Manipulating Ig production in vivo through CD180 stimulation

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A dissertation
submitted in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy

University of Washington

2012

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Program authorized to offer degree:

Molecular and Cellular Biology

University of Washington

Abstract

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CD180 is homologous to TLR4 and regulates TLR4 signaling, yet its function is unclear. This thesis reports that injection of anti-CD180 mAb into mice induced rapid polyclonal IgG, with up to 50-fold increases even in immunodeficient mice. Anti-CD180 rapidly increased transitional B cell number in contrast to anti-CD40 which induced primarily FO B cell and myeloid expansion. Combinations of anti-CD180 with MyD88-dependent TLR ligands biased B cell fate toward synergistic proliferation. Thus, CD180 stimulation induces B cell proliferation and differentiation, causing rapid increases in IgG, and integrates MyD88-dependent TLR signals to regulate proliferation and differentiation.

This thesis also reports that targeting Ag to CD180 rapidly induces Ag-specific IgG. IgG responses were robust, diverse, and partially T cell-independent, as both CD40- and T cell deficient mice responded after CD180 targeting. IgG production occurred with either hapten- or OVA-conjugated anti-CD180, and was specific for Ag coupled to anti-CD180. Simultaneous BCR and CD180 stimulation enhanced activation compared to

either stimulus alone. Adoptive transfer experiments demonstrated that CD180 expression was required on B cells but not on DCs for Ab induction. Surprisingly, Ag-targeting was also efficient in BAFF-R KO mice despite their lack of mature B cells. Ag-anti-CD180 induced rapid and robust expansion of Ag-specific B cells with a germinal center phenotype and differentiation to plasma cells. Mice preimmunized with Ag-anti-CD180 displayed Ag-specific IgG forming cells when boosted, demonstrating that Ag-anti-CD180 induces immunologic memory. A weak but significant memory response was evident even in CD40 KO mice. Targeting Ag to CD180 may provide a benefit in therapeutic vaccination or for the immunocompromised.

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List of Abbreviations

Ab	**A**nti**b**ody, or antigen-specific immunoglobulin
AFC	**A**ntibody **F**orming **C**ells
APC	**A**ntigen **P**resenting **C**ells
APRIL	**A** **Pr**oliferation **I**nducing **L**igand
BAFF	**B** cell **a**ctivating **f**actor, BLyS / TNFSF13B
BCR	**B**-**c**ell **r**eceptor for antigen
BrdU	**Br**omo**d**eoxy**u**ridine
CFA	**C**omplete **F**reund's **A**djuvant
CFSE	5,6-**C**arboxy**f**louresceindiacetate **s**uccimidyl **e**ster
CGG	**C**hicken **g**amma **g**lobulin, chicken IgY
CI	**C**ombination **I**ndex, Chou & Talalay's quantitative measure of
DC	**D**endritic **c**ell
FO	**Fo**llicular
HEL	**H**en **e**ggwhite **l**ysozyme
IFN	**I**nter**f**ero**n**
Ig	**I**mmuno**g**lobulin
IL-	**I**nter**l**eukin
i.p.	**I**ntra**p**eritoneal
ITAM	**I**mmunoreceptor **t**yrosine-based **a**ctivation **m**otif
ITIM	**I**mmunoreceptor **t**yrosine-based **i**nhibitory **m**otif
LPS	**L**ipo**p**oly**s**accharide
i.v.	**I**ntra**v**enous, tail-vein injection
mAb	**M**onoclonal **a**nti**b**ody
MFI	**M**ean **f**lourescence **i**ntensity
MHC	**M**ajor **h**istocompatibility **c**omplex
MyD88	**My**eloid **d**ifferentiation primary response gene **88**
MZ	**M**arginal **Z**one
NiP-	4-hydroxy-3-**i**odo-5-**n**itro-**p**henacetyl-
NP-	4-hydroxy-3-**n**itro-**p**henacetyl-
OVA	Chicken eggwhite **ova**lbumin, Serpin B14
p.i.	**P**ost **i**njection
PBS	**P**hosphate **b**uffered **s**aline
RPMI	**R**oswell **P**ark **M**emorial **I**nstitute
SPF	**S**pecific **p**athogen **f**ree

T1	**T**ransitional **1**
T2	**T**ransitional **2**
TACI	**T**ransmembrane **A**ctivator and **C**AML **I**nteractor / TNFRSF13B
TCR	**T c**ell **r**eceptor for antigen
TD	**T**hymus-**d**ependent/**T** cell-**d**ependent
Th1	**T h**elper cell type **1**
Th2	**T h**elper cell type **2**
TI	**T**hymus-**i**ndependent/**T** cell-**i**ndependent
TIR	**T**oll/**I**L-1-**r**eceptor
TRIF	**TIR**-domain-containing adapter-inducing **i**nter**f**eron-β
TLR	**T**oll-**l**ike **r**eceptor
TNF	**T**umor **n**ecrosis **f**actor
WT	**W**ild **t**ype (parent strain, either C57BL/6 or BALB/c)

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Acknowledgements

My sincere appreciation to Professor Edward Clark for believing in both my ability and character, and especially for opening his laboratory to me. I am also indebted for the support and direction of Professor Jeffrey Ledbetter during the early investigation of CD180's polyclonal effects.

I also wish to thank Kevin E. Draves for managing the mouse colony and making the impossible happen regularly, Sarah Grewal, and Amy E. Steadman for excellent laboratory assistance, and Rohan Damle for help with synergy analysis.

This thesis would never have been completed without the constant encouragement and support of my wife Pauline.

Chapter 1: Introduction

1.1 Lymphocytes require co-stimulatory signals for activation

The immune system has the remarkable ability to defend against a diversity of microbial pathogens, yet not normally respond to self despite the continual presence of self-antigens. The discovery of the lymphocyte antigen (Ag) receptors (B cell and T cell receptors for antigen, BCR and TCR respectively) provided an explanation for the specificity and diversity of both humoral and cellular responses. In experiments with lymphocytes from naïve animals, stimulation of these receptors by cognate antigen induces activation and transition from the resting G_0 state of the cell cycle into the G_1 state, however unless the Ag was administered with pathogen derived molecules (LPS, CFA, etc.) activation and proliferation were aborted. Furthermore, studies (1) have suggested that antigen alone is not sufficient to drive the full activation of naive T cells; these findings eventually led to the two-signal model of lymphocyte activation. According to this model, naïve lymphocytes require engagement of their Ag receptors (signal 1) together with a costimulatory receptor (signal 2) to fully differentiate into effector cells. In addition, seminal studies (2, 3) demonstrated that TCR-mediated activation of T cells in the absence of this 'costimulation' resulted in antigen-specific unresponsiveness (termed anergy), rendering the cells unable to respond to subsequent Ag exposure. Conversely, the addition of costimulatory signals was sufficient to convert anergy-inducing responses to immunity-inducing responses (4). Thus, costimulation was postulated to have a pivotal role in determining whether the outcome of lymphocyte encounter with Ag would be activation or anergy.

The critical role of costimulation in regulating the immune response is of both fundamental biological and therapeutic interest. Early studies using naïve T cells defined the function of the CD28 receptor (5), and identified the B7 family members CD80 (B7-1) and CD86 (B7-2) as ligands for CD28 (6-8). The interaction between CD28 and CD80/CD86 fulfilled many of the requirements for the costimulatory signal postulated by Lafferty, Schwartz and colleagues (2, 3). The CD28 homolog cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) (9) was later found to be a higher affinity-binding partner for CD80 and CD86, compared with CD28 (10). It was presumed that CTLA-4 would also be a stimulatory receptor; however, the dramatic fatal inflammatory phenotype of CTLA-4-deficient (KO) mice revealed the critical inhibitory function of CTLA-4 (11, 12). Furthermore, the phenotype of CTLA-4 KO mice demonstrated that these signaling networks included mechanisms to block costimulation and halt immune responses that had run their course as a means to limit damage from chronic inflammatory or autoimmune processes.

While the CD80/CD86:CD28/CTLA-4 pathway is the best characterized costimulatory pathway, there are now many additional costimulatory pathways recognized for both B and T lymphocytes. These pathways fall into several major families: the immunoglobulin (Ig) superfamily, which includes the B7/CD28-like grouping; the tumor necrosis family (TNF)/TNF receptor superfamily, which includes the CD40L/CD40 pair; the pattern recognition receptor (PRR) superfamily, which includes the leucine-rich repeat toll-like receptor (TLR) family; and soluble mediators such as cytokines that signal to further enhance or skew the responses of effector cells. Until the discovery of the innate immunity-mediating TLRs, most costimulation literature was

focused on pathways propagating a cell-fate decision of whether B cell or T cell responses led to anergy or to effector responses and immunologic memory (e.g. presence or absence of B7 family costimulation on APCs), but little was known about how costimulatory pathways are primed during first contact with Ag.

1.1.1 Examples in T and B cell activation pathways

After discovery of the B7/CD28 system, similar mechanisms were sought for licensing of effector functions in lymphocyte populations. Analogous to the B7 family on APCs activating T cells and enhanced by this action (32), a similar process was uncovered where CD40L, which is transiently expressed on activated T cells, provides costimulatory signals through CD40 to B cells (13, 14); CD40 ligation induces B cell survival after BCR stimulation, class switch recombination, somatic hypermutation, and differentiation into memory B cells (15-26). Both mice deficient in CD40 or CD40L (27, 28) and humans with similar deficiencies (hyper-IgM syndrome, 29-31) demonstrate immunopathology with the same hallmarks. Regardless of the number of doses or formulation used, vaccination attempts in hyper-IgM patients consistently fail to produce Ag-specific IgG, affinity maturation of Ab, or immunologic memory (31).

The TNF receptor associated factor (TRAF) family of signaling adaptors largely mediates these functions of the CD40 pathway (22). Originally thought to be specific to TNF receptor family members, TRAF adapters are sporadically used in other signaling pathways (33), but CD40 signaling utilizes all but one of the six known TRAF family members (34, 35). Oligomerization induced by ligand binding directs TRAFs 2, 3, and 6 to bind directly to the cytoplasmic tail of CD40 and induce signaling via p38 MAPK, Akt, JNK and NF-κB (36). TRAF2 mediates Ig production/secretion and proliferative

effects via activation of the NF-κB pathway (37-40); TRAF6 mediates anti-apoptotic (survival) effects through phosphoinositide-3-kinase (PI3K) activation of the PKB/AKT kinase as well as induction of IL-6 production, upregulation of CD80, affinity maturation, and CD40-mediated rescue from CD95-induced apoptosis (33, 41-45). TRAF3, in contrast, appears to play a negative regulatory role by restraining the NF-κB2 signaling pathway (46). Thus, as demonstrated by patients with hyper-IgM syndrome, the costimulatory function of CD40 is considered to be essential for effective humoral immunity. As activated T cells are a primary source of CD40L, this costimulatory pathway is an essential component of T cell regulation of B cell and DC effector functions.

Further experiments with blocking costimulation in models of autoimmunity and transplantation (47-53) and studies in which costimulatory signals were added to poorly immunogenic self-Ag as cancer therapies (54-56) underscored the therapeutic potential of regulating costimulatory pathways.

While these studies demonstrated a need for additional signals along with Ag and how costimulatory signals are propagated between activated and naïve leukocytes, it was uncertain what provided the initial costimulatory signals required during initial (primary) Ag encounter. For instance, the CD28 costimulation required to fully activate T cells required the prior upregulation of either CD80 or CD86 while ligation of APCs by CD40L on activated T cells drove the upregulation of CD80 and CD86 (57, 58); however, neither CD86 nor CD40L are expressed on resting lymphocytes, so it was unclear exactly how this “reciprocal amplification” cycle was primed. Certain compounds (LPS, CFA, alum; collectively termed adjuvants) were known to produce this

priming effect and regularly used in inducing robust immune responses but it was unclear what specific receptor or pathway mediated this initial priming.

1.1.2 Inflammation as a priming co-stimulatory signal

A feature shared by many adjuvants is that they all cause either local or systemic inflammation, characterized by a combination of pain, heat, redness, and swelling. These are a nearly invariant result of innate immune responses, occurring with most instances of acute inflammation resulting from infection or adjuvant administration. The programming of adaptive immune responses by inflammatory processes has been recognized as early as 1884, when it was reported that coincident infections with *Streptococcus pyogenes* sometimes led to spontaneous cancer remissions (59). These observations led William Coley to administer extracts of heat killed *S. pyogenes* and *S. marcescens* under the term "Coley's toxins" for the treatment of cancer between 1893 and 1963 with mixed success. While remissions were documented they were infrequent, the treatment was harsh, and there was no clear mechanism as to how a high fever would cause elimination of the cancer that had previously grown unfettered in the host. Recent reexamination of the approach has implicated bacterial components as priming inflammation and delivering initial costimulatory signals (60, 61), thereby converting an anergic response to tumor-associated antigens into an effective anti-cancer immune response.

1.2 Structure/function of TLR family members and the homolog CD180

The innate immune response is the first line of defense against pathogenic microbial organisms (62-64). After recognition of pathogens and their products, the innate immune system responds to produce a variety of antimicrobial peptide 'defensins' (65) and

various proinflammatory cytokines (66), and to induce adaptive immune responses (67, 68). An important consequence of innate immune responses is polyclonal activation of B cells leading to the production of germline-encoded, polyreactive antibodies that are believed to be an essential part of the first line defense against systemic bacterial and viral infections (69).

As LPS was long known to induce inflammation and prime for adaptive immune responses as an adjuvant, several groups utilized naturally occurring mutants to isolate the innate immune receptor responsible for LPS signaling. Positional cloning of a LPS unresponsive strain of mice, C3H/HeJ, revealed a point mutation of proline at codon 712 to histidine in the signaling domain of TLR4 (70, 71). Another LPS non-responsive mouse strain, C57BL10/ScCr, lacked the entire genomic region of TLR4 (71). This was confirmed by the generation of mice lacking TLR4 by gene targeting (72), which were non-responsive to LPS.

The first reported human Toll-like receptor was described in 1994 (73) and mapped to a chromosome in 1996 (74). Because the immune function of Toll in *Drosophila* was not then known, it was assumed that TIL (now known as TLR1) might participate in mammalian development as Toll did for Drosophila. However, in 1991 (prior to the discovery of TIL) it was observed that a molecule with a clear role in immune function in mammals, the interleukin-1 (IL-1) receptor, also had homology to drosophila Toll; the cytoplasmic portions of both molecules were similar (75). First linked to innate immunity in mammals in 1997 (76, 77), the TLRs are one class of innate immune receptors that recognize invariant molecular patterns unique to entire classes of microbes. Such pathogen-associated molecular patterns (PAMPS) are found in essential structural

components of bacteria and viruses such as the membrane components LPS, bacterial lipoproteins, and lipoteichoic acids; the flagellin required for microbial motility; and non-eukaryotic nucleic acids such as unmethylated bacterial CpG DNA or viral RNA (78). Different pathogen-associated molecular patterns are recognized by different TLR, e.g., LPS is recognized by TLR4; unmethylated CpG DNA is recognized by TLR9; and flagellin is recognized by TLR5. Most TLRs are plasma membrane-bound receptors, but a few, such as TLR3, -7, and -9, are found in the endosomes. All TLRs share common structural features including extracellular domains with leucine-rich repeats and a highly conserved cytoplasmic domain consisting of the Toll/IL-1 receptor (TIR) domain that is critical for signal transduction. The single known exception is the orphan TLR family member, CD180, also known as Bgp-95 or RP105, which is a homolog of the extracellular domain of TLR4 but does not contain a TIR domain and cannot signal through the common TLR adaptor molecules MyD88 or TRIF (79).

1.2.1 Ligand-driven dimerization leads to accumulation of adapters

Signaling by TLRs requires ligand-induced dimerization of the extracellular domains that drives the recruitment of various intracellular signaling adaptors to the scaffold of paired cytoplasmic TIR domains (80, 81). Indeed, gain-of-function mutations naturally occurring in Drosophila Toll are produced by unpaired cysteines in the extracellular domain that lead to constitutive dimerization (82). This paired-TIR domain scaffold mediates interactions with either or both of two intracellular TIR-containing adapter molecules: MyD88 and its facilitator MyD88 adaptor-like (MAL), or TIR domain-containing adaptor inducing IFN-α (TRIF) and its facilitator TRAM (83-85). Various other adapter molecules such as the IL-1R-associated kinases (IRAKs), TRAF6,

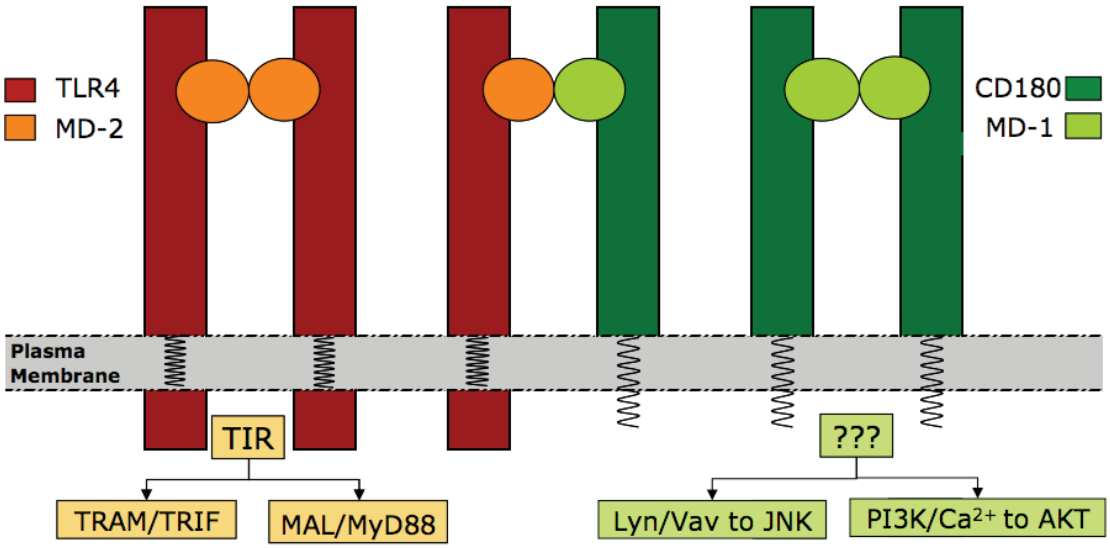
and TRAF-associated NFκB activator-binding kinase 1 can then associate with specific receptor complexes, leading to the activation of IFN regulatory factor 3 (IRF3), p38 MAPK, other MAPK pathways, and NF-κB (86). The two primary adapters, MyD88 and TRIF, mediate different responses: MyD88 recruits TRAF6 and largely drives production of pro-inflammatory cytokines (IL-6, IL-12 and TNFα) while TRIF recruits both TRAF3 and TRAF6 to drive interferon production (87). Inclusion of TRAF6 in the TLR signaling pathway mediates their proliferation and anti-apoptotic functions as it does for CD40 stimulation. The activation of these pathways then regulates the expression of cytokine receptors and other effector genes. However, the full nature of the signaling pathways used by TLRs to stimulate the release of cytokines and chemokines is still not fully understood.

1.2.2 CD180 is specifically homologous to but distinct from TLR4

While the TLR family is diverse, with significant and even dispersal from the common ancestor TLR gene (88), each individual TLR family (e.g. TLR2 vs. TLR4) clusters tightly with little variation between organisms as diverse as humans and chickens. This is ascribed to strong selective pressure to maintain recognition of the PAMPS, however this rationale breaks down with respect to CD180 and TLR4.

Among the reported TLR family members, TLR4 and CD180 have unique structural characteristics in that they associate with molecules which are required for their function, MD-2 and MD-1 respectively (Fig. 1.1, 89, 90). Indeed, it is the MD-2 protein that directly binds LPS in the TLR4 complex. Additionally, while there is high variability in both length and sequence between TLR families (88), the extracellular domains of CD180 and TLR4 both contain 613 amino acids with a 61% similarity and 29% identical

Figure 1.1



sequence (as determined by ClustalX utilizing UniProt sequences Q9QUK6 [murine TLR4] and Q62192 [murine CD180], original sequence published in 91). Based on this similarity the CD180/MD-1 complex was postulated to bind LPS and form signaling heterodimers with TLR4/MD-2 (92). Yet the CD180/MD-1 complex cannot bind LPS, as MD-1 contains a binding pocket incapable of containing LPS (93). Crystallographic studies have consistently found poorly packed endogenous lipids from the producing cell line in the MD-1 binding pocket but all attempts to load it with pathogen-related lipids have failed and both hexa-acyl and penta-acyl LPS forms are too bulky to fit (94, 95). Multiple attempts have been made to stimulate CD180 with LPS (90) or pulldown CD180 with biotinylated-LPS (79) however all have failed, while attempts co-immunoprecipitate TLR4 and CD180 are unsuccessful without overexpressing both with added affinity tags (79). As CD180/MD-1 clearly does not bind LPS and whether it can form a heterodimer with TLR4 under physiological conditions in undocumented, it is unknown why it has maintained such high homology to TLR4 in the face of different

ligand specificity. Even TLRs 7 and 8 which both recognize RNA are more divergent than CD180 and TLR4 (88). Additionally, as CD180 lacks the TIR domain required for TLR signaling, any heterodimer formed with a TLR would not be capable of signaling via the adaptors TRIF and MyD88 as the required paired scaffold of TIR domains would not be formed (79). This difference in the signaling domains led to a second postulated function, that CD180/MD-1 formed heterodimers with TLR4/MD-2 to block excess signaling induced by LPS (79). Both postulates have supporting data and several shortcomings.

