h with mouse IgG2a anti-CD5 and anti-BTLA antibodies and frequencies of $CD4^+$ subsets were assessed by flow cytometry. Treatment with anti-CD5 antibody

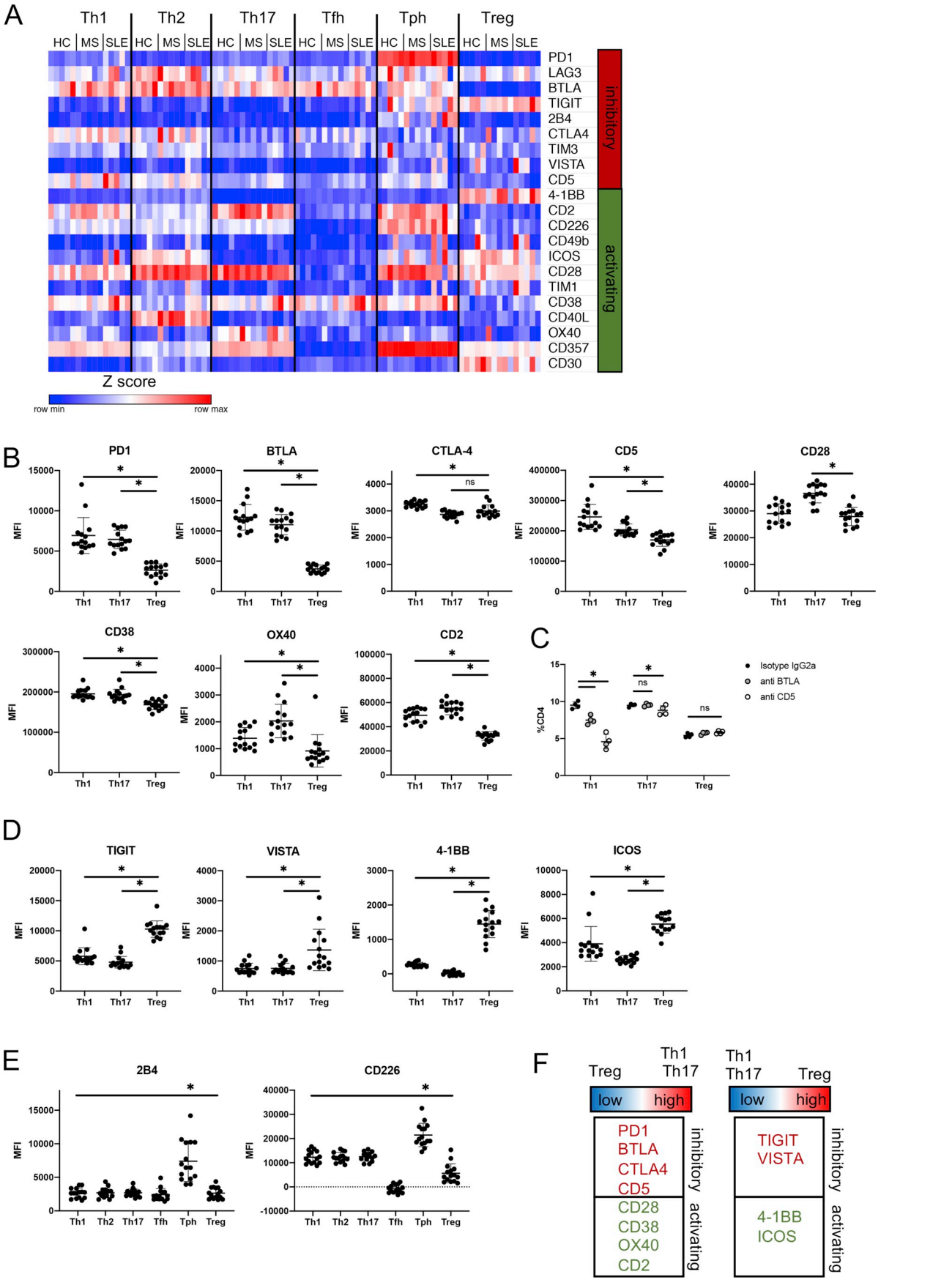

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Fig. 1. Differential expression of 21 activating and inhibitory receptors in CD4$^+$ T cell subsets. (A) Heat map depicting relative marker expression. Each column represents a patient. Green and red bars indicate functional classification of receptors. (B, D) MFI for each indicated marker in Th1, Th17, and Treg cells. Each dot represents a subject. Mann-Whitney *U* test was used to compare Treg to Th1 cells and Treg to Th17 cells ($n = 15$, $^*P < .0001$). (C) Frequencies of Th1, Th17, and Treg subsets as a percentage of total CD4$^+$ T cells following 24 h incubation with anti-BTLA, anti-CD5, or mouse IgG2a isotype control. Each dot depicts a replicate (2 donors, 2 technical replicates) ($n = 2$, $^*P < .05$). (E) MFI for each indicated marker for all investigated CD4$^+$ Th cell subsets. Mann-Whitney U test was used to compare Tph to Treg, Tfh, Th17, and Th1 subsets. (n = 15, $^*P < .05$). Bonferroni's correction was applied to adjust the *P* value for multiple comparisons by multiplying *P* by the number of parameters tested, which is 21. (F) Schematic depicting receptors that are elevated in Th1 or Th17 subsets (left panel) or elevated in Tregs (right panel). Red and green text indicates inhibitory and activating function respectively.

