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Immune-modulation via IgD B-cell receptor suppresses allergic skin inflammation in experimental contact hypersensitivity models despite of a Th2-favoured humoral response

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Short title: Anti-IgD ameliorates allergic skin inflammation in vivo

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Highlights

- The novel therapeutic effect of anti-IgD treatment is more efficacious than anti-CD20 treatment in suppressing allergic skin inflammations in murine models of chronic contact hypersensitivities (CHS).
- Anti-IgD effectively suppresses allergic skin inflammation despite an enhanced Th2-skewed antibody response in vivo.
- Anti-IgD selectively depletes ‘initiator’ mature CD19⁺IgD^{hi} B cells while promotes regulatory B cells and T cells in vivo.
- Collectively, these findings highlight the therapeutic potentials of targeting IgD B cell receptor with a monoclonal antibody as a novel treatment of chronic atopic dermatitis.

ABSTRACT

Atopic dermatitis (AD) and allergic contact dermatitis (ACD) are common skin inflammatory conditions. B and T cells are strongly implicated in allergic contact hypersensitivity (CHS) conditions. Activation of IgD B-cell receptor (BCR) by anti-IgD stimulation depletes mature B cells and modulates T-helper cell type 1/2 (Th1/2) responses in vivo. It is not known whether these effects by anti-IgD exacerbates or ameliorates chronic skin inflammations. This study investigated the effects of anti-IgD and B-cell depleting anti-CD20 antibody on skin inflammation in CHS murine models. Chronic CHS were induced by challenges with allergens trimellitic anhydride (TMA) or 2,4 dinitrochlorobenzene (DNCB). Mice were treated with an anti-IgD or anti-CD20 at various time-points following allergen challenges. This study revealed that early therapeutic treatments with anti-IgD at 4 hour after allergen challenge significantly reduced skin inflammation in both TMA- and DNCB-induced CHS models ($P < 0.05$). In contrast, anti-CD20 treatment exacerbated skin inflammation in DNCB-induced CHS despite of an extensive B cell depletion ($P < 0.05$). Anti-IgD treatment depleted mature $CD19^+IgD^+$ B cells but enhanced allergen-specific IgM and total IgE productions, suggesting a Th2-favoured humoral response. Anti-IgD reduced neutrophilic infiltrations but increases accumulation of mast cells in dermal tissues. The anti-inflammatory effects of anti-IgD were supported by evidence of an increase in the percentage of regulatory B cells and T cells. Collectively, this study demonstrates that immune-modulation by anti-IgD treatment suppresses Th2-mediated allergic skin inflammation in murine models despite a skew toward a Th2-favoured humoral response and therefore may present a novel treatment for chronic human AD and ACD.

Abbreviation: ACD, Allergic contact dermatitis; AD, Atopic dermatitis; BAFF, B-cell activating factor; BCR, B-cell receptor; Breg, regulatory B cells; CD40L, CD40 ligand; CHS,

Contact hypersensitivity; DNCB, 2,4 dinitrochlorobenzene; FcRs, Fc receptors; FcγRβII, Fc gamma receptor beta isoform II; HRP, horseradish peroxidase; mAb, monoclonal antibody; Immunoglobulin D, IgD; TMA, trimellitic anhydride; Th1/Th2/Th17/Th22, T helper cell type 1, 2, 17 and 22; Treg, regulatory T cells.

Keywords: Atopic dermatitis, Contact hypersensitivity, IgD, Psoriasis, B cell.

1. Introduction

Atopic dermatitis (AD) and allergic contact dermatitis (ACD) are common inflammatory skin conditions that affect about 10% of the general population worldwide and carry significant socioeconomic costs [1]. Clinical manifestations of inflamed skin lesions are often caused by disruption of normal skin barrier function resulting in chronic exposures to sensitizing allergens, which in turn provoke a dysregulated immune response to contact allergens [2-4]. Although many patients with AD and ACD respond well to conventional therapies consisting of topical steroids and non-steroidal inhibitor treatments, chronic and severe cases remain a clinical challenge because of the limited efficacies of current therapies and the undesirable side-effects associated with their chronic uses [2-4]. Therefore, there is a medical need for a safer and more effective therapy for chronic AD and ACD patients.

