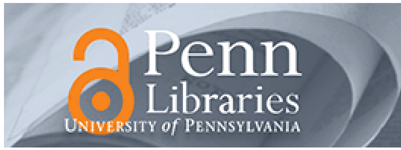

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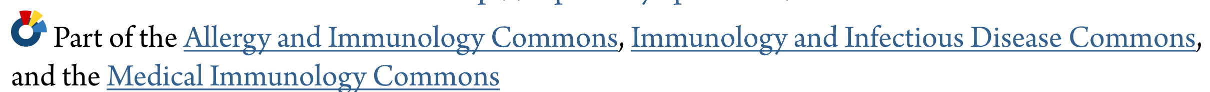
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Interplay of Il-4, Il-21, and Ifnγ on Memory B Cell Fate Decisions

Abstract
The ability to establish a durable pool of memory B (BMEM) cells is not only a key feature of adaptive immunity but also critical for host survival upon secondary infection. Depending on the nature of the pathogen, preimmune B cells differentiate into various BMEM cells associated with a particular immunoglobulin isotype. Moreover, cytokines dictate this process via the induction of transcription factors resulting in a stable lineage. Recently, the transcription factor, T-BET, has been implicated in reinforcing BMEM cells of the IgG2c isotype. Further, phenotypically similar cells express the integrin, CD11c, and appear in humoral autoimmunity and aged mice. However, the activation requisites and extrinsic cues driving T-BET and CD11c expression remain poorly defined. T follicular helper (TFH) cells instruct B cells to adopt various BMEM cell fates via the production of cytokines—specifically IL-4, IL-21 and IFNγ. Here we reveal a novel interplay among these cytokines in determining T-BET+ B cell fate. We find that IL-21 or IFNγ directly promote T-BET+ B cells in the context of TLR engagement. Further, IL-4 antagonizes IL-21-induced T-BET expression, but augments that of IFNγ. Finally, IL-21, but not IFNγ, promotes CD11c expression. Using well-defined infections that drive IL-21 and robust IFNγ or IL-4 production, we show that these same cytokine interactions function in vivo to determine T-BET and CD11c expression. We elaborate a model in which abundant IFNγ will drive T-BET+ B cells; however, in the absence of IFNγ, IL-21 and IL-4 reciprocally regulate both T-BET and CD11c. Importantly, CD11c expression is restricted to BMEM cells, which phenotypically resemble Age-associated B cells (ABCs). In accord with our infection results, we show that T-BET+CD11c+ ABCs are likely a pool BMEM cells. Consistent with this idea, ABCs are somatically mutated, class- switched, and require the ability to present antigen and receive costimulation to form. These findings suggest that T-BET+ B cells seen in health and autoimmunity share the common initiating features of TLR driven activation within this circumscribed cytokine milieu.

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INTERPLAY OF IL-4, IL-21, AND IFNγ ON MEMORY B CELL FATE DECISIONS

Martin Souren Naradikian

A DISSERTATION

in

Immunology

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy

2016

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DEDICATION

I dedicate my thesis to my family.

To my parents, Iordanka & Souren Naradikian, who taught me the value of unwavering perseverance in the face of incredibly difficult odds. I hope this work adequately memorializes their resolve in providing the best opportunities for my brother and me. Without their sacrifice by emigrating from Bulgaria, none of this would have been impossible.

To my brother, Markar Naradikian, who has always been there for me even though at times it results in permanent scars.

To my grandmother, Penka Naradikian, who as a lifelong primary school teacher always stressed the importance of education.

To the loved ones I lost during this journey. First, my grandmother, Mitra Stoyanova Shopova, passed away due to a hospital-acquired infection following surgery. Although her life as farmer in rural Bulgaria precluded access to the type of education and training I have received at Penn, she too always emphasized the importance of education above all else. Second, to Raymond Hill, who lost the fight against addiction. Although his life was tragically cut short, I am lucky to have known such a brilliant individual. I dedicate my thesis to you both in the hope that future scientific discoveries will prevent such loss.

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Lastly, to my mentor, Michael Paul Cancro, thank you for taking a chance on me when others doubted my abilities.

ABSTRACT

INTERPLAY OF IL-4, IL-21, AND IFNγ ON MEMORY B CELL FATE DECISIONS

MARTIN SOUREN NARADIKIAN

MICHAEL PAUL CANCRO

The ability to establish a durable pool of memory B (B_{MEM}) cells is not only a key feature of adaptive immunity but also critical for host survival upon secondary infection. Depending on the nature of the pathogen, preimmune B cells differentiate into various B_{MEM} cells associated with a particular immunoglobulin isotype. Moreover, cytokines dictate this process via the induction of transcription factors resulting in a stable lineage. Recently, the transcription factor, T-BET, has been implicated in reinforcing B_{MEM} cells of the IgG_{2c} isotype. Further, phenotypically similar cells express the integrin, CD11c, and appear in humoral autoimmunity and aged mice. However, the activation requisites and extrinsic cues driving T-BET and CD11c expression remain poorly defined. T follicular helper (T_{FH}) cells instruct B cells to adopt various B_{MEM} cell fates via the production of cytokines—specifically IL-4, IL-21 and IFNγ. Here we reveal a novel interplay among these cytokines in determining T-BET^{+} B cell fate. We find that IL-21 or IFNγ directly promote T-BET^{+} B cells in the context of TLR engagement. Further, IL-4 antagonizes IL-21-induced T-BET expression, but augments that of IFNγ. Finally, IL-21, but not IFNγ, promotes CD11c expression. Using well-defined infections that drive IL-21 and robust IFNγ or IL-4 production, we show that these same cytokine interactions function *in vivo* to determine T-

BET and CD11c expression. We elaborate a model in which abundant IFNγ will drive T-BET^{+} B cells; however, in the absence of IFNγ, IL-21 and IL-4 reciprocally regulate both T-BET and CD11c. Importantly, CD11c expression is restricted to B_{MEM} cells, which phenotypically resemble Age-associated B cells (ABCs). In accord with our infection results, we show that TBET^{+}CD11c^{+} ABCs are likely a pool B_{MEM} cells. Consistent with this idea, ABCs are somatically mutated, class-switched, and require the ability to present antigen and receive costimulation to form. These findings suggest that TBET^{+} B cells seen in health and autoimmunity share the common initiating features of TLR driven activation within this circumscribed cytokine milieu.

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LIST OF ABBREVIATIONS

(c)DNA	(complementary) Deoxyribonucleic Acid
(c)RNA	(complementary) Ribonucleic Acid
ABC	Age-associated B Cells
ADCC	Antibody-Dependent Cell mediated Cytotoxicity
AID	Activation-Induced Deaminase
APC	Antigen Presenting Cell
APRIL	A Proliferation Inducing Ligand
BAFF	B cell activating factor
BAFFR	BAFF Receptor
BCMA	B Cell Maturation Antigen
BCR	B Cell Receptor
CDR	Complementarity Determining Region
CFSE	Carboxyfluorescein Succynimmidyl Ester
CSR	Class-Wwitch Recombination
CTL	Cytotoxic T Lymphocyte
DC	Dendritic Cell
dsDNA	double-stranded DNA
EAE	Experimental Autoimmune Encephalomyelitis
FACS	Fluorescence Activated Cell Sorting
Fc	Fragment Crystallizable
FcR	Fragment Crystallizable Receptor
FcγR	Fragment Crystallizable gamma Receptor
FO	Follicular
GC	Germinal Center
HAU	Hemagglutination Units
HP	*Heligmosomoides polygyrus*
ICAM	Intercellular Adhesion Molecule
IFNγ	Interferon gamma
Ig	immunoglobulins
i.n.	Intranasal
IL	Interleukin
IMM	Immature
i.p.	Intraperitoneal
i.v.	Intravenous
JAK	Janus kinase
KO	Knockout
LLPC	Long-Lived Plasma Cell
LPS	Lipopolysaccharides
MACS	Magnetic Activated Cell Sorting
MHC	Major Histocompatibility Complex
MZ	Marginal Zone
MΦ	Macrophage
NP-OVA	4-Hydroxy-3-nitrophenyl acetyl-Ovalbumin

PAMP	Pathogen-Associated Molecular Patterns
PR8	Influenza strain A/PR/8/34
PRR	Pattern Recognition Receptor
SHM	Somatic Hypermutation
SLE	Systemic Lupus Erythematosus
SLPC	Short-Lived Plasma Cell
STAT	Signal Transducer and Activator of Transcription
T-BET	T-box Expressed in T cells
TACI	Transmembrane activator and cyclophilin interactor
$TCID_{50}$	50% Tissue Culture Infective Dose
TCR	T Cell Receptor
TD	Thymus-dependent
T_H	T Helper
TI	Thymus-independent
TLR	Toll Like Receptors
TNF	Tumour Necrosis Factor
TR	Transitional
VCAM	Vascular Cell Adhesion Molecule
VCT	Violet Cell Trace

1. OVERVIEW OF HUMORAL IMMUNITY

1.1 The innate and adaptive immune response

Protective immunity relies on establishing appropriate immune effector functions during primary responses, then sustaining these qualities in effector and memory cell pools. While appropriate effector functions promote lifelong immunity, aberrant effector choices can yield failed pathogen clearance, chronic inflammation, or autoimmunity. Current ideas suggest that cooperation between the innate and adaptive immune systems rids the vertebrate host of pathogens while maintaining tolerance to self. Indeed, cells in these two branches establish a complex network of activating and regulatory interactions that includes both cell-to-cell contacts and extracellular signals. Myeloid cells including DCs, eosinophils, basophils, neutrophils, MΦs, mast cells, comprise the innate system and are considered the rapid, first line of defense against tissue-invading pathogens. Further, myeloid cells express germline-encoded PRRs imparting recognition of and specificity for evolutionarily conserved PAMPs (1). Importantly, although PAMPs are typically associated with microbes, mammalian cellular products can also trigger PRRs. Regardless of the source of PAMPs, engagement with PRRs communicates activation signals. Once initiated, these responses foster differentiative programs that direct particular effector choices for the subsequent activation and regulation of the adaptive immune response (2, 3).

In contrast to innate lineages, two lymphoid lineages, T and B cells, are the primary mediators of adaptive immune responses. Each builds a unique antigen receptor (TCRs and BCRs, respectively) via a process of gene

rearrangement. In accord with the clonal selection paradigm (4), these somatically generated receptors are clonally distributed among lymphocytes such that each T and B cell expresses only one antigen receptor specificity. Moreover, receptor engagement beyond a requisite threshold affords specificity and triggers robust intracellular signaling cascades. Once activated, lymphocytes possess the unique capacity to generate long-term memory cells of related pathogen-reactive clones, which often impart protection against secondary infection. Importantly, lymphocytes accomplish their effector functions while also maintaining tolerance to the host. Thus, the tenets of the adaptive immune system–specificity, inducibility, memory, and tolerance—are key aspects of cell-mediated and humoral immunity as established by T and B cells, respectively.

In order to orchestrate cell-mediated immunity, T cells recognize antigenic peptides presented on MHC molecules via the TCR. The coreceptors, CD4 and CD8, define two functionally distinct subsets of T cells that engage antigen in the context of MHCII and MHCI molecules, respectively. CD8 T cells, or CTL, are responsible for lysing cells that present non-self peptides in the context of MHCI on their surface. Further, nearly every nucleated cell expresses MHCI molecules, which are loaded with peptide via the cytosolic pathway, thus allowing for broad surveillance of intracellular protein synthesis. Conversely, CD4 T cells, or helper T cells, generally orchestrate immune responses by recognizing non-self peptides in the context of MHCII on the surface of APCs such as DCs, B cells, or MΦs. Importantly, CD4 T cells monitor extracellular proteins since

MHCII expression is restricted to phagocytic cell types and peptide loading occurs via the endocytic pathway. Furthermore, helper T cells have the capacity to differentiate into several effector subsets: T_H1, T_H2, T_H17, T_{FH}, or T_{reg} (5-8). The cues guiding these cell fate decisions likely involve the nature of the pathogen, the PAMPs it carries, and how innate and adaptive cells integrate PRR signals. Further, master transcriptional regulators that reinforce particular fates frequently characterize these effector differentiation patterns. Thus, preimmune CD4 T helper cells adopt alternative effector fates based on TCR signal strength, costimulatory cues, and cytokine milieu (9-12).

Analogously, B cells can adopt a variety of distinct effector fates that are similarly guided by BCR signal strength coupled with additional cues that foster key transcriptional programs to establish humoral immunity. Importantly, B cells also express certain PRRs and respond to PAMPs. Coupled with the BCR, integration of these signals ultimately drives antibody production, antigen presentation and costimulatory interactions with CD4 T cells, and cytokine secretion. Principally among these functions, the Ig produced consists of identical sets of heavy and light chains joined together by disulfide bonds. Both chains combine to form the antigen binding or CDR, whereas only the heavy chain forms the Fc region which imparts antibody effector functions. Five main heavy chain isotypes, μ, δ, γ, ε, and α, combine with either a λ or κ light chain to make an IgM, IgD, IgG, IgE, or IgA respectively. Moreover, the IgG isotypes can be subdivided into IgG_1, $IgG_{2a/c}$ (IgG_{2a} for BALB/c and IgG_{2c} for C57BL/6, (13)), IgG_{2b}, or IgG_3. Similar to cell fate decisions in T helper cells, B cells adopt an

isotype based on the prevailing cytokine meilieu and subsequently induced master transcriptional regulator. The nature, levels, and persistence of Ig heavy chain isotypes that are provoked have profound implications for response quality. Indeed, nearly all heavy chain isotypes link adaptive humoral responses with a circumscribed group of innate inflammatory cells and systems important for pathogen clearance, such as complement fixation, opsonization, degranulation, and ADCC. These effector processes rely on isotype-specific Fc receptors that are differentially distributed among innate, myeloid, and other cell types. Since FcR can be either activating or inhibitory, Ig isotype engagement can either enhance or limit the activation and behavior of these cells (14). While marshaling these FcR effector systems is critical to effective humoral immunity, the same effector mechanisms mediate autoimmune and chronic inflammatory pathologies. Thus, the importance of these Ig:FcR interactions are apparent, yet to generate a protective antibody of the proper effector isotype requires strict regulation of B cell development, homeostasis, and activation.

1.2 B lymphocyte commitment, development, and homeostasis

B cells can be separated into two lineages: B-1 and B-2. Debate remains as to whether B-1 and B-2 cells derive from a common progenitor and diverge based on antigen-driven selection, or instead reflect the products of distinct, lineage-restricted progenitors (15-17). Regardless of their exact origins, each lineage plays distinct yet overlapping roles in humoral immunity, reflecting differences in their generation, antigen receptor diversity, and anatomic niche. Murine B-1 cells are derived primarily from the fetal liver, and are sustained

largely by self-renewal in the periphery (15, 16, 18, 19). In contrast, continuous B-2 cell production yields a much larger steady-state pool throughout life (20). B-2 cell subsets are defined by surface marker criteria that are correlated with developmental stage, activation status, recirculation properties, and anatomic locale. **Table 1** summarizes these criteria, features, and transcriptional regulators for developing and preimmune B cell subsets. In healthy adults, B cells are generated continuously from hematopoietic stem cells in the bone marrow (BM), where Ig heavy and light chain gene rearrangements are completed during the pro- and pre-B cell stages respectively (21) (reviewed in (22, 23)). Commitment to the B lineage involves both the acquisition of a B cell transcriptional program and the suppression of programs leading to other hematopoietic fates (24). Accordingly, adoption of B lineage fate involves the expression of PAX5 (25-27), EBF1 (28-31), and E2A proteins (32, 33), which establish a transcriptional circuit that not only induces and reinforces B lineage genes, but also represses transcription factors driving alternate lineages (34). Upon acquisition of surface IgM, developing B cells enter the IMM subset. These cells migrate to the periphery via the blood and pass through the TR developmental stages before entering the comparatively long-lived mature FO or MZ B cell pools (35-40). The cues required to adopt the MZ instead of FO B cell fate include tonic BCR signal strength and transcriptional programs driven by Notch2 (41) interactions with Delta-Like-1 (42, 43) reviewed in (44).

Stringent selection based on BCR specificity occurs during both the IMM and TR stages: only 10% of IMM cells produced survive to exit the marrow, and

Table I: Features of developing and preimmune B cell subsets

Locale	B cell subset	Surface phenotype	Transcription Factors	BLyS receptors	Survival cytokine
BM	**pro-B**	CD19^{+}, CD43^{+}, CD93^{+}, IgM^{-}, IgD^{-}	PAX5	none	IL7
	pre-B	CD19^{+}, CD43^{-}, CD93^{+}, IgM^{-}, IgD^{-}	PAX5	none	?
	IMM	CD19^{+}, CD93^{+}, IgM^{hi}, IgD$^{lo/-}$	PAX5	BR3/TACI	?
Spleen/ Blood	**TR**	CD19^{+}, CD93^{+}, CD43^{-}, IgM^{hi}, IgD$^{lo/-}$, CD21^{-}, CD23^{-}	PAX5	TACI/BR3	BLyS
Blood, lymph, spleen	**FO**	CD19^{+}, CD93^{-}, CD43^{-}, IgM^{lo}, IgD^{hi}, CD21^{lo}, CD23^{+}	PAX5	TACI/BR3	BLyS
	MZ	CD19^{+}, CD93^{-}, CD43^{-}, IgM^{hi}, IgD^{lo}, CD21^{+}, CD23^{lo}	Notch targets	TACI/BR3	BLyS
Lymph nodes, Spleen	**GC (DZ)**	CD19^{+}, IgM^{-}, IgD^{-}, PNA^{+}, CD95^{+}, CXCR4^{+}, CD83^{-}	BCL6	BR3	BLyS
	GC (LZ)	CD19^{+}, IgM^{-}, IgD^{-}, CD23^{+}, PNA^{+}, CD95^{+}, CXCR4^{-}, CD83^{+}	BCL6	BR3	BLyS
Spleen Blood, BM	**SLPC**	CD19^{+}, B220^{+}, IgM$^{+/-}$, IgD^{-}, CD138^{hi}	BLIMP1	TACI/ BCMA	APRIL? BLyS? IL6
	LLPC	CD19^{-}, B220^{-}, IgM^{-}, IgD^{-}, CD138^{hi}	BLIMP1	BCMA	APRIL?
	Sw Bmem	CD19^{+}, IgM^{-}, IgD^{-}, CD38^{+}, PDL2$^{+/-}$, CD73$^{+/-}$, CD80$^{+/-}$	?	BR3	?
	IgM Bmem	CD19^{+}, IgM^{+}, IgD^{-}, CD38^{+}, PDL2$^{+/-}$, CD73$^{+/-}$, CD80$^{+/-}$	?	BR3	?
Spleen. Blood, BM, Tissue	**ABC**	CD19^{+}, CD93^{-}, CD43^{-}, IgM$^{+/-}$, IgD$^{+/-}$, CD21^{-}, CD23^{-}, CD11c$^{+/-}$	T-BET	BR3/TACI	?

only 30% of the TR cells thus generated survive to join the FO or MZ pools (36). These profound cell losses reflect both negative and positive selection based on BCR signal strength (45-51), presumably reducing the frequency of polyreactive and self-reactive specificities (46, 48, 52), as well as selecting for cells with optimal sub-threshold signal strength (47, 51, 53, 54). Indeed, multiple studies now indicate that the representation of frankly autoreactive B cell specificities in each pool decreases at each successive differentiation stage (55, 56). Thus, among peripheral B cells, the TR pool is a rich source of autoreactive BCR specificities compared to most other B cell subsets (57).

The steady-state sizes of preimmune FO and MZ B cell pools are controlled by the TNF family member, B cell activating factor (BAFF) (58, 59) (reviewed in (60)). This cytokine sustains FO and MZ B cell survival by signals delivered through BAFF receptor (BAFFR) (61-65). All TR, FO, MZ B cells express BAFFR (66, 67) and compete continuously for BAFF to survive, such that available systemic BAFF determines their lifespan and thus overall FO and MZ pool sizes ((62, 63), reviewed in (68)). Because the steady-state size of pre-immune B cell pools is driven by the need for adequate surveillance and hence organismal size, the idea that BAFF governs this parameter predicts that total systemic BAFF must be relatively constant and that the predominant source must be tied to organism volume. Indeed, bone marrow radiation chimeras revealed that a radioresistant, non-hematopoietic sources of BAFF fully supported primary B cell homeostasis (69). More recent studies suggest that fibroblastic reticular cells are the primary source of system BAFF (70). These observations

strengthen the idea that systemic BAFF levels are a surrogate for biological space, since BAFF is produced by cells whose numbers will scale with organism size. Thus, BAFF depletion yields profoundly reduced TR, FO, and MZ B cell numbers, yet spares developing bone marrow B cell subsets. Further, the stringency of BCR-mediated selection among TR B cells is governed by BAFF; increasing BAFF lets TR B cells normally lost to negative selection survive (56, 71, 72). Together, these observations favored the notion that systemic BAFF governs pre-immune B cell numbers by controlling TR cell throughput and mature B cell survival, thereby acting as the molecular surrogate for homeostatic 'space' in the pre-immune B cell niche (68).

1.3 Thymus dependent and independent B cell activation

Among mature primary B cells, BCR ligation initiates downstream signaling systems that foster activation. The type of antigen, the participating B cell subset, the avidity of BCR cross linking, and the intercellular interactions involved dictate the characteristics of the subsequent humoral immune response. In general, B cell responses follow the two signal paradigm (73), whereby BCR ligation (signal one) must be followed by additional activation and differentiation cues (signal 2) that are delivered via other cells or molecules. Two broad categories of humoral responses are defined based on the source of second signals. The thymus-dependent (TD) response involves second signals that are delivered when B cells internalize, process, and present protein antigens to CD4 helper T cells. In contrast, the second signals in thymus-independent (TI) responses are delivered through innate immune receptors such as TLRs

expressed by the B cells themselves (TI-1), or through exceptionally intense BCR cross linking alone (TI-2).