CD180/MD-1 cannot bind LPS and there is no known ligand. Consequently, all studies on stimulation of CD180 have been done using agonistic anti-CD180 mAbs that force dimerization and induce signaling in the absence of natural ligand. Conversely, mAbs to the MD-1 accessory protein of CD180 seem to either induce the wrong orientation of induced dimers or fit between the bound complexes such that activation of CD180 is blocked (81). When stimulated by anti-CD180 primary B cells and certain B cell lines (A20) respond with a MyD88 independent signal (96). While the proximal signaling adaptor that interacts with CD180's six amino acid tail is unknown, the tyrosine kinase / calcium flux cascade is similar to that of the BCR. Various experiments have demonstrated involvement of the following components commonly linked to BCR stimulation: Src-family kinase Lyn, Bruton's tyrosine kinase (Btk), CD19, CD45, Protein Kinase C (PKC β1/2), guanine nucleotide exchange factor for the Rho family (Vav1), Phosphoinositide-3-kinase p110δ, Protein Kinase B (PKB/Akt1), Phospholipase Cγ2, Mitogen-activated protein kinase kinase (MEK), Mitogen-activated protein kinase 1 (MAPK1 / ERK2), the c-Jun N-terminal kinases (JNK1/2), and NF-κB (96 - 102).

1.3 CD180 regulates B cell activation

It is not surprising that CD180 ligation drives proliferation and survival of B cells since it delivers signals known to induce proliferation (NF-κB pathway) and enhance cell survival (Akt kinase pathway). These two responses were used in hybridoma screens to establish the mAbs against human and mouse CD180 (97, 103); these reagents were then used to the determine N-terminal amino acid sequence of CD180 then clone the gene (91) and generate CD180 deficient mice (92).

While rescuing B cells from radiation or dexamethasone induced apoptosis had been established as an effect of ligatingCD180 with mAbs, a single report placed CD180 as a regulator of BCR-induced apoptosis. When mouse B cells were treated with anti-IgM their viability decreased if they had been pre-treated with anti-CD180 but not if they had been pretreated with anti-CD40 (104). This observation was not extended with co-treatment, however, in the initial report of CD180, it had been shown that stimulating human dense tonsillar B cells with both anti-IgM and anti-CD180 resulted in a synergistic increase in proliferation and survival (103).

Several approaches seemed to validate the postulate that CD180/MD-1 acted as an alternative or co-receptor specifically for LPS. At this time no structural data on the ligand-binding pocket was available and it was not known that TLRs required dimerization of TIR domains to signal, making it an attractive idea based on extracellular domain homology alone. To test the impact of CD180 on LPS induced signaling both CD180 KO and MD-1 KO mice were generated, however, after determining that the phenotypes of these mice were equivalent, only CD180 KO mice were examined (89, 92). These mice have normal spleen sizes and distributions of B cells with no basal

differences other than reduced serum IgG3 levels compared to WT mice of the same background. CD180 KO mice do indeed have a selective defect in their Ab response to LPS-based T cell independent type-1 (TI-1) Ags and transfection of Ba/F3 cells with both TLR4 and CD180 resulted in greater NF-κB activation following LPS stimulation than either alone. While this effect was initially reported to be specific for LPS signals and therefore, for CD180's homolog TLR4, later CD180 was reported to be required for optimal signaling through TLR2 as well (105). This study did not discuss the original homology-based heterodimer model as TLR2, and proposed no further model to explain the observed signaling enhancement. Ten years after the initial claim of specific heterodimerization of CD180 with TLR4, a TLR with low homology to TLR4 as well as a different ligand-binding domain and cellular localization, TLR9, was found to interact with CD180 as stimulation of both receptors resulted in far greater B cell proliferation that either stimulus alone (106). It is clear that CD180 signals influence the outcome of signals from multiple TLRs, despite the low likelihood of physical association, implying that CD180 has a functional rather than physical interaction with TLRs. While CD180 expression clearly impacts the function of multiple TLRs, it is difficult to compare the effects as each study utilized a different readout.

Recent studies have implicated marginal zone (MZ) B cells in the impact CD180 has on responses to TI-1 Ag (107). IgM production of sorted primary mouse MZ and follicular (FO) B cells was compared following stimulation with lipid A (the active component of LPS), anti-TLR4 mAb, anti-CD180 mAb, or both mAbs together. While the combination of both mAbs stimulated equivalent proliferation in both B cell subsets, only the MZ B cells significantly expressed CD138, a marker of plasmablast

differentiation. Interpreting this distinction between MZ and FO B cells is complicated as no attempt to distinguish or remove "immature" or transitional B cells was made in these experiments. Multiple reports demonstrate that transitional 1 (T1) B cells rapidly convert to IgM secreting AFCs after either TLR or combined TLR/BCR stimulation (108, 109). Furthermore, despite claims that the combination of anti-TLR4 and anti-CD180 substitutes for an intact LPS (or lipid A) signal, both B cell proliferation and IgM production were two to three fold greater than that produced by saturating concentrations of lipid A.

Multiple attempts were made to implicate CD180 as a constitutive source of low-level (or "tonic") B cell activation signals using CD180 KO mice crossed to autoimmune disease-prone mice. A role for CD180 in tonic, TLR-independent signaling was supported by experiments using CD180 KO mice crossed with MRL/lpr mice, which demonstrated a slightly slower progression of disease compared to MRL/lpr controls; however, while splenomegaly and blood urea nitrogen were improved in the CD180 KO/MRL/lpr mice, they displayed no changes in either levels of autoantibodies or glomerulonephritis (110). Additionally, CD180 deficiency had no effect on NZB x NZW F1 mice, a model of systemic lupus erythematosus (SLE, 110) that more closely recapitulates SLE pathology than the MRL/lpr model. Conversely, CD180 KO mice backcrossed onto a DBA/1 background and treated with collagen in CFA displayed an accelerated development of collagen induced arthritis, a model of rheumatoid arthritis, worsened symptoms and increased autoantibody production (111). Differences in these models - defective autoreactive lymphocyte apoptosis in MRL/lpr mice, lowered activation thresholds and loss of tolerance in NZB x NZW F1 mice, or the action of CFA

- make comparisons difficult; nevertheless, there is no clear and simple role for CD180 common to these autoimmune models.

While abandoned by the "LPS co-receptor" proponents, the homology-based heterodimer model is currently used to explain CD180-induced suppression of LPS signaling. This model postulates that when LPS binds TLR4/MD-2 and induces dimerization, it can either homodimerize with another TLR4 to signal effectively or heterodimerize with CD180/MD-1 and thus fail to signal; in this case, CD180 would function as a decoy or sink for LPS and reduce inflammatory responses (79). This model was tested both in transfected cell lines and in CD180 KO mice. Independent transfection of CD14, TLR4, MD-2, CD180, and MD-1 into HEK293 cells led to less activation of NF-κB following LPS stimulation than with HEK293 cells transfected with only CD14, TLR4, and MD-2 (79). However, there are a number of technical concerns with this study including: 1) the high total number of independent transient transfections; 2) the difference in the number of transfections between the groups; and the absence of irrelevant sequence transfections to control for transfection toxicity effects. The authors also reported co-immunoprecipitation between CD180/MD-1 and TLR4/MD-2 but with overexpression of all components in epitope tagged formats under non-stoichiometric conditions. Studies attempting to immunoprecipitate CD180/MD1 and TLR4/MD-2 from primary cells have been universally negative (92). CD180, however, was unambiguously shown to restrain cytokine production, as both bone marrow-derived dendritic cell cultures treated with LPS and CD180 deficient mice inoculated with LPS produced significantly more TNFα, IL-6 and IL-12p70 than wild type (WT) equivalents (79).

A study attempting to extend the homology-based TLR4-suppressing heterodimer model of CD180 to the role of CD180 in infection was inconsistent. CD180 KO or WT mice were infected with Leishmania major, which requires TLR4 for efficient control (112, 113); while low dose infection resulted in worsened symptoms and faster disease progression in CD180 KO mice than in WT, the opposite result was observed with high dose infection (114).

Experiments have also been conducted to try to reconcile the conflicting roles of CD180 in both promoting B cell proliferation and Ig production in response to LPS with CD180 suppressing DC cytokine production in response to LPS. B cells from WT and CD180 KO B cells were adoptive transferred into B cell deficient (μMT) recipients; eight weeks later the proliferation induced by LPS was measured and the defect in CD180 KO-derived MZ B cell proliferation to LPS was significantly reduced. However this increased proliferation of CD180 KO MZ B cells following transfer may also be due to the fact that both genotypes of MZ B cells (WT and CD180 KO) had expanded fifty fold more than FO B cells demonstrating that they were already proliferating (115). With the discovery that CD180 KO mice have higher constitutive levels of BAFF, a B cell stimulatory cytokine, the authors ascribed the defective B cell response to LPS in CD180 KO mice to changes in DC cytokine production and concluded that CD180 had no intrinsic effect on B cells. This is logically inconsistent, as increases in BAFF would be expected to lead to increased activation and proliferation of B cells rather than defects in proliferative responses. In addition, this group also asserts, in spite of significant evidence to the contrary, that there is no direct evidence of CD180-induced signaling in B cell lines. While no signaling was observed in Ba/F3 cells transfected with CD180/MD-1, clear

phosphorylation of eight different signaling components was observed after CD180 ligation of the A20 B cell line (96).

Throughout the over 20 years of investigations into the function of CD180, nearly all experiments have been done comparing the absence of CD180 to WT; despite the availability of an agonistic mAb. At the beginning of the work described in this dissertation, no studies had been reported examining the role of CD180 activation in vivo or the effect of combining CD180 and TLR signals. Additionally, as $CD180^{+/-}$ (heterozyogote) mice have not been used in the published studies and only at most two doses of TLR stimuli have been reported, there are no data on the dose-dependence of signaling components for the interactions observed between CD180 and various TLRs. These are significant knowledge gaps for a cell surface receptor that may play a significant role in both B cell activation and the regulation of innate immune stimuli that regulate both advantageous and autoimmune responses.

1.4 Antigen targeting strategies for generating immunity

As CD180 has an effect on B cell activation and IgG3 production, we also considered targeting antigens (Ags) to CD180. As discussed here Ag targeting is the process of delivering Ag to target leukocytes by covalently attaching the Ag to a mAb specific for receptors on the cells of interest (116). Upon binding the Ag-mAb is internalized and the Ag processed for presentation. In some cases the mAb may also deliver a stimulatory or inhibitory signal as another effect of ligating its target. These approaches can sometimes reduce the dose of Ag required for effective immunization by 100 to 1,000 fold and may direct Ag into certain processing and presentation pathways in order to tailor the outcome of the response to Ag. This is a distinct approach from using mAbs to deplete cell

populations (such as targeting B cells with Rituximab, 117, 118) or from genetically modifying certain cell populations by directing engineered viruses to them via mAbs incorporated into the viral envelope (119, 120).

1.4.1 Ag-targeting can enhance humoral or cellular immunity

To date, Ag has been targeted to over 40 different surface molecules with strikingly diverse results. Seemingly small changes in the target, even within the same cell type, can produce immune responses that are qualitatively completely different. For instance, the receptors Dectin-1, DEC205, DCIR2 and Clec9A are all expressed on DCs, yet delivery of Ag to Dectin-1 induces strong CD4+ T cell and weak Ab responses if administered with adjuvant (121); targeting to DEC205 induces strong CD8+ T cell responses with adjuvant but low Ab responses (122); targeting to DCIR2 in the absence of adjuvants generates strong CD4+ T cell and extrafollicular IgG1 Ab responses (123); and targeting to Clec 9A generates CD8+ T cell responses with adjuvant and efficiently activates T follicular helper (Tfh) cells for Ab production in the absence of adjuvant (124). Some of these differences result from targeting to distinct subsets of DCs. However, enough DC surface molecules have been targeted to show that there is also an activation component that varies depending on which receptor is targeted and ligated. This difference is apparent in targeting to the FIRE and CIRE receptors that are both expressed on the CD8- dendritic cell subset, despite targeting the same cell there are clear differences in magnitude and duration of the Ab response induced (125).

1.4.2 Experience with pan-APC targeting, DC-specific, and B cell-specific targeting

The earliest example of Ag targeting was neither intentional nor specific. The first subunit vaccine administered to humans was the diphtheria toxin (DT)-antitoxin immune

complex championed by Theobald Smith in 1909. In order to neutralize the DT, Smith mixed it with serum from horses immunized with sub-lethal doses of toxin (126). When injected these complexes strongly bind Fcγ receptors, stimulate DCs and macrophages, and are rapidly internalized for processing and presentation to CD4 T cells (127). The unfortunate side effect of sensitization to horse proteins rapidly made this approach impractical (128). However immunizations with these immune complexes stimulated far greater and more durable immunity than current Ag in alum immunizations for DT (129). Many of the early intentional Ag targeting approaches were directed toward B cells. A few of these targets were ineffective (targeting IgA, B220 or FcγRII; 130 and 131) but many were moderately effective at inducing Ab production (targeting IgM, IgG, IgD, CD19 or protein A which binds to IgG; 130 - 133). While these approaches rapidly generated T cell help by loading many B cells with Ag, the amount of Ab induced was lower than that achieved when molecules that are broadly expressed on APCs were targeted (immune complexes which bind to multiple Fcγ receptors, as well as direct ligation of FcγRI, MHC II, CD11c, CD23, CD40, CD45, transferrin receptor, VCAM, TLR2 or complement components; 134 - 143). This led to a gradual shift away from B cells as targets and a focus on myeloid cells, and specifically DCs, as the cells of interest for Ag targeting.

The current list of myeloid cell targets for Ag is long: Dectins 1 and 2, DEC205, DCIR 1 and 2, DC-SIGN, Clec9A and Clec12A, CD103, CD163, CD317, mannose receptor, sialoadhesin, FIRE and CIRE, MGL, DC-ASGPR, Siglec-H, Langerin, and multiple chemokine receptors (121, 123, 125, 144 - 158). The C-type lectin receptor (CLR) family comprises a large portion of these targets as they are pathogen recognition

receptors similar to TLRs that bind fungal Ags and activate the DC in the process. CLRs are primarily DC restricted, readily internalize following ligation to interact specifically with components of the Ag processing pathways, and many CLRs transduce strong stimulatory signals via Syk and CARD9 (159, 160). TLR agonists also easily enhance the stimulation of DCs by C-type lectins as the pathways display synergistic activation (161). The vast majority of these DC targets are highly efficient at inducing T cell responses, especially CD8 T cell responses, and targeting to a few of these receptors induce large quantities of Ab (DCIR2 and Clec9A). However, the majority induce only modest quantities of Ab and require adjuvants to do so. Additionally, as these Ag conjugates target pathways that most likely go from DC to T cell to B cell, their use in immunization is unlikely to be effective in patients with immunodeficiencies including T cell defects or dysfunctional CD40 signaling.

The difference in cell type targeted may not be the primary difference in the Ag targeting efficacy gap between B cells and DCs. Many of the DC receptors targeted, when ligated activate Ag processing and presentation pathways, while the ones targeted on B cells largely do not. While BCR components (IgD, IgM, IgG, CD19) are clearly involved in activating B cells, the high serum levels of IgM and IgG make them unattractive targets, and targeting Ag to BCR-associated components may not add any more stimulus than Ag alone or in the case of CD22 and FcγRIIb may be inhibitory. The result of targeting Ag to B cells via a receptor that adds a second or costimulatory signal, distinct from the BCR pathway, has not been reported.

1.5 Questions to address

This dissertation will examine the relationship of CD180 signals, rather than deficiency, with TLR signals and more broadly how stimulation of CD180 impacts B cell function and immunity. In particular, it will focus on how CD180 signals affect immunoglobulin production either alone or in combination with TI and TD Ags in vivo. Both polyclonal, innate immune-like B cell responses and Ag-specific responses will be investigated in the context of common imunodeficiencies (CD40 KO models of hyper-IgM syndrome and T cell deficient models of DiGeorge syndrome). It will also explore the efficacy of using CD180 as a potential Ag targeting vaccination strategy.

Chapter 2: Materials and Methods

Mice

C57BL/6, CD40 knockout (KO), OT-I ovalbumin (OVA)-specific CD8 T cell receptor (TCR) transgenic, OT-II OVA-specific CD4 TCR transgenic, B cell-deficient (μMT), and T cell-deficient (TCRβ/δ KO) mice were from Jackson Laboratory (Bar Harbor, ME) and all strains were on the C57BL/6 background unless otherwise noted. CD180 KO, MHC II KO, and type 1 interferon (IFN) α/β receptor (IFNα/βR) KO mice were gifts from S. Skerrett, P. Fink and K. Murali-Krishna, respectively (University of Washington, Seattle, WA). OX40L KO mice were a gift from A.H. Sharpe (Harvard University, Cambridge, MA). BAFF-R KO mice were a gift from K. Rajewsky (Harvard Medical School, Boston, MA). B6.SJL-B1-8^{hi} knockin Ly5.1 mice with B cells specific for the 4-hydroxy-3-nitrophenyl acetyl (NP) hapten were a gift from M. Nussenzweig (Rockefeller University, New York, NY). IL-4 KO mice on a BALB/c background were a gift from S. Ziegler (Benaroya Research Institute, Seattle, WA), and WT control BALB/c mice were purchased from the Jackson Laboratory. TRIF KO spleens were a gift from D. Rawlings (Children's Research Institute, Seattle, WA). MyD88 KO mice and TLR2/4 KO spleens were gifts from K. Elkon (University of Washington, Seattle, WA). All mice were sex and age matched and used at six to twelve weeks of age, except for the memory recall studies which utilized 60-week-old mice. The University of Washington Institutional Animal Care and Use Committee approved all animal work.

Cell preparation and culture

For total splenocyte preparations and B cell or T cell purifications, spleens were processed by mechanical disruption and erythrocytes were depleted by Gey's lysis. For

DC purifications, spleens were processed by Liberase (Roche, Indianapolis, IN) digestion. B cells, T cells, or DCs were isolated by three successive rounds of negative selection enrichment (STEMCELL technologies, Vancouver, BC, Canada) and purity exceeded 99% as assessed by flow cytometry with CD19/CD3/CD11c staining.

Total splenocytes or purified cells were cultured in complete medium (RPMI-1640 supplemented with 10% fetal calf serum [Hyclone, Logan, UT], 4 mM glutamine, 1 mM pyruvate, 1 x Non-Essential Amino Acids, 100 IU/ml penicillin-streptomycin [Invitrogen, Carlsbad, CA], and 50 uM 2-ME [Sigma-Aldrich, St. Louis, MO]) in the presence of stimuli at a final cell density of 1×10^6/mL for 64 hours at 37 °C.

ELISA measurement of total or Ag-specific antibody or cytokines and ELISPOT analysis

Sera were obtained after injection of mice with mAbs and/or TLR agonists. Polystyrene plates were coated with 2 μg/mL anti-mouse IgG (H+L) (Jackson ImmunoResearch, West Grove, PA) for total Ig or 20 μg/mL Ag (NiP-BSA from Biosearch Technologies, Novato, CA, or OVA from Sigma-Aldrich) for Ag-specific determinations. Affinity determinations were performed as described previously (123, 187), using custom NP_2- and NP_{20}-BSA prepared by conjugation to the succinimidyl ester of NP (Biosearch Technologies) according to manufacturer instructions. After blocking with 4% nonfat dry milk in PBS-Tween, serial dilutions of serum were added. Abs were detected with isotype-specific HRP conjugates (anti-IgG1, anti-IgG2b, and anti-IgG3 from ICL, Newberg, OR; anti-IgM and anti-IgG2c from Southern Biotech, Birmingham, AL) and absorbance was compared with standard curves generated from mouse monoclonal standards (IgG3 from BioLegend, San Diego, CA; IgM from Jackson ImmunoResearch; IgG2c from Southern Biotech; IgG1 and IgG2b standards were

purified in our laboratory) for absolute quantitation. No cross-reactions between standards for the IgG subclasses, IgM, or the injected rat IgG2a mAbs were observed. Relative concentrations of serum IgA and IgE were detected, following light chain or antigen capture, with anti-IgA and anti-IgE HRP direct conjugates (ICL) and compared to pre-bleed serum (for polyclonal serum comparisons) or Ag-isotype injected (for Ag-αCD180 response) values due to lack of appropraie standards for full quantitation. Total *in vitro* Ig production was assessed as above after culturing $5x10^5$ splenocytes/ml with the indicated stimuli for 72 hours at 37 °C.

IL-6, IL-10, and TNF-α concentrations in 24-hour supernatants from cultures of purified cells were measured by ELISA (DuoSets from R&D Systems, Minneapolis, MN) per the manufacturer's instructions.

ELISPOT analysis was performed as previously described (Goins et al., 2010) except for the use of a CTL-ImmunoSpot S5 Core Analyzer ELISPOT reader with ImmunoSpot Academic V5.0 software for quantitation of spot number and size (Cellular Technology Ltd., Shaker Heights, OH).

Analysis of lymphocyte subsets and proliferation

Flow cytometry analyses were performed on either a standard FACScan or FACSCanto (Becton Dickinson, Franklin Lakes, NJ). Minimums of 30,000 cells of the final gated population were used for all analyses. Data analysis was performed with FlowJo (Tree Star, Ashland, OR) software. Staining was performed for: CD3, CD24, CD80, and CD95 (Becton Dickinson clones 145-2c11, M1/69, 16-10A1, and Jo2); CD4, CD8α, CD8β, CD19, CD21, CD23, CD25, CD11b, CD11c, F4/80, and CD69 (BioLegend clones RM4-5, 53-6.7, YTS156.7.7, 6D5, 7E9, B3B4, 3C7, M1/70, N418,

BM8, and H1.2F3); CD5, CD45R/B220, GL7, Ly5.1, and CD86 (Clones 53-7.3, RA3-6B2, GL-7, A20, and GL1 from eBioscience, San Diego, CA); Peanut Agglutinin from Vector Labs (Burlingame, CA); MHC II (NIMR-4 from Southern Biotech, Birmingam, AL); Mouse anti-rat IgG secondary Ab was from Jackson ImmunoResearch; and TACI/TNFSF13b (166010 from R&D Systems, Minneapolis, MN). NP-APC was prepared by conjugation of APC (Sigma-Aldrich) to NP as described for NP_2-BSA above. All isotype control mAbs were from BioLegend and unconjugated APC was used as the staining control for NP-APC.