The clinical manifestation of AD and ACD in human and animal models involves the recruitment and activation of innate (macrophages, basophils, neutrophils, mast cells, and dendritic cells) and adaptive (B cells, CD4⁺ and CD8⁺ T cells) immune cells at the site of inflammation [5-7]. Chronic skin exposure to allergens activates keratinocytes and resident immune cells (such as mast cells, dendritic cells and Langerhans cells) to produce a myriad of pro-inflammatory cytokines and chemokines that recruit a large influx of neutrophils and T

cells to inflamed skin tissues. These events in turn amplify the dysregulated inflammatory immune responses to the allergens that manifest in inflamed skin lesions [5]. Clinical and experimental evidence demonstrate a mixed features of CD4⁺ T helper cell type-1, type-2 and type-22 (Th1, Th2, Th 22) cytokine with a polarization toward Th2 response in the pathogenesis of AD while Th1 and Th17 cytokine and chemokine responses are predominantly upregulated in the inflamed skin tissues of ACD, demonstrating an important and complex role of T-cell responses in both of these conditions [5,8-13]. In addition, the pathological role of B cells in AD and ACD may involve antibody-dependent (via productions of allergen-specific IgM and IgE) as well as antibody-independent modulation of T-cell and innate cellular responses [14-16].

Contact hypersensitivity (CHS) murine models are commonly used as animal models of human ACD, and recently AD. In the ACD model, challenges with contact allergens such as 2,4-dinitrochlorobenzene (DNCB) elicit a predominantly Th1-mediated skin inflammation that resembles many common clinical features of human ACD lesions [11]; whereas chronic challenges with the respiratory allergen trimellitic anhydride (TMA) was used in AD murine model [13]. Skin sensitization to TMA elicits a mixture of both Th1/Th2 responses but with a skew toward Th2-biased features that is evidenced by an increase in total IgE production and thickening of the skin tissue layer reflecting clinical features of human AD[13,17,18]; whereas chronic skin exposure to DNCB produces pronounced local and systemic T-cell responses of a predominant Th1 phenotype associated with skin tissue thickening and epidermal hyperplasia and apoptosis (flaky skin) that resembles the pathological features of chronic ACD and psoriasis [11,17]. In both animal models, dermal infiltrations of immune cells results in severe skin inflammation. These clinically relevant features of murine models of TMA- and DNCB-induced CHS make them valuable models to study the mechanism of human diseases and to evaluate new therapies [11,13].

Membrane immunoglobulin D (IgD) is a major component of the B-cell receptor (BCR) complex expressed on maturing B cells. Treatment with anti-IgD antibody activates a broad polyclonal B-cell response which leads to a selective depletion of mature CD19⁺IgD⁺ B cells and activations of a broad mixture of Th1/Th2 responses but with a skew toward Th2 phenotypes in vivo [19-23]. In addition, soluble IgD has been shown to bind to the cell surfaces of CD14⁺ monocytes, Fc epsilon receptor isoform I positive (FcεRI⁺) basophils and mast cells thereby making these innate immune cells a target for immune-modulation by anti-IgD treatment in vivo [22-25]. Therapeutic treatment with anti-IgD can alleviate Th1-mediated arthritic inflammation in the collagen-induced arthritis (CIA) mouse model [23], an autoimmune disease that involves both humoral and cellular immune responses [26,27]. Given the broad effects of anti-IgD on the innate and adaptive immune responses in vivo and in the context of the intricate roles of mast cells, basophils, Th1 and Th17 responses in ACD, and the implication of pathological role of Th2-mediated immune responses in AD, it is not known and intriguing whether the systemic effects of anti-IgD on the immune responses can potentiate or suppress chronic inflammatory CHS responses in vivo. This study was therefore undertaken to investigate the effects of anti-IgD treatment on allergic skin inflammation in the chronic TMA- and DNCB-induced CHS mouse models. The finding of study demonstrates for the first time that anti-IgD antibody can suppress allergic skin inflammation in both murine models of CHS.