Important differences between TD and TI responses include the naïve B cell populations that participate, the antibody isotypes generated, the response duration, the ultimate antibody affinity, and the extent of immunological memory established. In general, B-2 cells, particularly those in the FO compartment, are the major contributors to TD responses. In contrast, TI responses arise primarily from either B-1 cells or the B-2 lineage MZ subset (74). These differences likely reflect the BCR signaling characteristics and differentiative potential of these pools, as well as the nature of inducing signals. TI responses are of short duration and tend to be skewed towards IgM production, whereas TD responses are more protracted and will usually culminate in IgG. Within days after TI antigen challenge, substantial numbers of antibody secreting plasma cells (PCs) appear in the splenic extrafollicular regions (75, 76). The antibodies made by this extrafollicular response are largely IgM, and display comparatively low affinity for antigen. Conversely, TD responses display a gradual but profound increase in average antibody affinity – a process termed affinity maturation (77). Moreover, both types of responses can generate relatively long-lived antibody forming cells and memory B cells (78-81). As with TI responses, within days of TD antigen challenge, substantial numbers of PCs that generate low-affinity IgM appear in splenic extrafollicular regions. However, a few days later clusters of proliferating B cells appear at the borders of B cell follicles and T cell zones in the lymph nodes and spleen (82, 83). These are germinal centers (GCs), transient

structures wherein the unique functional features of TD responses emerge, including affinity maturation (84, 85), as well as efficient long-lived plasma cell generation (LLPC) (86).

GC formation requires a series of cognate, bi-directional interactions between activated CD4 T cells and activated and antigen-presenting B cells beyond the initial T cell priming event with a dendritic cell (87, 88). These include MHCII-restricted presentation by the B cell, costimulation via CD40-CD40L, and key cytokines. Together, these interactions result in the adoption of a GC B cell transcriptional program driven largely by Bcl-6 (89-92). A key gene upregulated in GC B cells is activation-induced deaminase (AID), which creates point mutations in Ig V regions (93, 94). This so-called somatic hypermutation (SHM) mechanism results in clonal variants of GC B cells with altered antigen affinity and specificity (95). Through selective competition and survival, clonal variants with higher affinity for antigen are preserved, whereas those with lower affinity are at a disadvantage and die (96). While these processes are thought to occur most efficiently and frequently in GCs, affinity maturation and class-switch recombination (CSR) in extrafollicular sites have been reported (97, 98). The details surrounding this preferential survival remain an area of intense investigation, but clearly involve competition for antigen and subsequently presenting said antigen (99). Currently popular models posit that the anatomically defined GC light zones are where competition for antigen and T helper cell survival factors and instructive cues occur; whereas proliferation and AID-mediated SHM occur in GC dark zones (100). Importantly, the CD4 helper T

cell subset responsible for proper GC reactions is transcriptionally and anatomically distinct from other T helper lineages (101). As the moniker suggests, T follicular helper (T_{FH}) cells migrate to the B cell follicle via CXCR5 (102), select high affinity GC clones into effector pools via BLyS secretion (103) and direct immunoglobulin isotype determination.

1.4 Activated B cells differentiate into discrete effector subsets

Circulating antibodies have biological half-lives in the range of days to weeks depending on heavy chain isotype; thus, long term protective immunity must rely on either LLPCs that persist indefinitely without seeding from B_{MEM} (104-106), or upon the sustained generation of SLPCs from B_{MEM} precursors driven by persistent antigen. To adopt activated and effector fates, preimmune B cells must integrate instructional cues from a variety of signals, sustain the mutational stress of CSR and SHM, and undergo profound morphological changes to produce antibody. To support these functions, activated B cells undergo significant transcriptional reprogramming based on the cues they receive. Though initially thought to be required for PC commitment (107), the transcription factor BLIMP1 is necessary for *bona fide* PC differentiation (108). Given that PCs bear little morphological resemblance to their clonal progenitors, they are transcriptionally distinct because BLIMP1 extinguishes the B lineage determining transcription factor PAX5 (109, 110). In this regard, these cross competing transcription factors facilitate a gene expression program to direct mature B cells through activation, selection, and terminal effector function. No master regulator of the B_{MEM} cell lineage is yet identified; however, T-BET and

RORα associate with $IgG_{2a/c}$ and IgA isotypes, respectively, are indeed critical for the formation and maintenance of some B_{MEM} subsets (111).

The homeostatic regulation of antigen experienced B lineage pools is less well understood, and likely involves a more complex set of players than the comprehensive role assumed by BLyS for preimmune pools. Indeed, the heterogeneity in lifespan and composition of B_{MEM} and PC subsets has only begun to be appreciated over the last several years, and will likely expand further. For example, recent conceptual advances challenge the notion that PCs are a monolithic population. Instead, T cell independent PCs are longer lived than previously thought (81); new subset markers have revealed phenotypic heterogeneity and complex turnover kinetics in BM PC pools (112, 113); and some PCs secrete cytokines and antimicrobial agents in addition to antibodies (114). Despite this growing complexity, several factors that govern PC longevity and homeostasis have nonetheless been identified. LLPC survival depends, at least in part, on BAFF and/or APRIL signaling via the BCMA and/or TACI receptors (115, 116). In this regard, LLPCs are thought to occupy survival niches independent of the primary pool. Homing to and occupation of these longevity-fostering niches is still an active area of research especially given newly discovered subsets and isotype-associated functional differences (117).

Similarly, several B_{MEM} subsets have been defined, based on the presence of different isotypes and further markers (**Table 1**). The immunological role of B_{MEM} cells may appear ostensibly redundant with simultaneous, clonally similar, and elevated antibody titers; however, recent evidence highlights a

distinct B_{MEM} cell role in response to pathogenic variants that have escaped the neutralizing capacity of the primary antibody response (118). Moreover, delineating which B_{MEM} subsets rapidly differentiate into PCs or engage in further GC formation upon secondary antigen challenge is an area of intense investigation. While some evidence suggests that isotype determines B_{MEM} cell subset and properties (119, 120), more recent work shows that surface markers such as PD-L2 and CD80 predict functional outcomes irrespective of isotype (121). Furthermore, the maintenance of these pools is independent of BAFF, antigen, or T cells (122-124). *In toto*, these observations indicate that PCs and B_{MEM} cells play non-redundant roles in immunity and occupy distinct homeostatic niches.

1.5 Scope

The work presented herein describes the cytokine signals sufficient for the acquisition of a B_{MEM} cell subset characterized by the expression of the transcription factor T-box Expressed in T cells (T-BET, encoded by the gene *Tbx21*). In the first section, we describe how novel interactions between canonical T_{FH} cell cytokines in concert with innate signals drive T-BET expression in the B lineage. Further, since T-BET^{+} B_{MEM} cells are phenotypically related to Age-associated B Cells (ABCs), we interrogated whether our model predicts age-related dysregulation of immune function in the second section.

2. IL-21 AND IL-4 RECIPROCALLY REGULATE T-BET IN B CELLS

2.1 INTRODUCTION

2.1.1 T-BET in immunity and B cells

The transcription factor T-BET is part of an ancient family of 18 T-box transcription factors important for regulating many tissues and organs during metazoan development (125). Although originally discovered in its role for T_H1 lineage commitment and optimal IFNγ production (126-128), T-BET expression arises in many immune cells and orchestrates cellular functions beyond specific cytokine production (129, 130). Indeed, NK cell maturation and homeostasis, and proper CD4 T cell chemotaxis all require T-BET expression (131, 132). Thus, this transcription factor's pleiotropic effects extend to a variety of immune cells. B cell intrinsic T-BET expression fosters switching to IgG_{2a} (111, 133-135), an isotype associated with both T_H1-driven antibody responses and humoral autoimmunity (126, 136). Moreover, T-BET is required for the generation of ABCs, which are transcriptionally distinct from other B cell subsets and have also been associated with both viral clearance and humoral autoimmunity (137-139). A more detailed introduction on ABCs is provided in chapter 3.

Despite growing appreciation for the importance of T-BET-driven B cell fates, the exact signals that yield B lineage effectors characterized by T-BET expression – as well as how these regulate appropriate versus pathogenic outcomes – remain poorly defined. Candidates include cell-intrinsic signals from adaptive and innate receptors, including the BCR and TLRs, as well as signals from T_{FH} cells. In this regard, several T_H1 cytokines, including IL-12, IL-18, and

IFNγ, can induce T-BET in activated B cells (126, 136). Nonetheless, the roles and interactions of canonical T_{FH} cell cytokines – IL-21, IL-4, and IFNγ – in regulating T-BET expression have not been systematically interrogated (101, 140, 141).

2.1.2 Cytokines regulate B cell responses

Generally, intercellular communication occurs via cell-to-cell contact or secreted messengers. The latter includes a loosely categorized group of small proteins called cytokines (~5-20kDa). Importantly, these include interleukins, chemokines, tumor necrosis factors, and interferons but generally not hormones or growth factors (142). Thus, depending on type of cytokine produced, cytokine-secreting cells can regulate and instruct the behavior of other cells. In this regard, T_{FH} cells guide and direct B cell responses via the production of IFNγ, IL-4, and IL-21. Beyond their capacity in driving affinity maturation, T_{FH} cells instruct isotype switching via these cytokines. Indeed, the effects of IFNγ and IL-4 on antibody isotype determination have been long appreciated (140, 141, 143-145). However, in addition to the effects of the hallmark cytokine of the T_{FH} lineage, IL-21 (146-149), how B cells integrate cytokine signals to achieve appropriate isotype and fate decisions remains unclear (150, 151). Thus, careful consideration of each cytokine is necessary to understand its attributed effects on B cells.

While originally named for its capacity to promote antiviral activity in influenza virus infected chick embryos (152, 153), IFNγ-producing cells regulate a variety of cell-mediated immune responses. Indeed, natural killer (NK), CD8,

and T_H1 cells all produce IFNγ against intracellular pathogens and tumors (154). IFNγ functions by binding the IFNγR-α and -β chains, which drives JAK-induced phosphorylation of STAT1 (pSTAT1). The resulting pSTAT1 homodimer translocates to the nucleus and initiates a change in gene expression (155). For B cells, IFNγ drives CSR to the $IgG_{2a/c}$ and IgG_3 isotypes (145, 156-158). Moreover, IFNγ receptor deficient mice fail to induce a normal intracellular pathogen IgG_{2a} antibody response (159). These observations lead to the presumption that T_{FH} cell-derived IFNγ drives antiviral antibody responses; however, while global IFNγ is necessary for normal IgG_{2a} responses, T cell derived IFNγ seems redundant in this capacity (140). Thus, while IFNγ is important for normal antibody responses against intracellular pathogens, the cellular source acting directly on B cells remains unclear.

In contrast to how IFNγ was discovered, IL-4's immunological role was first shown by its direct action on B cells. IL-4 signals by binding the IL-4Rα and IL-2 common-γ chain receptor, which drive JAK-mediated phosphorylation of STAT6 (pSTAT6). The resulting homodimer translocates to the nucleus and drives transcription (160). Two research groups independently discovered IL-4 via its capacity to enhance B cell activation and drive CSR to IgG_1 and IgE (161-163). Further, *in vivo* evidence also confirms and extends IL-4's role in inducing these isotypes against extracellular pathogens (141, 143, 164); however, IL-4 is not critical for host survival against these infections.

In addition to the well-characterized roles of IFNγ and IL-4 in isotype determination, IL-21 also regulates B cell responses (165). IL-21 functions by

binding the IL-21R and IL-2 common-γ chain receptor, which drives JAK-mediated phosphorylation of STAT1, 3, 5a, 5b (pSTAT1, 3, 5a, 5b). The resulting pSTAT homodimers and heterodimers (pSTAT1:pSTAT3) translocate to the nucleus and initiate gene expression changes (166). While originally described for its role in NK cell expansion *in vitro* (167), subsequent serum isotype analysis revealed a critical role for in regulating IgG_1 production (168). In this regard, IL-21 was thought to account for the residual IgG_1 observed in IL-4 or STAT6 deficient mice. Moreover, subsequent reports demonstrated how IL-21 induces the mutually antagonistic transcription factors BLIMP1 and BCL6, which are critical for B cell fate determination (169). Indeed, given BCL6's association with the GC B cell fate, *in vivo* evidence also confirms IL-21's direct role in GC B cell maintenance presumably through BCL6 induction (150, 151). Thus, IL-21 regulates not only B cell fate decisions but also isotype determination.

2.1.3 CD11c expression in B cells

In addition to guiding cell fate decisions, cytokines also direct immune cells to cites of inflammation via the induction of chemokine receptors and integrins. Indeed, IL-4, IFNγ, and IL-21 have all been shown to modulate expression of trafficking proteins (170-173). Integrins are a family of heterodimeric cell adhesion receptors that mediate cellular migration, trafficking, and cell-to-cell contacts via binding to cell-surface or extracellular matric ligands. The heterodimers consist of α and β subunits. CD11a, CD11b, CD11c, and CD11d are all α subunits that heterodimerize with the β2 subunit (CD18) (174, 175). Originally, CD11c was thought to demarcate DCs with high fidelity (176),

and thus later developed into a DC-ablative tool (177); however, it is now apparent that B and other cells normally express CD11c. Thus, it is important to consider what this integrin does beyond its capacity to identify specific cell types. Functionally, CD11c facilitates efficient phagocytosis and cell mobility via its binding capacity of a variety of ligands including adhesion molecules (e.g. ICAMs and VCAMs), LPS, complement protein, and matrix proteins such as fibrinogen and collagen (175). In accord with these capacities attributed to CD11c, deficient mice have compromised immune function. Indeed, CD11c deficient mice are resistant to models of disease such as EAE and atherosclerosis and conversely mount reduced responses to pathogen challenge (178-181). In the B lineage, CD11c expression associates with viral or bacterial infections, autoimmunity, and neoplasia (138, 182-187), although its function is unclear. Thus, the signals driving B cell intrinsic CD11c expression and the integrin's role in normal or aberrant immune responses are of interest.

2.1.4 Summary of findings

Here, we show that B cells integrate IL-4, IL-21, and IFNγ signals to adopt distinct TBET$^+$ effector fates. In the context of TLR engagement, both IL-21 and IFNγ directly drive FO B cells to express T-BET *in vitro*. However, IL-4 antagonizes IL-21-driven TBET upregulation, but enhances IFNγ-induced T-BET expression. Moreover, IL-21 but not IFNγ promotes CD11c expression. Consistent with these *in vitro* results, the *in vivo* frequencies of GC B cells and B_{MEM} cells expressing T-BET or CD11c vary based on the prevailing cytokine milieu. Finally, using viral and helminthic infections in single and double cytokine

KO mice, we show that the relative abundance of these cytokines governs whether GC and B_{MEM} cells generated during ongoing immune responses express T-BET and CD11c. Together, these findings reveal a previously unappreciated interplay between key T_{FH} cytokines that, in concert with innate sensors, controls the adoption of T-BET^{+} fates among antigen-experienced B cells.

2.2 MATERIALS AND METHODS

Mice

Tbx21$^{-/-}$, *Stat6*$^{-/-}$, *Tbx21*$^{f/f}$*Cd19*$^{Cre/+}$, C57BL/6, and BALB/c mice were maintained and used in accordance with the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC) guidelines. University of Pennsylvania IACUC approved all animal experiments. *Il4*$^{-/-}$ mice were a gift from Dr. Paula Oliver. *Ifng*$^{-/-}$ mice were a gift from Dr. Edward Behrens. *Il4*$^{-/-}$*Ifng*$^{-/-}$ double deficient mice were bred in house. *IL21r*$^{-/-}$ and *Il21*Tg spleens and sera were shipped overnight on ice from Dr. Warren Leonard's mouse colony at the NIH. All experimental mice were 2-6 months of age.

Infections

Mice were infected by oral gavage with 200 infectious larvae of *Heligmosomoides polygyrus* (HP), which were generated as previously described (188). Mice were infected by intranasal (i.n.) infection with 30 $TCID_{50}$ of influenza strain A/PR/8/34 (PR8) (ATCC). Spleens and sera were harvested on days indicated in figure legends.

In vitro cultures

CD23^{+} splenic B cells were enriched by positive selection using MACS bead system (Miltenyi Biotec), labeled with either Violet Cell Trace (VCT, Invitrogen) or carboxyfluorescein succynimmidyl ester (CFSE, eBioscience) and were stimulated with 1uM ODN1826 (Invitrogen), 1ug/mL CLO97 (InvivoGen), $F_{(ab)'2}$ fragments of 10 µg/mL anti-IgM (Jackson ImmunoResearch Laboratories), 10 µg/mL anti-CD40 (clone HM40-3; BL), 25 ng/mL IL21, 10 ng/mL IL4, 10 ng/mL

IFNγ (Shenandoah Biotechnology) at 37°C and 5.5% CO_2 in RPMI supplemented with 10% characterized FBS (HyClone), 1% HEPES (Gibco), 1% glutamine (Invitrogen), 50 μM 2-mercaptoethanol (Gibco), 1% minimal essential amino acids (Gibco), and 1% OPI supplement (Sigma).

Flow Cytometry

Spleens were harvested at indicated days post immunization or infection, mashed between frosted glass slides, and filtered through 0.2 micron mesh. Antibodies or reagents reactive to the following antigens were purchased from BioLegend (BL), Beckton-Dickenson (BD), or eBioscience (eBio): T-BET (4B10, BL), CD11c (N418, BL), IgM (R6-60.2, BD), CD38 (90, eBio), CD138 (281-2, BL), IgD (11–26c.2a, BL), CD4 (RM4-5, BL), B220 (RA3-6B2, BL), CD62L (MEL-14, eBio), TCR-β (H57-597, BL), CD19 (6D5, BL), CXCR5 (L138D7, BL); PD-1 (RMP1-30, BL); and PNA-FITC (Sigma), CD8 (53-6.7, eBio), CD4 (H129.19, BL), F4/80 (BM8, eBio), Ly-6G/GR1 (RB6-8C5, eBio). Exclusion based on Zombie Aqua (BL) positivity was used to identify live cells, and doublets were excluded by forward and side scatter height versus width analyses. Cells were analyzed on an LSRII or sorted on an Aria II (BD), and data analyzed using FlowJo software (Tree Star). Intracellular stains were performed using the eBioscience Foxp3 transcription factor kit.

Serum antibody titers and PR8-specific ELISA

For ELISA, Immunosorb plates (Corning) were coated with 20 hemagglutination units (HAU) PR8 or 10 μg/ml anti-mouse heavy and light chain, and blocked with PBS containing 2% BSA. Virus used for coating ELISA plates was inactivated by

incubation with 0.1% beta-propiolactone (BPL; Sigma) and 0.1 M HEPES (Cellgro) overnight at 4C followed by a 90 minute incubation at 37C. Diluted sera were incubated for 1 hour at room temperature, detected with horseradish peroxidase conjugated goat anti–mouse IgG_{2a}, IgG_{2b}, IgG_{2c}, or IgG_1 antibodies (SouthernBiotech), and developed with using 3,3', 5,5' tetramethylbenzidine (TMB) substrate (BD). Color development was terminated with 2M H_2SO_4 then read on an E_{Max} microplate reader (Molecular Devices) at 450nm.

Quantitative PCR analysis

RNA was extracted with the RNeasy kit (QIAGEN) and reverse transcribed using SuperScript II Reverse transcription (Invitrogen) according to the manufacturer's protocols. cDNA was amplified using TaqMan Universal Master Mix (Applied Biosystems) and Taqman probes for various genes (Applied Biosystems). Real-time PCR was performed with an ABI 7300 (Applied Biosystems). Relative expression ($\Delta\Delta C_t$) was calculated using *Gapdh* (Mm99999915_g1) expression as an endogenous control for cells that were FACS sorted *ex vivo* or stimulated *in vitro*. Probes: *Il4* (Mm00445260_m1), *Ifng* (Mm00801778_m1), *Il21* (Mm00517640_m1), *Tbx21* (Mm00450960_m1), *Aicda* (Mm00507774_m1).

Transcriptional profiling and functional enrichment analysis

After 20 hours *in vitro* stimulation as described above, RNA was isolated using the miRNeasy kit (Qiagen). Biotin-labeled complementary RNA (cRNA) was generated using the Illumina TotalPrep RNA amplification kit (Ambion). Illumina MouseRef-8 v2.0 expression Beadarrays were hybridized with cRNA. Scanned images were converted to raw expression using GenomeStudio v1.8 software

(Illumina). Data analysis was carried out using the statistical computing environment, R (v3.0.2). Differentially expressed genes (1.5-fold, FDR ≤ 0.05) were identified by linear modeling and Bayesian statistics using the Limma package (189). Clusters of co-regulated genes were identified by Pearson correlation using the hclust function of the stats package in R. Data have been deposited on the Gene Expression Omnibus (GEO) database for public access (Accession # GSE77145).

Statistics

Student's t-test was used to generate all P-values, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. All error bars are S.E.M. based on biological replicates.

2.3 RESULTS

2.3.1 IL-21, IL-4, and IFNγ differentially regulate T-BET and CD11c expression

In preliminary *in vitro* studies, we established that IL-21 drives TBET expression in FO B cells responding to TLR9, but neither BCR (α-IgM) nor CD40 signals (**Figure 1A**). To explore these interactions further, we cultured FO B cells with IL-4, IL-21, or IFNγ in the presence of TLR7 or TLR9 agonists. Both *Tbx21* transcripts and TBET protein increased markedly in FO B cells cultured with IL-21 or IFNγ (**Figures 1B and C**), but IL-4 influenced these outcomes differently. IL-4 blocked IL-21-driven TBET upregulation, but enhanced IFNγ-mediated TBET upregulation (**Figures 1B and C)**. Furthermore, IL-4 mediated these effects in a dose dependent manner when applied concomitantly with ODN1826 and IL-21 (**Figure 1D**).