BrdU analysis was performed according to the kit manufacturer's instructions (Becton Dickinson) following a one hour pulse delivered by i.p. injection on d 3 post anti-CD180 injection.

CFSE (Invitrogen) labeling of cells was performed with a final concentration of 0.8 μM CFSE and $1.6x10^7$ cells/ml in 37°C PBS for four minutes. Proliferation Index was calculated by dividing the geometric MFI for gated live unstimulated singleton B cells by the geometric MFI of equivalently gated cells from the stimulated sample. This measurement simultaneously captures both percent proliferating cells and the average number of divisions per cell. A Proliferation Index of 1 indicates equivalence to unstimulated culture.

Synergy determinations and calculation of the combination index

The Combination Index (CI), a quantitative definition of synergy or antagonism, was calculated by the method of Chou and Talalay (21) through the use of CalcuSyn software (Biosoft, Cambridge, United Kingdom). As the CI method is based on the median effect principle of the mass action law, it is mechanism-independent.

Other antibodies and reagents

The anti-CD180 (RP/14) hybridoma was a gift from K. Miyake (University of Tokyo, Tokyo, Japan) and the rat IgG2a isotype control (9D6) hybridoma was a gift from R. Mittler (Emory University, Atlanta, GA). We used our previously generated hybridoma (1C10, rat IgG2a) to produce anti-CD40 mAb. To ensure equivalence these mAb were sequentially purified on the same protein G column, followed by LAL gel-clot assays in GlucaShield buffer (Associates of Cape Cod, East Falmouth, MA) and rejected if endotoxin levels above 0.025 EU/mg protein were detected. mAbs were conjugated to NP as described for NP_2-BSA above. Final NP conjugation ratios to mAb for use as antigens ranged from NP_6 to NP_{19} as determined by spectrophotometry and NP ratios were always higher for the paired isotype than anti-CD180 to control for any possibility of T Cell Independent Type-2 Ag effects. Chicken OVA (Sigma-Aldrich) was conjugated to mAbs as previously described (189) with an average conjugation ratio of 2 OVA per mAb. Amount of conjugate administered is referenced as the mAb component, i.e. 100 μg OVA-αCD180 contains a total mass of 156 μg OVA-αCD180 due to addition of 56 μg OVA to 100 μg of αCD180. Alum-precipitated antigens were prepared with Imject (Thermo Fisher Scientific) according to the manufacturer's instructions and administered in fixed 200 μl final volumes i.p.

LPS (L2143) was from Sigma-Aldrich. Synthetic TLR agonists Pam_2CSK_4, Pam_3CSK_4, CL097, and CpG ODN1826 were from InvivoGen (San Diego, CA).

Statistical analyses

Raw data of experimental groups were analyzed either by one-way ANOVA followed by Bonferroni's Multiple Comparison Test (GraphPadPrism software, version

4.0a for Macintosh, San Diego, CA) or by two-tailed, type two Student's *t*-test. Columnar data are represented as mean + standard error (SEM). A value of $p < 0.05$ was considered to be statistically significant and assigned *, while $p < 0.01$ and $p < 0.001$ were assigned ** and ***, respectively.

Chapter 3: Anti-CD180 (RP105) activates B cells to rapidly produce polyclonal Ig via a T cell and MyD88-independent pathway

Introduction

CD180 (RP105) was originally identified as a B cell surface molecule mediating activation and proliferation and was later recognized as a TLR homolog (91, 103). It is a leucine-rich repeat type 1 membrane protein with high extracellular homology to the LPS receptor (91), but unlike TLR4 its expression is restricted to APCs (B cells, macrophages, and dendritic cells (DCs)) (79). CD180 and TLR4 are also similar in that mAbs to these receptors cause B cell proliferation and upregulation of costimulatory molecules (CD86) (89). However, while TLR4 agonists induce only a subset of B cells to proliferate (15%)(162), anti-CD180 activates over 85% of both human and mouse B cells *in vitro*, causing extensive proliferation.

TLRs recognize conserved microbial components to initiate rapid responses that both prime and skew adaptive immunity (163). Each TLR binds specific ligands via its extracellular domain to initiate dimerization, recruitment of MyD88 and/or TIR-domain-containing adapter-inducing interferon-β (TRIF) intracellular adaptors to the Toll/IL-1-Receptor (TIR) domain, and downstream signaling (164, 165). While each TLR exhibits specificity for agonists with conserved molecular structures, combinations of TLR signals mediate much more robust and specific responses appropriate for fine-tuning against particular pathogens (166, 167). As CD180 is classified as a TLR it is essential to understand its interaction with other TLRs, as multiple TLRs are often required in concert to mediate full physiological function.

In CD180 KO mice B cell responses to LPS are impaired and constitutive serum IgG3 is reduced (105). These data supported a model of CD180 as a required co-receptor for B cell responses to bacterial cell wall components (92), binding LPS and forming heterodimers with TLR4 to enhance signaling. However, while the natural ligand of CD180 is unknown it is not LPS. CD180 does not bind LPS (79) and structural analysis of the CD180 complex revealed that it does not contain the required LPS binding pocket (168) as only four, not six, acyl chains can fit (93). Therefore, while CD180 regulates B cell sensitivity to LPS the mechanism of CD180 support for TLR4 signals remains unknown. Because CD180 lacks the cytoplasmic TIR domain common to all other TLRs, appearing instead to initiate an antigen receptor-like phosphotyrosine and calcium based signaling cascade in B cells (103, 96, 97, 102), there is no known point of interaction between the CD180 and TLR pathways. Furthermore, it is unclear how a scaffold of dimerized (169, 170) TIR domains forms with inclusion of CD180 in a heterodimer with TLR4. This led to an opposing model where CD180 forms inactive heterodimers with TLR4 and specifically attenuates LPS responses in myeloid cells, with only artifactual stimulation of B cells (79).

Current literature is confusing as it ascribes opposing functions for CD180, both as a required co-receptor for LPS / stimulator of B cells (105) and also as a specific TLR4 inhibitor in DCs with no physiological effect on B cells (114). While CD180 deficiency has been characterized (79, 105), neither CD180 stimulation *in vivo* nor the integration of CD180 and TLR signals has been studied (with the single exception of noting increases in $CD138^{+}$ B cells in spleen sections following anti-CD180 injection (105)). Here we report that anti-CD180 mAb *in vivo* induces rapid polyclonal B cell expansion and

striking Ig production, especially of the IgG1 and IgG3 subclasses. This Ig production is inhibited by co-administration of diverse TLR ligands. In contrast, anti-CD180 synergizes with ligands for all MyD88-dependent TLRs to increase B cell proliferation. While anti-CD180 in combination with TLR signals augmented cytokine production from purified B cells, it does not by itself induce cytokine production. Our data indicate that CD180 signals act directly on B cells to induce strong polyclonal B cell proliferation and Ig production, and that integration of TLR and CD180 signals through MyD88 skews B cells toward proliferation and cytokine production rather than differentiation.

Results

Anti-CD180 injection induces polyclonal Ig production of multiple isotypes

Because CD180 KO mice have low serum concentrations of IgG3 (105), we examined Ig concentrations of WT mice at 3, 7, 10, and 14 d after injection with either anti-CD180 or isotype-matched control mAb (the anti-CD180 antibody is an agonistic rat IgG2a that was not expected to deplete target cells). Dose response assays from 10 μg to 250 μg of anti-CD180 were performed and 100 μg gave a less pronounced effect than 250 μg for both splenic expansion and Ig production (data not shown). All subsequent *in vivo* assays utilized a 250 μg dose of anti-CD180. At no point did the anti-CD180-injected mice show any evidence of distress, unlike after injection of TLR4 agonists that rapidly induce septic shock.

Anti-CD180 alone increased serum Ig concentration of nearly every isotype and subclass by d 3, with increases for IgG1, IgG2c, and IgG3 that were both rapid and also dramatic in magnitude (12, 9.5, and 56-fold average increases at d 10, respectively), while changes in serum concentration of IgM were rapid (11-fold increase at day 3) but

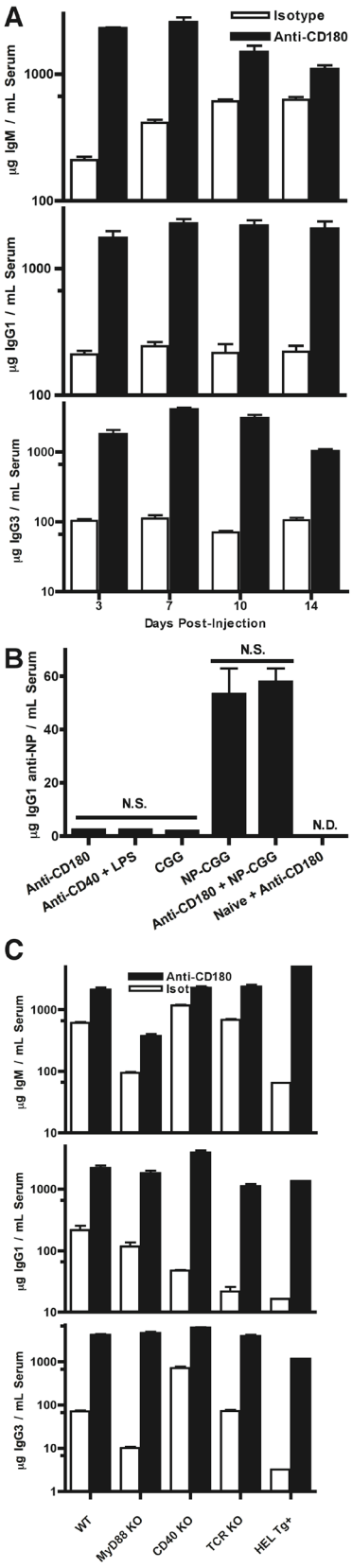


Figure 3.1. Anti-CD180 rapidly induces Ig production independently of memory recall, T cell help, or MyD88 signals. A) WT mice received 250 μg anti-CD180 or isotype control mAb, bled at indicated timepoints, and total serum Ig analyzed by ELISA. **B**) WT mice were immunized with 50 μg NP-CGG in alum, rested, and challenged with either 250 μg anti-CD180, 100 μg anti-CD40 plus 10 μg LPS, 25 μg uncongugated CGG, 10 μg NP-CGG, or anti-CD180 plus NP-CGG, and bled at d 10 for NP-specific Ig analysis. Non-immunized mice for the naïve group were age matched. **C**) WT, MyD88 KO, CD40 KO, TCR KO, and HEL Tg^+ mice were injected, bled at d 10, and total serum Ig analyzed. *p* value between paired columns < 0.001 unless otherwise noted. Four mice per timepoint, representative of four experiments for each panel.

transient (2.4-fold increase at day 10) and IgG2b varied with an average of 1.5-fold reductions (Fig. 3.1A and data not shown). ELISAs for serum IgA and IgE from d 10 bleeds indicate that IgA concentrations were equivalent to pre-injection bleeds in isotype control and anti-CD180 treated mice, while IgE concentrations increased roughly 12-fold in the anti-CD180 group but not the isotype group (Fig. 3.2A).

We examined whether rapid production of IgM and IgG1 after CD180 stimulation was due to reactivation of memory B cells. WT mice were immunized with NP-CGG in alum and

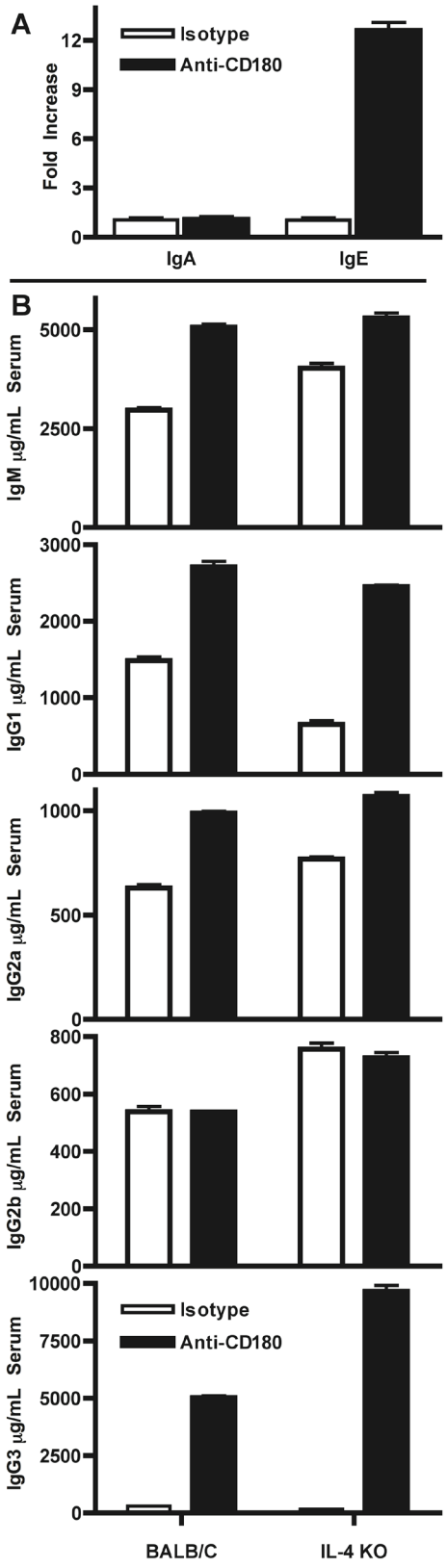


Figure 3.2. Anti-CD180 rapidly induces IgE but not IgA production, Ig production is independent of IL-4 and the C57BL/6 background. A) C57BL/6 mice received 250 µg anti-CD180 or isotype control mAb, bled at day 10, and total serum IgA and IgE were analyzed by ELISA and compared to pre-injection bleeds for fold induction. Four mice per group, representative of two experiments. **B**) BALB/C and IL-4 KO mice on the BALB/C background received 250 µg anti-CD180 or isotype control mAb, bled at day 10, and total serum Ig analyzed by ELISA. Four mice per group, representative of 4 experiments.

rested for 50 weeks before injection of recall stimuli. While recall Ag administration without adjuvant produced robust NP-specific IgG1, neither anti-CD180 nor inflammatory stimuli (LPS plus anti-CD40) induced significant recall compared to unconjugated CGG (Fig. 3.1B). Addition of anti-CD180 stimulation with Ag did not significantly impact recall IgG1 responses.

Anti-CD180-induced Ig production did not require T cells, CD40, IL-4, or TLR signaling as the increase in IgG concentrations still occurred at d 10

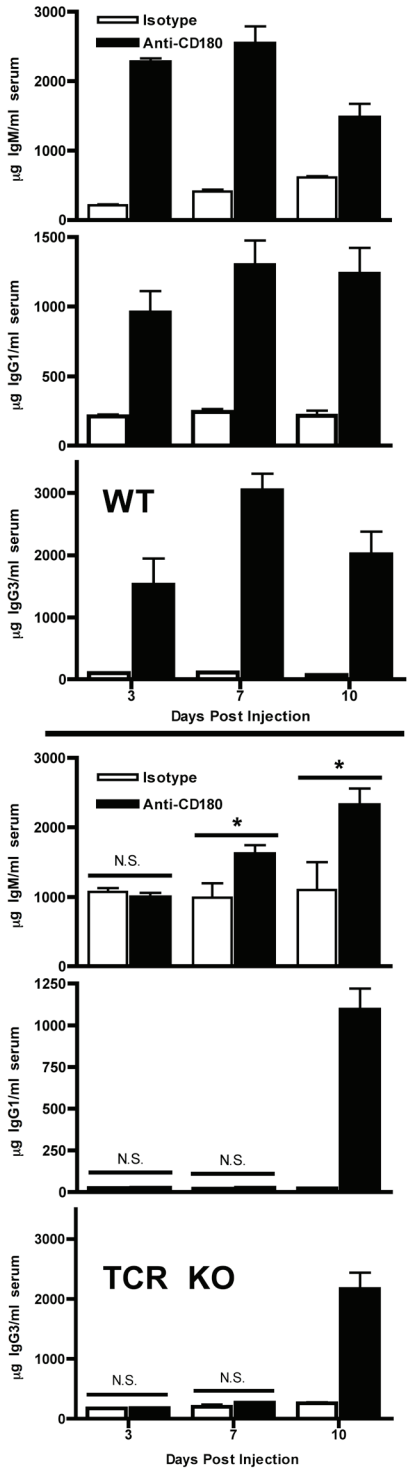


Figure 3.3. T cell deficiency delays, but does not prevent, anti-CD180-induced Ig production. WT and TCR KO mice were injected with 250 μg anti-CD180 or isotype control mAb and total serum Ig analyzed by ELISA. *p* value between paired columns < 0.001 unless otherwise noted. Four mice per group, representative of 3 experiments.

following injection of TCR KO, CD40 KO, IL-4 KO, MyD88 KO, or HEL Tg^+ mice (Fig. 3.1C and Fig. 3.2B). IgM production was largely bypassed and IgG production was strikingly delayed in TCR KO mice, indicating a supportive role for T cells despite dispensability for the overall anti-CD180-induced Ig effect (Fig. 3.3).

To assess whether anti-CD180-induced Ig is polyclonal or merely an extensive Ag-specific response, we examined antigen-specific responses in combination with CD180 signaling. We measured Ag-specific Ig produced following co-administration of anti-CD180 with T cell-independent (TI) antigens, the Ig produced against the rat IgG2a anti-CD180 mAb itself, and whether

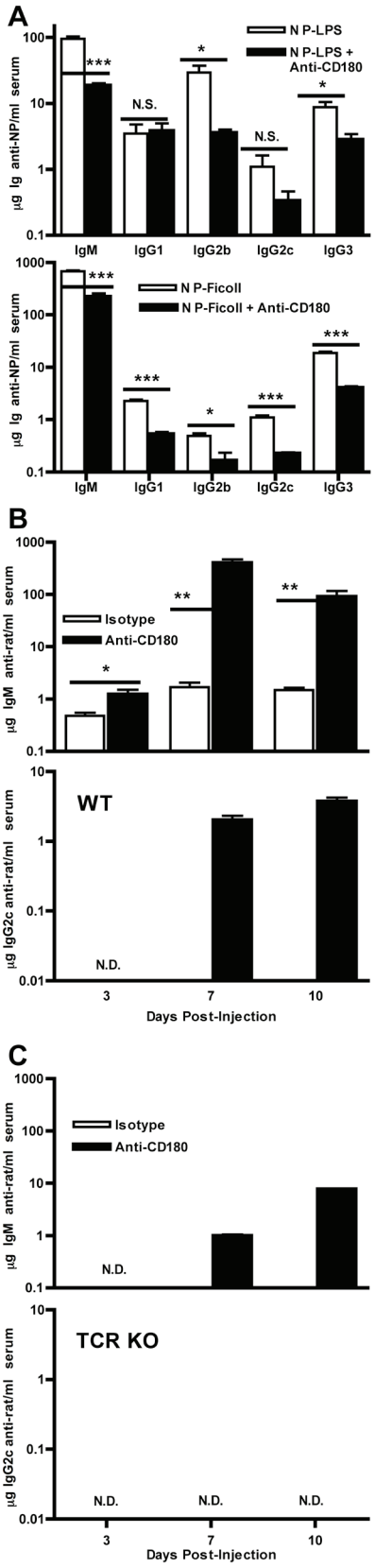


Figure 3.4. T-Independent Type-1 and 2, but not T-Dependent antigen specific antibody, are decreased by co-administration of anti-CD180. A) WT mice were injected with 1 μg NP-LPS (0.7 NP/LPS) (TI-1), or 20 μg NP-Ficoll (152 NP/Ficoll) in combination with 250 μg anti-CD180 or isotype control mAb, bled on d 10, and serum analyzed for NP-specific antibody. **B**) WT and **C**) TCR KO mice were injected with 250 μg anti-CD180 or isotype control mAb, and serum analyzed for anti-rat Ig-specific IgM and IgG2c antibody from d three, seven, and 10 time points. *p* value between paired columns < 0.001 unless otherwise noted. Four mice per group, representative of four experiments for each panel.

autoantibodies developed in anti-CD180 injected mice. Ag-specific antibodies of all isotypes were reduced or unchanged after addition of anti-CD180 mAb to immunization with either the TI-1 Ag NP-LPS or the TI-2 Ag NP-Ficoll (Fig. 3.4A). Although more anti-rat Ig was generated against the anti-CD180 mAb than the mAb isotype control it was never more than 7 d to peak while total IgM is essentially maximal by d 3 (Fig. 3.4B). Class-switched anti-rat Ig was predominately of the IgG2c subclass and was not produced against the isotype control mAb. T cell-deficient mice also

produced IgM specific for anti-CD180, but not class- switched Ig of any subclass (Fig. 3.4C). Auto-reactive antibody, as determined by semi-quantitative antinuclear antibody immunoflourescence, did not increase after anti-CD180 injection (data not shown).