decreased the frequency of Th1 and Th17 cells, and treatment with anti-BTLA decreased the frequency of Th1 cells, while the frequency of Treg cells remained unchanged with both treatments (Fig. 1C). Of note, treatment with anti-CD5 depleted Th1 and Th17 cells more potently than anti-BTLA. We hypothesize that high expression of BTLA on B cells outcompetes antibody binding to T cells at low antibody concentrations. In contrast, CD5 expression is restricted to T cells (Supplementary Fig. 2C).

An inverse expression pattern was identifiedfor the inhibitory receptors VISTA and TIGIT and the activating receptors 4-1BB and ICOS, which are more highly expressed in Tregs and lower on Th1 and Th17 cells (Fig. 1D). This data is in line with previous reports [50–52]. The inhibitory receptor 2B4 and the activating receptor CD226 were selectively elevated in T peripheral helper cells (Fig. 1E).

3.3. Activating and inhibitory receptors are differentially expressed in effector and memory T cell subsets

Chronic inflammation is thought to be driven by T_{EFF} and long-lived T_{EM} cells, which produce cytokines and traffic to peripheral tissues [4]. In contrast, T_{CM} home to lymphoid tissues and exhibit little effector function but can be activated to differentiate into T_{EFF} cells. An ideal therapeutic for autoimmune conditions would target T_{EM} populations while sparing T_{CM} and naïve T cells, in order to maintain T cell mediated protection against pathogens. To determine differential expression of activating and inhibitory receptors across naive, effector, and memory T cell subsets, CD4$^+$ and CD8$^+$ memory populations were subclassified using the markers CD45RA and CCR7 (Supplementary Figs. 1A and 2B). MFIs for markers of interest were compared across subsets (Fig. 2A). While no receptors were identified with high expression exclusively in T_{EM}, several markers displayed biased expression towards T_{CM} and T_{EM} subsets. Specifically, inhibitory receptor PD-1 and activating receptors CD2, OX40, and CD28 were expressed at higher levels in T_{CM} and T_{EM} subsets compared to naïve and T_{EFF} populations (Fig. 2B). Expression levels of the activating receptors 4-1BB, ICOS, and CD40L and the inhibitory receptor VISTA were considerably higher on CD8$^+$ than on CD4$^+$ populations (Fig. 2C). Activating receptor CD38 expression was slightly biased towards T_{EFF} and T_{EM} subsets, while the inhibitory receptor CD5 displayed the opposite pattern of expression across subsets (Fig. 2D).

3.4. A subset of activated CD8$^+$ T_{EM} cells is diminished in MS patients

Expanded Th1 and Th17 populations have been described in autoimmune conditions. We therefore sought to determine the frequencies of these subsets in our cohort (Supplementary Fig. 4, Supplementary Table 3). We observed a non-significant trend towards increased Th17 as well as Tfh populations in MS and SLE. Four out of five MS but not SLE patients had increased frequencies of Th1 cells compared to healthy controls, however this difference did not reach statistical significance either. Expanded T peripheral helper cells have been associated with SLE disease activity [53]. Here we also observed a trend towards more Tph cells in SLE, as Tph frequencies were elevated in three out of five of our SLE but not MS patients (Supplementary Fig. 4, Supplementary Table 3).

To identify previously unknown T cell subsets with differential abundance in health and disease we used Citrus, a high-dimensional analytical method to perform unsupervised clustering of all CD3$^+$ T cells by 21 markers (excluding live/dead stain and CD3 from clustering). Despite the relatively low number of patients, it was possible to identify a CD8$^+$ T cell population that differed between healthy and MS patients. Our model had a false discovery rate below 1% and a cross validation error rate of 20%, indicating moderate predictive power (Supplementary Fig. 3A, left panel). In contrast, Citrus analysis of the T cell panels comparing healthy to SLE returned a model with false discovery rate above 20% and a 40% cross validation error rate (Supplementary Fig. 3A, right panel). The identified CD8$^+$ T cell population comprises a cell cluster located at the end of a hierarchical branch (Fig. 3A). The population is depleted in all five MS patients (Fig. 3B). Cells in this cluster are CD8$^+$, CD45RA$^-$, and CD62L$^-$, indicating an antigen-experienced memory T cell phenotype that is capable of egressing from the lymph nodes and trafficking to the periphery (Fig. 3C, D) [54]. Intriguingly, CXCR3 expression was increased in this cluster. CXCR3 is involved in CD8$^+$ T cell differentiation into T_{EFF} phenotype and is implicated in trafficking to peripheral sites of Th1-type inflammation [55,56]. Egress from the blood to inflammatory CNS lesions might explain a diminished population in the periphery of our MS patients. The cluster is also characterized by elevated expression of CD69, expressed by recently activated T cells. Expression of CD25, CD127 and CTLA4 does not align with a CD8$^+$ Treg phenotype [57–59], but the inhibitory receptors PD-1 and 2B4 were elevated in this cluster.