To determine whether IL-21 and IL-4 directly regulate TBET in B cells, either *Il21r*$^{-/-}$ or *Stat6*$^{-/-}$ B cells were co-cultured with wild type (WT) B cells and stimulated as above. Since STAT6 is the key signal transducer of IL-4 and IL-21R is required for IL-21 signaling (167, 190), we reasoned that co-culturing these mutants with WT cells would reveal any secondary *trans* effects of cytokine treatment. In order to track cell type and expansion, WT or KO cells were labeled with VCT or CFSE, respectively (**Figure 2A, top row**). While IL-21-induced TBET expression in WT B cells, the co-cultured *Il21r*$^{-/-}$ B cells remained TBET negative, despite dividing to a similar extent. Analogously, although IL-21-driven TBET upregulation in WT B cells was reversed by IL-4, co-cultured *Stat6*$^{-/-}$

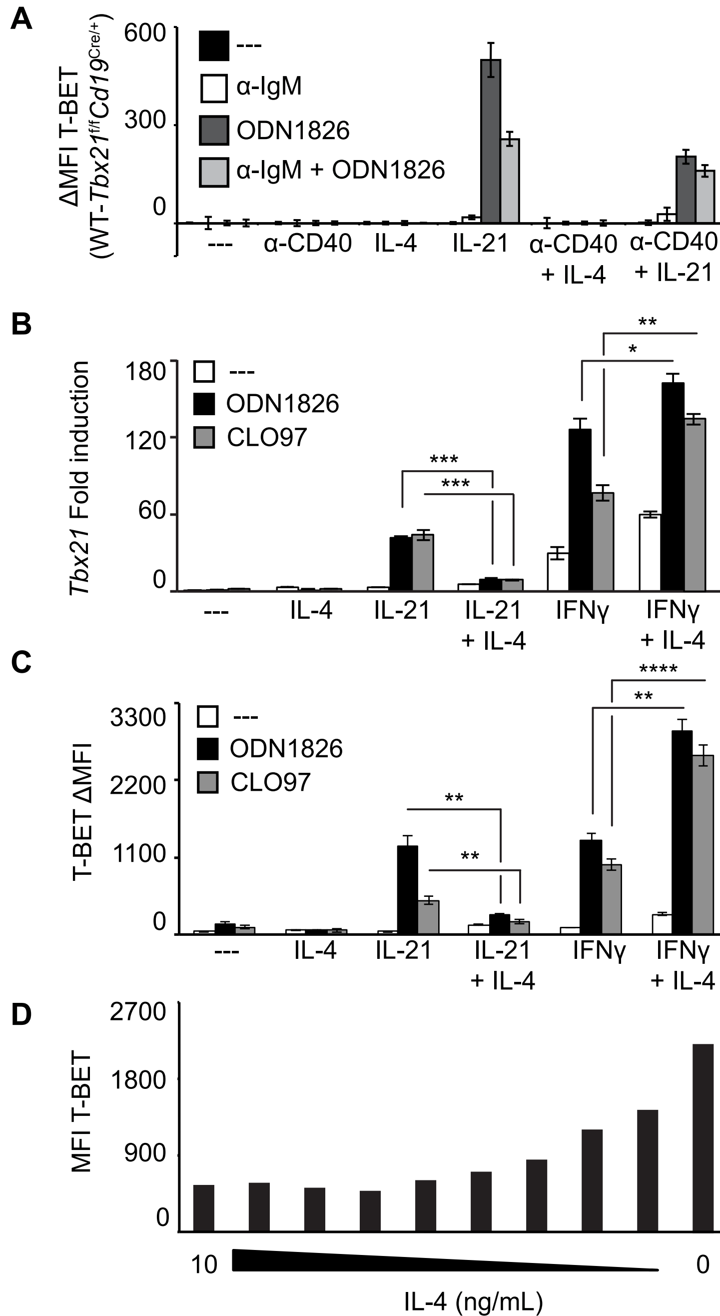

Figure 1. IL-4 and IL-21 reciprocally regulate T-BET expression *in vitro*. All in vitro experiments used magnetically enriched CD23⁺ splenocytes (FO B cells). (**A**) WT or *Cd19*$^{cre/+}$*Tbx21*$^{f/f}$ B cells were cultured with indicated stimuli for 48hrs and probed for T-BET (ΔMFI=WT-mutant). (**B**) *Tbx21* Gene expression analysis ($\Delta\Delta C_t$) from WT B cells treated for 20hrs with indicated stimuli. (**C**) FO B cells treated as in (**B**) for 48h and probed for T-BET. ** P <0.01, *** P <0.001, n = 3 independent experiments. (**D**) WT FO B cells cultured with ODN1826 and IL-21 with IL-4 titration and probed for TBET. (**B**-**C**) Data are presented as means of biological replicates ± SEM.

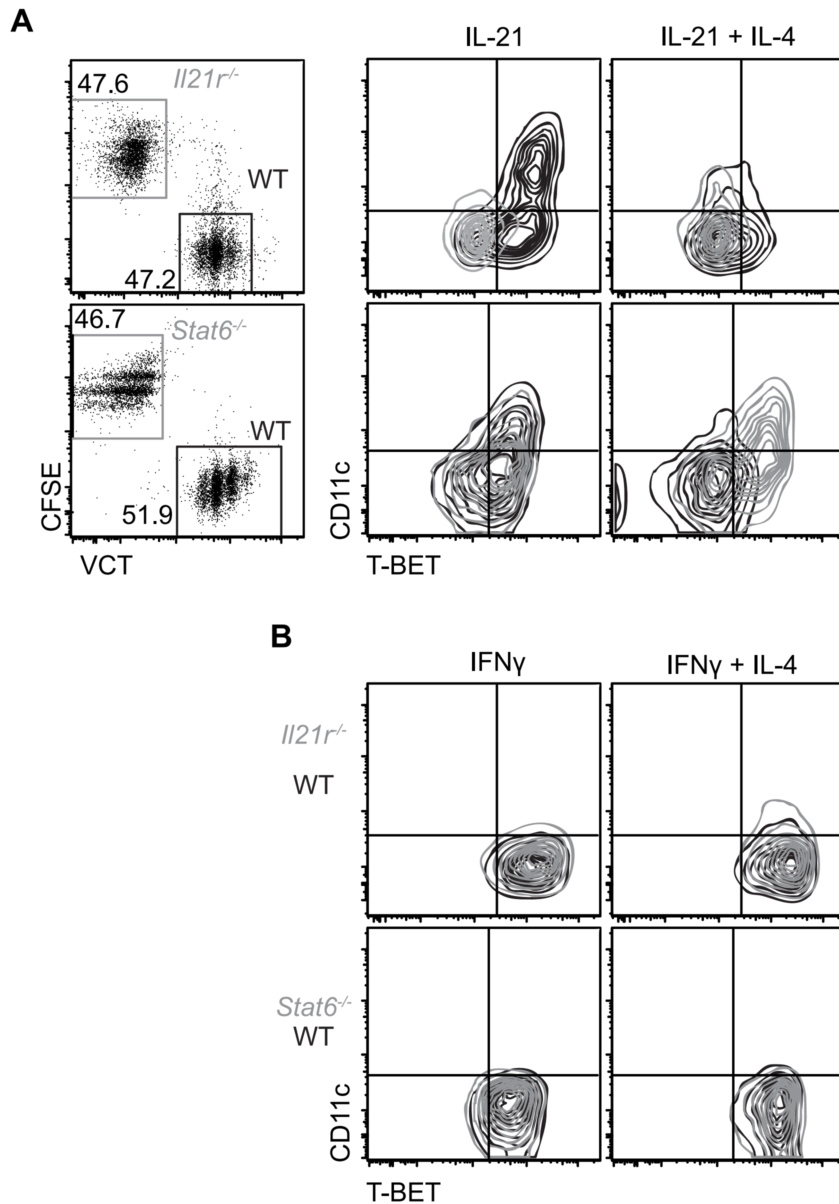

Figure 2. IL-4 and IL-21 act in a cell intrinsic manner to regulate T-BET expression *in vitro*. All *in vitro* experiments used magnetically enriched $CD23^+$ splenocytes (FO B cells). (**A** and **B**) FO B cells from WT, *Il21r*$^{-/-}$, or *Stat6*$^{-/-}$ spleens were labeled with either CFSE or Violet Cell Trace (VCT), treated with ODN1826 and indicated cytokine combinations for 48h, and assessed for CD11c and T-BET. n = 3 independent experiments.

cells were refractory to this negative regulatory effect **(Figure 2A, bottom row**).

These results show that B cell intrinsic IL-21 and IL-4 signals directly and reciprocally regulate TBET expression in the context of TLR9 driven activation. Importantly, IFNγ treatment induced TBET irrespective of *Il21r* or *Stat6* deficiency (**Figure 2B**). The converse effects of IL-4 on IFNγ- vs IL-21-induced TBET expression suggests that unique, TBET-associated fates are likely facilitated by each cytokine. We further interrogated this possibility in several ways. First, since previous studies have linked TBET with CD11c expression (138), we asked whether IFNγ or IL-21 influence CD11c differently. The results show that whereas IL-21 drives CD11c expression, IFNγ does not (**Figure 2**). Further, similar to its effects on TBET, IL-4 blocks IL-21-induced CD11c expression. These findings indicate that IL-21 and IFNγ drive TBET expression through distinct mediators. Further, TBET expression is not sufficient for the induction of CD11c. Thus, TBET and CD11c are each directly but separately regulated by IL-21 and IFNγ.

To further interrogate the distinct $TBET^+$ B cell fates driven by IL-21 versus IFNγ, as well as to distinguish TBET-dependent and -independent effects of each cytokine, we performed genome-wide transcriptional profiling on WT or *Tbx21*$^{-/-}$ B cells stimulated with either IFNγ or IL-21. Principal components analysis shows that 82.7% of variance in these data was explained by the cytokine employed, while *Tbx21* genotype accounted for 6.3% of the variance (**Figure 3A**). Further, each cytokine induces a unique transcriptional profile, including some T-BET-dependent shifts in gene expression (**Figure 3B** and **Table I**). We confirmed that

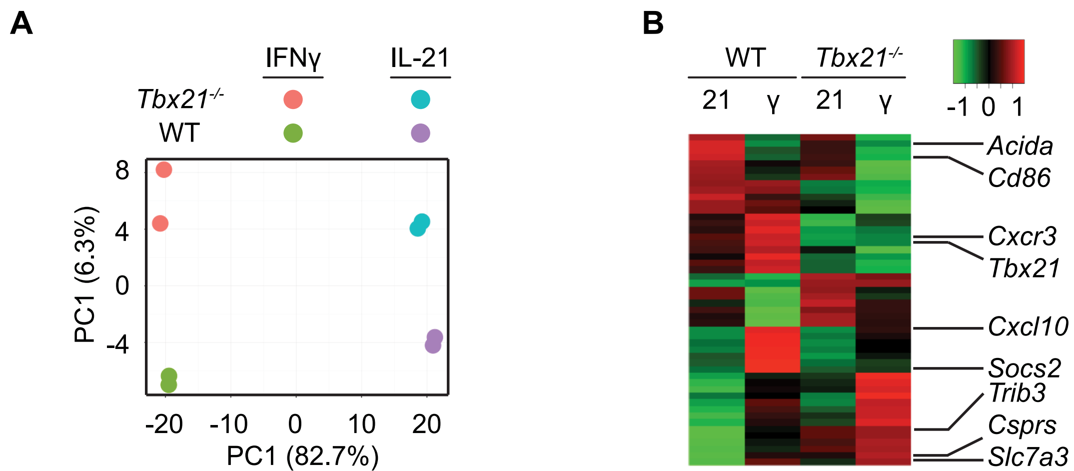

Figure 3. IL-21 drives T-BET-dependent shifts in gene expression distinct from IFNγ. All *in vitro* experiments used magnetically enriched $CD23^{+}$ splenocytes (FO B cells). WT or *Tbx21*$^{-/-}$ FO B cells were cultured for 20h with IL-21 (21) or IFNγ (γ) with ODN1826, RNA was harvested, and principal component (**A**) and Z-score (**B**) analysis performed according to materials and methods.

		WT		Tbx21 KO	
diffSymbols	diffEntrez	**IL21**	**IFNG**	**IL21**	**IFNG**
Hbb-bt	101488143	10.72	9.22	9.96	8.72
Pld4	104759	12.26	10.23	11.88	9.63
Csprs	114564	9.13	9.81	9.79	10.17
Adssl1	11565	10.19	8.76	9.56	8.38
Aicda	11628	10.62	8.16	9.57	8.16
Alas2	11656	8.70	8.70	8.17	8.08
Slc7a3	11989	9.58	11.94	10.89	12.33
Camk2b	12323	8.77	9.35	8.47	8.34
Cd86	12524	11.94	11.29	11.48	10.21
Cebpb	12608	9.80	10.40	10.26	11.03
Cxcr3	12766	8.59	8.89	7.96	8.02
Dapk2	13143	8.42	10.36	8.29	9.05
Ddx6	13209	10.43	10.85	11.02	11.22
Dmwd	13401	9.25	9.53	8.79	8.56
Igf2bp3	140488	9.90	9.52	9.19	8.80
Gcnt1	14537	10.30	9.69	10.42	10.28
Gfi1	14581	8.96	10.06	8.90	9.03
Slc6a9	14664	8.70	9.74	9.32	10.30
Gpr65	14744	10.08	9.98	9.66	9.18
Hba-a1	15122	12.98	12.81	11.08	10.61
Hhex	15242	12.03	11.50	11.87	10.90
Cxcl10	15945	8.07	11.26	8.05	9.70
Mrc1	17533	8.07	9.20	8.07	8.44
Enpp1	18605	8.96	10.10	9.03	9.44
Lgals3bp	19039	9.52	10.47	10.33	11.35
Sdc3	20970	8.69	8.48	9.34	9.22
Socs2	216233	8.25	9.21	8.31	8.39
Phf11a	219131	10.63	9.91	10.99	10.68
Trib3	228775	9.52	10.37	10.74	10.94
Oasl1	231655	8.69	9.41	9.08	10.03
Blvrb	233016	12.25	12.07	11.82	11.13
Rsad1	237926	8.98	9.49	8.36	8.71
Oas1g	23960	9.16	8.62	9.85	9.44
Usp18	24110	9.01	10.82	9.69	11.67
Ifnlr1	242700	8.45	10.02	8.60	10.64
Asns	27053	9.86	10.49	10.92	11.14
5031414D18Rik	271221	11.08	10.45	11.34	11.07
Abi2	329165	11.08	11.46	10.70	10.77
Tbx21	57765	10.68	12.07	8.29	8.51
Fkbp11	66120	9.11	9.92	8.93	9.26
Ifitm3	66141	9.95	10.52	10.39	11.68
Serpinb1a	66222	9.26	9.64	9.24	10.42
Sec11c	66286	10.14	10.37	9.88	9.74
Entpd4	67464	11.25	11.29	11.96	11.96
Chac1	69065	10.34	11.39	11.82	12.07
Tmem110	69179	10.22	10.43	10.07	9.67
Endod1	71946	11.34	10.31	11.42	10.96
Hvcn1	74096	12.52	11.50	12.40	10.68
Ly6k	76486	9.26	9.57	8.67	8.97
Lbh	77889	13.53	12.33	13.72	12.93

Table II: Top 50 differentially expressed genes.
Top 50 genes differentially expressed according to stimulation and genotype generating the heatmap in **Figure 3B**.

Tbx21 expression was high in WT mice and low in KOs thus validating our dataset. We find that IFNγ strongly and uniquely induced some genes, including *Cxcl10* and *Socs2*, which were highly T-BET dependent. Moreover, IL-21 also induced its own unique set of T-BET dependent genes including *Aicda* and *Cd86*. Additionally, there are some T-BET sensitive genes, such as *Cxcr3*, that were induced by both cytokines. Lastly, in accord with T-BET's role as a transcriptional repressor (191, 192), we also found some genes that were potently induced in the absence of T-BET such as *Trib3*, *Csprs*, and *Slc7a3*. Thus, although IFNγ and IL-21 overlap in regulating some T-BET dependent genes, each drives a unique transcriptional profile.

2.3.2 Relative abundance of IL-21, IL-4, and IFNγ regulate T-BET expression *in vivo*

Our *in vitro* findings suggest that IFNγ, IL4, and IL21 interact to modulate TBET and CD11c expression in B cells. As an initial assessment of whether this relationship exists *in vivo*, we surveyed GC B and B_{MEM} cells for TBET expression in C57BL/6 (B6) versus BALB/c mice (**Figure 4A**), because these strains display inherent T_H1 versus T_H2 skewing, respectively (193). We reasoned that if TBET expression is promoted by milieus rich in IFNγ, but repressed in those with plentiful IL-4 and little IFNγ, then the frequencies of $TBET^+$ B cells in these two strains should differ. In agreement with this prediction, most GC B cells in B6 mice are $TBET^+$ (**Figure 4B**), and variable CD11c expression was restricted to B_{MEM} cells (**Figure 4C**). In contrast, BALB/c mice lack $TBET^+$ B cells (**Figure 4B**), despite a small cohort of $TBET^-CD11c^+$ B_{MEM}

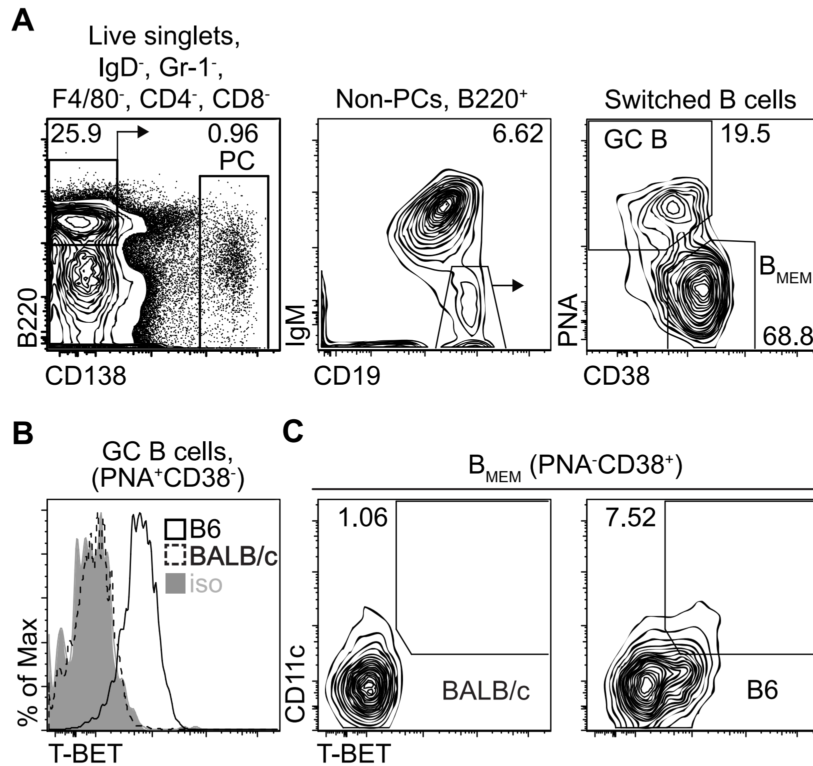

Figure 4. Genetic background determines T-BET expression *in vivo*. (**A**) Representative FACS gating strategy for activated splenic B cell subsets including PCs, GC B, and B_{MEM} cells. (**B**) Representative intracellular T-BET staining on C57BL/6 (B6) or BALB/c GC B cells and (**C**) T-BET and CD11c on B_{MEM} cells. n = 3 experiments.

cells (**Figure 4C**). These findings are consistent with the notion that IFNγ and IL-4 levels regulate TBET expression in GC B cells. To probe the impact of IL-21 on this overall relationship, we next asked whether extra-physiological levels of IL-21 would foster accumulation of $TBET^{+}CD11c^{+}$ B cells. Although the partially activated state of B cells in these mice confounds conventional phenotyping strategies (**Figure 5A**), nearly all mature B cells in *Il21*-Tg bear a $CD23^{-}CD21^{-}$ phenotype identical to the TBET-dependent ABC subset (169, 194). Furthermore, surface IgD and IgM expression on B-2 B cells reveals that excess IL21 does not drive profound switching to other isotypes (**Figure 5A, far right**). Regardless, profound increases in both TBET and CD11c expression were seen in all splenic B-2 B cells in *Il21*-Tg mice (**Figure 5B**), which is consistent with our *in vitro* results suggesting that IL-21 drives both TBET and CD11c expression. Furthermore, analysis of antigen-experience subsets indicates that $TBET^{+}CD11c^{+}$ accumulate in many different activated B cell subsets (**Figures 6A&B**). Finally, consistent with TBET's role in fostering class switch recombination to $IgG_{2a/c}$, we observed a marked increase of $IgG_{2a/c}$ but not IgG_{1} serum antibody titers in *Il21*-Tg compared to WT mice (**Figure 5C**).