2. Materials and Methods

2.1. Antibodies

Low endotoxin and azide-free (LEAFTM) grade-purified mouse anti-mouse IgD^a monoclonal antibody (clone AMS 9.1, mouse IgG_{2b}) and rat anti-mouse CD20 (clone AISB12, rat IgG1) and mouse IgG_{2b} isotype control (clone MPC-11) were all purchased from

BioLegend (San Diego, USA) with functional grade quality (low endotoxin and azide-free) suitable for in vivo injection. The characteristics of anti-mouse IgD^a clone AMS9.1 and its in vivo biological activities have been described and used in previous studies [21,23,36]. Clone AMS9.1 was used in our studies due to its ability to activate B cells in vivo and because it is a mouse IgG_{2b} it does not elicit an allogeneic response as a foreign antigen. Fluorescent-conjugated and biotinylated antibodies against mouse B220, CD19, CD4, IgD, IL-10, FoxP3, and nuclear staining buffer kits for flow cytometry were all purchased from eBioscience (San Diego, USA). Capturing antibodies specific for mouse IgM, IgG1 and IgE and horse radish peroxidase (HRP)-conjugated secondary detecting antibodies for ELISA detection were purchased from Jackson ImmunoResearch Laboratory (PA, USA).

2.2. *Reagents*

TMA and DNCB were purchased from Sigma Aldrich (NSW, Australia). For CHS induction, 4% DNCB and 10% TMA solution (w/v) was made up freshly on the day of the experiment by dissolving in 4:1 acetone/olive oil (v/v).

2.3. *Animals*

Young male BALB/c mice (6-8 weeks old) were used as an animal model of allergic skin conditions because they have high prevalence of dermal mast cells [28], and have tendencies to produce a strong mixture of Th1/Th2 and IgE responses when challenged with TMA, and a strong Th1 response to DNCB challenges [11,13,18], and their susceptibility to 'initiator' B-cell-mediated CHS [29]. Mice were maintained under a sterile standard animal housing condition. All animal studies were approved by the Royal North Shore Hospital Animal Care and Ethic Committee (ACEC-1311-010A).

2.4. *CHS induction and antibody treatment*

Chronic TMA- and DNCB-induced CHS in BALB/c mice were performed as previously described with some modifications [11,13]. Briefly, mice were sensitized with 50 μ l of 10% TMA or 40 μ l of 4% DNCB on the shaven back on day 0. From day 5-10, mice were daily challenged with 20 μ l of 3% TMA or 10 μ l of 0.8% DNCB on the back of the left ear. In preventive anti-IgD treatment, mice were given 3 consecutive daily tail vein injections with PBS, isotype control, 2 mg kg⁻¹ of anti-IgD or anti-CD20 as a preventive treatment on day 2-4. Early and late therapeutic treatment started at 4 or 24 hours respectively after the first allergen challenge, from day 5 to day 7. Ear swelling was measured with a digital micrometre calliper. Net increase in ear swelling was calculated by subtracting the thickness of the right ear (solvent control) from the left ear (allergen-challenged).

2.5. Immunohistology and histopathological analysis

Ear tissues were fixed in 10% formalin overnight and stored in 70% ethanol prior to paraffin-embedding. Four μ m sections of tissue were stained with H&E to examine morphological changes and infiltrations of neutrophils or were stained with toluidine blue for detection of mast cell infiltration. For immunohistochemistry, paraffin-embedded cross sections of ear tissues were stained with antibodies against mouse CD4 (4SM95), CD8 (4SM15), Ly6G (RB6-8C5) (eBioscience) and labelled with HRP-conjugated antibodies. Antibody-labelled tissue sections were visualized with HRP-substrate reaction and counter-stained with haematoxylin. Positively stained cells were counted under high magnification (20X) of 3 different fields of view (FoV) along the cross sections of ear lobes from 3 different mice in each group.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Total and TMA-specific IgM, IgG and IgE in mouse serum were measured by an in-house ELISA. Briefly, solution of 10% TMA (dissolved in acetone/olive oil) was diluted