3.5. Expression of activating and inhibitory receptors varies across B cell subsets

Pathogenic anti-nuclear and anti-dsDNA autoantibodies are a hallmark of SLE [60]. However, clinical trials using B cell depleting antibodies directed against CD20 have not met primary endpoints in SLE patients [41]. PB/PC subsets are the main antibody secreting cells but express only very low levels of CD20 and are therefore not the primary target of anti-CD20 therapies. Depleting PB/PC via CD19 or pursuing more targeted approaches against PB/PC via functional immunomodulatory receptors hold promise in SLE.

In MS the pathogenic relevance of specific autoantibodies is controversial. However, intrathecal oligoclonal immunoglobulins (commonly referred to as oligoclonal bands) are detected in 95% of patients with clinically definite MS and are supportive of the diagnosis [44,61]. In addition, B cell depleting therapies have proven to be highly effective in MS and gained FDA approval in 2018 [62], which sparked the emergence of numerous B cell-targeted therapies including additional anti-CD20 antibodies (ofatumumab, ublituximab), an anti-CD19 antibody (inebilizumab), which might show superiority over anti-CD20 antibodies by including CD20^{low} PB and PC [63], and agents targeting the signaling pathways of the B cell growth factors including BAFF and APRIL [64]. Class-switched memory B cells and PB are prone to cross the blood brain barrier and traffic into the CNS [38], suggesting that these subsets are the main pathogenic B cell subsets in MS.

We were interested if immunomodulatory receptors were differentially expressed across B cell subsets, which could be leveraged for selective therapeutic targeting. Similar to our reasoning for T cells, antibody-producing PB and PC would be preferred target cells, while