Together, our *in vitro* and *in vivo* observations prompt a model in which the relative availability of IL-4, IL-21, and IFNγ govern the likelihood of establishing B_{MEM} cells expressing TBET and CD11c. Further, they suggest that abundant IFNγ will drive a $TBET^{+}CD11c^{-}$ B cell fate regardless of IL-4 or IL-21 levels, but that in the absence of IFNγ, the $TBET^{+}CD11c^{+}$ fate will be reciprocally regulated by IL-21 versus IL-4. We therefore evaluated these predictions by

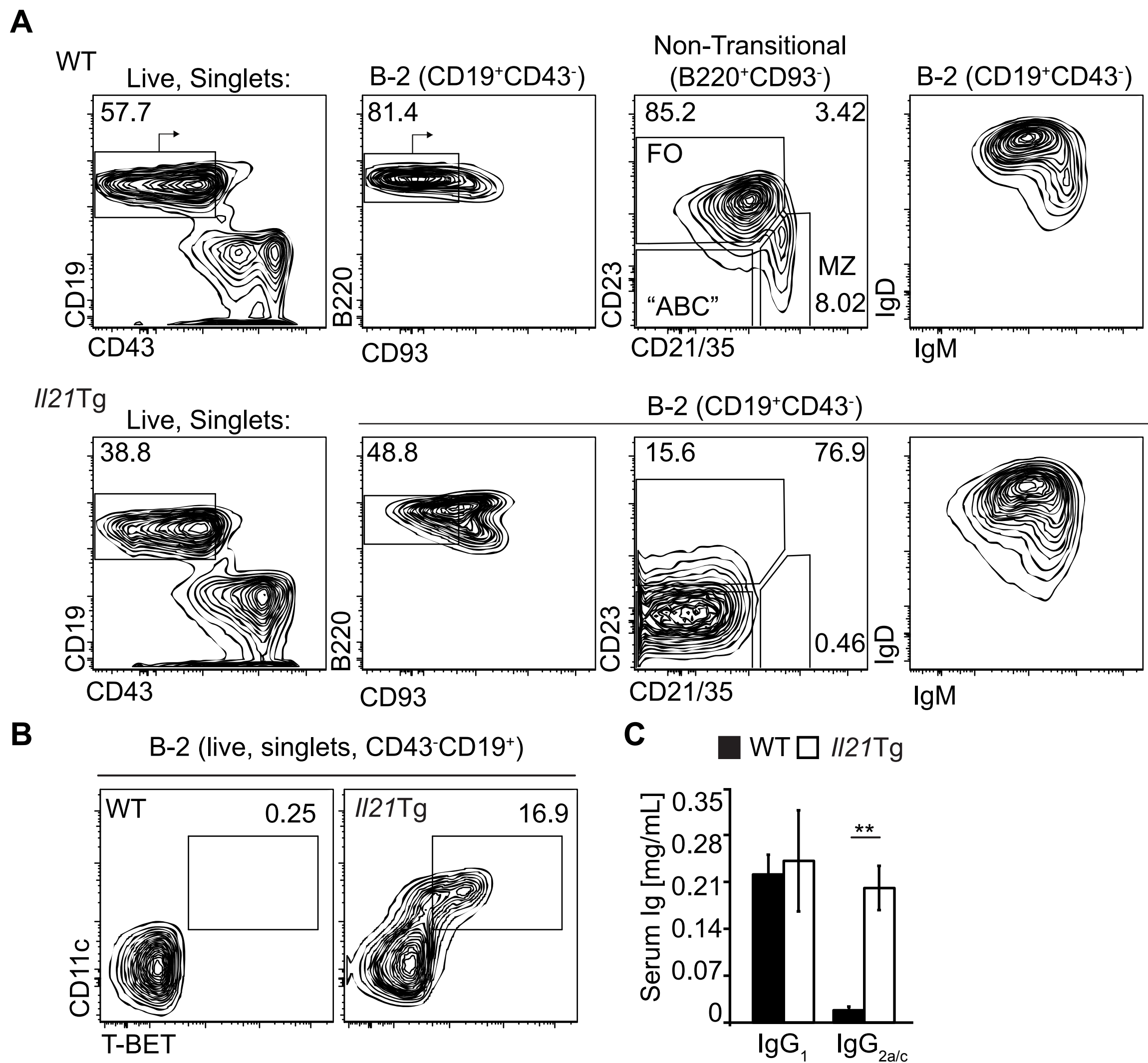

Figure 5. IL-21 overexpression in vivo generates T-BET⁺CD11c⁺ pre-immune B cells. (**A**) Representative FACS gating strategy for activated splenic B cells from WT and *Il21*Tg mice including FO, MZ, and "ABCs". (**B**) Representative FACS staining for T-BET and CD11c on splenic B-2 B cells . n = 3 independent experiments with 3 mice per group. (**C**) Total serum IgG_1 or $IgG_{2a/c}$ (IgG_{2a} + IgG_{2c}) titers between WT and *Il21*Tg mice. ** P <0.01, n = 2 independent experiments with 2-4 mice per group. Data are presented as means of biological replicates ± SEM.

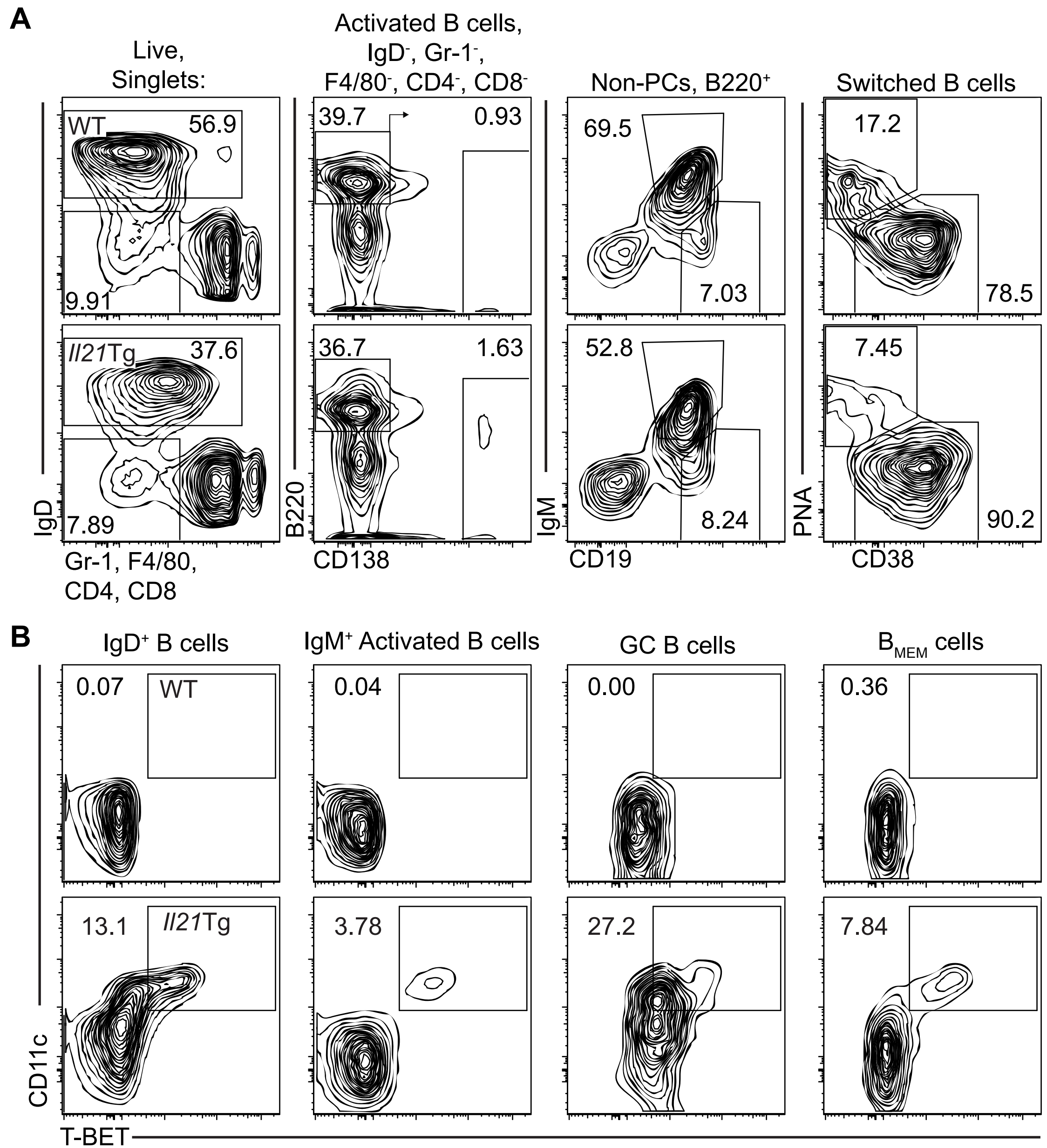

Figure 6. IL-21 overexpression in vivo generates T-BET⁺CD11c⁺ antigen experienced B cells. (**A**) Representative FACS gating strategy for activated splenic B cells from WT and *Il21*Tg mice including PCs, GC B, and B_{MEM} cells. (**B**) Representative FACS staining for T-BET and CD11c on splenic from indicated subsets. n = 3 independent experiments with 3 mice per group.

tracking the immune responses to either influenza virus or *H. polygyrus* infections in mice where cytokine availability could be experimentally manipulated.

2.3.3 Influenza virus infection generates T-BET$^+$CD11c$^+$ B_{MEM} in the absence of IL-4 and IFNγ

Influenza virus infection results in a well-characterized T-dependent and T_H1-skewed response, in which responding T_{FH} cells produce copious IFNγ as well as IL-21 and IL-4 (140). Thus, we reasoned that IFNγ would induce T-BET expression in GC B and B_{MEM} cells, but in the absence of IFNγ, IL-4 would prevent TBET expression. Accordingly, WT or *Ifng*$^{-/-}$ mice were infected with the A/Puerto Rico/8/1934 H1N1 influenza virus strain (PR8). As expected, WT animals mounted a robust GC B cell response to PR8 (**Figure 7A**), and these GC B cells expressed T-BET (**Figure 7B**). In contrast, GC B cells in *Ifng*$^{-/-}$ mice failed to express T-BET (**Figure 7B**) even though the magnitude of the GC B cell response was similar to WT (**Figures 7A&C**). Although the splenic PC numbers were reduced in *Ifng*$^{-/-}$ mice, B_{MEM} cell numbers remained intact across genotypes (**Figures 7D & 7E**). Thus, although the magnitude of the response varied slightly across gentoypes, all infected mice mounted a response above non-infected controls.

To confirm the intracellular T-BET staining and assess the cytokine profile of T_{FH} cells, we sorted both GC B cells and T_{FH} cells from each genotype (**Figure 8A**). Indeed, gene expression of *Tbx21* from FACS sorted GC B cells in WT and *Ifng*$^{-/-}$ mice confirmed our intracellular T-BET stain (**Figure 8B**). Next, we confirmed that FACS sorted T_{FH} cells from WT and *Ifng*$^{-/-}$ mice expressed equal

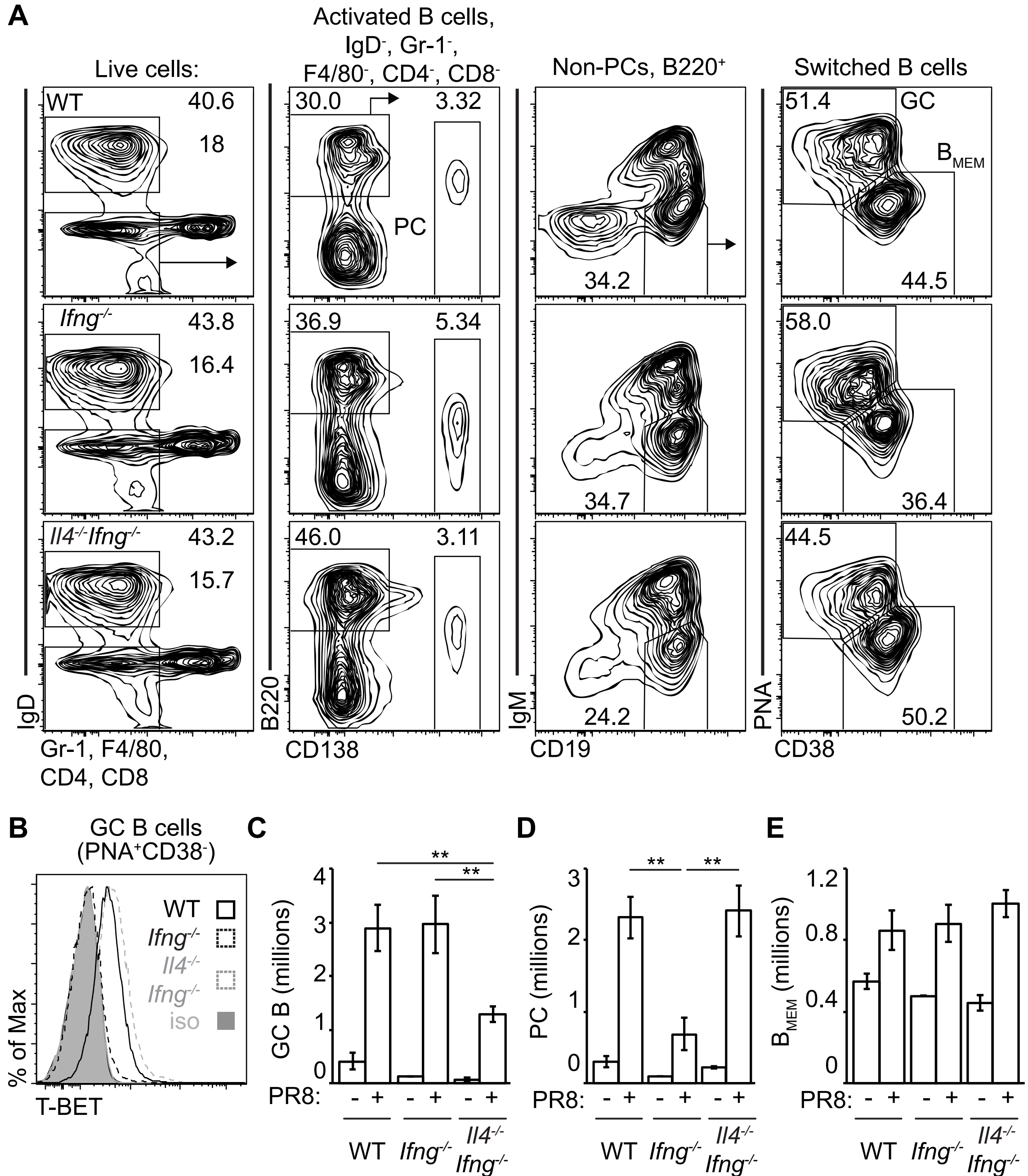

Figure 7. Influenza virus infection drives T-BET⁺ GC B cells formation in the absence of IFNγ and IL-4. All figures based on FACS stained splenocytes from non-infected or day 10 post i.n. 30 TCID50 PR8 infection in WT (n=21), *Ifng*$^{-/-}$ (n=13), and *Il4*$^{-/-}$*Ifng*$^{-/-}$ (n=13) mice across 3-7 experiments with ≥3 mice per group, ** P <0.01. (**A**) Representative FACS gating strategy for PCs, GC B, and B_{MEM} cells. (**B**) Representative FACS staining for T-BET on GC B cells. Enumeration of (**C**) GC B cells (**D**) PCs, and (**E**) B_{MEM} cells.

levels of *Il4* and *Il21* transcripts (**Figures 8C & D**). Moreover, although reduced in magnitude, *Ifng*$^{-/-}$ mice mounted a comparable T_{FH} cell response to WT controls (**Figure 8E**). These results are consistent with the idea that in the absence of IFNγ, IL-4 blocks T-BET expression in response to IL-21. To directly test this, we infected *Il4*$^{-/-}$*Ifng*$^{-/-}$ double deficient mice with PR8. While *Il4*$^{-/-}$*Ifng*$^{-/-}$ mice mounted a blunted GC B cell response (**Figures 7A&C**), these cells nonetheless clearly expressed T-BET (**Figures 7B & 8B**). Furthermore, only the GC B cell response was blunted in *Il4*$^{-/-}$*Ifng*$^{-/-}$ mice (**Figure 7C**), whereas the PC and B_{MEM} were comparable to WT (**Figures 7D&E**). Lastly, *Il4*$^{-/-}$*Ifng*$^{-/-}$ mice mounted a comparable T_{FH} cell response with the same levels of *Il21* transcripts as WT (**Figures 8D&E**).

We next assessed B_{MEM} cells for T-BET and CD11c expression and probed the serum for IgG_{2c} or IgG_1 PR8-reactive antibody given our initial phenotypic studies (**Figures 4C & 5C**). The composition of the B_{MEM} cell pool differed according to genotype in response to infection (**Figures 9A&B**). While WT mice generated some T-BET$^+$CD11c$^+$ B_{MEM} cells, *Ifng*$^{-/-}$ mice produced almost none above non-infected controls, suggesting the dominance of IL-4 in the absence of IFNγ. Lastly, *Il4*$^{-/-}$*Ifng*$^{-/-}$ mice produced the most TBET$^+$CD11c$^+$ B_{MEM} cells, presumably reflecting IL-21 activity in the absence of any repressive IL-4 effects. Furthermore, we assessed PR8 reactive serum antibody titers of the IgG_{2c} and IgG_1 isotypes and asked whether they correlated with T-BET expression. Consistent with the role of IgG_{2c} in antiviral immunity (195, 196), WT mice produced the most PR8-reactive IgG_{2c} compared to *Ifng*$^{-/-}$ and *Il4*$^{-/-}$*Ifng*$^{-/-}$

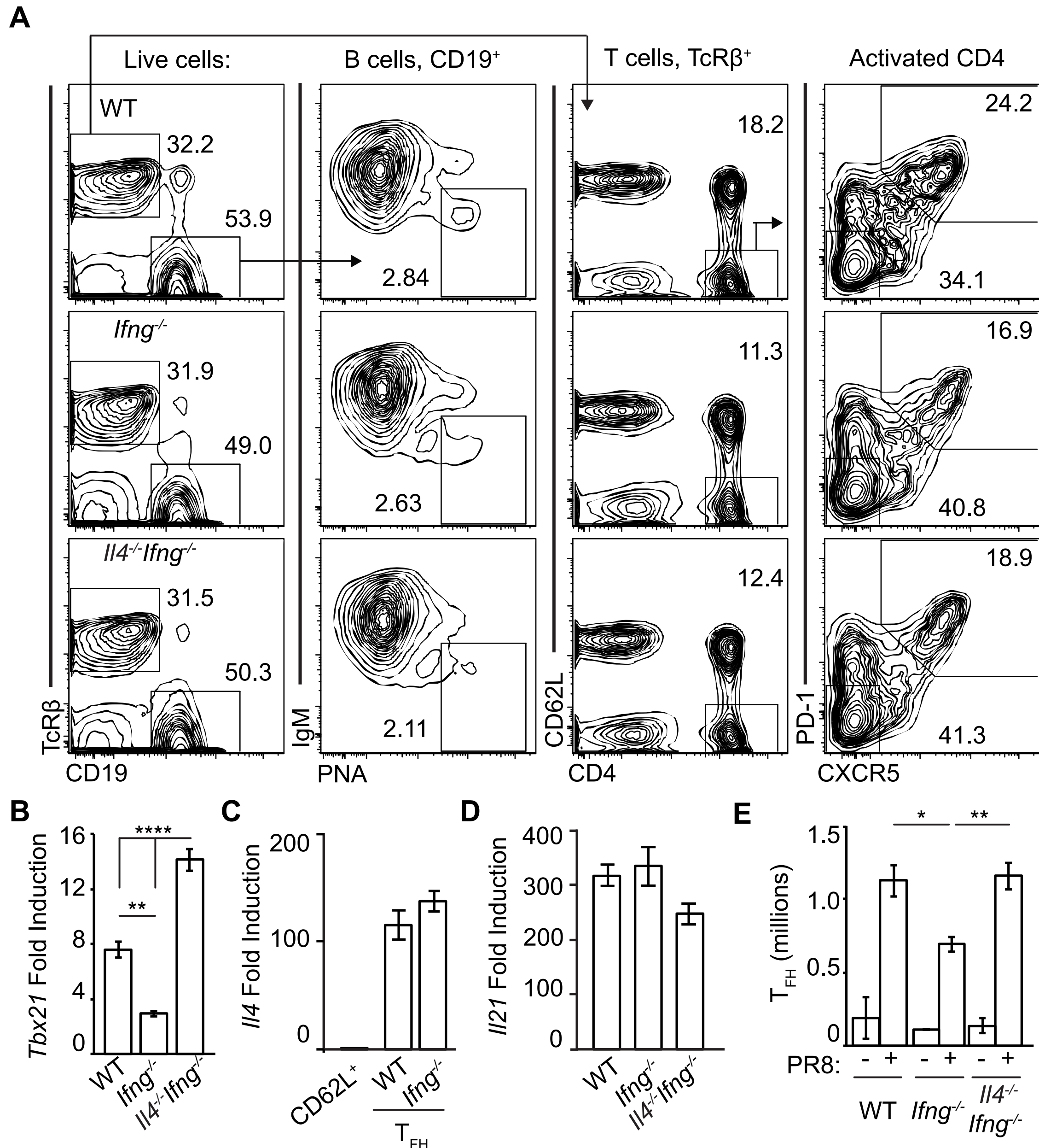

Figure 8. T_{FH} cells in influenza virus infection produce IL-4 and IL-21. All figures based on FACS stained splenocytes from non-infected or day 10 post i.n. 30 TCID50 PR8 infection in WT (n=21), *Ifng*$^{-/-}$ (n=13), and *Il4*$^{-/-}$*Ifng*$^{-/-}$ (n=13) mice across 3-7 experiments with ≥3 mice per group, * P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001. (**A**) Representative sort strategy for GC B and T_{FH} cells. Gene expression analysis ($\Delta\Delta C_t$) for (**B**) *Tbx21* from GC B cells and (**C**) *Il4* (**D**) *Il21* from T_{FH} cells. (**E**) Enumeration of T_{FH} cells.