Anti-CD180 injection expands splenic B cells

Three days after injection the spleens of anti-CD180-treated mice were enlarged nearly 3-fold compared to control mice (data not shown). Absolute splenic mononuclear cell numbers increased approximately 2.5-fold from controls (Fig. 3.5A). B cells ($CD19^+$) contributed the majority of the change by expanding 7, 9, and 2.5-fold in transitional 1 (T1), transitional 2 (T2), and follicular (FO) subsets respectively, while the marginal zone (MZ) B cell subset did not change significantly in number (Fig. 3.5A). To assess whether this was a survival/redistribution effect or if cells were actually induced to proliferate *in vivo* by anti-CD180, we inoculated mice with BrdU 3 d after anti-CD180 injection and one hour later harvested spleens and quantified $BrdU^+$ cells (Fig. 3.5B). Anti-CD180 induced significant proliferation in T1, T2, FO and MZ B cells. Although absolute numbers of MZ B cells did not increase after anti-CD180 treatment, MZ B cells proliferated more than the other B cell subsets, even exceeding the BrdU incorporation of the bone marrow cell positive control. Furthermore, T cell numbers also expanded significantly (Fig. 3.5A), but did not incorporate BrdU. In order to assess possible non-specific effects from the rat IgG2a anti-CD180, we compared anti-CD180 treated mice to mice injected with the same dose of a rat IgG2a anti-CD40 mAb (1C10). While anti-CD40 stimulated B cells as expected, its effects were distinct from those of anti-CD180. Anti-CD40 expanded FO B cells and myeloid cells (Fig. 3.6A) and induced increases in both CD80 and CD95/FasR expression (Fig. 3.6B). In contrast, anti-CD180 preferentially

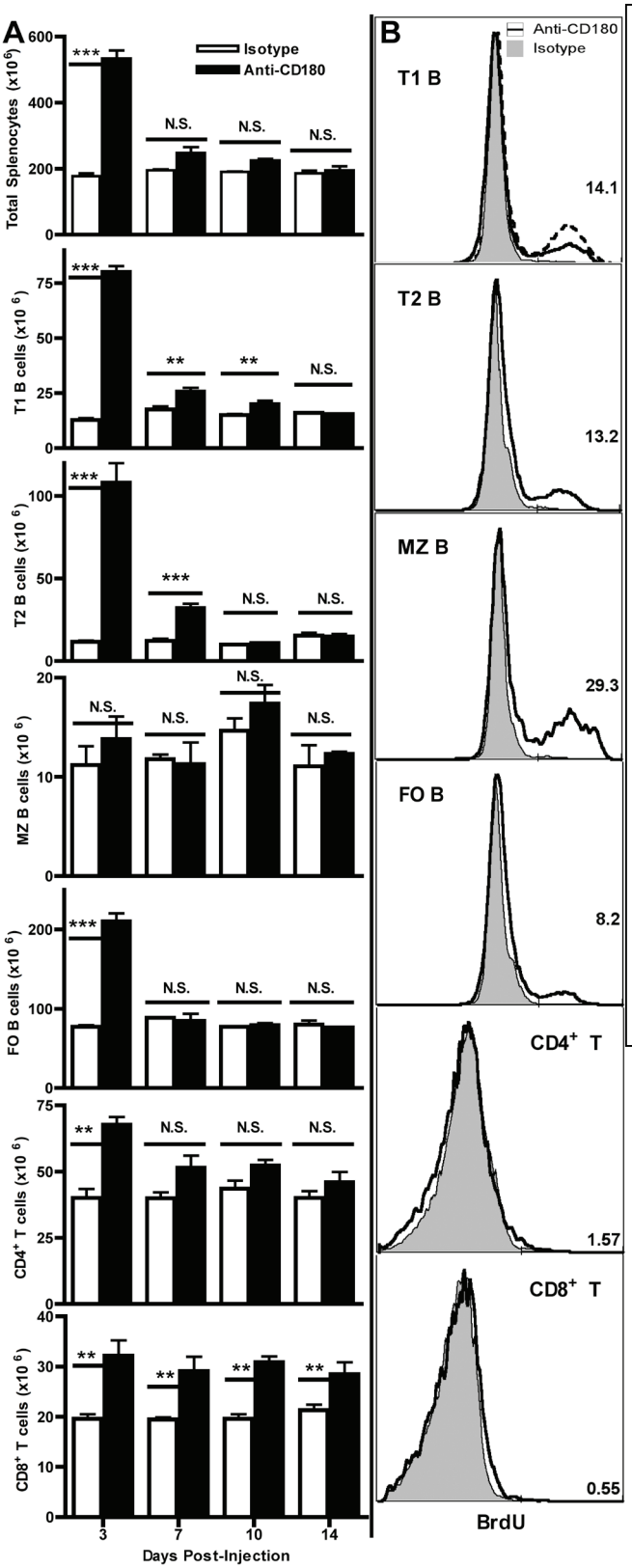


Figure 3.5. Anti-CD180 injection expands splenic B cells *in vivo* by inducing proliferation. A) Spleens were harvested and cells enumerated 3, 7, 10, or 14 d following injection of 250 μg anti-CD180 or isotype mAb. Total splenocytes were subsetted by standard CD21/23/24 staining for B cell subsets and CD3/4/8β staining for T cell subsets. Three mice per timepoint, representative of three experiments. **B**) Following a 1 h BrdU pulse on d 3 post injection of anti-CD180 or isotype control, spleens were harvested, stained for BrdU incorporation, and subsetted for analysis. The dashed line in the first panel indicates BrdU uptake in the bone marrow positive control sample (27%). Three mice per timepoint, representative of two experiments.

induced large increases in numbers of transitional B cells and increases in BrdU uptake in both transitional and MZ B cells, as well as CD86 expression in total $CD19^+$ cell populations, but minimal increases in FO B cells

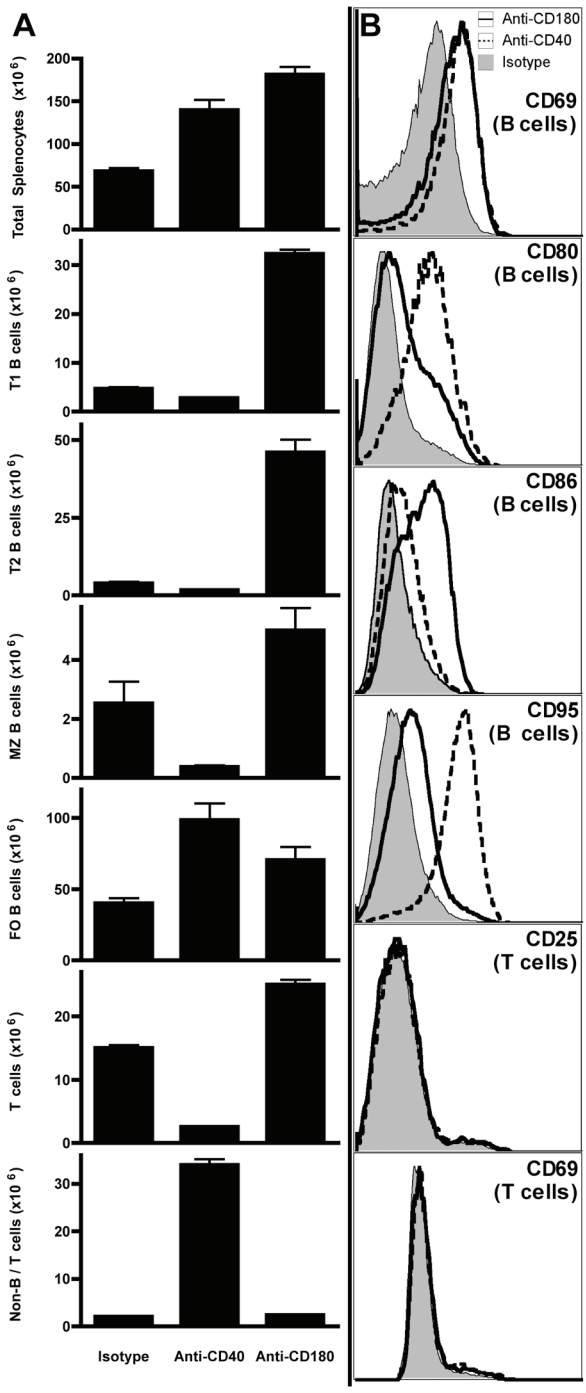


Figure 3.6. Anti-CD180 produces different expansion and activation patterns than anti-CD40. A) Spleens were harvested and cells enumerated 3 d following injection of 250 μg IgG2a anti-CD180, anti-CD40, or isotype control mAb. Total splenocytes were subsetted as in Fig. **3.5A**. **B**) Gated B cells ($CD19^+$) and T cells ($CD3^+$) were analyzed by flow cytometry for expression of common activation markers. Three mice per group; representative of two experiments for each panel.

and CD80 or CD95 expression. Both anti-CD180 and anti-CD40 upregulated CD69 on B cells but did not induce upregulation of either CD25 or CD69 on T cells. The differential effects of CD40 or CD180 stimulation on expression of the B7 family members CD80 and CD86 may lie in the type of stimulation they mimic; anti-CD40 mimics a portion of the highly coordinated costimulatory cascade between B and T cells during adaptive immune processes and induces the expression of CD80 which by itself produces only modest costimulation, while anti-CD180 mimics a Pathogen

Recognition Receptor response and induces CD86 expression which can lead to rapid and adjuvant-free B cell responses.

The lymphoid cell expansion induced after anti-CD180 injection was transient, as cell numbers were lower at d 7 and essentially normal by d 14. The single exception was $CD8^+$ T cells, which remained expanded through d 14. The kinetics of cell expansion paralleled binding of the anti-CD180 antibody, as determined by anti-rat IgG staining *ex vivo,* which demonstrated maximum binding at d 3, minimal binding at d 7, and undetectable binding on d 14 (data not shown).

Expansion of B cells was still evident in TCR KO mice (Fig. 3.7A) and showed equivalent kinetics (data not shown), indicating that T cells are not required for either expansion or contraction of B cell populations *in vivo* following anti-CD180 injection. However, T cell expansion is dependent upon B cells, since T cells did not expand in B cell-deficient mice after anti-CD180 treatment. This T cell expansion occurred even though T cells do not express CD180, and thus is an indirect effect requiring B cells. Unlike the B cell expansion, which clearly involves proliferation, T cells did not incorporate significant BrdU despite expanding in number nor display markers of activation. As T cell numbers increase without evidence of either stimulation or proliferation this may be due to enhanced retention of circulating T cells simply by increased numbers of B cells or increased expression of CD34 driving recruitment of naïve T cells. The selective effects of anti-CD180 upon transitional and MZ B cells is also not predicted by the level of CD180 expressed on B cell subsets, as these have at best equivalent CD180 expression with FO B cells yet FO B cells both proliferate and accumulate less extensively (Fig. 3.5AB, and Fig. 3.7B).

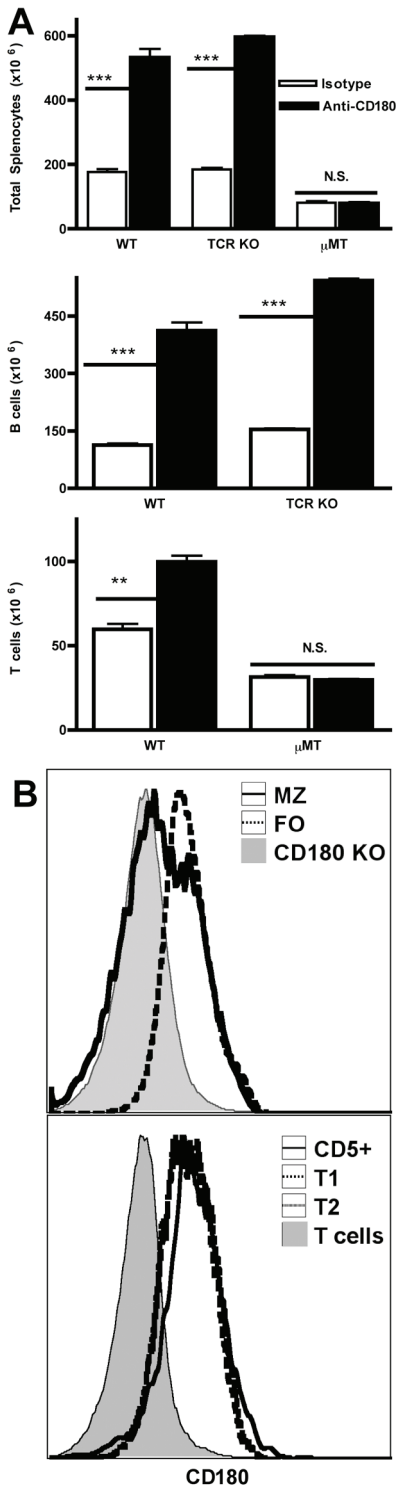


Figure 3.7. Anti-CD180 splenic expansion requires B cells but not T cells. A) WT, T cell-deficient (TCR KO), and B cell-deficient (μMT) mice were injected with 250 μg anti-CD180 or isotype control mAb and splenocytes counted and analyzed at the d 3 timepoint as in Fig. **3.5A**. **B**) Unstimulated WT cells or those of a CD180 KO (FO B subset) control were stained *ex vivo* for CD180 expression and subsetted as in Fig. **3.5A**. Three mice per group; representative of two experiments for each panel.

Combinations of TLR and CD180 signals reduce B cell differentiation and enhance proliferation

Due to the known interaction between CD180 and TLR4, we compared Ig production induced by anti-CD180 alone to co-injection with various TLR ligands. Combinations of anti-CD180 and LPS did not augment but instead resulted in decreased or unchanged Ig production (Fig. 3.8A) resulting in serum concentrations intermediate to anti-CD180 or LPS alone. This effect was also observed with Pam_3CSK_4 (a TLR2:1 ligand) and CpG (a TLR9 ligand), indicating a general effect of TLR signals rather than a specific interaction between CD180 and TLR4.

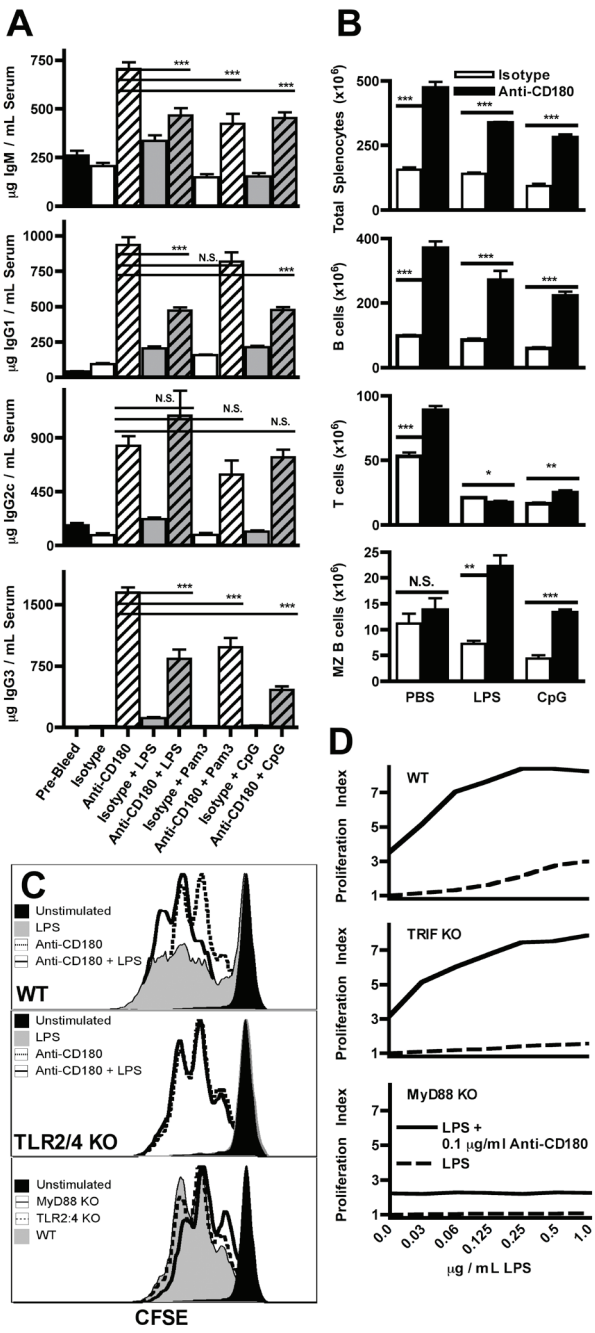


Figure 3.8. TLR signals reduce anti-CD180 induced Ig production but augment proliferation in a MyD88-dependent manner. A) WT mice were injected with the following TLR agonists in combination with either 250 μg anti-CD180 or isotype control mAb: 1 μg LPS, 2 μg Pam_3CSK_4, or 10 μg CpG. Sera were obtained at d ten and analyzed by ELISA. Four mice per group, representative of four experiments. **B**) TLR ligands LPS (10 μg), CpG (25 μg), or an equivalent volume of PBS were co-injected with either anti-CD180 or isotype and splenocytes were analyzed at the d 3 timepoint as in **Figure 3.5A**. Three mice per group, representative of four experiments. **C**) CFSE labeled splenocytes from TLR2/4 KO, MyD88 KO, or WT mice were cultured with anti-CD180 (0.2 μg/ml), LPS (0.5 μg/ml), or both. B cells were gated (FSC/SSC, $B220^+$) and CFSE dilution analyzed. B cells from all three genotypes stimulated with anti-CD180 are overlaid for a direct comparison of TLR and MyD88 requirements in CD180 signaling. **D**) CFSE labeled splenocytes from WT, TRIF KO, or MyD88 KO mice were cultured with graded doses of LPS alone or in combination with a constant 0.1 μg/ml dose of anti-CD180. Proliferation Index is graphed against the corresponding LPS concentration. One of three experiments with similar results.

We also injected anti-CD180 in combination with TLR ligands (LPS or CpG) to determine whether these combinations changed how splenic lymphocytes expanded. Compared with anti-CD180 injection alone, mice injected with anti-CD180/TLR agonist combinations showed roughly equivalent B cell expansion (3.5 fold) but had reduced expansion of T cells (Fig. 3.8B). Despite a lack of MZ B cell expansion with anti-CD180 alone, combinations of CD180 and TLR signals increased splenic MZ B cell populations.

As CD180 KO B cells have diminished proliferative responses to LPS (92), we examined possible reciprocal dependence of CD180 signals on TLR2 and TLR4 as well as the TLR adapter protein MyD88. In WT splenocyte cultures the combination of CD180 and TLR4 stimulation augmented B cell proliferation compared to either stimulus alone, increasing both the percentage of B cells proliferating and the average number of cycles (Fig. 3.8C). Deficiency of TLR2 and TLR4 had little or no effect on proliferation of B cells in response to anti-CD180, and as expected TLR4 deficient B cells did not respond to LPS. Similar results were obtained for MyD88 KO B cells. Thus, CD180 and MyD88-dependent TLRs provide distinct, non-redundant, and mutually reinforcing signals for B cell proliferation.

To identify the intersection of CD180 and TLR4 signaling pathways we assayed B cell proliferation with graded doses of LPS, with or without a fixed dose of anti-CD180, in splenocytes from WT, TRIF KO, and MyD88 KO mice (Fig. 3.8D). Despite minimal proliferation to LPS alone, robust augmentation of anti-CD180 on LPS-induced proliferation was still present in B cells from TRIF KO mice but not from MyD88 KO mice. While MyD88 is not required for CD180 signals to induce B cell proliferation, it is required to mediate the CD180 augmentation of TLR4 signals.

Anti-CD180 synergizes with multiple MyD88-dependent TLR ligands for B cell proliferation

Since Ig production decreased after TLR ligands were co-injected with anti-CD180, we examined the effects of TLR ligands on anti-CD180 induced differentiation and proliferation in quantitative *in vitro* systems to determine the nature and magnitude of signal interactions. *In vitro* Ig production experiments reproduced the decreasing trend observed *in vivo*, with the largest IgG production occurring with anti-CD180 alone, and decreasing as increasing doses of LPS were added to the cultures (Fig. 3.9). To assess proliferative effects from these combinations B cells were isolated and both anti-CD180 and TLR agonists were titrated, first separately (TLR agonist or anti-CD180 alone) and then together (TLR agonist plus anti-CD180) at equivalent dilution ratios. In addition to TLR4 (LPS), the interactions of CD180 with TLR2:1 (Pam_3CSK_4), TLR2:6 (Pam_2CSK_4), TLR7 (CL097), TLR9 (CpG ODN1826), and BCR ($F(ab')_2$

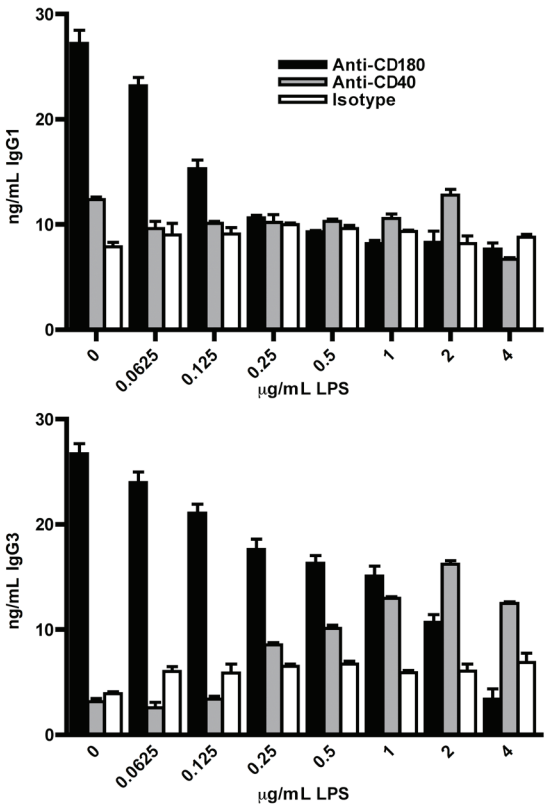


Figure 3.9. In vitro IgG production induced by anti-CD180 is diminished by LPS in a dose dependent manner. Total splenocytes were cultured for 3 days with the indicated stimuli (0.2 μg/ml anti-CD180, 10 μg/ml anti-CD40 or isotype control) with addition of graded doses of LPS and supernatants analyzed by ELISA for IgG1 and IgG3 production. Samples run in triplicate, representative of 2 experiments.