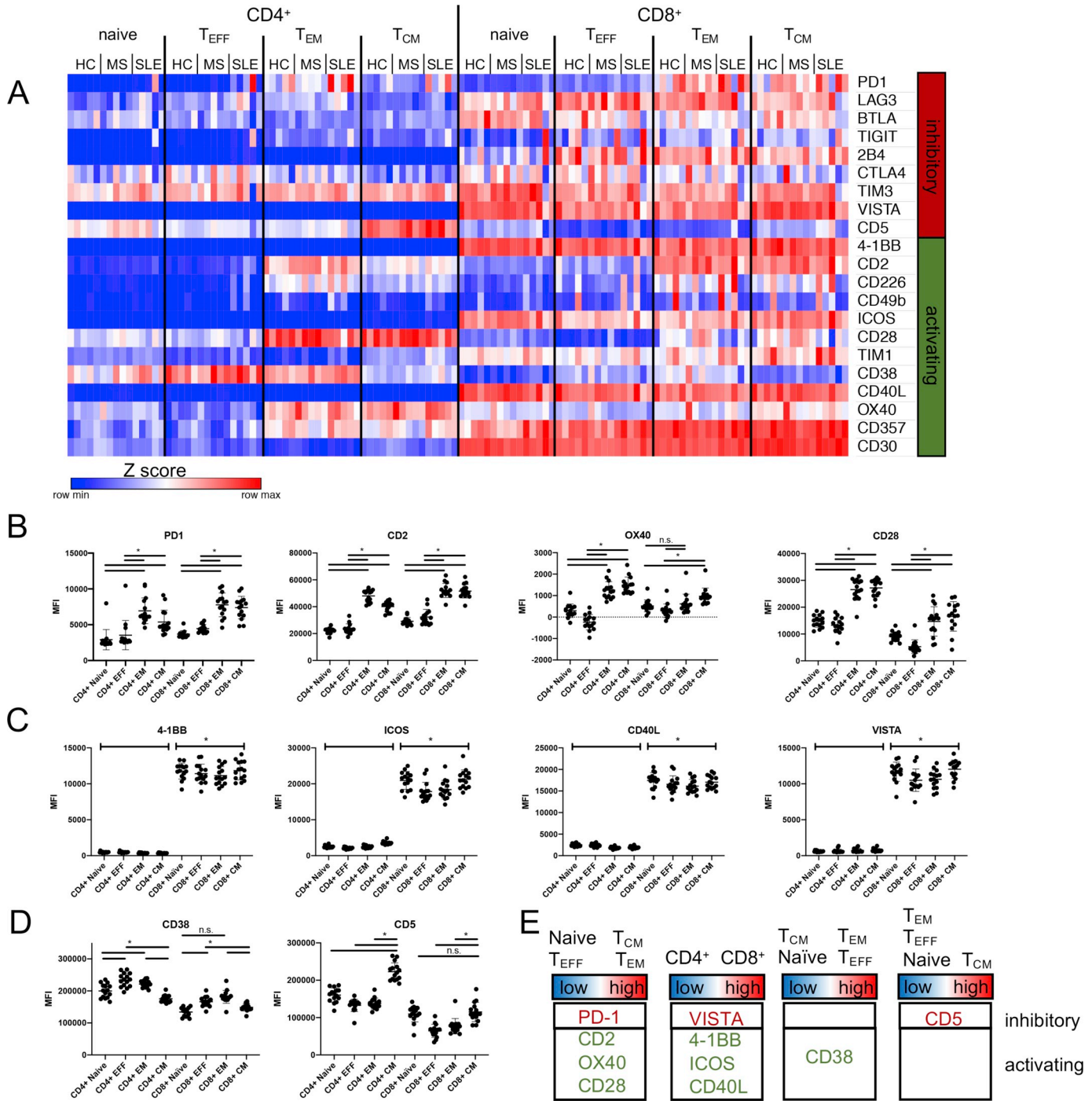


Fig. 2. Differential expression of 21 activating and inhibitory receptors in CD4$^+$and CD8$^+$$T_{NAIVE}$, T_{EM}, $T_{EFF}$and T_{CM}subsets. (A) Heat map depicting relative marker expression. Each column represents a patient. Green and red bars indicate functional classification of receptors. (B, C, D) MFI for each indicated marker in T_{NAIVE}, T_{CM}, T_{EFF} and T_{CM} subsets. Each dot represents a subject. Mann-Whitney U test was used to compare subsets and Bonferroni's correction was applied as in Fig. 1 (n = 15, *P < .05). All bars below the asterisk indicate a significant difference. (B) T_{NAIVE} was compared to T_{EM} and T_{CM} subsets and T_{EFF} was compared to T_{CM} and T_{EM} subsets. (C) MFI in CD4$^+$ subsets were averaged and compared to the average of the CD8$^+$ subsets. (D) CD38 expression in T_{NAIVE} was compared to T_{EFF} and T_{EM}, and T_{CM} was compared to T_{EFF} and $T_{EM.}$ CD5 expression in T_{CM} was compared to T_{NAIVE}, T_{EFF}, and T_{EM}. (E) Schematic depicting receptor expression bias across subsets. Red and green text indicates inhibitory and activating function respectively.

naïve and memory B cell subsets would ideally be spared to alleviate the detrimental effects of antibody secretion and T cell stimulation while maintaining a B cell pool that would replenish and maintain humoral immunity directed against pathogens. To determine receptor expression across B cell subsets, B cell populations were gated manually (Table 1, Fig. 4A, Supplementary Fig. 1B) and MFIs were compared. PB and PC share most of their lineage-defining cell surface markers (CD19$^+$ CD20^{low} CD27$^+$ CD38$^+$) and differed only in the expression of CD138, which is high on PC. As PC reside in the bone marrow and are scarce in our blood samples, both populations were combined into one PB/PC population for the analysis. CD27 and CD38 are used to define PB/PC subsets, hence their expression was high in this group. As both are also activating receptors, they need to be considered as candidates for targeting PB/PC, as are CD86 and CD43, the other two activating