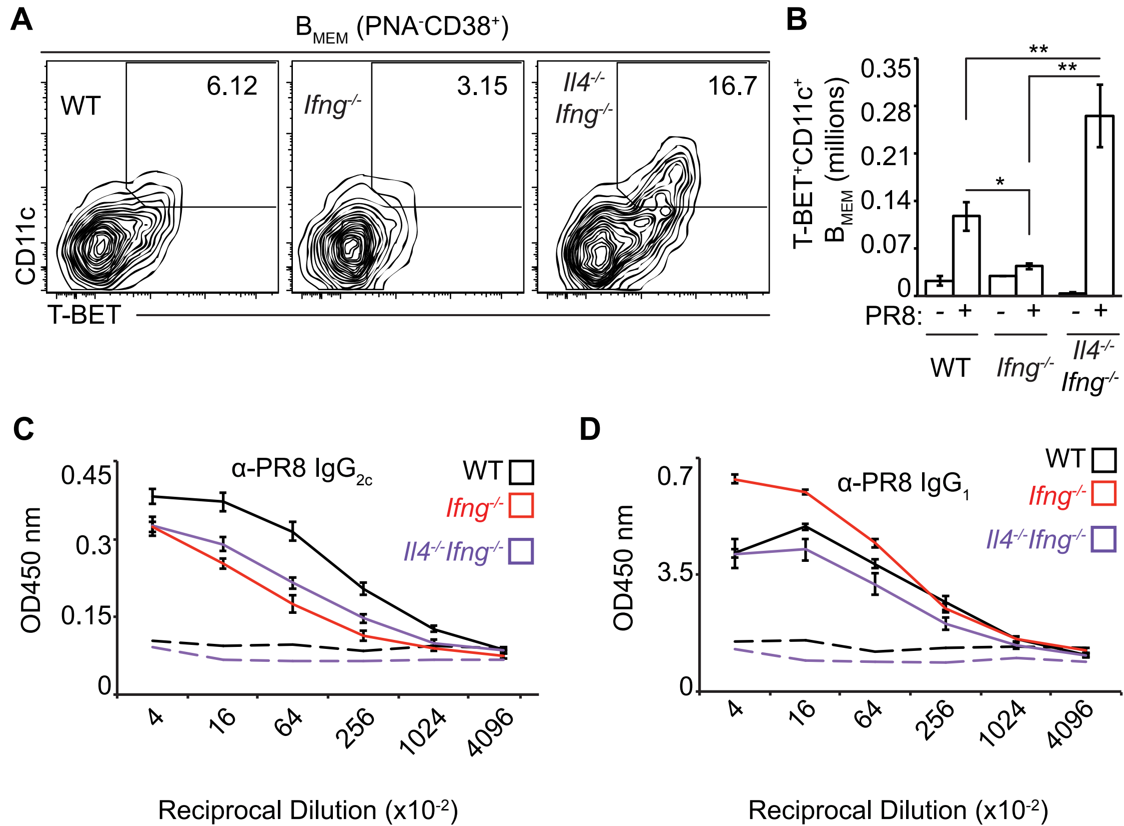

Figure 9. Influenza virus infection drives TBET⁺CD11c⁺ B_{MEM} cell formation in the absence of IFNγ and IL-4. All figures based on FACS stained splenocytes from non-infected or day 10 post i.n. 30 TCID50 PR8 infection in WT (n=21), *Ifng*$^{-/-}$ (n=13), and *Il4*$^{-/-}$*Ifng*$^{-/-}$ (n=13) mice across 3-7 experiments with ≥3 mice per group, * P <0.05, ** P <0.01 (**A**) Representative FACS staining for T-BET and CD11c on B_{MEM} cells as defined in Figure 7. (**B**) Enumeration of T-BET$^+$CD11c$^+$ B_{MEM} cells. PR8 reactive serum antibody titers of (**C**) IgG_{2c} and (**D**) IgG_1 isotypes.

mice (**Figure 9C**). Importantly, a substantial amount of antigen specific IgG_{2c} is independent of both IFNγ and B cell intrinsic T-BET expression. Lastly, PR8-reactive IgG_1 was most prominent in *Ifng*$^{-/-}$ mice, which is consistent with an IL-4 dominant response. Overall, these findings confirm and extend our *in vitro* findings, since the same interplay of cytokines directs T-BET expression among B effectors *in vivo*. Further, our observations suggest that T-BET$^+$CD11c$^+$ B_{MEM} cells will be fostered in immune responses where IL-4 and IFNγ are limited.

2.3.4 Il-4 deficiency is sufficient to induce T-BET$^+$CD11c$^+$ B_{MEM} independently of IFNγ in *Heligmosomoides polygyrus* infection

Results with influenza virus infection are consistent with the notion that IFNγ drives T-BET expression irrespective of concomitant IL-4 or IL-21, and that eliminating IFNγ creates a situation where the relative levels of IL-4 and IL-21 govern the T-BET$^+$CD11c$^+$ phenotype. However, this subtractive approach does not necessarily show that in responses where IFNγ is normally absent, the sole determinant of a T-BET$^+$ fate is IL-4 availability. Accordingly, we asked whether IL- deficiency is sufficient to permit T-BET expression in GC B cells during a T_H2 response, using *Heligmosomoides polygyrus* (HP). This intestinal helminth induces IL-4 and IL-21 production by T_{FH} cells, which drives a robust IgG_1 response (141). Thus, we hypothesized that in the absence of IL-4, IL-21 would be sufficient to induce T-BET expression in B effectors. To test this idea, we infected WT or *Il4*$^{-/-}$ mice with HP, and probed GC B cells for T-BET. As expected, WT mice mounted a GC B cell response that lacked T-BET expression (**Figures 10A-C**). Conversely, although blunted in magnitude, *Il4*$^{-/-}$ mice initiated

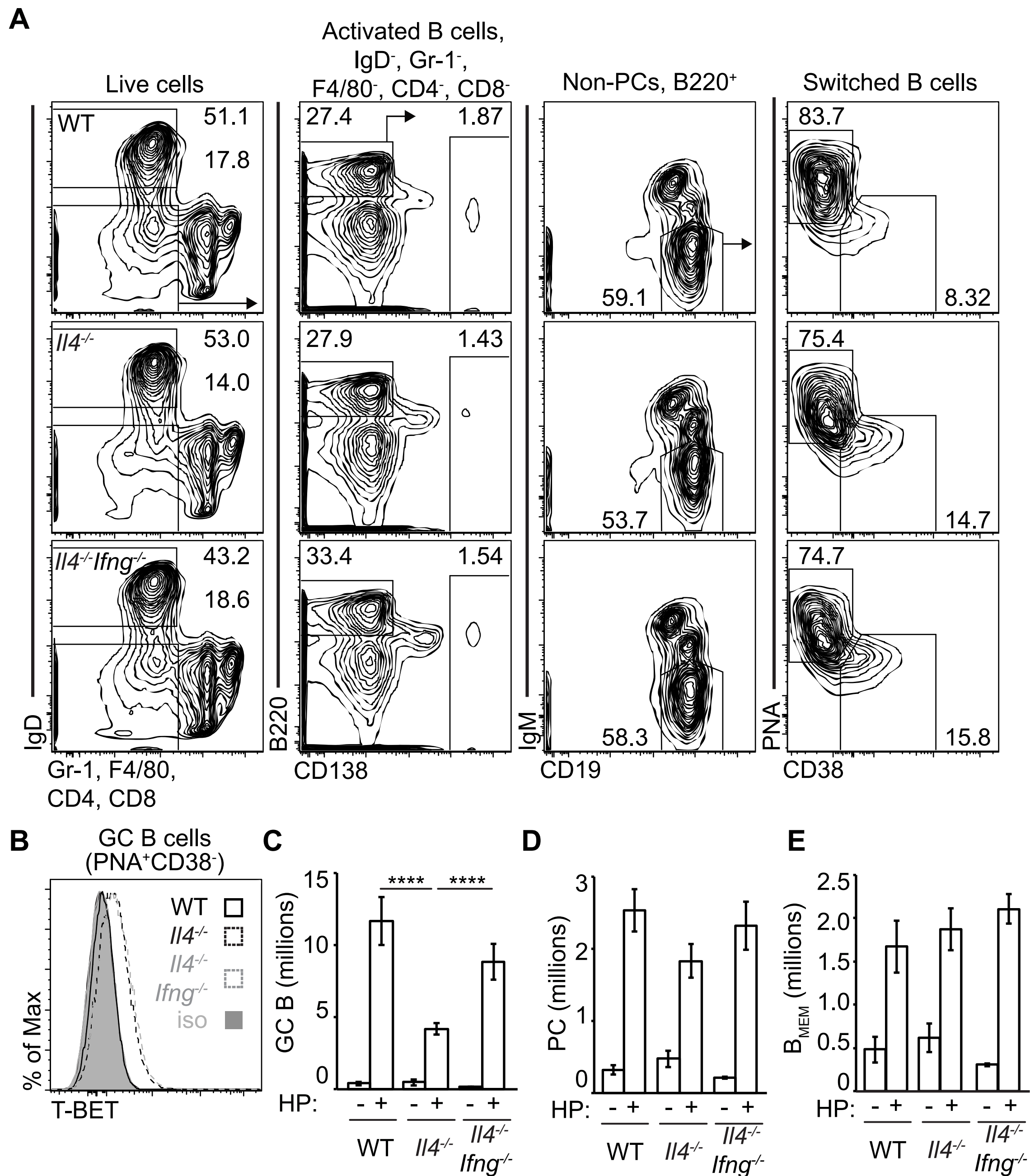

Figure 10. *Heligmosomoides polygyrus* (HP) infection drives T-BET+ GC B cells formation in the absence of IFNγ and IL-4. All figures based on FACS stained splenocytes and sera from non-infected or day 14 post oral gavage of 200 HP in WT (n=20), *Il4*$^{-/-}$ (n=22), and *Il4*$^{-/-}$*Ifng*$^{-/-}$ (n=11) mice across 3-6 experiments with ≥3 mice per group, **** P <0.0001. (**A**) Representative FACS gating strategy for PCs, GC B, and B_{MEM} cells. (**B**) Representative FACS staining for T-BET on GC B cells. Enumeration of (**C**) GC B cells (**D**) PCs, and (**E**) B_{MEM} cells.

a TBET$^+$ GC B cell response. To eliminate the possibility that excess IFNγ in *Il4*$^{-/-}$ mice explains these phenotypes, we infected *Il4*$^{-/-}$*Ifng*$^{-/-}$ mice with HP. The GC B cell response in *Il4*$^{-/-}$*Ifng*$^{-/-}$ mice was restored to WT levels (**Figure 10C**) but maintained T-BET expression independently of IFNγ (**Figure 10B**). Importantly, the magnitude of the PC and B_{MEM} cell response remained intact across genotypes (**Figures 10D&E**).

To confirm the intracellular T-BET staining and assess T_{FH} cell cytokine profile, we sorted both GC B and T_{FH} cells from each genotype (**Figure 11A**). *Tbx21* gene expression from FACS sorted GC B cells in WT, *Ifng*$^{-/-}$, and *Il4*$^{-/-}$*Ifng*$^{-/-}$ mice confirmed the intracellular T-BET stain (**Figure 11B**). Further, T_{FH} cells from both *Il4*$^{-/-}$ and *Il4*$^{-/-}$*Ifng*$^{-/-}$ mice produced less *Il21* even though these mice generated comparable or more T_{FH} cells than WT (**Figures 11C&D**). Finally, we again assessed B_{MEM} cells for TBET and CD11c expression and probed the serum for serum IgG_{2c} or IgG_1. We observed a similar alteration in the B_{MEM} pool according to cytokine availability. Whereas HP-infected WT mice did not generate T-BET$^+$CD11c$^+$ B_{MEM} cells, both *Il4*$^{-/-}$ and *Il4*$^{-/-}$*Ifng*$^{-/-}$ mice did—again suggesting an IL-21-driven phenotype independent of IFNγ (**Figures 12A&B**). Isotype representation varied with T-BET expression: whereas WT mice produced >95% IgG_1, over half of the serum antibodies in *Il4*$^{-/-}$*Ifng*$^{-/-}$ and *Il4*$^{-/-}$ mice were IgG_{2b} and IgG_{2c} (**Figure 10C**). Overall, the HP infection data are consistent with our model inasmuch as in the absence of IFNγ production, we observe T-BET and CD11c expression that is sensitive to IL-4. Moreover, the consistent relationship of these cytokines to T-BET and CD11c expression in both types of

infection suggest a common feature to most humoral immune responses.

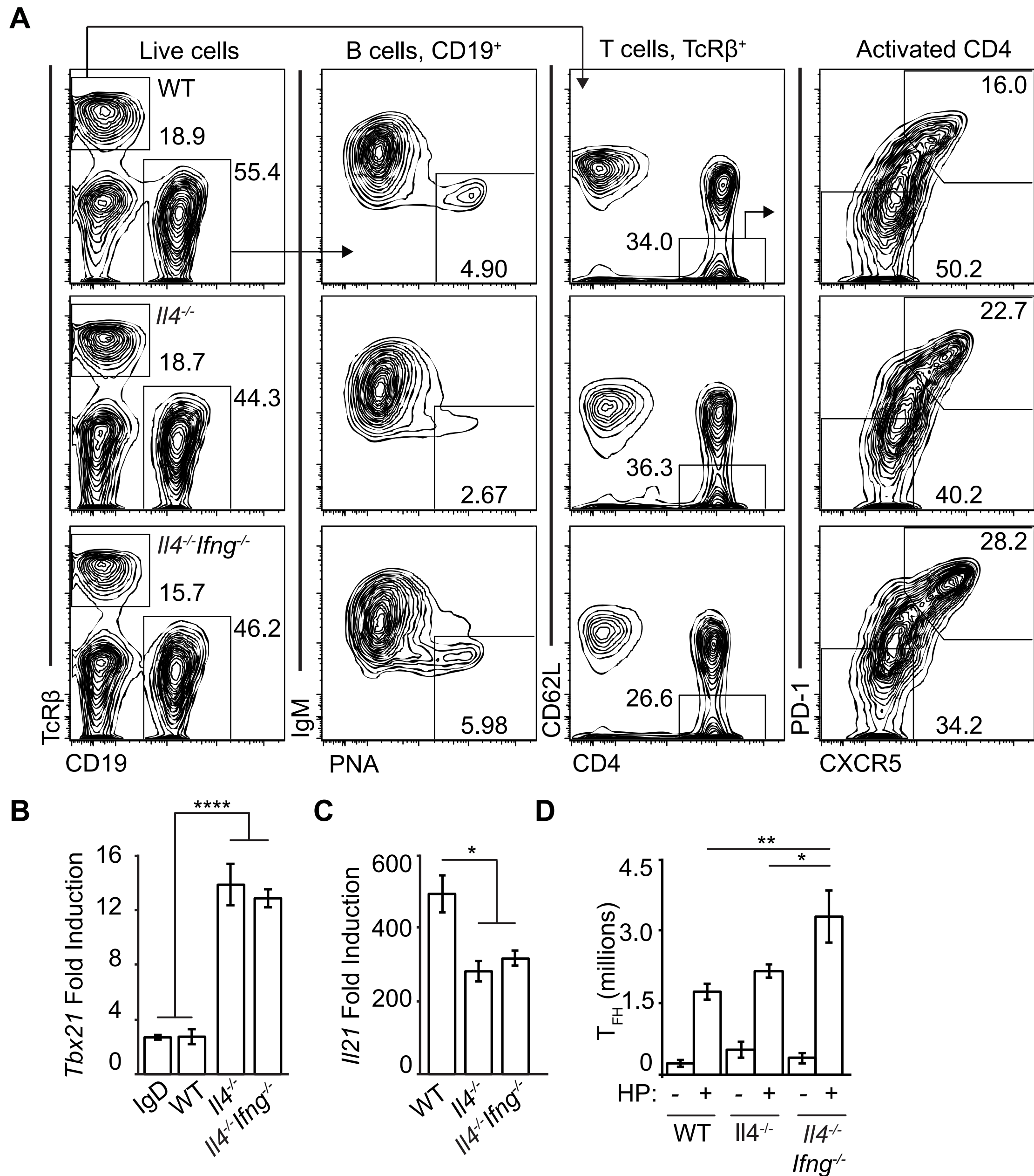

Figure 11. T_{FH} cells in *Heligmosomoides polygyrus* (HP) infection produce IL-21. All figures based on FACS stained splenocytes and sera from non-infected or day 14 post oral gavage of 200 HP in WT (n=20), *Il4*$^{-/-}$ (n=22), and *Il4*$^{-/-}$*Ifng*$^{-/-}$ (n=11) mice across 3-6 experiments with ≥3 mice per group, * P <0.05, ** P <0.01, **** P <0.0001. (**A**) Representative sort strategy for GC B and T_{FH} cells. Gene expression analysis ($\Delta\Delta C_t$) for (**B**) *Tbx21* from GC B cells and (**C**) *Il21* from T_{FH} cells. (**D**) Enumeration of T_{FH} cells.

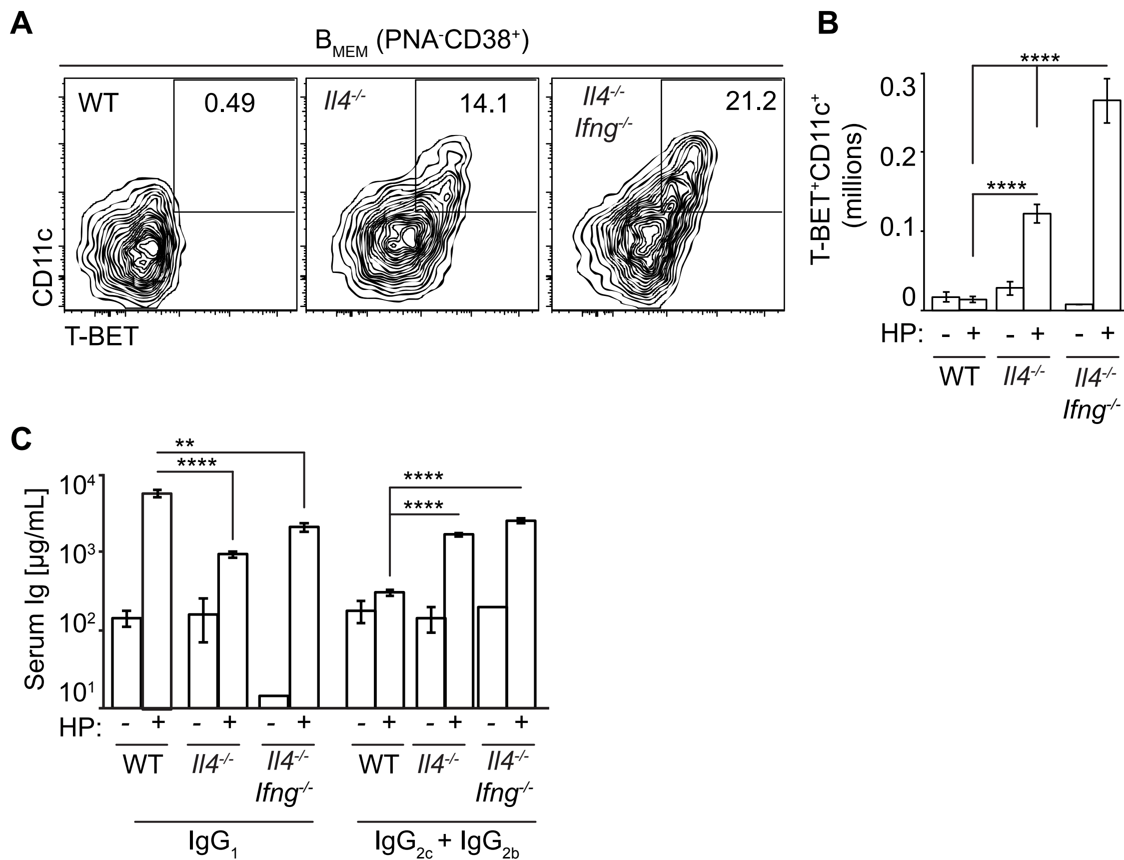

Figure 12. *Heligmosomoides polygyrus* (HP) infection drives T-BET$^+$CD11c$^+$ B$_{MEM}$ cell formation in the absence of IFNγ and IL-4. All figures based on FACS stained splenocytes and sera from non-infected or day 14 post oral gavage of 200 HP in WT (n=20), *Il4*$^{-/-}$ (n=22), and *Il4*$^{-/-}$*Ifng*$^{-/-}$ (n=11) mice across 3-6 experiments with ≥3 mice per group, ** P <0.01, **** P <0.0001. (**A**) Representative FACS staining for T-BET and CD11c on B$_{MEM}$ cells as defined in **Figure 10.** (**B**) Enumeration of T-BET$^+$CD11c$^+$ B$_{MEM}$ cells. (**C**) Total serum IgG$_1$ and IgG$_{2c}$ + IgG$_{2b}$ titers.

2.4 DISCUSSION

Together, these results show that in the context of TLR7 or TLR9 engagement, the aggregate of IFNγ, IL-21, and IL-4 signals determines whether B cells adopt a T-BET$^+$ fate. We provide *in vitro* evidence for a novel route to T-BET induction via the hallmark T_{FH} cell cytokine, IL-21. Importantly, this occurs robustly in the context of TLR engagement and independent of antigen receptor engagement or costimulation. Furthermore, concomitant IL-4 signals antagonize IL-21-induced T-BET expression but enhance IFNγ-driven T-BET. Moreover, IL-21, but not IFNγ, promotes CD11c expression independent of T-BET. Using influenza virus and *H. polygyrus* infections, we show that these interactions function *in vivo* to determine whether T-BET$^+$ and CD11c$^+$ B cells are formed. These findings suggest that T-BET$^+$ B cells seen in health and disease share the common initiating features of TLR driven activation within this circumscribed cytokine milieu.

TLR engagement appears necessary to position activated B cells for these fates upon subsequent IL-21 signaling. We have obtained similar results with the TLR2/4 ligand LPS (**Figure 13A**), suggesting pathways common to most TLRs, and perhaps other innate receptors, provide these key initial signals. We speculate that these innate signals alter gene loci accessibility for subsequent T_{FH} cytokine cues since there are reports of crosstalk between the TLR-MyD88 and IL-21r-STAT3 pathways in B cells (197, 198). Indeed, prior reports that CD11c$^+$ or TBET$^+$ B cells emerge in responses to a variety of viral and bacterial infections are consistent with this idea (137, 183). Furthermore, while BCR and

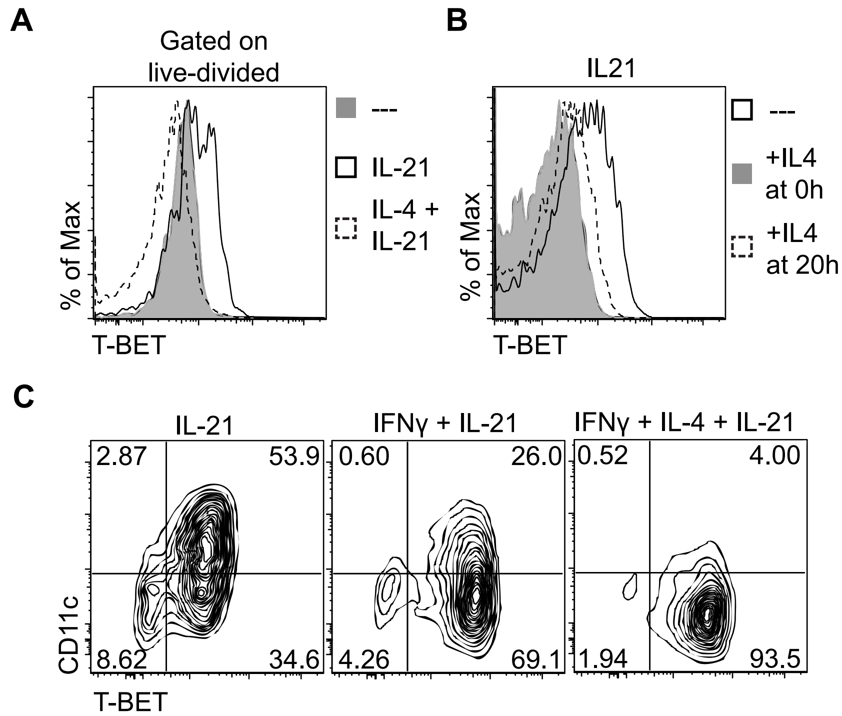

Figure 13. Dominant IFNγ-driven T-BET expression. All *in vitro* experiments used magnetically enriched $CD23^{+}$ splenocytes (FO B cells). FO B cells treated with LPS (**A**) or ODN1826 (**B**&**C**) and indicated cytokine combinations for 48h, and assessed for T-BET and CD11c. n = 3 independent experiments.