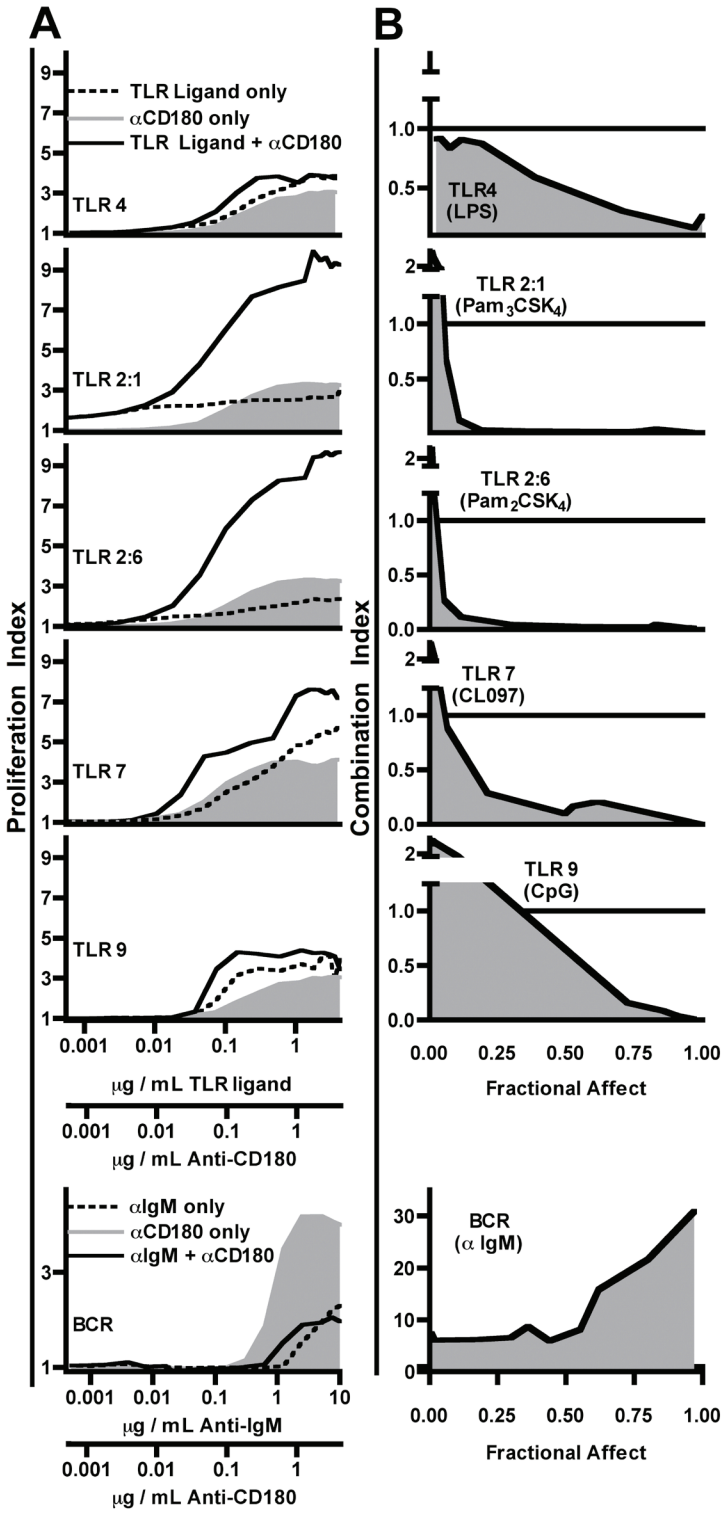


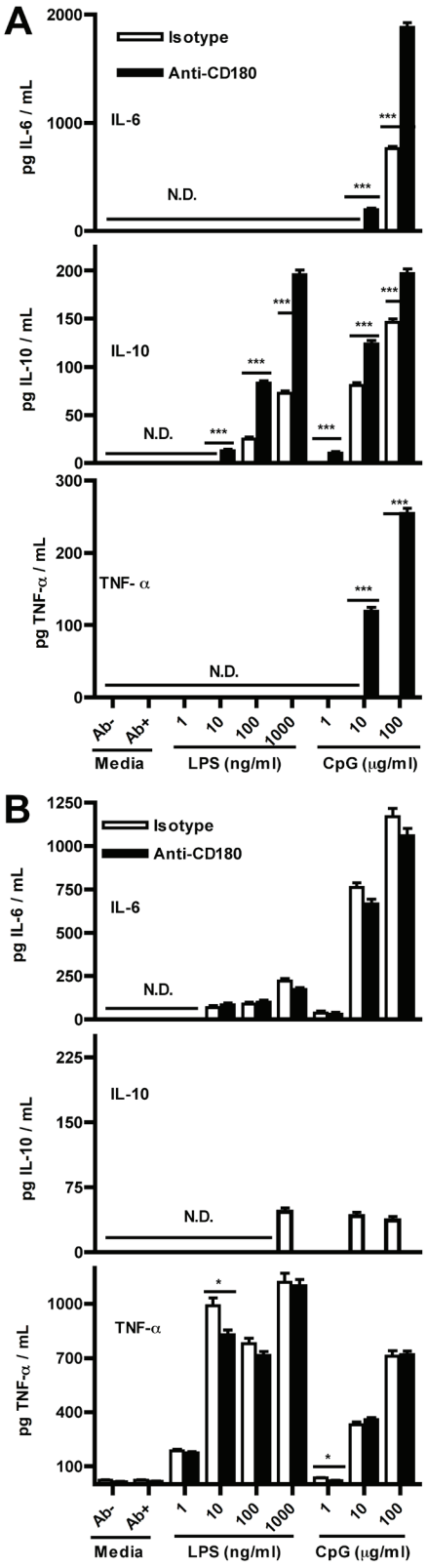
Figure 3.10. Anti-CD180 synergizes for proliferation with all TLR ligands that signal through MyD88. **A**) Purified **(99+ %)** WT splenic B cells were stimulated with either TLR agonist alone, anti-CD180 alone, or both in constant ratio combinations. Proliferation Index was calculated for each series and all curves graphed against the corresponding TLR agonist concentrations. The known antagonism of anti-CD180 for anti-IgM-induced proliferation (12) is included for comparison. **B**) The three Proliferation Index curves were transformed into a single Combination Index (CI) curve as described in "Materials and methods". Combination Index values of 1 (reference bar) indicate simple additive effect (no interaction), CI values < 1 indicate synergy (greater than additive effect), and CI values > 1 indicate antagonism / inhibition. One of four experiments with similar results.

anti-IgM) were also analyzed (Fig. 3.10A). The proliferation of B cells to combinations of anti-CD180 mAb and TLR agonists was augmented for all combinations. In contrast, antagonism was observed with BCR stimulation.

To extract quantitative information from the titration series of anti-CD180 and TLR (or BCR) interaction, the three separate titration curves were transformed into a single curve (Fig. 3.10B) by the Combination Index (CI) analysis method (171). The resulting graph displays signal interaction over the entire titration range, with CI = 1 indicating no interaction (mere additive effect), CI < 1 indicating synergy (greater than additive effect), and CI values > 1 indicating antagonism. Despite the previously reported selective relationship between CD180 and TLR4, we demonstrate strong synergy (CI << 1) for all MyD88-dependent TLR agonist combinations with anti-CD180. While CD180 is described as a specific regulator of TLR4, our analyses show significantly greater synergy with ligands of TLR2 or TLR7. Surprisingly, at very low Fractional Affects (relative doses) all combinations other than LPS revealed antagonism. As these experiments used isolated B cells (> 99% pure) the observed interactions are likely intrinsic to B cells, rather than indirect contributions from signaling in rare non-B cells.

Anti-CD180 augments cytokine production by TLRs in isolated B cells

We next examined cytokine production from isolated B cells treated with anti-CD180 or control mAb alone or in combinations with LPS or CpG (Fig. 3.11A). No production of IL-6, IL-10, or TNF-α was observed with anti-CD180 alone; however there was clear augmentation of cytokine production in combination with TLR ligands. Concentrations of IL-6 and IL-10 were substantially augmented even at LPS concentrations that alone resulted in no effect. Similar augmentation of cytokine



production was seen in combinations with CpG and included strongly increased production of TNF-α. Isolated DCs similarly did not produce cytokines after CD180 stimulation alone (Fig. 3.11B). Rather than the enhancement with CD180/TLR combinations observed in isolated B cells, however, DCs tended to have reduced cytokine production. This divergent behavior of B cells and DCs following CD180 stimulation is consistent with prior literature and underscores the differences between CD180 and TLR signals; TLR ligands would stimulate both B cells and DCs, both alone and in concert with other TLR ligands, while CD180 acts as a potentiator of TLR-induced cytokine expression for B cells but not for DCs.

Figure 3.11. Anti-CD180 does not induce cytokine production by B cells but augments induction by TLRs. A) Purified WT splenic B cells were seeded at $1x10^6$ cells/ml in media with indicated stimulants. Overnight (24 hour) culture supernatants were assayed by ELISA. **B**) Purified WT splenic DCs were treated as in **A**. Differences between paired columns are not significant unless otherwise noted. One of two experiments with similar results, all samples run as triplicates.

Discussion

Collectively, our data indicate that CD180 signals induce an extensive and rapid burst of polyclonal proliferation and activation in naïve B cells, proceeding to IgG production within 3 d. CD180 has been implicated in induction of IgG3 antibodies since constitutive serum concentrations of IgG3 in CD180 KO mice are approximately one-tenth that of WT mice (105). Our results broaden this interpretation as anti-CD180 mAb injection caused very rapid and large increases in serum concentrations of multiple Igs, with IgM, IgG1, IgG2c, and IgG3 concentrations each reaching or exceeding 1 mg/ml within 10 d of injection. IgG3 concentrations had the largest change with a > 50-fold increase over basal concentrations. While robust, this response was transient and reminiscent of an extrafollicular response (172) as concentrations of all isotypes had peaked and begun to decline by d 14. $CD138^+$ B cells in the spleens of anti-CD180 treated mice are significantly increased relative to isotype injected mice in the spleen at day 7, but are not detectable in the spleen on day 3, raising the possibility that peritoneal B cells may contribute significantly to the initial production of anti-CD180-induced Ig. (Data not shown.) However, the production of IgG2c is inconsistent with Ig production from peritoneal B cells so other populations (splenic, etc.) are likely to be involved. Further studies are needed to define the initial Ab producing cells in anti-CD180-stimulated mice and the mechanisms involved in their rapid activation.

The anti-CD180-induced Ig is polyclonal and not merely the result of an unexamined Ag-specific response. As exceptionally rapid production of Ag-specific Ig can occur with either TI-1 or TI-2 antigens, and cellular debris may stimulate B cells for these responses, we examined the effect of anti-CD180 on antigen specific responses to

NP-conjugated model antigens. The doses of NP-LPS were low and induced little polyclonal Ig but a robust NP-specific response. It is unlikely that cellular debris is stimulating Ig production as Ag-specific antibody was reduced by co-administration of anti-CD180. Also, antinuclear antibody did not increase with anti-CD180 treatment (data not shown). While Ag-specific responses to independent but co-administered Ag decreased, Ig specific for the anti-CD180 mAb itself was increased, though not to more than 16% of the total IgM in the serum following anti-CD180 injection. As the bulk of the IgM and essentially all of the IgG produced by anti-CD180 treatment is neither from memory recall nor specific for concomitantly present antigens, it is likely to be truly polyclonal. Anti-CD180 induced IgG production in knockin mice containing only an anti-HEL BCR confirms this.

Injection of anti-IgD similarly induces polyclonal B cell activation and production of high serum IgG1 concentrations (173, 174), and is the closest known parallel for the effects of anti-CD180 *in vivo*. However, there are notable differences between the effects of anti-CD180 and anti-IgD. Anti-IgD induced polyclonal Ig was restricted to IgG1 and IgE isotypes, and required T cell help and specifically IL-4 (175, 176). In contrast, anti-CD180 injection increased all isotypes and subclasses except for IgG2b and IgA, the two prototypic TGF-β induced Ig classes (177) – an effect that did not require the C57BL/6 background, T cells, CD40, IL-4, or MyD88-dependent signaling. As B cell class-switch recombination is thought to require either T cell help or MyD88-dependent TLR/TACI signals (178), anti-CD180-induced Ig production may involve an unrecognized pathway for class-switch induction. Notably, anti-CD180 treatment is remarkable among polyclonal activators by virtue of its profound and rapid induction of diverse Ig classes

and subclasses, including those that are normally counter-regulated or poorly induced by polyclonal stimuli. Additionally, while Ig production by anti-IgD required higher order clustering produced by either multiple mAbs or polyclonal sera (173, 175), a single anti-CD180 mAb induces Ig production, suggesting that only dimerization is required. While our data do not support the idea of CD180 signaling via IgD, we cannot rule out the involvement of other BCR signaling components (179). Despite the significant differences between CD180 and IgD as mediators of polyclonal activation, they still may be classified together in that both induce potent effects but have no confirmed function despite their discovery over 20 years ago.

Injection of anti-CD180 mAb resulted in a rapid increase in splenic cellularity; 3 d after injection T1, T2, and FO B cell subsets expanded 7-, 9-, and 2.5-fold, respectively, whereas neither MZ nor $CD5^+$ B cells expanded in number. While these lymphocyte expansions conflate survival and tissue homing effects with proliferation, the uptake of BrdU after a short pulse indicates that B cells were induced to proliferate *in vivo* and that the expansions are not simply a result of enhanced survival. MZ B cells proliferate the more extensively than any other subset following anti-CD180 injection, displaying even greater BrdU uptake than the rapidly cycling stem cells in the bone marrow positive control (29.3% $BrdU^+$ MZ B cells vs. 27.1% $BrdU^+$ ungated bone marrow). As the total number of MZ B cells did not increase in number the fate of the proliferating MZ B cells is unknown. As MZ B cells are the primary source of IgG3, one possibility is that after anti-CD180 treatment MZ B cells become highly activated, proliferate quickly, rapidly produce extrafollicular Ab, and then undergo apoptosis. Further studies are in progress to monitor the fate of the CD180-activated MZ B cells. While T cells do not express CD180

and neither become activated nor proliferate after anti-CD180 stimulation either *in vivo* or *in vitro,* their numbers are significantly increased in the spleen following anti-CD180 injection. This suggests that circulating T cells are increased in the spleen by passive mechanisms such as enhanced retention due to an indirect effect mediated by anti-CD180 activated B cells. Regardless of the mechanism, the expansion of T cells in the spleen is abrogated in B cell-deficient μMT mice, indicating that B cells are required for the effect on T cells and not other $CD180^+$ cells (DCs, macrophages). Increases to both B and T cell numbers were transient, approaching normal numbers by d 7 after injection; only $CD8^+$ T cell numbers remained increased through d 14. The function of these persistent $CD8^+$ T cells is unknown, as anti-CD180-induced expansion and contraction of B cells were equivalent in WT and T cell-deficient mice. It is possible that the prevalence of activated B cells is mediating memory T cell reactivation without the presence of cognate antigen.

The effects induced by anti-CD180 injection *in vivo* are not simply a byproduct of injecting a B cell-binding mAb. Anti-CD40 mAb, another known B cell activator, unlike anti-CD180, which expanded mainly transitional and MZ B cells, induced significant expansion only in FO B cells and myeloid cells (Fig. 3.6). Additionally, while anti-CD40 increased expression of both CD80 and CD95, classic markers of germinal center formation, anti-CD180 increased neither of these markers, but rather selectively upregulated CD86.

The combined injection of anti-CD180 with LPS, both inducers of polyclonal Ig, did not further increase Ig in serum but instead resulted in a reduction of Ig levels to concentrations intermediate to those seen with either stimulus alone. A similar effect was seen with co-injection of anti-CD180 with either TLR9 or TLR2:1 ligands (CpG or

Pam_3CSK_4). This result was duplicated with an *in vitro* system, where increasing doses of LPS decreased anti-CD180 induced IgG production in a concentration dependent manner. The suppression of anti-CD180 induced Ig by divergent TLR ligands might suggest either a restraining effect from TLR activated non-B cells or an intrinsic negative regulation by TLR signals of CD180 stimulated B cell differentiation. Neither of these models seem likely as TLR ligands usually increase Ig production when used as adjuvants in the majority of systems. Rather, our data support a model where combinations of CD180 signals and all MyD88-dependent TLR signals drive greater B cell proliferation at the expense of differentiation and Ig production – the combination effect is stimulatory, but at the expense of rapid differentiation. An extended timecourse for CD180/TLR agonist interaction may well show combinations to drive early proliferation leading to delayed but larger final Ig production.

Our data regarding B cell proliferation to anti-CD180 and LPS are not consistent with models suggesting CD180 functions by forming heterodimers specifically with TLR4 and regulating the canonical LPS signal (89, 92). Unlike LPS, the B cell proliferative response to anti-CD180 does not require MyD88, TRIF, or TLR4. Indeed, in a series of experiments, the impact of MyD88 deficiency on the anti-CD180 induced proliferation index was minimal while LPS induced proliferation was essentially abrogated. However, CD180 and TLR signals appear to be integrated through MyD88 because the combination of anti-CD180 and LPS signals augments B cell proliferation in TRIF-deficient but not MyD88-deficient B cells. Taken together, these results indicate that CD180 signals augment, but are independent from, those of TLR4. Given these findings, we hypothesized that stimulation of other MyD88-dependent TLRs (e.g. TLR9,

TLR7, TLR2:6, and TLR2:1) would also enhance B cell proliferation to CD180 ligation. Indeed, strong augmentation was evident with anti-CD180 and all TLR ligands tested. This effect may not have been detected in previous studies, which used only single concentrations of ligand combinations; saturation concentrations may have resulted in an insignificant augmentation unlike sub-maximal doses. As TLR7 and TLR9 are largely endosomal (180), and not at the cell surface like CD180, our data are not consistent with models of CD180 function requiring direct interactions with TLRs to augment B cell proliferation.

Our analysis allowed the use of the mathematical transformation described by Chou and Talalay (171) to quantify synergy over broad dose ranges. Synergy is highest between anti-CD180 and the TLR2 ligands, followed by TLR7, then by TLR9, with the least synergy between CD180 and TLR4. The analysis also revealed previously unreported antagonism between anti-CD180 and all MyD88-dependent TLR ligands, excluding LPS, at very low doses. Existing models of CD180 as a selectively forming heterodimers with TLR4 predict neither of these patterns, regardless of whether the interaction is stimulatory or inhibitory. Whether CD180 acts as a specific TLR4 “decoy” receptor in B cells, as proposed for DCs (79), or a required co-receptor for a single B cell LPS pathway (92), the effect should impact both the MyD88 and TRIF signaling pathways for LPS and no effect would be expected for other TLRs. Thus, our findings showing that CD180 synergizes with multiple TLR ligands in a MyD88-dependent TRIF-independent manner to enhance proliferation at nearly all dose levels suggest an alternative model where independent CD180 and TLR signals converge in B cells at the level of MyD88.

While anti-CD180 stimulation of purified B cells induced proliferation, it did not induce cytokine production. However, in combination with LPS, anti-CD180 stimulation increased production of IL-10 and IL-6, but not TNF-α, while anti-CD180 plus CpG increased production of all of these cytokines. The IL-10 concentrations were high (>1,000 pg/ml), suggesting that CD180 signals could be involved in development of anti-inflammatory IL-10 secreting B cells (181). Due to the complex effects of IL-10, both suppressing inflammation and activating B cells (182, 183), it is possible that combined CD180/TLR signaling may minimize TLR-induced inflammation while promoting select B cell functions. As with B cells, DCs failed to produce cytokines with anti-CD180 stimulation alone, however unlike B cells TLR-induced cytokine production was not augmented by the combination with anti-CD180. This finding is inconsistent with the inactive dimer model where CD180 suppresses TLR4 sensing of LPS (79); if anti-CD180's function were simply to sequester CD180 from TLR4 this would lead to an increase in DC sensitivity to LPS rather than the observed minimal decrease. A combination of evidence regarding anti-CD180 treatment - the lack of DC responsiveness, the requirement of B cells for splenic expansion, the production of high serum concentrations of Ig in both WT and T cell-deficient mice, and the proliferation of purified B cells *in vitro* - together suggest that CD180 stimulation is primarily mediated by, and intrinsic to, B cells. While our data cannot differentiate between a direct stimulatory effect upon B cells or the removal of a repressive signal the fact that anti-CD180 induces rapid expansions in B cell numbers, calcium flux, and enhanced expression of both costimulatory factors and cytokines, we think it is most likely that

regardless of mechanism the end effect of anti-CD180 treatment is intrinsic stimulation of B cells.

Our study of CD180 is unique in that use of an agonistic antibody allows us to perform quantitative interaction assays over broad dose ranges and characterize acute responses as opposed to genetic deletion that results in data singular in both dose and kinetics. Taken together, our results suggest that CD180 stimulation plays an important role in B cell proliferation, activation, and differentiation, and that these effects are significantly modulated by integration of MyD88-dependent TLR signals. While it remains to be determined whether the rapidly induced class-switched Ig also involves somatic hypermutation, it appears to be polyclonal. Finally, because anti-CD180 treatment induces immunomodulatory effects (augmenting anti-inflammatory IL-10, blunting Ag-specific responses, and producing polyclonal Ig which may clear apoptotic debris like natural antibody) it may have therapeutic potential in systemic autoimmune diseases; we are currently exploring this possibility in mouse models.