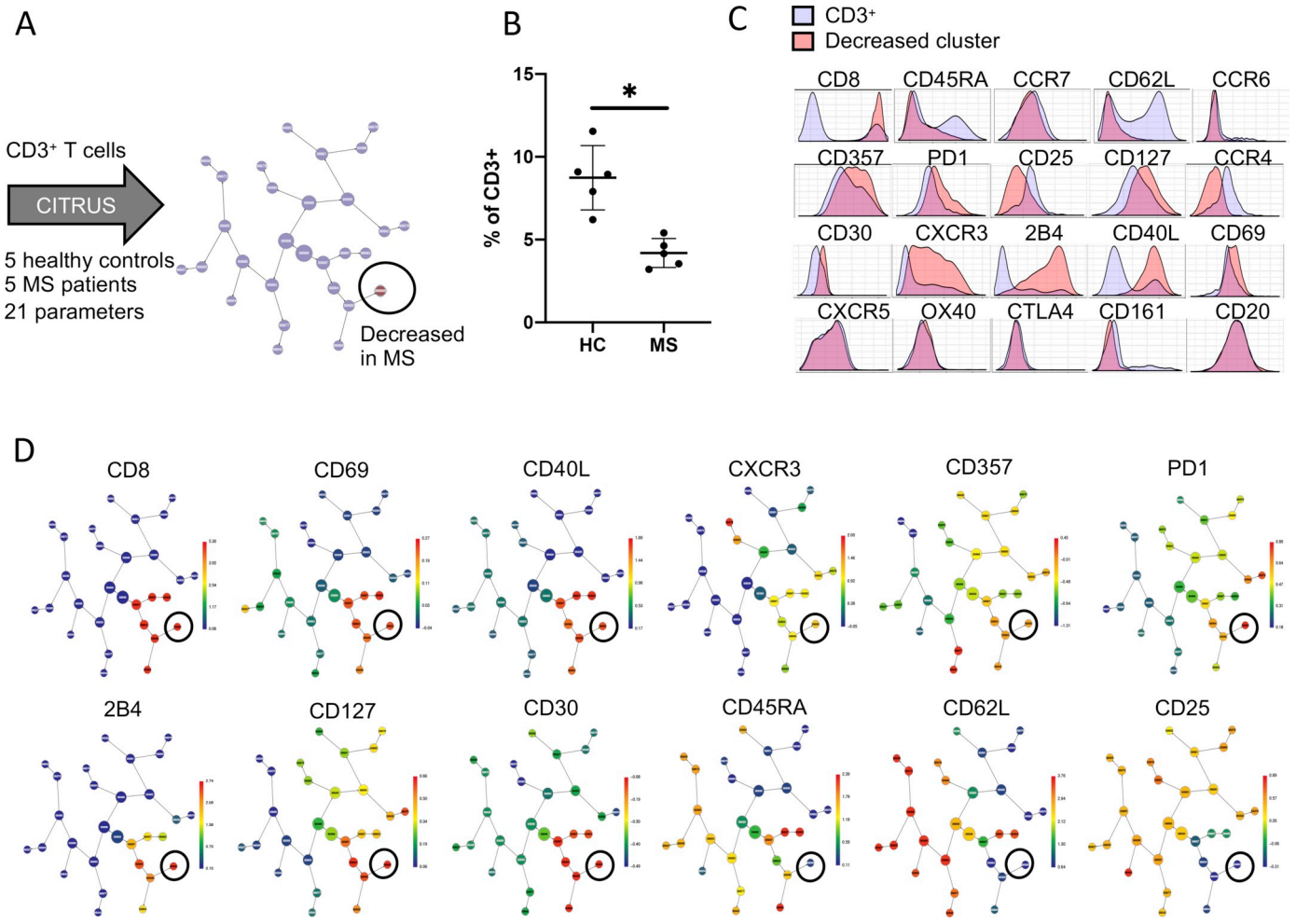


Fig. 3. Identification of a T cell population with decreased abundance in MS patients using Citrus. (A) Schematic depicting input for Citrus analysis. The hierarchical tree shows clusters of cells identified by Citrus. The cluster with differential abundance is circled. (B) Relative abundance of cells that fall within the indicated cluster out of total $CD3^+$ T cells. Each dot represents a subject. Statistical significance is defined according to the Citrus linear regression algorithm. (C) Histograms showing marker expression in the indicated cluster (red) compared to all $CD3^+$ T cells (blue). (D) Hierarchical tree as in A colored by relative marker MFI.

receptors that were highly expressed specifically in this population (Fig. 4B). Most of the other immunomodulatory receptors included in our panel, both activating and inhibitory, were downregulated in PB/PC.

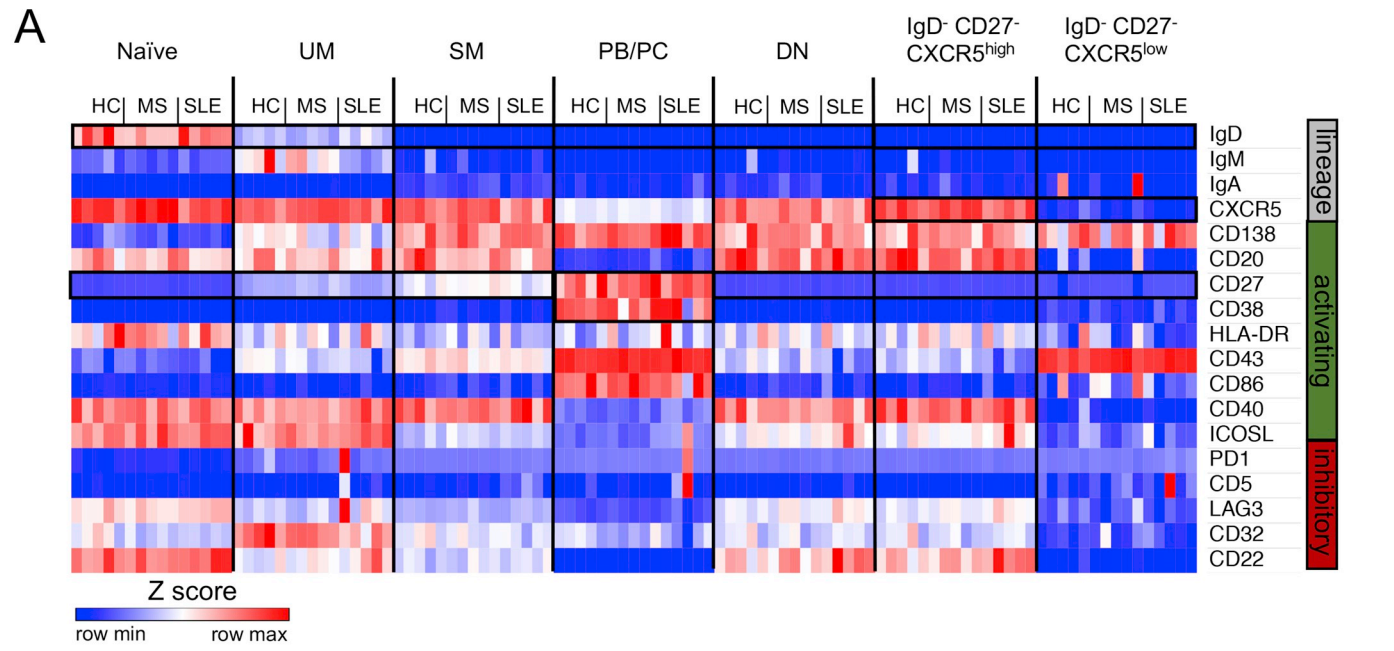
Double-negative (DN) B cells have been described as an exhausted and anergic subset in the context of infectious diseases, but recent reports suggest that they are more abundant in autoimmune disorders and promote inflammation [47,65]. In our study we could clearly subdivide the DN population by expression of CXCR5 into a $CD27^-$ IgD^- $CXCR5^{hi}$ and a phenotypically distinctive $CD27^-$ IgD^- $CXCR5^{low}$ population. Our observed $CD27^-$ IgD^- $CXCR5^{low}$ population differs from the DN2 population described by Jenks et al. [47], as these cells are $CD19^{dim}$ (Supplementary Fig. 1B). They are most likely CD27- pre-PC and their expression levels of immunomodulatory receptors are most closely related to the PB/PC population. Specifically, expression levels of CD20, CD40, ICOSL, LAG3, and CD22 align in PB/PC and $CD27^-$ IgD^- $CXCR5^{low}$ populations and differ significantly from expression levels in $CD27^-$ IgD^- $CXCR5^{hi}$ B cells (Fig. 4C).