CD40 engagement do not induce T-BET with IL-21, these signals do consistently down-modulate the levels of T-BET expression induced by ODN1826 and IL-21 (**Figure 1A**). This further suggests complexity among the NF-κB, STAT, and MyD88 signal cascades. Thus, understanding how B cells integrate these disparate signal transduction events should elucidate effector cell fate decisions.

The differential effects of IL4 on IL21 versus IFNγ suggest a complex interplay of STAT-dependent transcriptional regulation. The clear dose-response relationship of IL4-mediated effects is consistent with the idea that competitive relationships are involved (**Figure 1D**). Furthermore, IL-21-driven T-BET remains sensitive to IL-4 signals even 20 hours after it is induced (**Figure 13B**). This suggests that IL-21-induced T-BET fates are plastic *in vitro* and still subject to the repressive activity of IL-4. Although IL-4Rα and IL-21R both require common-γ chain receptor to phosphorylate their associated STATs (199), our *Stat6*$^{-/-}$ coculture data indicate that competition for membrane proximal receptor components is unlikely to explain these findings (**Figure 2**). If this were the case, then *Stat6*$^{-/-}$ cells would also be subject to IL4's repressive effects. Instead, downstream events are more likely candidates, including differential occupation of transcriptional regulatory sites, and altered stoichiometric relationships among the JAK-STAT proteins involved. In T cells, it is established that IL-21 can drive T-BET in a STAT1 dependent manner (200, 201); however, IL-21 transduced signals have not been so carefully characterized in the B lineage. Thus, assessing whether IL-21-driven pSTAT1 or pSTAT3 is altered by IL-4 or whether STAT6 binds the *Tbx21* locus could elucidate the mechanism behind the

interplay of IL-21 and IL-4 on T-BET expression.

The preferential induction of CD11c and differential effects of IL-4 stimulation lead us to the conclusion that IL-21 and IFNγ drive distinct T-BET$^+$ fates. Thus, since T-BET is a transcription factor, we explored how these two T-BET inducing stimuli differed in terms of gene expression in the presence or absence of T-BET (**Figure 3**). The most striking difference was between cytokine treatments as measured by principal component analysis (**Figure 3A**). However, each cytokine treatment induces and represses overlapping and distinct sets of genes in a T-BET dependent manner (**Figure 3B**). Specifically, in accord with its role in GC maintenance, IL-21 induced *Aicda* and *Cd86*, which are both critical for SHM and CSR (93, 202). Consistent with prior reports showing IL-21 driven *Aicda* and *Cd86* expression, and we further show that IL-21 induced *Aicda* and *Cd86* expression depends on T-BET (203, 204). Overall, IL-21 drives a gene expression profile most consistent with that of the GC B cell fate. Although overlap exists, the T-BET-dependent induction of *Cxcl10* by IFNγ and not IL-21 further confirms the notion that these two stimuli are distinct. Lastly, each cytokine represses a set of genes in a T-BET dependent manner. Although currently most of these genes have poorly defined immunological roles, *Trib3* has been shown to directly bind and disrupt to Akt kinase activity suggesting it may have a role in nutrient sensing. Overall, these transcriptional analyses further confirm that IFNγ and IL-21 drive distinct T-BET$^+$ fates.

Our findings reveal a T_{FH} cytokine network that governs T-BET$^+$ B cell fate decisions in the context of TLR stimulation. In accord with pervious reports, IFNγ

drives T-BET expression and is not appreciably influenced by the presence of either IL-4 or IL-21 (**Figure 13C**). Conversely, in the absence of IFNγ, IL-4 and IL-21 reciprocally regulate T-BET and CD11c expression both *in vitro* and *in vivo*. Since immune responses are rarely monolithic with regard to these three cytokines (140, 205), we suggest that the resulting multifunctional T_{FH} cells can generate a diverse set of B effectors. Consequently, altering the cytokine milieu affects the isotypes generated (**Figures 9C&D and 12C**) and the composition of the B_{MEM} pools (**Figure 9A&B and 12A&B**) while largely maintaining the magnitude of the response. Since T-BET^{+}CD11c^{+} B cells are observed in autoimmunity, viral infections, and aging, it is tempting to speculate that an underlying common feature is endosomal TLR engagement coupled with either copious IFNγ or abundant IL-21 with little IL-4. Indeed, both TLR7 and IL-21 deficiencies ameliorate disease in humoral autoimmunity models (206-209), and poor IL-4 production has been observed in T_{FH} from aged mice (210). Thus, understanding this interplay among IL-4, IL-21, and IFNγ might better define the etiology of humoral autoimmunity where ABC-like cells are observed (138, 187, 211, 212).

While it is clear that IFNγ and IL-21 differentially induce CD11c expression (**Figure 2**), the functional consequences of expressing this integrin remain elusive. Importantly, the restriction of CD11c expression to B_{MEM} cells is consistent with prior B_{MEM} subsetting studies in human tonsils and may thus define a tissue-homing population (185). While CD11c expression *per se* does not define tissue residency, future studies should assess whether altered integrin

and chemokine expression occurs on B_{MEM} cells generated in skewed cytokine milieus. Indeed, T_{FH} cells have been reported to produce every individual and combinatorial permutation of IL-4, IL-21, and IFNγ (140, 205). Thus, since a normal humoral immune response generates various pools of cytokine-skewed T_{FH} cells, they consequently drive a diverse array of B cell effectors. This assortment of effectors may be beneficial to host protection because adopting a singular strategy of immunity may be deleterious for the organism.

Finally, the most prominent B cell role, Ig production, is significantly affected in our infection models. In accord with previous reports, we observe that B cell intrinsic TBET expression during PR8 infection correlates with IgG_{2c} production in WT mice (111). However, even though *Ifng*$^{-/-}$ mice did not express TBET in B cells (**Figures 7B and 8B**), they produced as much PR8-reactive IgG_{2c} antibody as *Il4*$^{-/-}$*Ifng*$^{-/-}$ (**Figure 9C**) where prominent T-BET expression was observed. Thus, a substantial amount of IgG_{2c} is both IFNγ and T-BET independent. These observations are consistent with a prior report showing that TI-generated IgG_{2c} is T-BET-dependent whereas IgG_{2c} from TD responses is not (133). If the remaining IgG_{2c} PR8-reactive antibodies detected in *Ifng*$^{-/-}$ or *Il4*$^{-/-}$*Ifng*$^{-/-}$ mice are solely a result of a TD response, then the B_{MEM} clones and PR8 anti-sera in these mice should be of higher affinity than their WT counterparts. Although the HP infection experiments match current dogma regarding TBET expression and IgG_{2c} production (**Figure 12C**), these data also suggest that IL-21 can drive IgG_{2c} production independently of IFNγ. Again, how IFNγ and IL-21 drive different but related cell fates remains unclear but could reflect an

extrafollicular versus GC response. Overall, these studies expose a thus far poorly understood pathway to IgG_{2c} switching involving IL-21 and some transcriptional switch factor. Given role of IgG_{2c} autoinflammatory diseases (213), exploring how this pathway leads to IgG_{2c} production could underscore the etiology of some humoral autoimmune diseases.

Together, our *in vitro* and *in vivo* observations prompt a model in which the relative availability of IL-4, IL-21, and IFNγ govern the likelihood of establishing B_{MEM} cells expressing T-BET and CD11c. Further, they suggest that abundant IFNγ will drive a $T\text{-}BET^{+}CD11c^{-}$ B cell fate regardless of IL-4 or IL-21 levels, but that in the absence of IFNγ, the $T\text{-}BET^{+}CD11c^{+}$ fate will be reciprocally regulated by IL-21 versus IL-4 (**Figure 14**). Further, although both IFNγ and IL-21 induce TBET expression, IFNγ blunts IL-21-driven CD11c expression (**Figure 13C**). This reinforces the notion that IFNγ-induced TBET is refractory to the concomitant IL-4 or IL-21 signals, and therefore it is the dominant of the three cytokines for TBET induction.

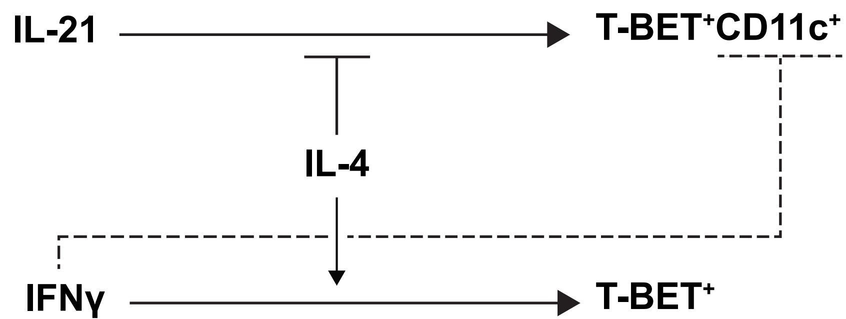

Figure 14. Model of IL-4, IL-21, and IFNγ interplay on T-BET expression. IL-21 uniquely induces both T-BET and CD11c expression. IL-4 blocks the capacity of IL-21 to drive TBET and CD11c expression but enhances the ability of IFNγ to induce T-BET. IFNγ either actively blocks IL-21-induced CD11c expression or dominantly drives T-BET expression alone.

3. ABCs IN PROTECTIVE AND AUTOREACTIVE RESPONSES

3.1 INTRODUCTION

3.1.1 Dysregulation of the immune system associates with age

Advancing age is accompanied by far-reaching shifts in immune system development and function, including reduced lymphopoiesis (214-217), blunted primary and recall immune responses (218-233), the appearance of autoantibodies (234-239), and increased frequencies of autoimmune and autoinflammatory conditions (240). Accordingly, shifts in the generation and relative representation of lymphocyte subsets have been scrutinized as potential mechanisms underlying these features. Within the T lymphocyte lineage, thymic involution, reduced T lineage specification, and an inversion in the ratio of naïve to memory T lymphocytes are established age-associated phenomena (215, 228, 236, 241-247), and more recent studies have linked these shifts to compromised vaccine and disease responses. Within the B lineage, similar reductions in early lineage specification and shifts in the sizes and kinetics of developing B cell pools have also been appreciated for some time (214, 216, 217, 222, 224, 226, 248-253). However, despite numerous clues indicating changes in the functional attributes of peripheral B cells with age (222, 223, 235, 252, 254-257), whether shifts in pre-immune and antigen experienced B cell subsets occur with age, as well as the functional consequences of such changes, remained unclear until recently. Within this context, a B cell subset with unique functional properties was identified and characterized.

3.1.2 ABCs emerge with age and have a unique surface marker phenotype

ABCs were first described in simultaneous reports from Hao et al and Rubstov et al, using distinct but largely complimentary phenotypic and functional criteria (194, 258) (reviewed in (259)). As their name implies, the ABC pool emerges in mid-life and continuously enlarges with advancing age. Thus, in healthy adult mice, ABCs are either absent or comprise a negligible proportion of peripheral B cells until about 12 months of age, then increase steadily in both proportion and number (194, 258-260). ABCs are observed in multiple inbred strains and F1 combinations, suggesting they are a common feature of the aging B cell pool (194). Although the tempo with which ABCs emerge varies considerably among individuals, they generally comprise 30-40% of mature recirculating B cells by 24-30 months of age (260). Moreover, ABCs tend to appear sooner and achieve higher numbers and representation in females (258), although the exact basis for this sex-associated dichotomy is unclear [discussed in (259)].

ABCs display a distinct surface phenotype in mice (**Table I**). They are mature B cells, as evidenced by their lack of CD93. Further, while positive for both B220 and CD19, they lack the canonical FO, MZ or B1 B cell markers CD23, CD21 and CD43, respectively. Detailed phenotypic, functional, and transcriptional analyses confirmed that ABCs differ from all previously defined B cell subsets. Whereas Hao et al (194) used this combination of features to define the population, Rubtsov et al (258) employed CD11c as a singularly characteristic marker. Thus, while the B cell population circumscribed by the

criteria in Hao et al includes the CD11c^{+} cells of Rubtsov et al, it also captures additional cells that lack CD11c. This distinction may underlie some apparent differences in functional attributes.

Anatomically, ABCs are found in the blood, spleen, and BM, but are rarely observed in lymph nodes (194). While these qualities suggest they are circulating cells, multiple observations suggest their trafficking and homing patters differ from FO B cells and other recirculating subsets. For example, expression of the follicle homing factor CXCR5 is reduced on ABCs (194), and more recent studies reveal they are enriched at the splenic T:B border, presumably reflecting migratory differences imparted by increased CCR7 expression (261). Based on these observations, it is tempting to speculate that ABCs may be involved in the age-associated alterations in MZ composition previously reported by Birjandi et al (262). Further, based upon their absence from the lymphatics, coupled with the lack of formal studies probing their circulatory properties, it remains possible that some ABCs are sessile tissue resident cells. In this regard, further phenotypic analysis of ABCs may reveal their origins and function in health and disease.

3.1.3 ABC activation and survival requirements differ from those of other B cell subsets

In addition to their distinct phenotype and localization characteristics, ABCs display unique activation requisites and functional attributes. Initial *in vitro* studies revealed that, unlike TR, FO, or MZ B cells, ABCs fail to proliferate after BCR cross-linking, but nonetheless survive (194). In contrast, they exhibit robust proliferative responses to stimulation via TLR9 or TLR7, and despite being

refractory to BCR cross-linking alone, concomitant BCR engagement potentiates their proliferative responses to TLR ligands (194, 258). Consistent with these observations, both TLR9 and TLR7 expression are elevated in ABCs (194). There is probably heterogeneity among ABCs in terms of relative responsiveness to TLR7 versus TLR9 ligation; cells included by the surface marker criteria of Hao et al show more extensive proliferation to TLR9 than to TL7 agonists, whereas cells defined by the criteria of Rubtsov et al display the reverse. Whether this reflects alternative routes of generation, different stages of the ABC differentiation pathway, or other distinctions remains unclear.

ABCs are unusual – and again unlike other B cell subsets - because they express both the BR3 and TACI receptors for BLyS but are independent of BLyS for their survival (194). Thus, BLyS neutralization *in vivo* spares ABCs, despite eliminating all FO and MZ B cells. Because ABCs display the same spectrum of BLyS receptors as FO and MZ B cells, they bind and sequester BLyS equivalently and can act as super-competitors for this limiting cytokine, inasmuch as they consume BLyS but do not need it to survive. Accordingly, as ABCs are generated and accumulate, they capture a progressively larger proportion of the BLyS-dependent niche at the expense of the FO compartment. Moreover, this capacity for BLyS-independent survival capacity is similar to what has been reported for B_{mem} cells, although currently defined B_{mem} pools do not express BR3 (263). Given the recent advances in delineating murine B_{mem} cell subsets (121, 264), exploring potential heterogeneity within the ABC pool may elucidate how ABCs fit into the broader immunological context.

3.1.4 ABCs exhibit a characteristic set of effector attributes

The unique functional attributes of ABCs extend to virtually all B cell effector mechanisms, including antigen presentation, cytokine secretion, kinetics of PC differentiation, and isotype switching preferences. Several studies have established that ABCs are effective APCs. Initial findings *in vitro* indicated that ABCs can serve as APCs, in accord with their comparatively high levels of MHC II, CD80, and CD86 (194). Further, ABC-mediated antigen presentation *in vitro* tends to skew naïve CD4 T cells to a T_H17 fate, although this was not exclusive of other CD4 T cell cytokine profiles (194). More recent findings indicate that ABCs are also potent APCs *in vivo* (261). When activated by either TLR7 or TLR9 agonists, ABCs secrete a variety of cytokines, including IFNγ, IL-4, IL-6, and IL-10. Finally, upon activation, ABCs rapidly differentiate to antibody secreting PCs and tend towards $IgG_{2a/c}$ class switching (137, 261), despite their broad surface IgM and IgD expression (194). Given their propensity to become antibody-secreting cells, ABCs may constitute a population of slowly accumulating B_{mem} cells generated in response to nucleic acid containing antigens. While it is unclear whether isotype or costimulatory molecule expression governs B_{mem} cell responses (119-121), most ABCs are unswitched and remain uncharacterized for PD-L2 and CD73 expression. Lastly, ABCs also express CD95 and CD138 based on microarray analysis (258), which are markers associated with the GC and PC cell fates respectively (see Table 1). Further characterization should distinguish the possibility of distinct ABC subsets or an atypical state of activation. *In toto*, ABCs effector mechanisms seem to be

largely normal, although skewed toward a type-I response.

3.1.5 ABCs are generated in response to intracellular infections

Early adoptive transfer studies revealed that FO B cells give rise to ABCs. In these studies, CFSE labeled FO B cells were transferred to congenic hosts and harvested 30 days afterwards, revealing that transferred cells which had extensively proliferated had acquired the ABC phenotype (194). This observation linked the generation of ABCs with extensive division, although the stimulating conditions driving the phenotype were unclear, and further functional characterization of these recently formed ABCs was hampered by the small cell numbers recovered in such experiments. Nonetheless, these findings suggest that ABCs are likely an antigen-experienced pool. Since B cell responses against viruses result in a protective, T-BET dependent $IgG_{2a/c}$ response, it was hypothesized that a viral infection would drive T-BET *in vivo*. Indeed, mice infected with gamma herpes virus, vaccinia, or LCMV all produced T-BET^{+}CD11c^{+} B cells (137). Of the TLR agonists used, TLR7 stimulation is the most effective at driving T-BET expression in the context of BCR and IFNγ stimulation. Lastly, B cell intrinsic T-BET expression played a non-redundant role in controlling viral and anti-viral $IgG_{2a/c}$ production. These findings have been extended to human studies where a subset of B cells in HIV-viremic individuals also display an ABC surface phenotype but are yet to be fully characterized for T-BET and CD11c (265). *In toto*, these data support the notion that ABCs are indeed antigen experience cells arising from immune responses characterized by nucleic acid containing antigens in the context of proinflammatory cytokines.

Given the similar nature of the antigens, nucleic acid containing self-antigens, like viruses, can drive a similar ABC-like program.

3.1.6 ABCs are associated with humoral autoimmune and autoinflammatory diseases

Beyond their initial identification in aged mice, ABCs prematurely accumulate in a variety of autoimmune prone mouse strains. A considerable ABC population accumulates as early as 6 months of age in mice lacking the tyrosine kinase, Mer, as well as in NZB/WF1 animals (258). In accord with these findings, ABCs constitute a higher proportion of total B cells in female RA patients (258). Moreover, before their detailed characterization in mice, ABC-like cells were also observed in common variable immunodeficient (212) and Sjögren's syndrome patients (187). It is unclear if the CD21$^{-/lo}$ cells in these individuals constitute a *bona fide* ABC population, since they were not characterized for CD11c, T-BET, or other subsequently appreciated ABC makers. In contrast, a more recent investigation of an early-onset Evan's syndrome patient exhibited premature senescence and an increased number of CD11c^{+}TBET^{+} B cells that correlated with antinuclear antibodies (211). Interestingly, duplicating the tripeptidyl peptidase II (TPP2) frame shift mutation in mice recapitulated both the disease and ABC phenotype. Thus, ABCs are implicated in both human autoimmune syndromes and in murine models of humoral autoimmunity. These finding prompt the question of how the dichotomous roles of ABCs – providing beneficial and appropriate effector functions during intracellular infection but also being associated with humoral

autoimmunity – can be mechanistically reconciled.

3.1.7 Summary of findings

In light of IL-21's novel TBET-inducing capacity described in the previous chapter, we sought to investigate whether the proposed model of cytokine interplay on $TBET^+$ B cell fates also holds true for ABC phenotype, formation, and presumed function. Thus, we hypothesized that ABCs would adopt a cell surface phenotype most compatible with B_{MEM} cells and accumulate into this antigen-experienced pool. Further, adoptive transfer studies suggest that ABC formation depends on MHCII antigen presentation and CD40 costimulation. Consistent with these results, we find that ABCs are somatically mutated. Although causal relationships between ABC formation and autoantibody production have not been established, we also observe that aged mice have increased serum $IgG_{2c,}$ and anti-dsDNA antibodies are primarily IgG_{2c}. Finally, T_{FH} cells in aged mice produce less IL-4, comparable IL-21, and more IFNγ than adult counterparts in response to alum immunization. The implications of this skewed T_{FH} cytokine profile and resulting effects on B cells are discussed.

3.2 MATERIALS AND METHODS

Mice

I-a$^{b-/-}$ C57BL/6 mice were gift from Dr. Terri Laufer; *Cd40*$^{-/-}$ spleens were shipped overnight from Dr. Mandy Ford's mouse colony; CD45.1 and CD45.2 C57BL/6, and NZBWF1/J mice were obtained from Jackson Laboratory. *Aid*$^{-/-}$ mice were bred at the National Institute on Aging (NIA). All animal protocols were reviewed and approved by the Animal Care and Use Committees at the NIA and the University of Pennsylvania.

Adoptive transfers

CD23^{+} splenic B cells were enriched by positive selection using MACS bead system (Miltenyi Biotec), subsequently labeled with CFSE (eBioscience) according to manufacturer's instructions, and 8 million were adoptively transferred into congenic hosts.

Flow cytometry

The same FACS reagents listed in chapter 2 were used with the following additions: CD45.1 (A20, BL), CD45.2 (104, BL), Fas/CD95 (clone Jo2).