with PBS to 1% solution which was then used for coating the 96-well plate. Wells were then blocked with PBS/5% bovine serum albumin (BSA). Serum samples were added to the well at 1/20 dilutions with PBS/0.5% BSA buffer and incubated at room temperature for at least 4 hours. Wells were washed 5 times with PBS/0.05% Tween-20 buffer. Serum mouse immunoglobulin isotypes were detected by polyclonal goat or donkey anti-mouse immunoglobulin horseradish peroxidase (HRP)-conjugated antibodies and detection was developed with Sigma-Fast substrate (Sigma Aldrich, Australia). All ELISA were repeated twice with similar results.

2.7. *Flow cytometry*

Fresh cells were isolated from the spleen on day 14 and were immediately labelled with antibodies for flow cytometry analysis as previously described [23]. Fluorescent-labelled cells were detected by FACS Calibur Flow Cytometer and analyzed by CellQuest Software (Becton Dickson, USA).

2.8. *Statistical analysis*

All statistical analyses were performed by GraphPad Prism Version 5 software. The two tailed Student *t*-test, *Mann-Whitney U* and *Kruskal-Wallis* tests were used for analysis and *P* < 0.05 was considered statistically significant, and ns = non-significance.

3. Results

3.1. *Preventive and early therapeutic treatment with anti-IgD reduce skin inflammation in mouse model of chronic TMA-induced CHS*

To examine the effects of anti-IgD treatment on the course of a predominantly Th2-mediated skin inflammation, mice with chronic TMA-induced CHS were treated with anti-

IgD as a preventive or an early and late therapeutic treatment (Fig. 1A). Both early and late therapeutic treatment significantly reduced ear swelling (Fig. 1A & 1B). Preventive treatment also significantly reduced ear swelling albeit to a lesser extent than therapeutic treatment (Fig. 1A). Histopathological analysis by haematoxylin and eosin (H&E) staining of ear tissue from 3 representative individual mice confirmed both preventive and early therapeutic treatment reduced thickening of epidermal and dermal tissue layers induced by repeated TMA challenges (Fig. 1B). Toluidine blue staining revealed chronic TMA-induced CHS resulted in some losses of chondrocytes and cartilage production in cartilage layer which were partially rescued by anti-IgD treatment (Supplementary Fig. 1). These data demonstrate the therapeutic effects of anti-IgD treatment in suppressing skin inflammation in chronic TMA-induced CHS in vivo.

3.2. *Anti-IgD treatment is more efficacious than anti-CD20 antibody in suppressing skin inflammation in mouse model of chronic DNCB-induced CHS*

B-cell depletion therapy with anti-CD20 antibody has been effective as a treatment for some autoimmune and some inflammatory conditions. Depletion of B cells with anti-CD20 treatment reduces T-cell responses in vivo [30]. This study next sought to compare the efficacy of B cell depletion by anti-IgD and anti-CD20 on the chronic DNCB-induced CHS mouse model, a predominantly Th1-mediated CHS animal model that resembles some of the clinical features of chronic human ACD. Unexpectedly, therapeutic treatment with anti-CD20 exacerbated ear swelling in comparison to untreated control (Fig. 2A). Therapeutic treatment with anti-IgD at earlier time points significantly reduced ear swelling (Fig. 2A). Histopathological analysis with H&E and toluidine blue staining confirmed the therapeutic effects of anti-IgD and the aggravated effects of anti-CD20 on the thickening of epidermal and dermal layers, epidermal hyperplasia and necrosis, loss of cartilages production within the elastic cartilage tissues (Fig. 2B; Supplementary Fig. 2). Collectively, these data

demonstrate the opposite effects of anti-IgD and anti-CD20 antibodies treatment on the Th1-mediated DNCB-induced CHS skin inflammation *in vivo*.