3.6. Identification of a switched memory B cell subset that is decreased in SLE

Given our small patient cohort, we could not detect major changes in the frequency of manually gated B cell subsets among the three groups (Supplementary Fig. 4C). However, consistent with previous reports, there is a statistically non-significant decrease in the frequencies of switched and unswitched memory B cells in SLE patients [66].

Because aberrant B cell activation and expansion is implicated in MS and SLE, we hypothesized that examination of functional markers in B cells would identify B cell subsets with functional significance in these autoimmune diseases. To identify B cell subsets with differential abundance in diseased and healthy conditions, we utilized Citrus to perform unsupervised clustering as in Fig. 3, with a 2% minimum cluster size. Comparison of $CD19^+$ B cells between healthy individuals and MS patients produced a non-significant model with a false discovery rate above 60%, (Supplementary Fig. 3B, left panel). In contrast, comparison of healthy controls to SLE generated a model with a false discovery rate below 1% and a 28% cross validation error rate, indicating acceptable predictive power for this model.

The model identified 6 clusters that are smaller in SLE patients vs. healthy controls. Proximal clusters have parental relationships and encompass all distant clusters. Here, the difference in abundance is dependent on the 4 distal clusters, named 1–4 (Fig. 5A), which are suppressed in all 5 SLE patients (Fig. 5B). These 4 clusters describe an $CD27^+$ IgD^{low} IgM^{++} unswitched memory B cell population (Fig. 5C,D). Unswitched memory B cells have been described previously to be diminished in peripheral blood of SLE patients and lower