Mutational Analyses

The following gating strategies were used for sorting: FO B cells, CD93 (AA4.1)$^{-}$ CD43^{-} B220^{+} CD21/35^{+} CD23^{+}; MZ B cells as CD93 (AA4.1)$^{-}$ CD43^{-} B220^{+} CD21/35^{+} CD23^{Lo}; and ABCs as CD93 (AA4.1)$^{-}$ CD43^{-} B220^{+} CD21/35^{-} CD23^{-}. Cells were lysed in Trizol and RNA was prepared. cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen). Immunoglobulin heavy (IgH) chain variable, diverse, and joining (VDJ) genes, and kappa light (Igκ) chain VJ

genes were amplified using Taq polymerase (TaKaRa, Clontech) with 5' degenerate primers specific to the framework 1 region of V genes and 3' primers located in the IgM or Igκ constant regions as previously described. PCR products were then cloned into the Strataclone TA cloning vector (Agilent Technologies) and sequenced. Only sequences with unique VDJ or VJ joins were counted. The sequences were blasted against the mouse Ig loci using IgBLAST from NCBI to identify V, D, and J gene segment usage and mutations. Lisa M. Russell Knode in the Gearhart lab at NIA performed these experiments.

Serum antibody titers and NP-specific ELISA

Same procedure as explained in chapter 2 except that ELISA plates were coated with 10 μg/ml NP_4-BSA, NP_{33}-BSA (Biosearch Technologies).

Quantitative PCR analysis

Same procedure as explained in chapter 2.

Statistics

Student's t-test was used to generate all P-values, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. All error bars are S.E.M. based on biological replicates.

3.3 RESULTS

3.3.1 ABCs are phenotypically heterogeneous and accumulate into B_{MEM} cell pools

Others and we previously established that ABCs are phenotypically distinct from other mature subsets and hypothesized that ABCs are a pool of antigen-experience, B_{MEM} cells (194, 259, 261). However, ABCs were reported to express CD95, which suggests a GC B cell phenotype. To interrogate this point, we performed a phenotypic analysis on ABCs using a variety of B cell activation surface markers. We find that ABCs bifurcate for CD95 (Fas) expression, stain highly for CD38, but are negative for PNA (similar results obtained with GL7) (**Figure 15A**). As a control, we used mature naïve B cells (FO and MZ) and GC B cells (background GCs from a young mouse). Thus, we confirmed prior reports of elevated CD95 expression, but the lack of PNA and GL7 suggests that ABCs are not *bona fide* GC B cells. Further, CD38 expression is consistent with a B_{MEM} cell phenotype since mature B and B_{MEM} cells express CD38 while GC B cells and PCs do not (266, 267). Overall, these data not only reinforce the idea that ABCs are phenotypically heterogenous, but also that they likely inhabit B_{MEM} cell pools.

In defining ABCs, other groups employed different gating strategies that included T-BET and CD11c. To assess how this phenotypic strategy differs from our own, we added these criteria to our FACS parameters. We find that our gating strategy encompasses T-BET^{+}CD11c^{+} ABCs as described by Rubtsov et al (258). Furthermore, T-BET and CD11c expression defines 3 distinct groups of

ABCs: T-BET$^-$CD11c$^-$, T-BET$^+$CD11$^-$, and T-BET$^+$CD11c$^+$ (**Figure 15B, left**). Thus, we asked whether T-BET and CD11c expression correlates with CD95, CD38, or IgM. The data show that all T-BET$^+$ ABC subsets express high levels of CD95 and CD38 regardless of CD11c levels, whereas the T-BET$^-$ subset is low to negative for both of these markers. Importantly, CD11c$^+$ ABCs had the lowest surface IgM expression, which is indicative of class switching (**Figure 15B, far right**). Overall, these data confirm that ABCs are phenotypically diverse with respect to isotype and canonical B cell activation markers. Further, the broad expression of surface IgM on ABCs previously reported can be attributed to distinct B_{MEM} cell subsets.

A hallmark of immunoscenece is the progressive accumulation of memory T cells with age (268); however whether B_{MEM} cells increase with age has not been interrogated. If ABCs are B_{MEM} cells that slowly accumulate over the lifetime of the host, we reasoned that the switched T-BET$^+$CD11c$^+$ ABCs would be a significant part of the B_{MEM} cell pool in aged mice. Using the phenotypic criteria described above, we observed an increased frequency of T-BET$^+$CD11c$^+$ into the switched, splenic B_{MEM} cell compartment using the gating strategy in **Figure 4A** (**Figure 15C**). Importantly, while the total number of B_{MEM} cells increases by 5-fold, we observed a 13- and 22-fold increase of T-BET$^+$ and -BET$^+$CD11c$^+$, respectively (**Figure 15 C & D**). Thus, these switched, T-BET$^+$CD11c$^+$ ABCs phenotypically resemble the IL-21 driven B_{MEM} cells described in the previous chapter.

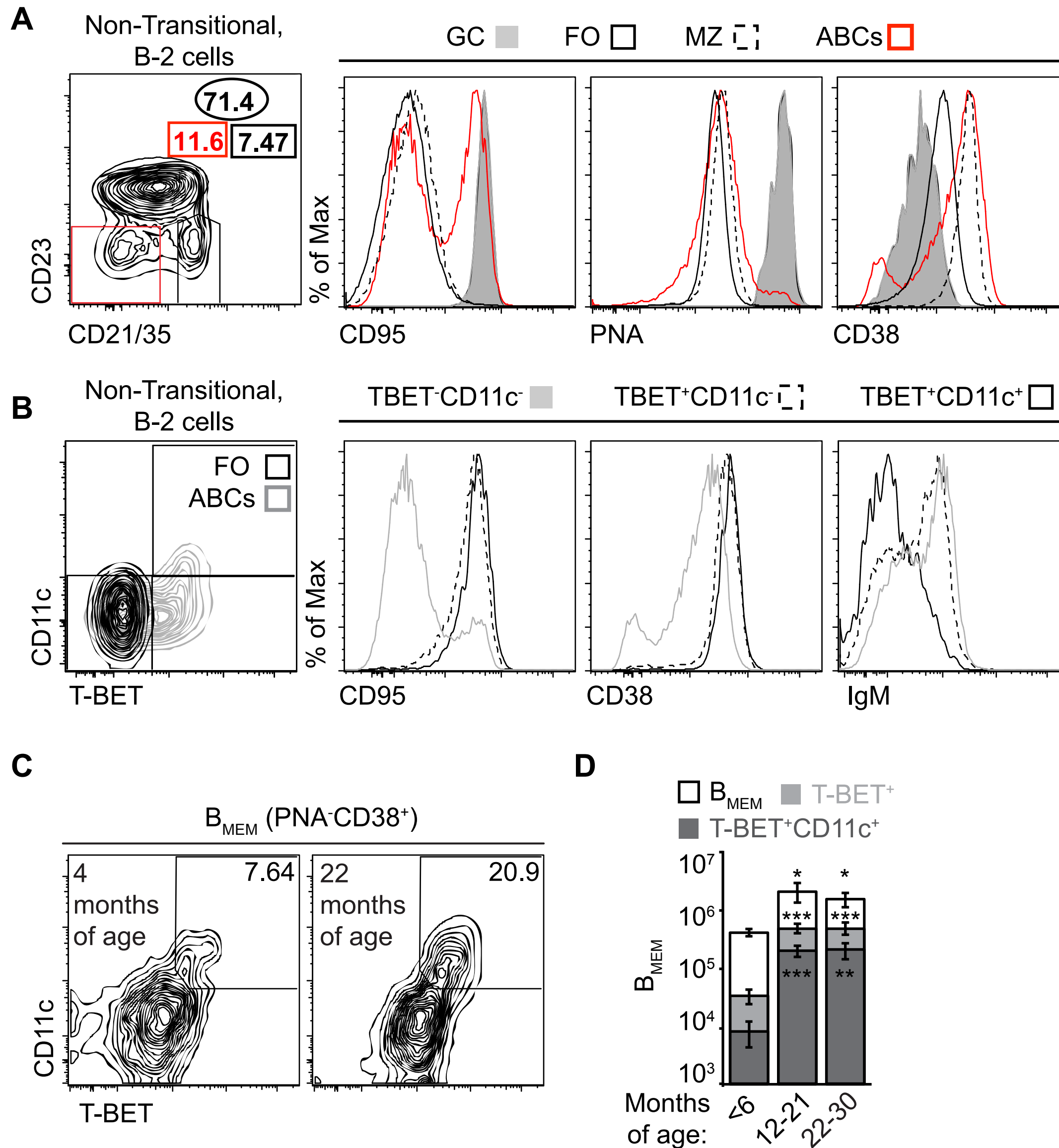

Figure 15. ABCs phenotypically resemble and accumulate into B_{MEM} cell pools. (**A**) Representative FACS plots for CD95, PNA, and CD38 stains on GC B, FO B, MZ B, and ABCs from 24 month old mice. GC B cells defined as $GL7^{+}IgM^{-}CD19^{+}B220^{+}CD138^{-}IgD^{-}DUMP^{-}$; FO B cells defined as $CD23^{+}CD21/35^{lo}CD93^{-}CD43^{-}B220^{+}CD19^{+}$; MZ B cells defined as $CD23^{lo}CD21/35^{+}CD93^{-}CD43^{-}B220^{+}CD19^{+}$; ABCs (in red) defined as $CD23^{lo}CD21/35^{lo}CD93^{-}CD43^{-}B220^{+}CD19^{+}$. (**B**) FO B cells and ABCs from (**A**) were further probed for T-BET and CD11c. n = 3 independent experiments with >3 mice per group. (**C**) Representative T-BET and CD11c staining on B_{MEM} cells from adult and aged mice gated as in **Figure 4C**. (**D**) Enumeration of populations in (**C**) across 3 independent experiments with 12, 10, and 11 mice aged <6, 12-21, and 22-30 months, respecively.

3.3.2 ABC formation requires MHCII and CD40 expression

We previously showed that ABC-like cells can arise from FO B cells following extensive *in vivo* expansion in adoptive hosts 30 days after transfer (194). In these experiments, the extensively divided cells downregulated CD23 and CD21/35 and thus resembled ABCs (**Figure 15A**). Reasoning that this is a proxy for ABC formation, we next tested whether surrogates of cognate help are critical for ABC formation. Thus, we transferred MHC class II (*I-a*$^{b-/-}$), *Cd40*$^{-/-}$, or WT B cells into WT congenic recipients. A month later, we observe a population of donor-derived (CD45.2) B cells (**Figure 16A**). Consistent with prior results, CFSE^{hi} cells represent the vast majority of transferred cells, whereas some B cells had extensively divided (CFSE^{lo}) and are low for CD23 (**Figure 16B, WT**). Moreover, these CFSE^{lo} B cells adopt an ABC phenotype, including expression of T-BET and CD11c (**Figure 16C, left panel**). In contrast, both MHCII and CD40 deficient B cells yielded fewer extensively divided cells (**Figure 16B**), and among these there was negligible TBET expression (**Figure 16C**). These results are consistent with the view that ABCs arise from B cells involved in TD immune responses because both antigen-presenting capacity and CD40 costimulation are required.

3.3.3 ABCs have undergone SHM and correlate with increased IgG_{2c} autoantibodies

The phenotypic similarity to IL-21 driven cell fates, accumulation into B_{MEM} cell pools, and requirement of cognate interactions to form suggests that ABCs originate from GC reactions. If so, then the V genes of ABCs should contain

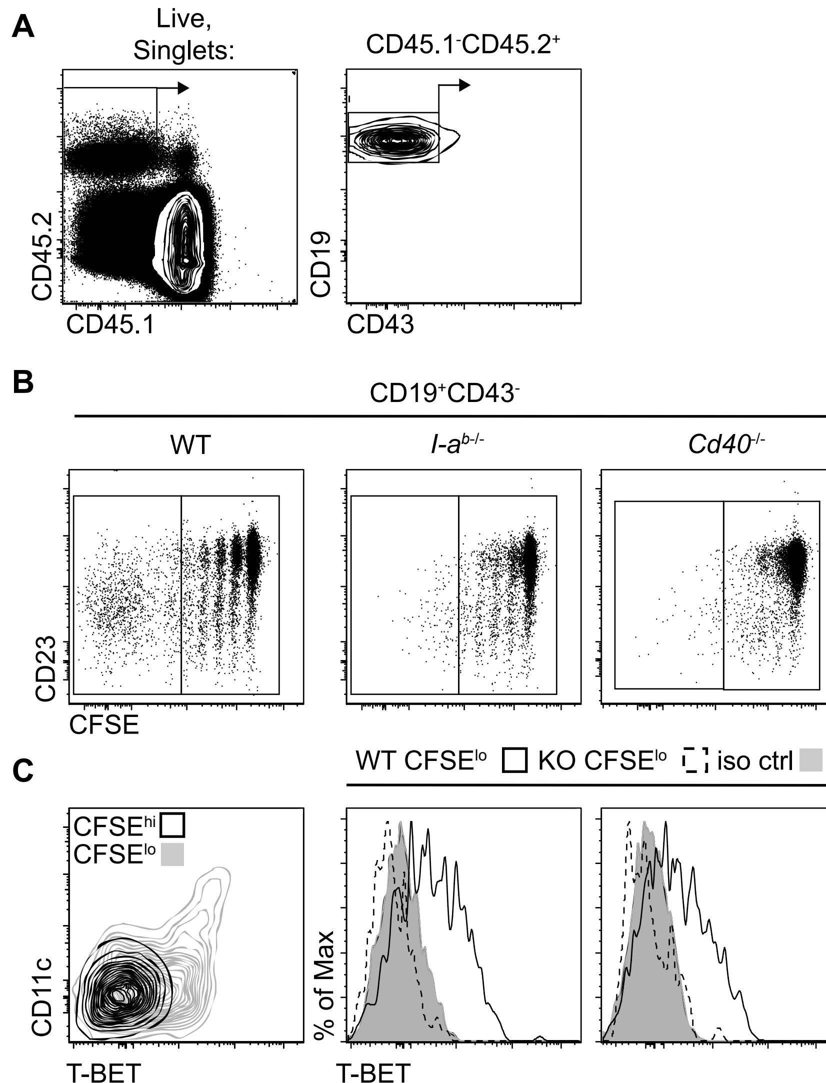

Figure 16. Interactions with *I-a*^{b} and *Cd40* drive the accumulation of ABCs. (**A**) CD23^{+} FO B cells from C57BL/6 mice (CD45.2) were labeled using CFSE and adoptively transferred into young congenic CD45.1 hosts. Recipient mice were analyzed one month later. Shown is a representative plot of the gating strategy of live, singlets of the recipients (CD45.1) and donors (CD45.2), and (**B**) representative dot plots showing the dilution of CFSE in wild type (n=19), *I-a*$^{b-/-}$ (n=11), *Cd40*$^{-/-}$ (n=17) mice across 3 independent experiments. CFSE^{lo} defined as more than 4 divisions. (**C**) Histograms show staining for T-BET in indicated populations.

increased frequencies of mutations compared to other subsets. In collaboration with the Gearhart lab, we counted the number of mutations in VDJ and VJ_{κ} exons amplified from sorted FO, MZ, and ABC B cell subsets, and compared the V, D, and J sequences to their germline counterparts to identify mutations. Sequences from ABCs had a significant, four-fold increase in mutations compared to FO cells and a significant two-fold increase compared to MZ cells (**Figure 17A**). As a control, V exons were sequenced from FO and MZ cells from young *Aid*$^{-/-}$ mice, which do not undergo hypermutation. The mutation frequency was approximately $2x10^{-3}$ mutations per bp for *Aid*$^{-/-}$ cells, which represents the background frequency of errors produced during cDNA synthesis and PCR amplification. Thus, ABCs have undergone AID-mediated somatic mutation.

In addition to SHM, AID also mediates CSR. Since B cell intrinsic T-BET expression fosters switching to IgG_{2c}, we reasoned that the accumulation of T-BET^{+} B_{MEM} cells would correlate with increased serum IgG_{2c} in aged mice. Indeed, analysis of serum IgG subtypes reveals that serum IgG_1 concentrations remain unchanged between young and aged mice; however, IgG_{2c} is nearly 10-fold higher in aged mice (**Figure 17B**). Finally, the emergence of increased serum anti-DNA autoantibodies titers is another well-documented hallmark of immunosenescence (234). Thus, we reasoned these autoantibodies in aged mice would also be of the IgG_{2c} isotype. Using the autoimmune prone mouse, NZ(BxW)F1 as a positive control, we observe that most of the dsDNA reactive antibodies are of the IgG_{2c} rather than IgG_1 isotype in aged mice, whereas young mice served as negative controls for both isotypes (**Figure 17C**). Overall, these

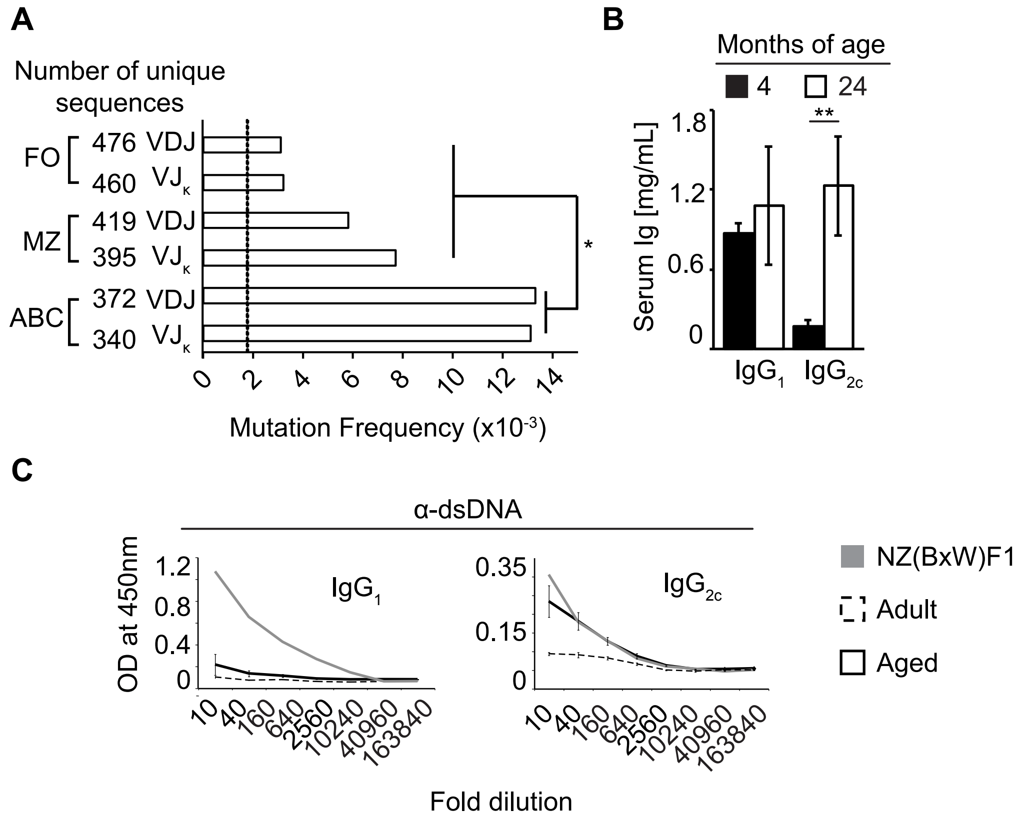

Figure 17. ABCs undergo SHM and correlate with IgG_{2c} autoantibody. (**A**) Only unique sequences were analyzed for mutations in the V, D, and J gene segments. Mean mutation frequencies (mutations/nucleotides) in the VDJ and VJκ exons of each B cell subset were calculated. The dotted line indicates the background mutation frequency in *Aid*$^{-/-}$ FO and MZ B cells from young mice. * = P < 0.0001, Chi-squared test. (**B**) Total serum IgG_1 and IgG_{2c} in 4 (n=11) and 24 (n=15) month old mice. (**C**) dsDNA reactive IgG_1 and IgG_{2c} antibody titers in aged (n=6), adult (n=5), and NZ(BxW)F1 (n=1) mice.

observations are consistent with the idea that ABCs are likely a population of somatically mutated, class-switched, B_{MEM} cells arising from interactions with IL-21 and IFNγ producing T_{FH} cells. However, there is no clear causal connection between the increase of both T-BET^{+} ABCs and IgG_{2c} autoantibodies.

3.3.4 T_{FH} cells produce less IL4 and more IFNγ in aged mice

As discussed previously, T_{FH} cells instruct antibody isotype determination via cytokine production. Therefore, given the skewing towards IgG_{2c} observed in aged mice, we asked whether these T_{FH} cells are skewed in their cytokine production profile. We reasoned that T_{FH} cells in aged mice would produce less IL4 or more IL21 and/or IFNγ. Therefore, we immunized mice with NP-OVA precipitated in aluminum salts, which drive a robust IgG_1 antibody response (although other isotypes are also produced) against the immunizing agent via the production of IL-4 from T_{FH} cells. Using the gating strategy shown in **Figure 8A & 11A**, we sorted T_{FH} cells from immunized mice. Consistent with prior aging reports, we observed a substantial decrease of naïve, CD62L^{+} CD4 T cells in aged mice compared to adults (**Figure 18A**). Next, we probed for IL-21, IL-4, and IFNγ mRNA from sorted T_{FH} cells. The data show that while IL-21 mRNA levels remained similar between aged and adult mice, T_{FH} cells in aged mice produced far fewer IL-4 and substantially more IFNγ transcripts (**Figure 18B**). These data suggest that the resulting antibody response would be skewed towards IgG_{2c} rather than IgG_1 in aged mice; however, this prediction does not hold true. Instead, consistent with many prior reports, the humoral immune response in aged mice is severely blunted. Using high (NP_{25}, **Figure 18C**) and

low (NP_4, **Figure 18D**) substitution ratios of hapten-carrier conjugates, we can detect low and high affinity antibodies, respectively. Thus, while adult mice produced affinity matured NP-reactive antibodies of both isotypes, aged mice produced far fewer antibodies of all affinities and isotypes. Thus, even though T_{FH} cells produce equivalent amounts of IL-21 between young and aged mice, reduced IL-4 production is not sufficient to drive IgG_{2c} in aged mice as observed in *Il4*$^{-/-}$ mice and HP infection (**Figure 12C**).