Chapter 4. Targeting antigens to CD180 rapidly induces antigen-specific IgG, affinity maturation, and immunologic memory

Introduction

Delivering antigens (Ags) directly to antigen presenting cells (APCs) such as dendritic cells (DCs) by coupling them to Abs specific for APC-restricted surface molecules is a method to efficiently induce immune responses (116, 184). While targeting Ag to all APCs by using antibodies against broadly expressed markers (e.g. anti-MHC II) is effective, many Ag-targeting approaches specifically direct Ag to DC subsets via mAbs specific for C-type lectin receptors (CLRs) (185). This method of immunization reduces the amount of Ag required and directs the immune response toward specific effector functions such that the final immune response to Ag can be altered. Selection of either the cell-surface receptor targeted by mAb or the epitope recognized by the targeting mAb can significantly change the nature of the response induced. For example: delivery of Ag to Dectin-1 induces strong CD4+ T cell if administered with adjuvant (121); targeting to DEC205 induces strong CD8+ T cell responses with adjuvant but low Ab responses (122); targeting to DCIR2 in the absence of adjuvants generates strong CD4+ T cell help and extrafollicular IgG1 Ab responses (123); while targeting to Clec9A generates CD8+ T cell responses with adjuvant and efficiently activates T follicular helper (Tfh) cells for Ab production without adjuvant (124).

CD180 was originally identified as a 95 kDa B cell-associated surface molecule designated Bgp95 or RP105, that when ligated with mAb could mediate B cell activation and proliferation (97, 103). CD180 is recognized as a homolog of the extracellular

domain of TLR4 (91), however it lacks the cytoplasmic TIR domain common to all TLRs and instead initiates a BCR-like signaling cascade that does not utilize TLR signaling adaptors (96, 97, 100 - 103). Despite having a signaling pathway overlapping that of the BCR, CD180 signaling also has been reported to be antagonistic to BCR signaling and to induce apoptosis of B cells following sequential stimulation of the BCR after CD180 stimulation (104).

Previously, we reported that inoculation of mice with anti-CD180 induces extremely rapid and robust polyclonal IgG production, even in the absence of CD40 signaling or T cells (186). Since anti-CD180 alone induced T cell-independent (TI) class switched Ab, we evaluated in this report whether Ag delivery to CD180 was able to induce Ag-specific IgG responses. As CD180 internalizes after ligation, we reasoned that Ag-anti-CD180 might be processed by DCs and/or B cells and activate CD4 T cell helper cells. We found that intravenous inoculation of mice with Ag-anti-CD180 induces both T cell-dependent (TD) and TI Ag-specific IgG responses. CD180 expression by B cells was required, whereas expression on non-B cells enhanced the response but was not required. Remarkably, targeting Ags via CD180 in a single inoculation without adjuvant primed mice to mount secondary immune responses, even in CD40-deficient mice. Our results show that coupling Ags to anti-CD180 is an effective means for raising Ag-specific Ab responses that may find efficacy for both therapeutic and prophylactic vaccines.

Results

Targeting antigen to CD180 induces specific and strong Ag-specific IgG responses

In a previous study we found that administration of a high dose of anti-CD180 mAb (250 μg) induced >15 fold increases in serum IgG through polyclonal Ig production

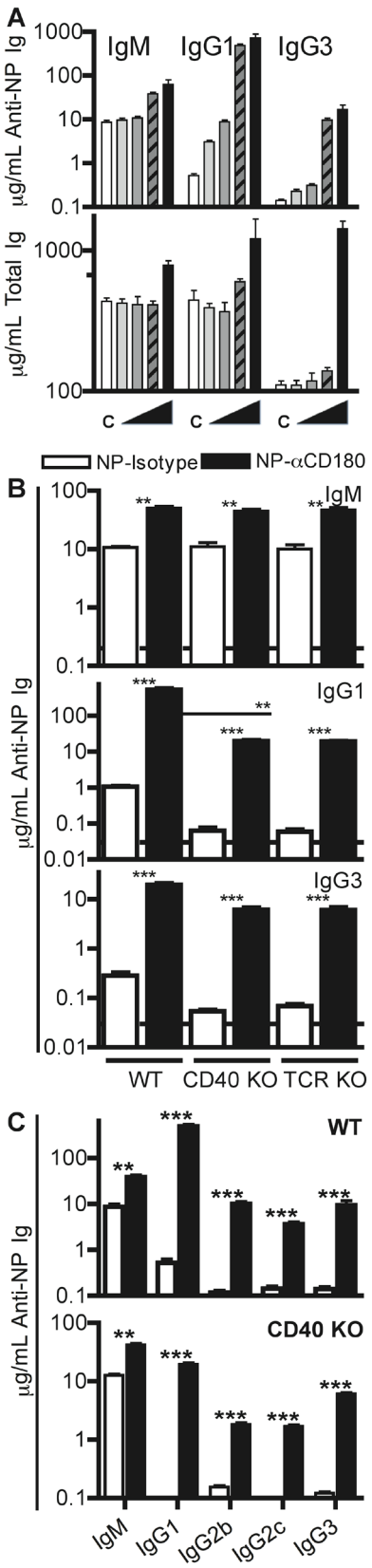


Figure 4.1. Targeting to CD180 induces Ag-specific Ig production independently of T cell help. A) WT mice received 100 μg of NP-isotype ("c", open column) or graded doses of NP-αCD180 (10, 30, 100, or 250 μg, light grey, medium grey, crosshatched grey, and black columns respectively) mAb i.v., bled at day 10, with total serum Ig and anti-NP Ig analyzed by ELISA. **B**) WT, CD40 KO, or TCR KO mice were inoculated with 100 μg NP-αCD180 or NP-isotype, and bled at d 10 for NP-specific Ig analysis by subclass. **C**) WT or CD40 KO mice were treated as in **B** for NP-specific Ig analysis by subclass. Three mice/group, representative of three experiments for **A**, **B**, and **C**.

in both WT mice as well as in CD40- and T cell-deficient mice (186). Thus, given this B cell stimulatory effect and the fact that CD180 is internalized following ligation by mAbs, we decided to examine whether Ag coupled to anti-CD180 could induce Ag-specific IgG responses in normal and immunodeficient mice. We conjugated the hapten NP to anti-CD180 (NP-αCD180) or to a non-binding rat IgG2a isotype control (NP-isotype) mAb and administered them in graded doses i.v. to WT mice. Analysis of day 10 bleeds indicates doses of 100 μg or less induced little or no polyclonal Ig production but did induce large NP-specific IgG responses in a dose dependent

manner (Fig. 4.1A). Reduced doses of NP-αCD180 produced similar effects when administered with a compensating amount of unconjugated anti-CD180 (data not shown), strongly suggesting that the requirement for large doses is due to the large number of potential binding sites rather than other considerations. Peak Ag-specific IgG production induced by NP-αCD180 was three fold higher than that normally achieved with immunization of NP-isotype precipitated in alum. Unless otherwise specified all subsequent inoculations utilized 100 μg Ag-αCD180.

Targeting Ag to CD180 induces Ag-specific IgG in the absence of T cell help

Targeting Ag to CD180 also induced Ag-specific IgM and IgG production in both CD40 KO mice and T cell deficient (TCR β/δ KO) mice (Fig. 4.1B). Anti-NP Abs were not observed in mice immunized with anti-CD180 mAb alone, therefore the response to NP-αCD180 is due to targeting of Ag rather than an adventitious Ag-binding product of polyclonal Ig production (data not shown). Ag-specific IgM levels were similar in WT and immunodeficient mice, but Ag-specific IgG levels were significantly lower in both CD40 KO and TCR KO mice. Despite the overall reduction in Ag-specific IgG, in immunodeficient mice the broad IgG subclass distribution was maintained and similar to

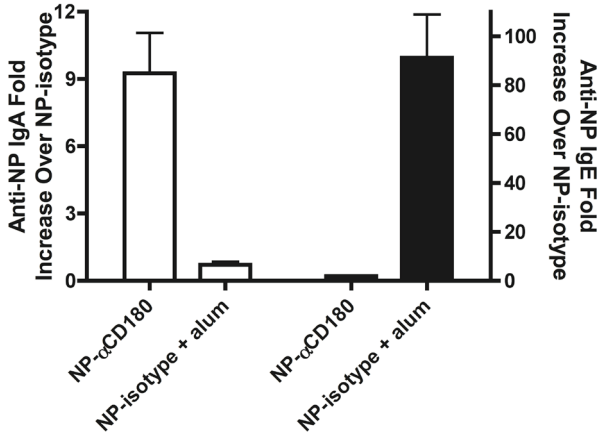


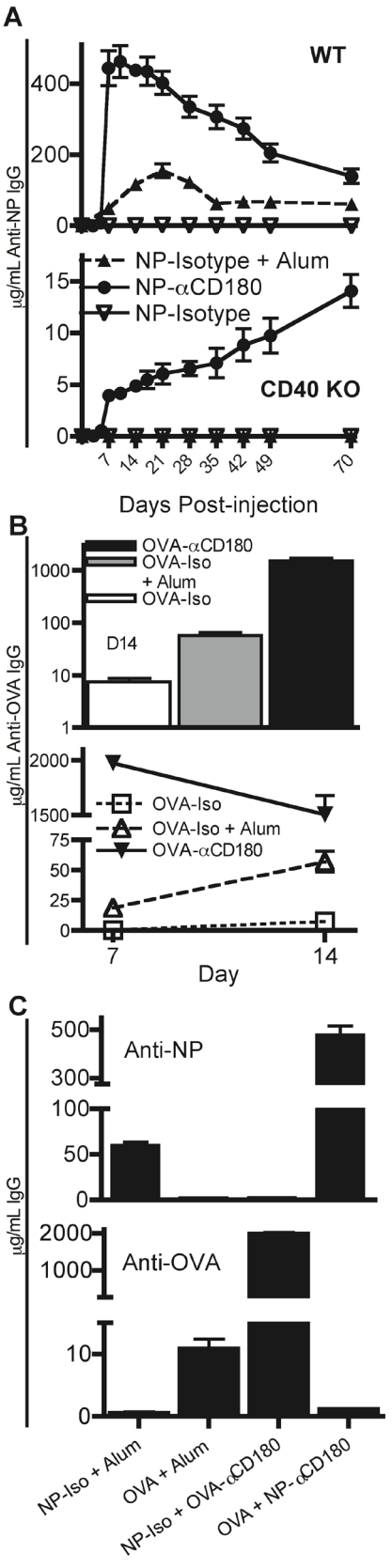
Figure 4.2. Ag-αCD180 rapidly induces IgA but not IgE Ag-specific Ab production. C57BL/6 mice received 100 μg NP-αCD180 or 100 μg NP-isotype in alum, bled at day 10, and NP-specific IgA and IgE were analyzed by ELISA and compared to mice injected with 100 μg soluble non-adjuvanted NP -isotype control mAb for fold induction. Three mice per group, representative of two experiments.

that in WT mice (Fig. 4.1C). In addition to Ag-specific IgG, NP-αCD180 also induced Ag-specific IgA, but not IgE (Fig. 4.2). This preferential induction of IgA responses rather than IgE is counter to the polyclonal effects of anti-CD180 (Fig. 3.2) and is likely due to the change in isotype class-switch regulation from stimulating CD180 and the BCR concurrently (with NP-αCD180), rather than CD180 alone as in Fig. 3.2. We conclude that targeting Ag to CD180 induces both TD and TI IgG antibody responses of exceptionally broad isotype subclass distribution.

CD180 targeting produces more extensive and rapid Ag-specific IgG than Ag in alum

We next determined the kinetics of Ag-specific IgG production following NP-αCD180 inoculation. We immunized WT or CD40 KO mice i.v. with NP-αCD180 or NP-isotype, or i.p. with the NP-isotype precipitated in alum. In WT mice, NP-αCD180 induced a far more rapid anti-NP IgG response that peaked 10 days p.i. as compared to Ag in alum (21 days to peak). Mice inoculated with NP-isotype alone did not produce more than 2 μg/mL anti-NP Ab at any timepoint (Fig. 4.3A, upper panel). As expected, CD40 KO mice immunized with Ag in alum did not make an NP-specific IgG response, however they did develop a significant and continually increasing amount of NP-specific IgG after CD180 targeting (Fig. 4.3A, lower panel).

Targeting to CD180 induces anti-protein IgG responses and requires linked Ag



To determine whether the strong Ab response to NP-αCD180 was also induced when we targeted protein antigens to CD180, we coupled whole OVA to anti-CD180 (OVA-αCD180) and isotype mAb (OVA-isotype) and immunized WT mice with one of these antigens or OVA-isotype in alum (Fig. 4.3B). As with NP-αCD180, OVA-αCD180 induced a rapid and strong Ag-specific IgG response with peak concentrations of nearly 2 mg/mL anti-OVA IgG at day 7.

As anti-CD180 alone stimulates B cells, possibly changing the context of how they present Ag even if it were administered in an

Figure 4.3. CD180 targeting rapidly and specifically induces high concentration Ag-specific IgG. A) WT or CD40 KO mice were inoculated with 100 μg NP-αCD180 or NP-isotype or 100 μg NP-isotype in alum, bled at the indicated timepoints and serum analyzed for NP-specific IgG. **B)** WT mice were inoculated with 100 μg OVA-αCD180, OVA-isotype, or OVA-isotype in alum, bled at the indicated timepoints and serum analyzed for OVA-specific IgG. **C**) WT mice were inoculated with 100 μg each of the indicated stimuli and bled on d 10 for Ag-specific IgG analysis. Three mice/group, representative of two experiments for **A**, **B**, and **C**.

unlinked fashion, we inoculated mice with crisscross combinations of NP- and OVA-based Ag where only one Ag was coupled to αCD180 (NP-αCD180 + OVA or OVA-αCD180 + NP-isotype). As expected, mice inoculated with NP-isotype or OVA in alum produced IgG only against NP or OVA, respectively (Fig. 4.3C). Mice inoculated with NP or OVA coupled to anti-CD180 along with soluble OVA or NP-isotype only made antibodies against the Ag coupled to anti-CD180 and not to the soluble, unlinked Ag. We conclude that during Ag targeting to CD180, only B cells specific for the Ag attached to anti-CD180 are driven to produce Ab. These results also suggest that both BCR ligation and CD180 binding on the same cell are required for specific Ab to be produced.

Ag-specific B cells are efficiently activated by linking BCR and CD180 stimuli

Since linked specific Ag and anti-CD180 is required to induce IgG Ab responses, we compared the early activation state of B cells ex vivo following stimulation in vivo through either the BCR, CD180, or through both receptors. We used B1-8^{hi} mice, which contain a knock-in IgH that is specific for NP if paired with a lambda light chain (190); groups of these mice were injected with either 100 μg NP-αCD180 or with NP-isotype and spleens harvested at the indicated timepoints. The NP-specific B cells (6-10%) were distinguished from total $CD19^+$ B cells by staining with NP-APC. Four groups of $CD19^+$ B cells were then analyzed for their expression of CD69, CD86, MHC class II, and TACI levels: unstimulated (NP^- B cells from NP-isotype treated mice), BCR stimulated (NP^+ B cells from NP-isotype treated mice), CD180 stimulated (NP^- B cells from NP-αCD180 treated mice), and BCR + CD180 stimulated (NP^+ B cells from NP-αCD180 treated mice). Compared to unstimulated B cells, B cells stimulated via either Ag or αCD180 had increased expression of CD86 and TACI (Fig. 4.4A); however, the levels of CD69,

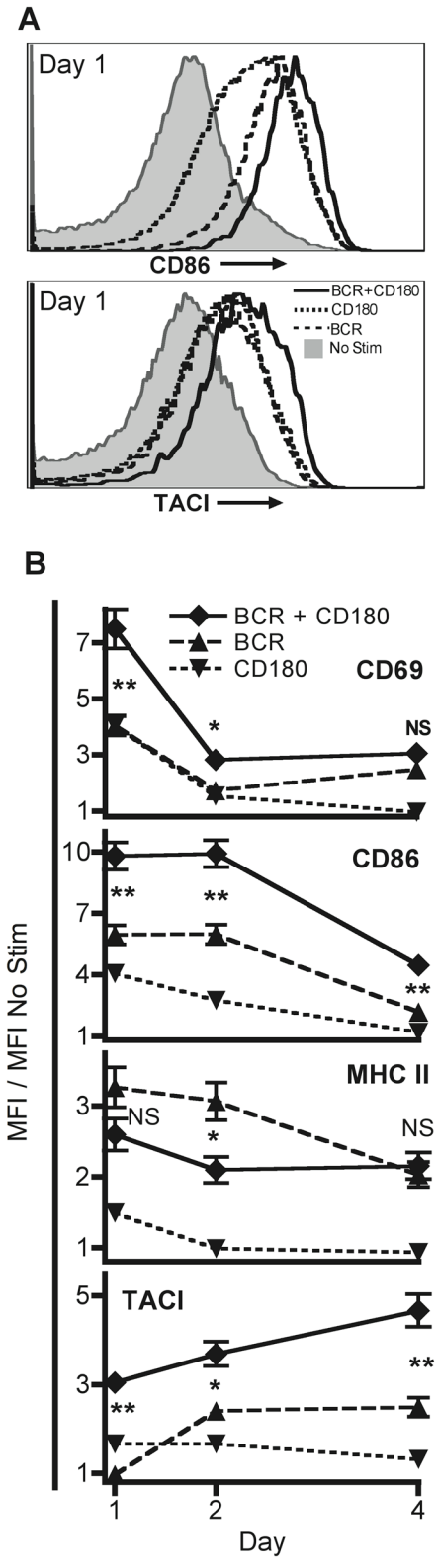


Figure 4.4. Ag-specific B cells are hyper-activated by linked BCR and CD180 stimuli **A** and **B**) B1-8^{hi} mice were inoculated with either 100 μg NP-isotype or NP-αCD180 and spleens harvested at the indicated timepoints. Total splenocytes were gated to isolate both non-NP binding B cells (CD19^{+} NP-APC^{-}, No Stim from NP-isotype mice and CD180 from NP-αCD180 mice) and NP binding B cells (CD19^{+} NP-APC^{+}, BCR from NP-isotype mice and BCR + CD180 from NP-αCD180 mice) to create the four groups for activation analysis displayed in **A** and graphed in **B**.

CD86, and TACI were both higher and more persistently increased on B cells stimulated through both the BCR and CD180 (Fig. 4.4B). As TACI upregulation favors further B cell activation, this effect may create a persistent and self-perpetuating activation as well.

B cell expression of CD180 is necessary and sufficient for Ag-αCD180 driven Ab responses

Since CD180 is expressed on both B cells and non-B cells and is internalized following ligation by mAb (data not shown), Ab responses induced by CD180 targeting may be mediated by delivery of

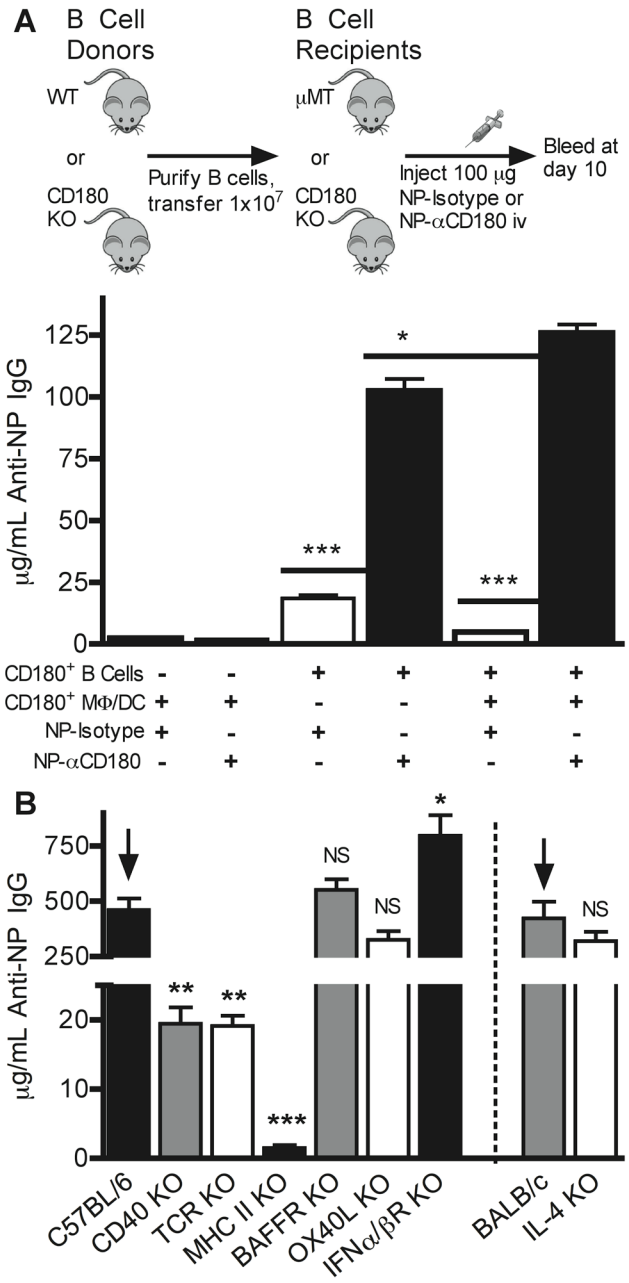


Figure 4.5. CD180 Ag-targeting responses require expression on B cells and function through T1 B cells. A) $1x10^7$ B cells purified from either WT or CD180 KO mice were transferred to μMT or CD180 KO recipients as indicated. 24 hours following transfer mice were inoculated with 100 μg of NP-isotype or NP-αCD180 and bled ten days later. **B)** WT (C57BL/6 and BALB/c) mice and paired KO were inoculated with 100 μg NP-αCD180 or NP-isotype, and bled at d 10 for NP-specific IgG analysis by ELISA. Three mice/group; representative of two experiments for **A** and **B**. All statistics relative to population indicated by arrow.

both Ag-mediated BCR signaling together with CD180 signals to Ag-specific B cells or alternatively, CD180 signals and Ag-independent delivery to non-B cells which then in turn stimulate Ag-specific B cell and T cell responses. To assess this possibility we conducted adoptive transfer experiments to establish mice that express CD180 only on B cells, only on non-B cells, or on both target cell populations. B cell deficient $CD180^+$ recipients (μMT) into which CD180 KO B cells were transferred (CD180 only on non-B cells) failed to make Ag-specific IgG after inoculation with NP-αCD180 (Fig. 4.5A), demonstrating that CD180 expression on B

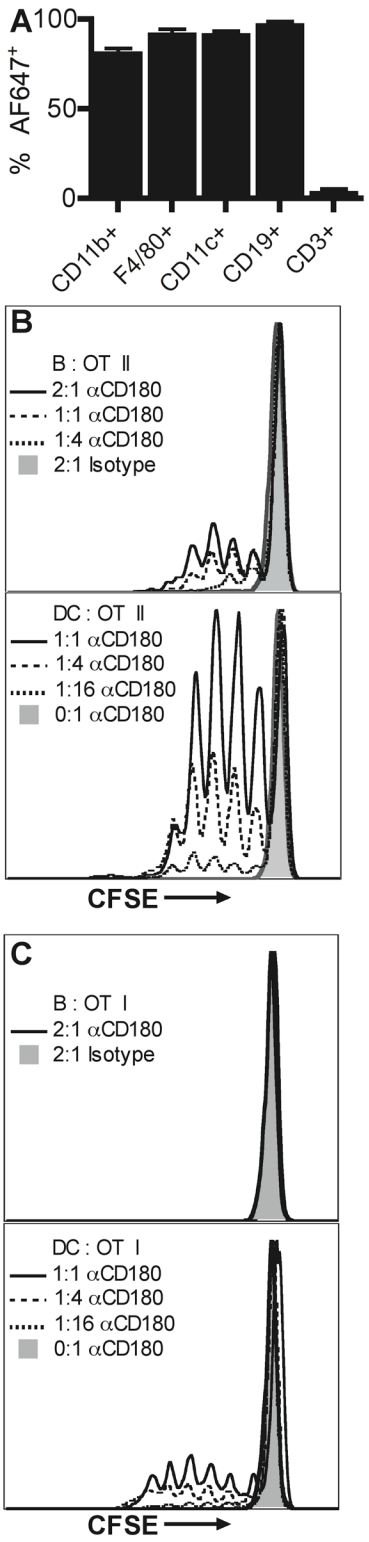
cells is necessary to generate the Ab response. In contrast, following immunization with NP-αCD180 the CD180 KO recipients into which $CD180^+$ B cells were transferred (CD180 only on B cells) made >75% of the NP-specific IgG produced by $CD180^+$ recipients into which $CD180^+$ B cells were transferred (CD180 on both B cells and non-B cells). These data show that CD180 expression on B cells is sufficient for αCD180-based targeting. However, CD180 expression on non-B cells such as DCs, while not essential for Ag targeting, influences the extent of IgG production.