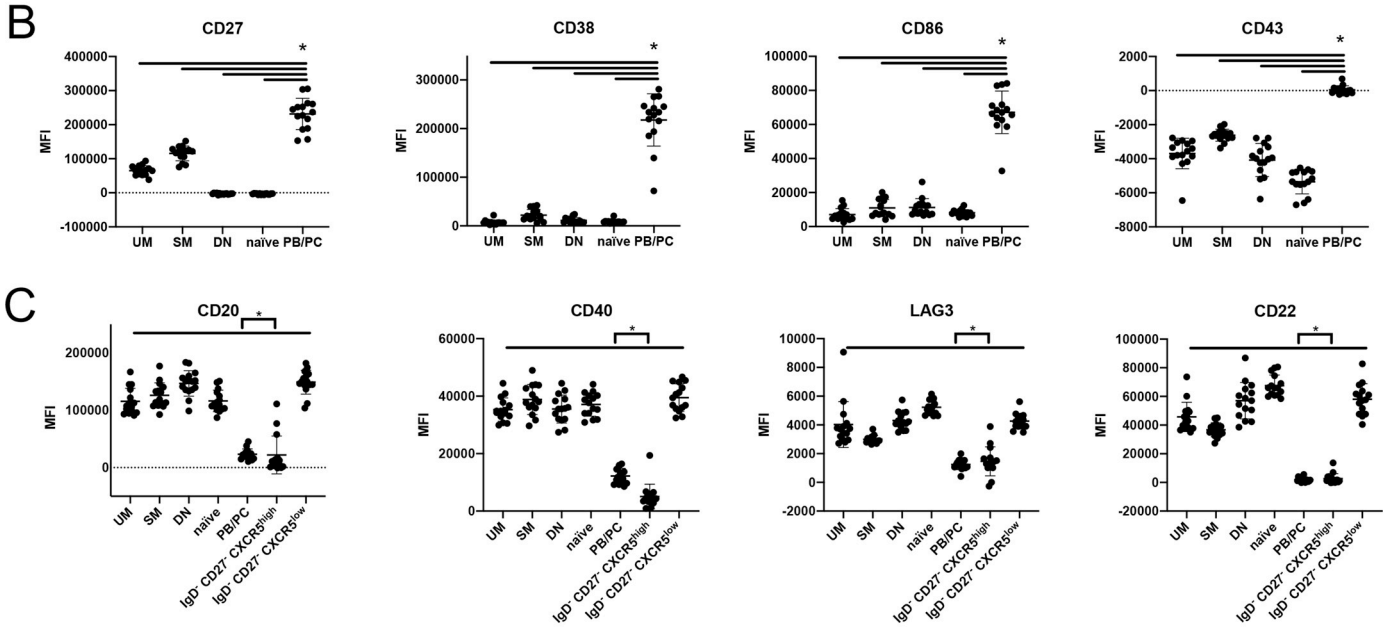


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Fig. 4. Differential expression of 13 activating and inhibitory receptors in B cell subsets. A) Heat map depicting relative marker expression. Each column represents a patient. Green and red bars indicate functional classification of receptors. Black boxes indicate markers used to gate each subset. (B, C) MFI for each indicated marker in B cell subsets. Each dot represents a subject. Mann-Whitney U test was used to compare subsets. (n = 15, $*P < .01$). Bonferroni's correction was applied to adjust the *P* value for multiple comparisons by multiplying *P* by the number of parameters tested, which was 13. (B) PB/PC was compared to UM, SM, DN, and naïve populations). (C) PB/PC was compared to UM, SM, DN, naïve, and IgD$^-$ CD27$^+$ CXCR5^{high} subsets. IgD$^-$ CD27$^+$ CXCR5^{low} was compared to IgD$^-$ CD27$^+$ CXCR5^{high}, UM, SM, DN, naïve subsets. Bracket indicates PB/PC and IgD$^-$ CD27$^+$ CXCR5^{low} populations are significantly different from UM, SM, DN, naïve, and IgD$^-$ CD27$^+$ CXCR5^{high} subsets. (D) Schematic depicting receptor expression bias across subsets. Red and green text indicates inhibitory and activating function, respectively.

unswitched memory B cell counts are associated with higher levels of ANA, anti-dsDNA and anti-ENA antibodies [67,68]. Interestingly, Citrus divides this population into 4 sub-populations based on diverging expression levels of IgA, CD138, ICOSL, and Lag3 (Fig. 5C,D). Higher expression of IgA and CD138 towards cluster 4 indicates that the higher-numbered clusters contain a more diverse population of cells, including those more advanced in their development towards switched memory B cells and PC. Interestingly, expression levels of the activating receptor ICOSL and the inhibitory receptor Lag3 appear to increase concurrently.

In contrast, population 5 describes a CD27$^-$ IgD$^+$ IgM$^+$ B cell population (Fig. 5A,C,D), which is increased in our cohort of SLE patients (Fig. 5B). Population 5 is a large population that includes 55–85% of B cells. The majority of population 5 are naïve B cells, but other smaller populations are likely included in this cluster as well, e.g. CD27$^-$ memory B cells and CD5$^+$ CD38^{int} pre-naïve B cells (cluster 13,314). Zhu et al. found naïve B cells to be increased in new-onset SLE patients, but not in chronic SLE patients [66]. Our patient cohort is too small to attribute much significance to this finding. The naïve B cell fraction, however, is subject of intense research, as abnormalities in B cell