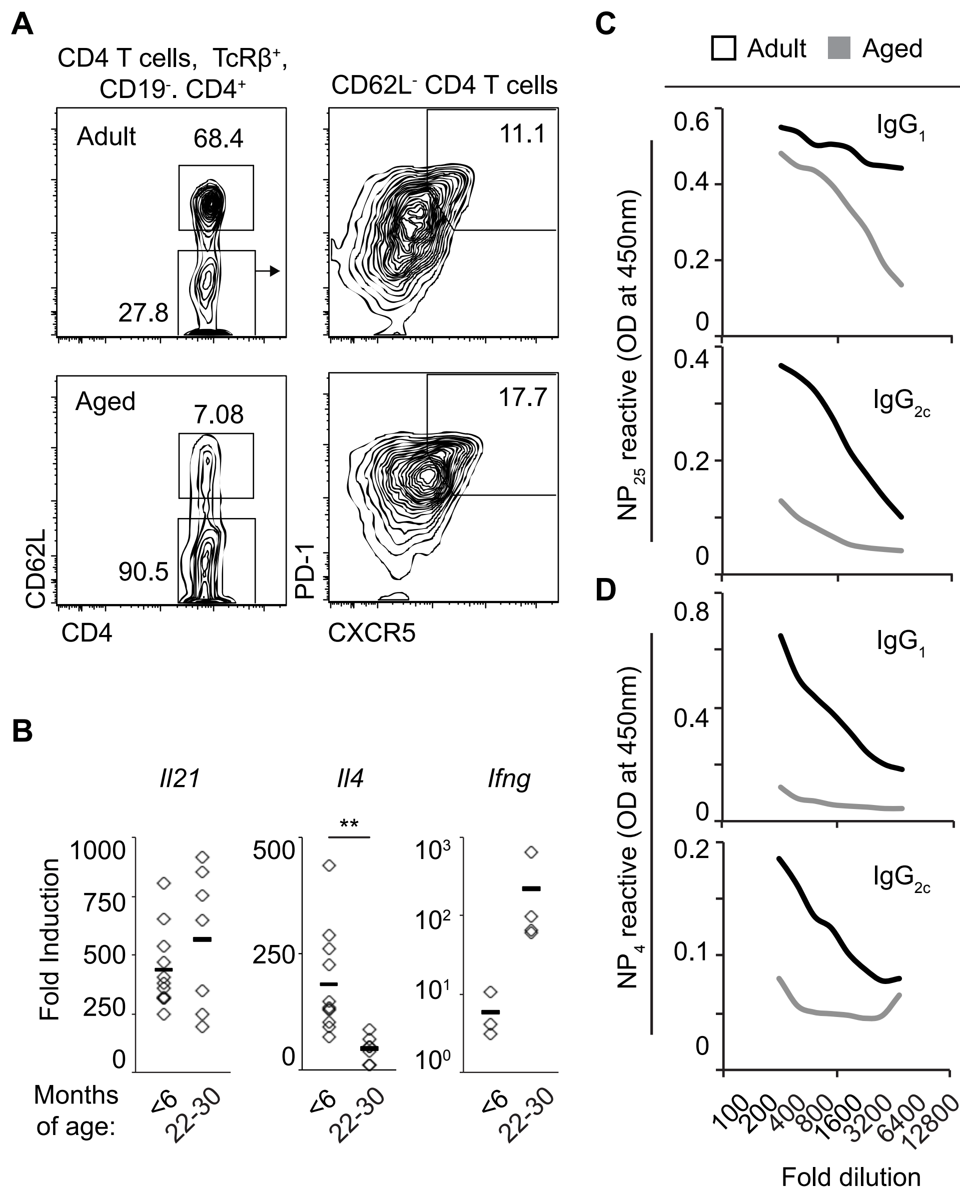

Figure 18. Aged T_{FH} cells produce less IL-4 and more IFNγ. (**A**) FACS stained splenocytes from day 14 post NP-OVA immunized adult and aged mice. (**B**) Gene expression analysis ($\Delta\Delta C_t$) for *Il21*, *Il4*, and *Ifng* from sorted T_{FH} cells from (**A**). Serum titers for (**C**) NP_{25} (low affinity) and (**D**) NP_4 (high affinity) reactive IgG_1 or IgG_{2c} antibodies from (**A**).. Figures are represenatative across 3 experiments with ≥ 3 mice per group.

3.4 DISCUSSION

Together these data suggest that ABCs constitute a pool of antigen-experienced, B_{MEM} cells that tend to express TBET. Phenotypic analysis reveals that ABCs are heterogeneous and accumulate into B_{MEM} cell compartments. Consistent with this idea, we used a proxy of ABC generation and determined that their formation is dependent on both antigen presentation via MHCII and costimulation through CD40. Moreover, the marked increase of somatic mutations in VDJ and VJ_{κ} exons further confirms that ABCs require T cell help for their formation and may originate from GC reactions. Furthermore, consistent with its role in class switching, T-BET expression in ABCs correlates with increased total and anti-dsDNA IgG_{2c}. Finally, cytokine profiling of T_{FH} cells in aged mice reveals that they lose the capacity to produce robust IL-4 but make substantial IFNγ. However, the antigen-specific humoral response is significantly reduced and not skewed towards the IgG_{2c} isotype as the T_{FH} cytokine profile would suggest. Overall, these data suggest that ABCs constitute a pool of B_{MEM} cells and require IL-4-limited T cell help to form.

Phenotypic analysis of ABCs provides important clues about their presumed function and origin. Using well-defined markers associated with GC B and B_{MEM} cell fates (**Table I**), we find that ABCs express CD95, but not PNA or GL7 suggesting they are not GC B cells (**Figure 15A**). Moreover, high expression of CD38 is consistent with a B_{MEM} cell phenotype, and CD95 expression has been previously reported on B_{MEM} cells (269). Importantly, $TBET^{+}$ ABCs uniformly express CD95. While we speculate that CD95

expression may functionally sensitize ABCs to FasL-mediated cell death, many other survival factors may overcome this extrinsic apoptosis pathway (270). Furthermore, initial ABC phenotypic analysis revealed that they bear broad surface IgM expression (194). Dividing them further by T-BET and CD11c reveals that the $TBET^{+}CD11c^{+}$ subset is mostly negative for IgM (**Figure 15B**). Although it is unclear whether this is due to a TD or TI response, the data indicate these ABCs have undergone CSR. Consistent with this, $T\text{-}BET^{+}CD11c^{+}$ ABCs accumulate into the switched B_{MEM} compartment (**Figure 15C & 15D**). Overall, these phenotypic data support the notion that ABCs are a heterogeneous population of B_{MEM} cells. However, the origins and necessary cellular interactions for ABC formation remain unclear.

When first described, ABCs were found to be sensitive to sub-lethal ionizing radiation and did not rebound from aged progenitor B cells in the BM (194). Moreover, ABC accumulation was proportional to a decrease in mature FO B cells. Thus, ABCs were thought to originate from mature B cell pools. Adoptive transfers of FO B cells into congenic hosts revealed that extensive division was sufficient to generate an ABC phenotype (i.e. $CD23^{-}CD21/35^{-}$). Positive staining for T-BET and CD11c further confirmed that these extensively divided B cells phenotypically resemble ABCs (**Figure 16C**). Importantly, extensive division and T-BET staining were largely abrogated when B cells could not present antigen or receive costimulation. In these cases, some cells did divide likely reflecting homeostatic expansion. Assuming that this assay is a proxy of ABC generation, these data indicate that cognate interactions between T

and B cells are required for ABC formation. In addition to the mutational analysis (**Figure 17A**), we further conclude that ABCs may originate from GC reactions. However, recent reports highlight an important role for the extrafollicular response in autoimmune etiology (271, 272). These responses also require IL-21 and MHCII expression on B cells for SHM and disease progression (273, 274). Therefore, since ABCs tend to accumulate more quickly in these autoimmune mice, both the extrafollicular and GC responses must be considered as sources of ABC generation. While the data presented here cannot distinguish between the two possibilities, we suggest that ABCs are a product of cognate T cell interactions with limited IL-4 and abundant IFNγ and IL-21.

A long-standing observation of age-related immune dysfunction is the emergence of autoantibodies (234). Although murine autoantibodies were reported to be IgG (237), subtype analysis was never performed. Thus, it is tempting to speculate that the increase in total and anti-dsDNA IgG_{2c} antibodies is due to T-BET^{+} ABC accumulation (**Figure 17B & 17C**); however, currently these associations are purely correlative. While the tempo of ABC accumulation dramatically increases in autoimmune prone mice, *bone fide* ABCs in otherwise healthy aged mice are yet to be rigorously tested for overt self-reactivity. Nonetheless, if ABCs constitute a B_{MEM} cell pool of autoreactive specificities, then it would also be worthwhile to investigate whether they maintain autoantibody titers by feeding into the PC compartment. Moreover, the preferential accumulation of IgG_{2c} autoantibodies is important because the type of IgG generated has profound effects on subsequent antibody effector function.

Indeed, while antigen-binding capacity is crucial, the heavy chain isotype determines functionality via binding to FcγRs, which are expressed on a variety of myeloid cells and enhance effector functions such as ADCC, phagocytosis, and opsonization (14). Moreover, FcγRs can be inhibitory or activating depending on whether the cytoplasmic tail bears an ITIM or ITAM, respectively (275). Importantly, IgG_{2c} binds to all 3 activating and 1 inhibitory FcγRs whereas IgG_1 only binds to one of each (276). Therefore, the result that most anti-dsDNA antibodies in aged mice are IgG_{2c} (**Figure 17C**) suggests that myeloid cells receive increasingly proinflammatory signals when DNA-IgG_{2c} complexes engage ITAM-bearing FcγRs receptors. Although the lack of standards makes absolute quantification impossible, whether the nearly 10-fold increase of total serum IgG_{2c} in aged mice is significantly composed of autoreactive specificities is an important future consideration (**Figure 17B**).

Overall, these data indicate that ABCs likely represent a pool of B_{MEM} cells resulting from cognate interactions with IFNγ or IL-21 producing T cells. Because these cytokines may originate from T_{FH} cells, we wanted to know whether T_{FH} cells in aged mice have a cytokine production profile that is permissive to the ABC phenotype. Indeed, T_{FH} cells from aged mice made less IL-4 and equivalent IL-21 to adults (**Figure 18B**), and thus we reasoned that the resulting NP-reactive antibodies would be IgG_{2c} instead of IgG_1 as we had observed with HP infection in *Il4*$^{-/-}$ mice (**Figure 12C**). Unsurprisingly, the most consistent result was a diminished antibody response both in terms of quantity and affinity regardless of isotype (**Figures 18C** & **18D**). While it is difficult to

dissect cause and effect, there exist a number of possibilities. We cannot conclude that aged T_{FH} cells are intrinsically incapable of producing IL-4, since improper priming in the aged microenvironment could also explain these data. Importantly, T_{FH} cells require two independent antigen presentation events from both a DC and B cell to adopt full effector status (87). Thus, currently it is impossible to know whether the aged T cell itself, DC, or B cell explain the decrease in IL-4 and robust IFNγ production. However, when used as APCs *in vitro*, ABCs have been shown to aberrantly skew T cells (194); therefore, it is tempting to speculate that ABCs may contribute to this shift in cytokine production *in vivo*. Lastly, aged T_{FH} cells made substantially more IFNγ than adults. It is documented that anti-IFNγ treatment reinvigorates the aged humoral response back to adult levels (277), thus we consider that excess IFNγ in aged mice may hinder the GC response. Overall these data indicate that T_{FH} cells in aged mice have a skewed cytokine profile, but that does not correlate with expected antibody isotype outcomes.

4. PERSPECTIVE

ABCs impact a broad spectrum of immunological phenomena, including protective immunity to some pathogen classes, immunosenescence, and autoimmunity. In mice, ABCs constitute an accumulating population of B cells with unique surface phenotype, signaling properties, and effector status. While initially described in aged mice, phenotypically and transcriptionally identical cells are observed in humoral autoimmunity and immune responses against intracellular pathogens in both mice and humans. These observations lead us to propose that ABCs are a memory B cell population, and that the signals and interactions that yield ABCs in both normal and autoreactive humoral responses are comparable; these consist of concomitant signals from the BCR, nucleic acid sensing TLRs, and inflammatory cytokines. Thus, understanding how responses to endogenous versus exogenous nucleic acid-bearing ligands are regulated in order to maintain tolerance yet allow appropriate responses to pathogens should yield insights relevant to both protective and autospecific humoral immunity.

Inasmuch as ABCs were first detected by virtue of their progressively increasing representation with age, the question arises as to whether they contribute to age-associated alterations in immune activity and immune responsiveness. This seems likely based on their unique spectrum of activation requirements, antigen presenting capacity, and effector cytokine profile, and several recent findings support this notion.

First, recent studies suggest that ABCs may be instrumental in the decreased B lymphopoiesis associated with age. Ratliffe *et al* assessed the

proportion of ABCs in mice at different ages, and found that the degree of B lymphopoiesis depression was proportional to ABC representation with age (260). Moreover, they showed that ABCs are a potent source of TNFα, and that this cytokine was in part responsible for inhibiting the survival of B lineage precursors *in vitro* and *in vivo*. These observations, coupled with the ability of ABCs to occupy homeostatic space to the detriment of preimmune FO B cells, are particularly intriguing in light of reports that B lymphopoiesis and robust humoral responses can be rejuvenated in aged individuals by B ablative regimes (278-282).

Second, recent studies have revealed aspects of T_{FH} generation that rely on cognate B cell interactions differ in young and aged mice. For example, several laboratories have shown that optimal IL-21 and IL-4 production among dendritic cell-primed T_{FH} cells only occurs following antigen presentation by B cells (87, 283, 284). However, under *in vivo* immunization conditions that normally foster this T_{FH} profile, aged individuals fail to generate T_{FH} cells with the characteristic upregulation of IL-4 (210). While causal links have not yet been forged, it is tempting to speculate that ABC antigen presentation may foster at T_{FH} cytokine profile that lacks IL-4. Alternatively, T_{FH} cells generated in aged individuals may intrinsically skew towards a cytokine profile lacking IL-4, which might be instrumental in driving ABC generation.

Third, in addition to the decline of B lymphopoiesis and functional T cell alternations, an increase in anti-dsDNA, anti-nuclear, and other autoantibodies occurs in both mice and humans (234, 237, 285, 286). Indeed, ABC

accumulation correlates with serum autoantibody levels, and ABCs isolated from aged mice and stimulated with a TLR7 agonist produced anti-chromatin (258). While not PCs, ABCs could constitute a pool of B_{MEM} cells that feed a population of autoantibody secreting PCs. Thus, while ABCs accumulation has a number of correlative associations with B cell development, altered T cell functionality, and autoantibody production, causality is yet to be established.

ABCs in aged, virally infected, and autoimmune-prone mice and humans share a common triumvirate of stimuli for their origin: BCR signals, nucleic acid sensor ligation, and a type-I inflammatory cytokine milieu. This commonality is likely the link that connects beneficial and pathogenic ABC formation. In particular, the role of endosomal nucleic acid sensors driven by BCR-acquired antigens may prove the most important feature. Over the past decade the contribution of nucleic acid sensing PRRs, particularly TLR7 and TLR9, to the etiology of autoimmune disease have come to forefront. Beginning with the seminal observation that BCR delivered nucleic acid ligands have distinct survival and proliferative potentials for B cells (287), it has become increasingly apparent that TLR7 and TLR9 both promote and regulate the progression of humoral autoimmunity. To test this idea, Shlomchik's group bred SLE-prone mice to either TLR7 or TLR9-deficient mice (208, 288). Because extra *Tlr7* gene copies results in an SLE-like disease (289), it was expected that TLR7 deficiency would ameliorate disease in autoimmune-prone mice. While this hypothesis was indeed validated, TLR9 deficiency unexpectedly exacerbated disease (206, 208). This suggests not only a regulatory role for TLR9 but also differential disease

outcomes based on whether autoreactive B cells can sense RNA or DNA moieties. These ideas are further confounded by the fact that TLR9 deficiency in other models of autoimmunity ameliorates disease (290). Though the mechanism by which TLR9 limits while TLR7 promotes disease remains unclear, recent findings suggest a role for type-I interferon signaling for disease etiology (291). Whether ABC formation occurs in these models of autoimmunity and whether TLR7 and TLR9 modulate ABC accumulation is yet to be established. Thus, the signals from the BCR, nucleic acid sensing TLRs, and inflammatory cytokines – while exactly those involved in appropriate ABC-mediated immunity – are exactly those that, when dysregulated, foster humoral autoimmunity.

While the interplay of IL-4, IL-21, and IFNγ dictate the assumption of T-BET^{+}CD11c^{+} B cells in the context of TLR engagement, the functional consequences of making such cells remains elusive. Our infection and aging studies reveal that ABCs phenotypically resemble B_{MEM} cells; however, *bona fide* B_{MEM} cell function is not shown. Adoptive transfers of T-BET^{+}CD11c^{+} B_{MEM} cells from PR8 primed *Il4*$^{-/-}$*ifng*$^{-/-}$ mice (**Figure 9A**) into naïve recipients followed by lethal PR8 challenge would begin to address whether these cells are sufficient to mediate protection. If the recipients survive, then ABC-like cells observed in viral infection do indeed constitute a functional pool of B_{MEM} cells. Moreover, challenge with a heterologous virus may also elucidate a non-redundant role for these cells independent of high affinity antibody. Indeed, it is suggested that B_{MEM} cells are less somatically mutated than their PC counterparts and are thus more promiscuous in their specificity. In this regard, the established PCs and

antibodies are blind to escape variants. Thus, B_{MEM} cells may be better suited to protect the host against variants of the original infectious agent.

T-BET expression may also provide further clues about ABC function. While T-BET's role in fostering CSR to $IgG_{2a/c}$ is well-established in B cells, other studies (133) and our data show that a substantial amount of PR8-specific IgG_{2c} is independent of T-BET (**Figure 9C**). Thus, while B cell intrinsic T-BET expression is necessary to control viremia (137), T-BET may be orchestrating other key cellular programs independent of CSR to $IgG_{2a/c}$. Indeed, T-BET has been shown to drive many cellular processes including proper chemotaxis, metabolism, maintenance, and homeostasis (111, 129, 131, 132, 292). Our transcriptionally analysis (**Figure 3B**) confirm T-BET's pleiotropic effects since many genes involved in trafficking and metabolism are affected by T-BET deficiency in addition to those necessary for SHM and CSR. Thus, does T-BET deficiency alter B_{MEM} cell trafficking patterns or homeostatic maintenance? To answer this question, $Cd19^{Cre/+}Tbx21^{f/f}$ mice infected with PR8 would reveal whether protective B_{MEM} cells traffic properly to sites of infection without T-BET, i.e. the lung. Moreover, establishing a pool of T-BET^{+} B_{MEM} cell pool via PR8 infection in hCD20-TamCre *x* $Tbx21^{f/f}$ mice followed by tamoxifen-mediated deletion of T-BET would address their maintenance requirements. Furthermore, long-term studies could also assess whether T-BET^{+} B_{MEM} cells are necessary for the maintenance of PR8 specific PC numbers and antibody titers. In each experiment, adoptive transfers B_{MEM} cells from PR8 immune mice where T-BET has been deleted into naïve recipients would be necessary to prove whether they

are protective independent of pre-established antibody titers. Thus, while our data establish a role for IL-21 in mediating this cell fate, more studies are necessary to determine functionality.

These observations and future directions lead us to propose the model depicted in **Figure 19**. In general, any nucleic acid containing antigen harbors the capacity to drive an ABC phenotype. However, growing evidence suggests that T cell derived proinflammatory cytokine signals, as well as cognate help, may be required to engender a long-lived ABC fate. In this regard, an activated B cell presenting viral peptides can efficiently receive all three signals required for recruitment into long-lived effector subsets. In contrast, autoreactive B cells binding nucleic acid containing self molecules, such as apoptotic debris or other sources, while receiving BCR and TLR signals will fail to receive further cues for survival, resulting in short-lived responses or death. However, inadvertent or aberrant recruitment of such short-lived autoreactive cells into long-lived effectors would thwart this peripheral tolerance system, and yield sustained autoantibody and auto inflammation driven by $IgG_{2a/c}$ production. Multiple routes to such dysregulation could be envisioned, including overabundance of autoantigen or deficiencies in the molecules that mediate these regulatory circuits.

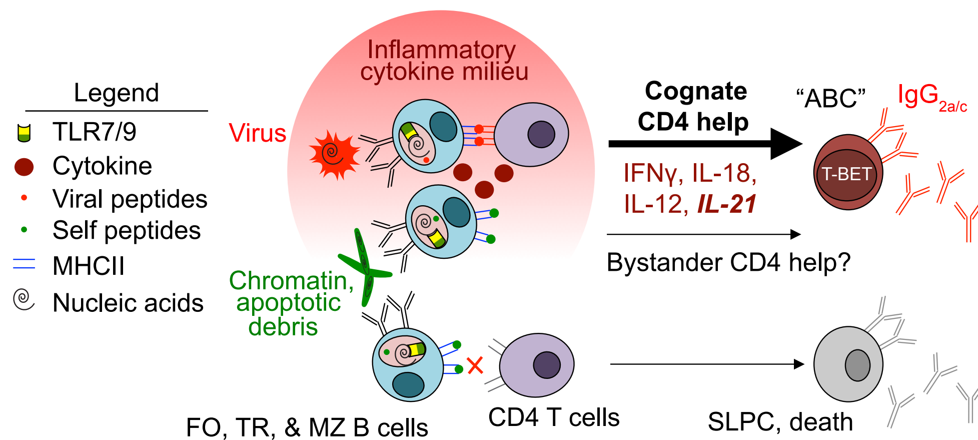

Figure 19. Both beneficial and pathogenic ABCs arise via a common triad of signals. Preimmune FO, MZ, and/or TR B cells bind, internalize and traffic antigens to endocytic compartments via the BCR. Pathogen degradation leads to viral nucleic acids engaging TLR7 or TLR9. Processing and loading of viral peptides onto MHCII molecules results in cognate CD4 T cell interactions and T cell derived inflammatory cytokine secretion. The combination of these signals leads to T-BET$^+$ memory and effectors, and to $IgG_{2a/c}$ isotype switching. Normally, B cells that internalize nucleic acid containing self-antigens fail to survive because they lack the cognate T cell interactions and appropriate cytokine signals to further differentiate and survive. However, inadvertent or aberrant receipt of these signals can afford survival of these self-reactive cells and enable their recruitment into a long-lived memory and effector ABC pool.

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