While it has been suggested that CD180 KO mice have LPS-sensing defects related to BAFF dysregulation (115), this experiment does not measure TI-1 Ag responses but only TD Ag responses which are uniformly reported to be normal in CD180 KO mice (92).

Th2 mediators, IFN-α/β, and mature B cells are not required for Ag-targeting to CD180

As another T cell-dependent B cell activator, αIgD, requires the action of IL4 (191), we compared Ab responses of WT, IL4 KO and OX40L KO mice inoculated with NP-αCD180. Neither IL-4 nor OX40L deficiency resulted in significant changes in anti-NP IgG titers compared to WT controls (Fig. 4.5B). In contrast, abrogating signaling through the IFN-α/βR significantly increased anti-NP IgG production, suggesting that type 1 IFNs may normally restrain Ab responses induced via CD180. Mice deficient in MHC II after immunization with NP-αCD180 had a similar, though more severe, reduction in anti-NP IgG production than either CD40 or TCR KO mice. Surprisingly, although BAFF-R KO mice have a near complete block in mature B cell development (192), they did not show any significant deficiency in anti-NP IgG production following

targeting to CD180, suggesting that while CD180 expressing B cells are required for Ag-αCD180 targeting they need not be mature B cells.



Targeting to CD180 in vivo leads to processing of Ag by both B cells and DCs

To assess which CD180$^+$ cells had the potential to take up, process, and present Ag to T cells after targeting by anti-CD180, we first measured the binding of anti-CD180 to WT splenocytes by tracking AF647-αCD180 30 min following i.v. injection. All CD180$^+$ APC populations (CD11b$^+$ or F4/80$^+$ macrophages, CD11c$^+$ DCs, and CD19$^+$ B cells) were >85% positive for AF647, with B cells being nearly 100%

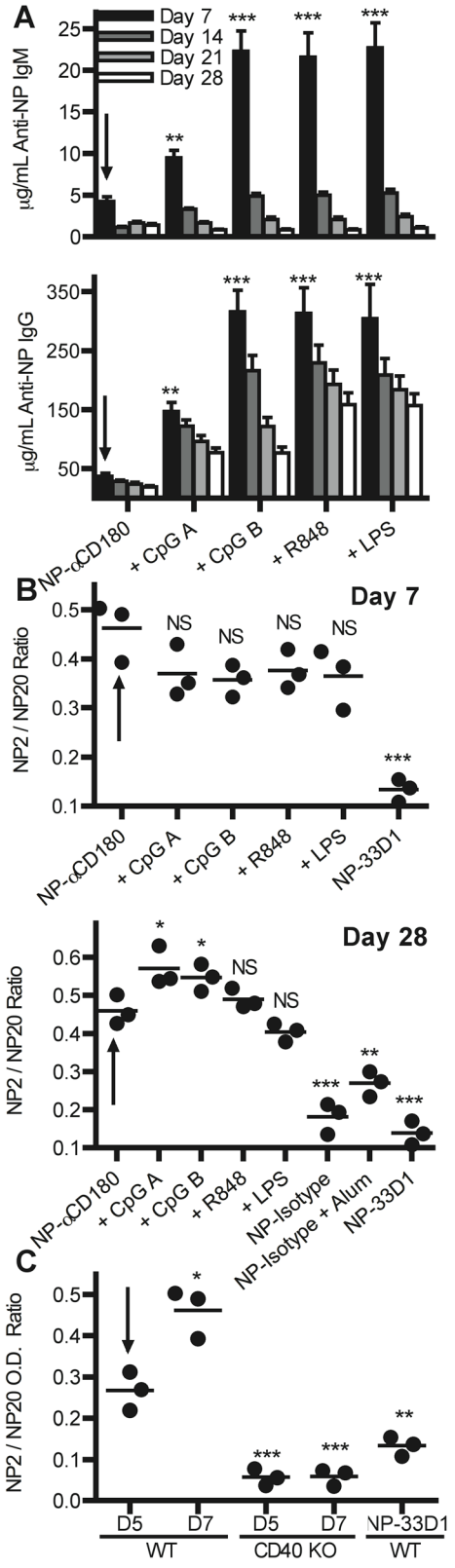
Figure 4.6. Targeting to CD180 expands Ag-specific T cells. A) WT mice were sacrificed 30 minutes following inoculation with 100 μg AF647-αCD180 and total splenocytes stained as indicated and analyzed for AF647 label on each gated cell type. Three mice/group, representative of two experiments. **B)** WT mice were inoculated with 100 μg OVA-αCD180 or OVA-isotype and spleens harvested 16 hours later. B cells and DCs were purified, seeded into culture with CFSE labeled purified OT II T cells at the indicated ratios, and CFSE dilution of CD4$^+$ Vα2 TCR$^+$ cells was assessed following 72 hours in culture. **C)** Performed as in **B** but with CD8$^+$ Vα2 TCR$^+$ OT I cells. Co-cultures performed in triplicate, representative of two independent experiments for **B** and **C**.

positive (Fig. 4.6A). T cells ($CD3^+$) do not express CD180 and showed minimal background staining. To determine which APC populations were most effective at priming T cells following targeting to CD180, WT mice were inoculated with either OVA-isotype or OVA-αCD180 and 16 hrs later B cells and DCs were purified by negative selection and co-cultured with CFSE labeled OVA-specific OT-II CD4 T cells. 72 hours later the levels of CFSE in the OVA-specific T cells were measured by flow cytometry (Fig. 4.6B). While OVA-αCD180 targeted B cells, unlike OVA-isotype primed B cells, clearly induced proliferation of Ag-specific CD4 T cells, OVA-αCD180 targeted DCs were much more effective at stimulating OT-II proliferation. In contrast, OVA-αCD180 targeted B cells, unlike OVA-αCD180 targeted DCs, failed to induce any proliferation of OVA-specific OT-I CD8 T cells (Fig. 4.6C), consistent with the poor cross-presentation of Ag by B cells to compared to DCs. Thus while DCs are not required for the Ag-specific Ab response induced by Ag-αCD180 they impact stimulation of Ag-specific T cell help.

TLR-based adjuvants enhance Ab responses to CD180-targeted Ag

To assess whether addition of adjuvants could affect the efficiency or quality of Ab responses after Ag-αCD180 targeting, we coadministered NP-αCD180 (50 μg) with adjuvants primarily influencing either DCs (CpG A), B cells (CpG B), or both (R848 and LPS). Each adjuvant, when combined with NP-αCD180, increased NP-specific IgM and IgG production 4 to 7 fold. CpG type A, which is known to efficiently stimulate DCs and not B cells, was the least effective (Fig. 4.7A).

We also examined the effect of TLR agonists on affinity maturation induced via NP-αCD180 using an assay measuring the relative binding of antisera to BSA with low



levels of NP bound (NP_2) vs. to BSA with higher levels of NP bound (NP_{20}). At d 7 Ab affinity was significantly higher than that of the NP-33D1 low affinity control (121) but inclusion of adjuvants did not significantly impact the Ab affinity (Fig. 4.7B, upper panel). By day 28 after immunization the Ab affinity of NP-αCD180 only inoculated mice had not

Figure 4.7. Adjuvants increase responsiveness to CD180 targeting and long-term affinity, but not IgG persistence. **A**) WT mice were inoculated with 50 μg NP-αCD180 or NP-isotype with the indicated adjuvants (50 μg CpG, 20 μg R848, or 4 μg LPS) bled at the indicated timepoints and serum analyzed for quantities of NP-specific Ig and **B)** WT mice were inoculated with 100 μg NP-αCD180, NP-isotype, or NP-isotype in alum with the indicated adjuvants (50 μg CpG, 20 μg R848, or 4 μg LPS) and analyzed for NP-specific IgG affinity as measured by NP_2/NP_{20} Ag-binding ratio. Sera from NP-33D1 inoculated mice included as a known low-affinity control. **C)** WT and CD40 KO mice were inoculated with 100 μg NP-αCD180, bled at the indicated timepoints, and serum analyzed for IgG affinity as measured by NP_2/NP_{20} Ag-binding ratio. Three mice/group; representative of two experiments **A**, **B**, and **C**. All statistics relative to population indicated by arrow.

increased beyond day 7 values, however the addition of CpG adjuvants significantly increased affinity while other adjuvants did not (Fig. 4.7B, lower panel). Compared to low-affinity Ab responses induced by targeting Ag to DCIR2 DCs (NP-33D1), NP-αCD180 induced a small but significant increase in Ab affinity as early as 5 to 7 days after immunization (Fig. 4.7C) which is absent in CD40 deficient mice.

Ag-αCD180 induces rapid expansion, GC phenotype, and predominately AFC differentiation

To follow expansion and differentiation of Ag-specific B cells we adoptively transferred $2x10^5$ splenocytes from Ly5.1$^+$ B1-8^{hi} mice into Ly5.2$^+$ WT hosts. Spleens were harvested at d 4 or d 7 following

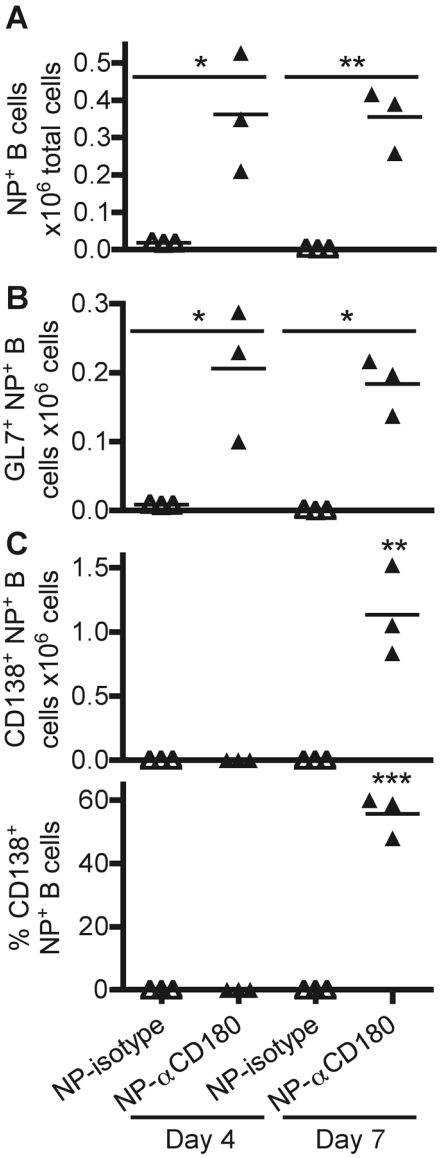
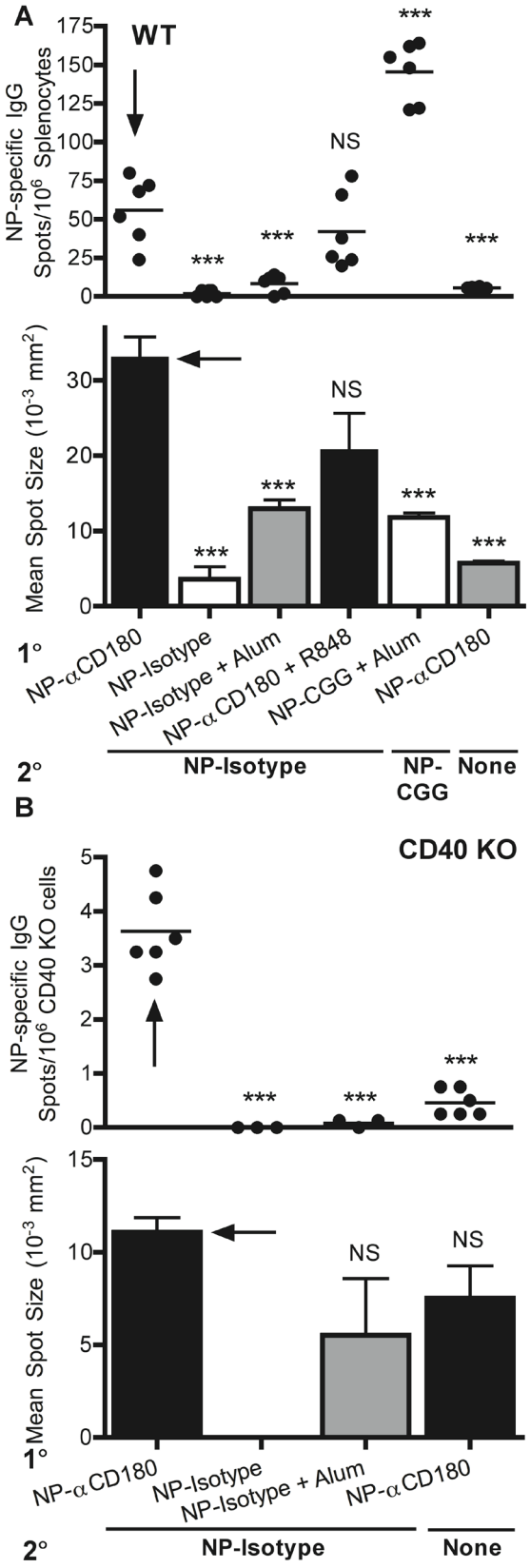


Figure 4.8. Ag-αCD180 induces rapid Ag-specific B cell expansion, acquisition of germinal center markers, and conversion to AFC. $2x10^5$ total splenocytes from Ly5.1$^+$ B1-8^{hi} mice were adoptively transferred to Ly5.2$^+$ WT recipients at day -1. On d0 the mice were inoculated with 100 μg of either NP-αCD180 or NP-isotype and spleens harvested at either d4 or d7 for flow cytometric analysis. All panels were gated for Ly5.1 (congenic marker for transferred cells), B220 (lineage marker for B cells), and NP-APC binding for antigen specificity. **A)** Total transferred NP-specific B cell numbers, **B)** B cells with a germinal center phenotype as measured by GL7, **C)** AFC numbers (upper panel) and percentage of total NP binding B cells (lower panel) as assessed by CD138/Syndecan-1$^+$ and B220^{lo} staining. Three mice/group; representative of two experiments.

inoculation with 100 μg NP-isotype or NP-αCD180 and analyzed by flow cytometry using sequential gating for $B220^{+}$, $Ly5.1^{+}$, and NP-APC binding. NP-isotype treated mice showed no expansion of Ag-specific $B220^{hi}$ B cells but NP-αCD180 treated mice showed approximately 20-fold expansion at both timepoints (Fig. 4.8A). Examination for germinal center markers ($GL7^{+}$ and PNA^{+}) indicated that greater than 50% of $B220^{hi}$ NP-specific B cells were $GL7^{+}$ (Fig. 4.8B) at both timepoints, indicating a rapid shift toward a germinal center phenotype. Using CD138 as a plasma cell marker on $B220^{lo}$ cells, we detected few NP-specific AFCs in NP-isotype treated or NP-αCD180 treated mice at d 4; however at d 7 NP-αCD180 treated mice had significant numbers of AFCs that outnumbered $B220^{hi}$ B cells nearly 3 to 1 (Fig. 4.8C).

Targeting to CD180 primes for immunologic memory even in the absence of adjuvants or CD40

To determine whether the Ag-specific B cell expansion induced by Ag-αCD180 extended to generating immunologic memory we immunized groups of WT and CD40 KO mice with NP-conjugated mAbs as above or with NP-CGG in alum as a positive control, rested them for 10 weeks and then boosted with matched soluble Ag (NP-isotype or NP-CGG) or with PBS as a negative control. Four days later spleens were harvested and the number and size of IgG-producing antibody forming cells (AFCs) was assessed using an NP-specific IgG ELISPOT assay. As expected, the NP-CGG-primed mice produced significant numbers of IgG-producing AFCs when given soluble Ag (Fig. 4.9A, upper panel). NP-αCD180-primed mice also rapidly produced AFCs upon Ag re-challenge. While the number of memory AFCs generated in mice primed with NP-αCD180 in the absence of adjuvant was roughly one third that of NP-CGG in alum



primed mice, the spot size - a measure of the amount of NP-specific IgG produced per AFC - was three times as large as spots from mice primed with NP-CGG (Fig. 4.9A, bottom panel). Addition of the adjuvant R848 during the primary immunization with NP-αCD180 neither increased the spot number nor spot size upon Ag re-challenge compared to mice primed with NP-αCD180 alone. Surprisingly, we also detected NP-specific IgG-secreting AFCs in CD40 KO mice primed with NP-αCD180 following Ag re-challenge (Fig. 4.9B). While the

Figure 4.9. Targeting to CD180 primes for memory in both WT and CD40 KO mice. A) WT and **B)** CD40 KO mice were primed as indicated, rested for 10 weeks, then boosted i.p. with Ag only (20 μg) or PBS. Four days post boost spleens were harvested and analyzed for NP-specific AFC by ELISPOT. Three or six mice/group; representative of two experiments. All statistics relative to population indicated by arrow.

number of NP-specific IgG-secreting AFCs in CD40 KO mice was roughly $1/15^{th}$ as numerous as in WT mice (3.7 per million cells as opposed to 55 per million cells), they were significantly higher than in PBS-boosted CD40 KO mice or CD40 KO mice primed with Ag in alum (0.08 per million cells and 0.46 per million cells, respectively). Our results demonstrate that Ag-αCD180 effectively primes for immunologic memory, even in the absence of CD40 signaling.

Discussion

Collectively, our data indicate that targeting Ags to CD180 induces rapid activation of Ag-specific B cells, leading to both significant affinity maturation and IgG production within 7 days (Fig. 4.3A, 4.4, 4.7C). Remarkably, a single injection of Ag-αCD180 without any additional adjuvant led to the development of immunologic memory (Fig. 4.9A). Furthermore, while severely impaired, Ag-specific IgG production and responses to secondary immunizations were retained in CD40 KO mice (Fig. 4.9B), even though CD40 KO mice did not make IgG or develop memory B cells in response to Ag in alum as has been reported previously (27). The Ab responses induced were specific for the Ags attached to anti-CD180 and could be induced to both haptens and protein Ags (Fig. 4.3B, C).

Several possibilities may explain why this mode of immunization is so effective in raising Ab responses. Our analysis of B cells activated in vivo revealed that stimulating the Ag receptor and CD180 together is more effective in activating B cells than either stimulus alone (Fig. 4.4). The large induction of CD86 expression may be a critical feature of targeting Ag to CD180, as CD86 is necessary for IgG responses to non-adjuvanted Ag (193). The TACI receptor was also induced to high and sustained levels

after CD180 targeting, and TACI has been implicated in class switching and high quantity IgG production (178). Furthermore, while responses to linked Ag occurred with both NP-αCD180 and OVA-αCD180, there was no significant response to coadministered soluble Ags (Fig. 4.3C). These results indicate that delivering the CD180 stimulus with Ag produces a substantially different activation signal than Ag and anti-CD180 in combination. That the targeting effect requires CD180 to be expressed on B cells also implies that simultaneous Ag receptor and CD180 ligation are necessary (Fig. 4.5A).

Early Ag targeting approaches utilized anti-Ig mAb to deliver Ag to B cells and speed expansion of Ag specific CD4+ T cells (130, 132). However, Ab responses induced by Ag-anti-Ig are weaker than those induced by targeting Ag to the pan-APC marker MHC II (134), while other B cell targets tested proved ineffective at generating Ab responses (B220, FcγRII; 131). The higher efficacy of Ag delivery to dendritic cells has lead to the majority of Ag-targeting approaches being focused on targeting myeloid cell subsets. We note that the B cell surface molecules chosen in prior studies were either a) not known to signal (B220); b) inhibitory receptors (FcγRIIb); or c) BCR components (IgD), such that targeting Ag to them was unlikely to produce additional stimulation beyond what Ag already provided.