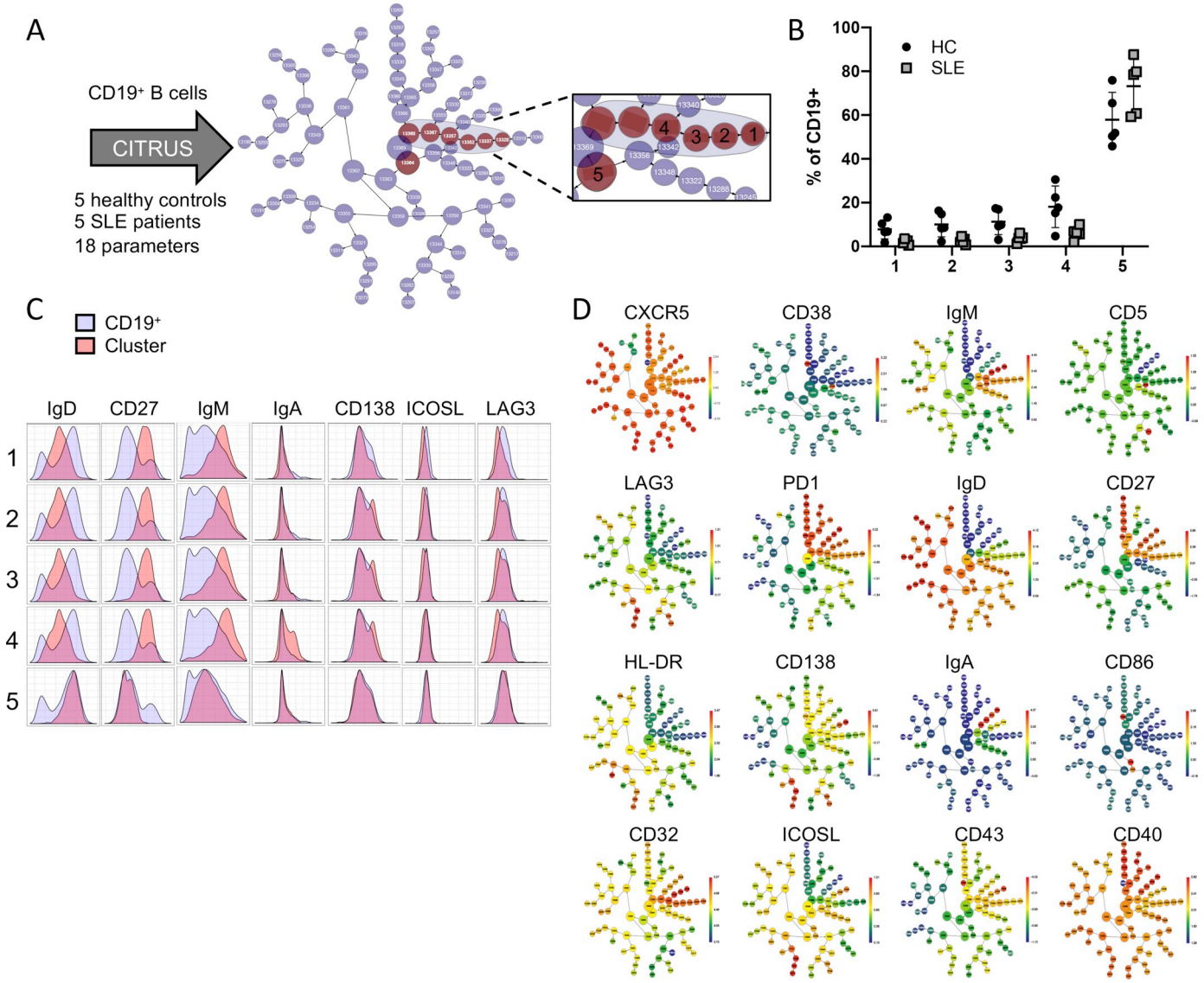

Fig. 5. Identification of B cell populations with differential abundances in SLE patients using Citrus. (A) Schematic depicting input for Citrus analysis. A hierarchical tree shows clusters of cells identified by Citrus. Clusters with statistically significant differential abundance are colored in red and clusters of interest are numbered 1 to 5. (B) Relative abundance of cells that fall within the indicated cluster out of CD19$^+$ B cells. Each dot represents a subject (circles: healthy controls, squares: SLE). Statistical significance is defined according to the Citrus linear regression algorithm. (C) Histograms showing marker expression in each cluster (red) compared to all CD19$^+$ B cells (blue). (D) Hierarchical tree as in A colored by relative marker MFI.

development contribute to SLE pathology. It has been shown that the fraction of naïve B cells that produce autoantibodies are 2–5-fold increased in SLE patients over healthy individuals [69]. Certain naïve subsets that are clearly elevated in SLE express more activation markers, escape peripheral anergy, and express autoreactive antibodies [70,71]. Fig. 3D adds to this research, as it points out that the naïve B cell pool is heterogeneous based on different expression levels of immunomodulatory receptors.

4. Conclusion

In this study we provide a detailed overview on the differential expression of immunomodulatory cell surface receptors on B and T cell subsets. We describe several activating and inhibitory receptors with high expression on Th1 and Th17 cells compared to low expression on regulatory T cells, which makes them ideal therapeutic targets in autoimmune diseases. Using BTLA and CD5 as examples, we demonstrate that differential receptor expression can be exploited to selectively deplete Th1 and Th17 cells while sparing Treg cells. Additionally, several immunomodulatory receptors were found with increased expression on T_{EM} cells compared to T_{NAIVE} and T_{EFF} cell subsets. On B cells we found four receptors that are exclusively elevated on PB/PC and we show that expression levels of multiple other immunomodulatory receptors correlate between PB/PC and IgD^- $CD27^-$ $CXCR5^{low}$ cells.

Interestingly, none of the investigated activating and inhibitory lymphocyte receptors showed remarkably diverging expression levels between healthy controls, MS, and SLE patients. We acknowledge that our limited sample size may have prevented detection of more granular differences. Nonetheless, our data suggests that the included immunomodulatory receptors are suitable for therapeutic utilization across disease boundaries.

We identified a subset of $CD8^+$ T cells that is decreased in MS patients compared to healthy controls. Cell surface marker expression of this subset does not suggest a $CD8^+$ Treg phenotype, but is consistent with an antigen-experienced memory T cell population that traffics to the periphery. The diminished occurrence in the blood of MS patients could therefore be due to egress to inflammatory CNS lesions. Additionally, we identified a population of unswitched memory B cells that is decreased in SLE patients compared to healthy controls. Given our limited sample size, larger studies are needed to verify the relevance of these cell populations and to determine their function.

The following are the supplementary data related to this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2019.108276.

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