Ag targeting to CD180, while requiring B cells, appears to not require mature B cells: BAFF-R KO mice mainly have transitional 1 (T1) B cells as they have a fivefold reduction in T2 B cells and are almost completely deficient of follicular and marginal zone mature B cells (192). Nevertheless, inoculation of Ag-αCD180 into BAFF-R KO mice produced as much Ag-specific IgG as in WT mice (Fig. 4.5B). This suggests that T1

B cells are a major target for Ag-anti-CD180. Although T1 B cells readily apoptose following BCR stimulation (194), T1 B cells also constitutively express activation-induced deaminase (AID) (108, 195) and can rapidly produce large quantities of IgG when triggered with a combination of BCR and TLR stimuli (108, 195 - 197). T1 B cells have also been implicated in the development of the B cell repertoire in both CD40 KO mice and hyper-IgM syndrome patients (198). Thus, our data suggest that AID+ T1 B cells signaled through concurrently through the BCR and CD180 may rapidly switch and mature into IgG-producing plasma cells. Further studies are in progress to define the B cell subsets and signaling pathways responsible to the rapid IgG response. As a further note, equivalent Ag-specific IgG production in BAFF-R KO mice following Ag-anti-CD180 immunization indicates that, contrary to the claims of Allen (115), the impact of CD180 signals are not solely from dysregulation of BAFF – changes in the expression of BAFF following Ag-anti-CD180 treatment, while not measured here, would not have significant impact in BAFF-R KO mice.

Our results indicate that Ag-αCD180 targeting generates long-lived plasma cells and switched memory B cells in both WT and CD40 KO mice. First, WT mice have an Ag-specific IgG t½ of 38 days (based on kinetics in Fig. 4.3A, upper panel) while catabolism of a discrete burst of IgG from a short-lived AFC response would have a t½ of 21 days. Additionally, Ag-specific IgG levels in CD40 KO mice continue to rise over time (Fig. 4.3A, lower panel). Both of these results require continual IgG production to slow or offset the constant elimination, so some Ab-producing cells must be being retained. Second, on day 4 an average of 57% of all $B220^{hi}$ B cells had an early GC phenotype ($GL7^{+}$, Fig. 4.8B) and the double positive ($GL7^{+}$ PNA^{+}) population increased

from d4 to d7. This GC phenotype suggests ample memory precursors are being generated. Third, both WT and CD40 KO mice reproducibly exhibit significantly more AFCs following Ag boost than with Ag-isotype priming or the no boost control (Fig. 4.9). Furthermore, the characteristics of the NP-αCD180 induced primary response carry over to the memory that it primes; NP-αCD180 primed AFCs produced large amounts of IgG per cell as shown by a spot size three times that of the controls even though recalled with the same soluble Ag. Despite the consensus that CD40 signals are required for memory B cell induction by TD or TI-2 Ag (199, 200), there are also reports of TI-1 Ag-induced memory in the absence of T cell help (201-203).

Although CD40 KO and TCR deficient mice still can make IgG after CD180 targeting, the amount of Ag-specific IgG is only about 10% of that in WT mice. Thus, T cells clearly are required for most of the IgG response. Since CD180 is expressed on both B cells and DCs and internalizes following ligation by mAb (data not shown), it was likely that CD180 targeting could deliver Ag both to Ag-specific B cells as well as to DCs that don't bind Ag. Indeed, this was the case and DCs targeted in vivo are more efficient than B cells in stimulating CD4 T cells. Ag targeting to CD180 only requires CD180 expression on B cells, however DCs are generally more effective than B cells at stimulating naïve T cell responses (204) and may lead to larger overall Ab responses by amplifying T help relative to targeting Ag only to B cells. After targeting to CD180 in vivo DCs clearly more efficiently took up and processed Ag and could stimulate T cells more effectively than B cells (Fig. 4.6B). This amplification of T help by DCs promotes a greater response to CD180 targeting in WT mice than if Ag were solely directed to B

cells (Fig. 4.5A). Ag-specific Ig was significantly reduced in both CD40 KO and T cell deficient mice (Fig. 4.1B).

The method of targeting Ag to CD180 has high potential impact as it induces both very rapid and large TD IgG Ab responses required for therapeutic vaccination as well as TI IgG Ab responses to normally TD Ags along with class-switched memory in immunodeficient hosts. As current vaccination methods and all dendritic cell-based Ag targeting strategies require functional T cell help Ag-αCD180 immunization provides a unique benefit. While effective without adjuvant in immunodeficient models, it is possible that the magnitude of these responses can be improved with the addition of appropriate adjuvants. Further research is needed on the impact of adjuvants, particularly non-TLR adjuvants, on optimizing the effects of CD180 targeting in immunodeficient mouse models. As vaccination for certain primary immunodeficiencies (Hyper-IgM, DiGeorge syndrome), or for the elderly where T cell help is limited, are currently areas needing effective methods further studies transitioning this method to human B cells and relevant pathogen models are in progress.

Chapter 5: Conclusions and Final Remarks

This study has identified CD180 as a molecule that influences the generation of immunoglobulins. When induced to signal strongly by high dose anti-CD180 mAb, the CD180 pathway is responsible for rapid generation of polyclonal Ab. When linked to Ag and administered at lower doses anti-CD180 triggers a robust Ag-specific class switched response. While CD180 is expressed on and appears to play a significant functional role in DCs, it also directs B cells including B cell sensitivity to TLR signals and B cell activation, proliferation, and Ab production. Thus CD180 signals can regulate both polyclonal Ig production and priming of adaptive immune responses. Further understanding of how CD180 signals function in these contexts may provide valuable insight for therapies based on both non-specific IgG production, such as IVIg replacement therapies, or for those aimed at enhancing protective immunity for various diseases via Ag-targeting.

Polyclonal T cell-independent IgG induced via CD180 stimulation

The contribution of CD180 to Ig production has already been suggested, as CD180 KO mice have decreased serum IgG3 concentrations and defective responses to TI-1 Ag. It has been proposed that defects in B cell functions in CD180 KO mice are due to defects in DC cytokine production rather than an ability of CD180 to provide a direct, functional signal to B cells (115). However, it is clear that there are signals generated by anti-CD180 mAbs in human B cells (99, 103), mouse B cells (186) and the B lymphoma cell line A20 (96). Furthermore, inoculating Ag-αCD180 in vivo activates B cells and leads to Ag processing by B cells as well as DCs (Fig. 4.6B). Therefore, the model that

CD180 functions mainly through regulating cytokine production is oversimplistic. Signals delivered by anti-CD180 mAb are substantially different than those of the TLR family and call into question whether CD180 is a co-receptor for TLRs or an entirely different signaling module that accentuates or bolsters TLR signaling in some fashion. As shown in Figure 3.10, CD180 stimulation combines with signals from five different TLRs to drive enhanced proliferation. This is not consistent with specific heterodimer formation, CD180 delivering a signal via the TLR signaling pathway, nor with CD180 blocking the ability of TLRs to signal. Given the nature of the signal initiated by CD180 in B cells it appears to elicit a separate signaling pathway which is able to amplify the action of any MyD88-dependent TLR signal. While it is possible that defects in B cell function within CD180 KO mice are related to overexpression of BAFF, as recently reported, experiments in this dissertation with purified WT B cells lacking any detectable DCs have also demonstrated that there are B cell intrinsic effects from CD180 stimulation. Thus, while loss of CD180 and any constitutive signaling or interaction with TLRs may result in B cell extrinsic defects, CD180 stimulation on WT B cells and B cell lines clearly delivers a B cell intrinsic stimulatory signal.

While an initial experiment of inoculating agonistic anti-CD180 mAb into WT mice showed an increase in Syndecan-1 (CD138) positive B cells, the magnitude and quality of the resulting immunoglobulin production was surprising. The production of near peak levels of IgG 3 days after inoculation indicates that CD180 signaling induces a rapid response pathway in B cells. Although the natural ligand for CD180 has not yet been identified, it is tempting to speculate that the CD180 pathway is part of rapid response pathway selected for during evolution possibly designed for the production of

germline encoded IgG and IgM Abs in response to pathogens. This 'innate B cell' pathway could well work in concert with TLR-induced Ab responses, comprising conserved germline encoded Igs with broad reactivity to carbohydrate and phosphorylcholine targets. However, the responses induced by anti-CD180 mAb stimulation are substantially different. Anti-CD180 mAb induces much more production of IgG than IgM, of a far greater magnitude, and without the systemic shock that would be induced by inoculation with an equally potent amount of a TLR agonist. Additionally, while TLR-induced IgG production is CD40-independent, such TLR-induced polyclonal IgG is clearly MyD88-dependent, as MyD88 KO mice also have defects in constitutive IgG3 production and insignificant production of Ab following inoculation of LPS or similar TLR agonists/TI-1 Ag (92). Interestingly, CD180 stimulation invokes the same magnitude of large and rapid polyclonal IgG production even in MyD88 KO mice – thus possibly providing an alternative pathway for a rapid response to pathogens.

The signaling pathway mediated through CD180 significantly impacts responsiveness to TLR agonists, yet is not restricted to TLR4 or even to the plasma membrane-restricted TLR1/2/4/6 grouping. Thus, it is very unlikely that CD180 signaling interaction requires formation of TLR:CD180 heterodimers. How might these signals interact once independently initiated? While CD180 does not require MyD88 for its own signaling pathway, the MyD88 adapter is required for CD180 enhancement of TLR signals. Several studies suggest a possible role for Btk. First, CD180 fails to signal in Btk deficient mice (97). Second, Btk was recently reported to be required for phosphorylation and full activation of MyD88 in B cells (205-207). Furthermore, the loss of Btk

significantly impacts production of IL-10 following TLR stimulation, while costimulation of CD180 and TLRs led to increases in IL-10 production (Fig. 3.11).

Results from this dissertation also suggest that CD180 signals interact with those of MyD88-dependent TLRs to bias the fate of responding B cells away from differentiation and rapid immunoglobulin production and toward extensive proliferation. This proliferation bias may be of value in rapid responses to pathogen encounter, such that the local B cells proliferate extensively to maximize opportunities to take up and present Ag.

Inoculation of anti-CD180 into HEL transgenic mice, whose B cells only express the transgene-encoded BCR specific for hen eggwhite Lysozyme (HEL), revealed that this CD180-driven response is truly polyclonal and likely drives germline Ig production without any Ag selection as no HEL was present in the inoculation or in the animal facility. Induction of anti-HEL Ab in these mice, thus, was completely BCR-independent. Inoculation of other KO mice showed that T cells, CD40, and MyD88 are not required for CD180-induced polyclonal Ig production; thus T cell help, TLR signaling, and TACI signaling are all dispensable for the anti-CD180 induction of class-switched Ab.

These studies of CD180 enhance our understanding of B cell activation and the consequences of a distinct mode of stimulation. CD180 delivers a distinctly different stimulus than other polyclonal B cell activators such as anti-CD40, anti-IgD and TLR agonists. Anti-CD40 induces proliferation and upregulation of costimulatory receptors primarily in myeloid cells and FO B cells, which upregulate CD80 and CD95; in contrast, anti-CD180 selectively impacts transitional B cells and upregulates CD86 while having a

minimal effect on CD80 and CD95 expression. Anti-IgD induces a large production of Ig that is restricted IgG1/IgE and IL-4-dependent, while anti-CD180 induces production of all Ig isotypes and subclasses (other than IgG2b and IgA) in an IL-4-independent fashion. Finally, TLR ligands induce large quantities of IgM and IgG3 and also toxic shock from cytokine production when administered in large doses; in contrast, anti-CD180 induces much more IgG1 than IgM and can be administered safely at large doses. This suggests that CD180 stimulation results in an innate-like but TLR independent pathway for B cell stimulation and rapid IgG production.

Ag-specific TD and TI IgG induced via Ag-antiCD180

In addition to the impact of CD180 signals on rapid B cell proliferation and polyclonal Ig production, this dissertation has also shown that linked stimulus of CD180 and the BCR drives enhanced TD and TI Ag-specific IgG responses. This Ab production can be induced with lower doses of anti-CD180 mAb and is both qualitatively and quantitatively different from the response induced by high doses of anti-CD180 alone. First, Ag-αCD180 induces 90% TD and CD40-dependent IgG rather than 100% TI like anti-CD180 alone and with different kinetics (peak IgG at d7 vs. d3). Additionally, a broader and different isotype subclass distribution is induced by Ag-αCD180 (mostly IgG1, some M, G2b, G2c, G3, and IgA vs. mostly IgG3, significant IgM and IgG1, with little G2c and IgE for anti-CD180 alone). Finally, Ag-αCD180 induces only Ag-specific Ab thus not bypassing the BCR specificity while essentially all IgG produced by anti-CD180 alone is BCR independent.

Although T cells and CD40 are required for most of the response, both IL-4 and OX40 signaling - key T cell derived supporting factors - are dispensable. Rather than decreasing IgG responses, the elimination of IFNα/β signaling in IFN R KO mice increased Ab production following immunization. This may be due to changes in GC formation or in TNFα production as TNFα and type I IFN appear to be counter-regulated. A broader examination is necessary to determine which costimulatory pathways Ag-αCD180 immunization can bypass. Given the IgG subclasses produced (Fig. 4.1C), the increase in TACI expression (Fig. 4.4) and the data in KO mice (4.5B) good candidates would be deficient in the cytokines IFNγ, TNFα, APRIL, (for their role in inducing Ig class switch toward the IgG1 subclass) or in the signaling mediators MyD88 and ICOS ligand (for their roles in mediating innate class switching and T cell-stimulation of B cell effector function, respectively). This robustness of Ag-αCD180 targeting in the context of defects that normally severely impair IgG Ab production gives it particular relevance to vaccination strategies for immunodeficient patients.

The ability of BAFF-R KO mice to effectively respond to Ag-αCD180 immunization is particularly interesting from both a mechanistic standpoint and as a potential benefit in vaccination protocols. Transitional 1 (T1) B cells comprise more than half of the B cells in these mice and are significantly responding to Ag-αCD180, yet current models of B cell differentiation state that T1 B cells have not passed a key tolerance checkpoint. Because of this, T1 B cells are sometimes viewed as a potential source of autoreactive B cells when the BCR repertoire is not effectively trimmed of potentially self-reactive clones. The induction of T1 B cells to become IgG-producing AFCs, thus potentially may have both hazardous and beneficial aspects. Further studies

are required to determine if Ag anti-CD180 induces production of autoantibodies while also recruiting Ag-specific B cells from a more diverse repertoire. If this is the case, then it may be necessary to define the conditions that skew the Ag-αCD180 responses away from autoAb induction; for instance, different epitope may be recognized or affinity may change over multiple immunizations so that only mature B cells are effectively stimulated. While we did not examine autoantibody production following anti-CD180 or Ag-αCD180 inoculation, we did not detect in treated mice any signs of distress or pathology up to 140 days following treatment.

Given the very rapid induction of Ag-specific B cells to the GC phenotype induced by Ag-αCD180 and the similarly fast transition to plasmablasts after only 7 days, further research is needed to determine how different B cell subsets respond to combined BCR and CD180 signals. Are T1 B cells being induced to form GCs with this combined stimulus or do T1 B cells differentiate directly to plasmablasts while other B cell subsets populate the GC?

Despite having a signaling pathway significantly overlapping that of the BCR, some studies have suggested that CD180 signals are antagonistic to BCR signaling and that apoptosis of B cells follows sequential stimulation of CD180 and the BCR (104). It is not clear why our results differ from these. At least 3 possibilities exist: 1) working in vivo with anti-CD180 stimulates both B cells and non-B cells in coordinated fashion; 2) Ag-αCD180 different from signals to CD180 and BCR when they are not brought into close proximity; and 3) constitutive cytokine production by non-B cells rescues B cells in vivo but not in purified B cell cultures in vitro. The first possibility seems unlikely as our

data from adoptive transfer experiments (Fig. 4.5A) shows that B cells respond to Ag-αCD180 effective even when DCs do not express CD180, therefore coordinated stimulation of non-B cells seems to be dispensable. Our studies extend BCR and CD180 signal interactions to include both unlinked but simultaneous BCR and CD180 stimulation (in co-titration experiments from Fig. 3.10) as well as linked stimulation (Fig. 4.3). When CD180 and BCR agonists are administered at the same time but unlinked, the B cells survive and proliferate but the stimulation observed does not exceed that of the BCR signal alone (Fig. 3.10). This implies that the BCR signal somehow prevents propagation of the CD180 signal, possibly by competition for recruitment of mediators required by both pathways and forcing the CD180 signal to abort due to a lack of signaling adaptors. Linked delivery shows significant enhancement over either stimulus alone (Fig. 4.3), indicating that when the signals are delivered together they are reinforcing. This would be the prediction if the signaling adaptors shared between the CD180 and BCR pathways have higher affinities for the BCR-related scaffold. If CD180 signaling first depletes the availability of adaptors for BCR signaling it could induce apoptosis due to aborted BCR signals. If treated with unlinked Ag and anti-CD180 mAb there would be intracellular competition for signaling adaptors, this would favor the BCR signal at the expense of the CD180 signal due to higher BCR affinity for the signaling adapters. Finally, linked administration of BCR and CD180 stimuli would draw the signaling adaptors to the joint signalosome and allow for synergistic crosstalk between the related pathways.

Unlike the majority of highly effective Ag-targeting strategies Ag-αCD180 requires B cells but not DCs. While DCs are not required for Ag-specific IgG production

following Ag-αCD180 immunization, they enhance the effect, likely because they efficiently amplify T cell help. DCs also cross-present Ag via MHC I when delivered by Ag-αCD180, even in the absence of adjuvants. Further examination of Ag-αCD180 immunization for stimulation of CD8 T cell anti-cancer and anti-viral responses is necessary. This ability of a B cell directed Ag-targeting strategy to produce such a robust IgG response may lay in the coordinated delivery of a strong independent activating signal along with the Ag. Previous attempts to target Ag to B cells utilized either BCR components (IgM, IgD, or CD19) or weakly signaling or inhibitory targets (CD45, MHC II) rather than a target that by itself could induce IgG production. While other strongly stimulatory surface receptors exist on B cells (such as TLR2, an Ag-delivery target previously investigated), many of them also mediate deleterious effects such as shock due to TLR stimulation and are thus of limited utility.

Most surprisingly, this study indicates that Ag-αCD180 can induce immunologic memory after a single adjuvant-free injection, even in CD40 KO mice. Two questions require further research. First, how do CD180 signals manage to substitute for CD40? Second, is there a way to enhance the CD40-independent memory? Dose response data are available for IgG production induced via CD180 targeting but not for priming of immunologic memory or in the absence of CD40. Would adding adjuvants increase priming in immunodeficient animals? If so which form of adjuvant (TLR, NLR, or others) would provide the greatest boost? Would it be advantageous to link that stimulus as well, such as with CpGs that could co-stimulate TLR9 once internalized to the endosome? If the ability to effectively immunize hyper-IgM patients now exists, how can it be optimized?

An overall question of both polyclonal and Ag-targeting approaches utilizing CD180 stimulation is left; how do CD180 signals interface with TLR and Ag receptor signals and how do they do so to generate such divergent responses in B cells vs. DCs? It has been suggested that the apparent discrepancy between B cell and DC function of CD180 is merely a result of dysregulated BAFF expression in the CD180 KO mice (115), however this interpretation rests on a weakly supported physical interaction model (TLR4:CD180 heterodimers) and the odd assertion that combining two mitogenic stimuli known to produce synergistic results when administered together (BAFF and LPS) somehow leads to a diminished response in this specific system. Rather than invoke physical associations between CD180 and TLRs or broad cytokine dysregulation, we suggest that the CD180 signal interacts with that of TLRs and the BCR. While Btk is an attractive candidate, as it is known to play a role in activation of B cells and macrophages but to be dispensable for DC function, it is more likely that Syk plays that role. Anti-CD180 stimulation has recently been reported to activate Syk (208), and the previously reported roles of both Lyn and Btk in CD180 signaling support this result as they are immediately upstream and downstream of Syk, respectively. The B cell phenotype in response to CD180 stimulation is explained by this model as Syk activation is known to have a strongly stimulatory role in B cell function, required for full cytokine expression and differentiation to Ig production, and also to potentiate TLR signals by phosphorylation of Btk. Moreover, Syk in known to have a complex role in DC signaling where some signals are stimulatory (CLRs stimulating DCs via Syk in complex with CARD9) while others are clearly and strongly inhibitory (such as the interaction between Syk-phosphorylated DAP12 and TLRs, dampening TLR-induced signals, 209). Thus, if

Syk is a mediator of CD180 signaling as reported, the known signaling behavior of Syk may explain the divergent responses between B cells and DCs to CD180 stimulation without invoking more complicated or untenable biology.

In conclusion, I have shown that CD180 controls a unique B cell intrinsic signal that strongly influences not only B cell proliferation but also differentiation and Ig class switching. CD180 stimulation has therapeutic potential to both induce polyclonal IgG, as a possible replacement for Intravenous IgG (IVIg) infusion therapy in certain primary immune deficiencies or autoimmune diseases, and also to rapidly induce high titer Ag-specific IgG for vaccination in therapeutic/post-exposure or immunodeficient settings. The results from this study illustrate the effect of a single determinant involved in producing Ig responses. Harnessing the full potential of CD180 signals to tailor Ig responses in vivo will likely require extensive investigation of the interaction of CD180 stimulation with non-TLR adjuvants to optimize efficacy as well as surveying diverse immunodeficiencies to determine the scope of immune signaling defects CD180 stimulation can circumvent. Given the large impact on both innate-like TI polyclonal Ig production and adaptive Ag-specific IgG production further studies into both the mechanism of action and medical application of CD180 signaling pathways and targeting are clearly warranted.

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