3.3. *The effects of anti-IgD and anti-CD20 on neutrophils, mast cells, CD4⁺ and CD8⁺ T cells infiltrations in inflamed dermal tissues*

Infiltration of immune cells in skin tissue is a hallmark of human diseases and in mouse model of AD and ACD [2,3,13]. As shown by H&E staining (Fig. 1B & 2B), there was extensive infiltration of neutrophils and eosinophils (stained dark blue) in inflamed ears tissues from both chronic TMA- and DNCB-induced CHS mice compared to naïve control. Therapeutic treatment with anti-IgD noticeably reduced the extensive loci of inflammation containing infiltrating immune cells in TMA- and DNCB-induced CHS as shown by H&E staining (Fig. 1B & 2B). Unexpectedly, anti-IgD significantly increased the number of mast cells accumulated in the dermal tissues of both chronic TMA- and DNCB-induced CHS mouse models (Fig. 3A & 3B; Supplementary Fig. 1 & 2). Staining of specific markers for neutrophils (Ly6G⁺) and T cells (CD4⁺ and CD8⁺) showed anti-IgD treatment led to a reduction in the number of infiltrating neutrophils but had no effects on CD4⁺ and CD8⁺ T cells (Fig. 3A & 3B). Anti-CD20 treatments markedly increased the extent of Ly6G⁺ neutrophil infiltration but did not affect the number of infiltrating mast cells or CD4⁺ and CD8⁺ T cells in dermal ear tissues (Fig. 3A & 3B).

3.4. *Treatment with anti-IgD enhances allergen-specific IgM and total IgE productions*

Allergen-specific IgE and IgM productions, which are driven by Th2-mediated responses, play an important role in sensitization and elicitation of skin inflammation in both human allergic conditions and CHS murine models [14,16]. Therefore, this study next examined whether the therapeutic effect of anti-IgD was associated with a reduction in Th2-

mediated allergen-specific antibody response. Unexpectedly, both preventive and therapeutic treatment with anti-IgD significantly increased serum levels of TMA-specific IgM (Fig.4A).

A marked increase in TMA-specific IgG1 and IgE responses was observed in anti-IgD treated group compare to PBS control but this result did not have a statistical significance (Fig. 4A). Anti-IgD treatments also significantly increased the serum level of total IgE responses to TMA challenges, demonstrating a promoting effect of anti-IgD on the Th2-mediated polyclonal humoral responses in vivo (Fig. 4A). In chronic DNCB-induced CHS model, anti-IgD treatment also significantly increased DNCB-specific IgM responses but did not affect IgG1 or IgE production (Fig. 4B). Collectively, these data suggest that the therapeutic effects of anti-IgD treatment do not involve the suppression of antibody responses against allergen challenges in chronic TMA-induced or DNCB-induced CHS murine models.

3.5. *Anti-IgD treatment selectively depletes mature B220⁺IgD⁺ and 'initiator' CD19⁺IgD^{hi} B cells but increases the percentage of regulatory B cells and T cells*

Previous studies have demonstrated that 'initiator' B cells, characterized as CD19⁺IgM⁺IgD^{hi} B cells, are partially responsible for the initiation of skin inflammation in DNFB-induced CHS [15,29]. Consistent with previous [21,23,33], both anti-IgD and anti-CD20 treatment significantly depleted B220⁺IgD⁺ population that includes 'initiator' CD19⁺IgD^{hi} B cells in chronic DNCB-induced CHS mice (Fig. 5A). However, anti-CD20 treatment depleted B cells more extensively than anti-IgD treatment as demonstrated by a higher reduction in total B220⁺ and CD19⁺ B cells (Fig. 5A).

Both regulatory B cells (IL-10⁺ B cells, Breg) and T cells (CD4⁺FoxP3⁺ Treg) have been shown to suppress inflammatory responses in human allergic conditions and CHS murine models [31-33]. Consistent with our previous findings and other studies [23,34-36], anti-IgD treatment augmented an increase in the percentage of CD19⁺IL-10⁺ Breg and CD4⁺FoxP3⁺

Treg cells in chronic DNCB-induced CHS mice (Fig. 5B). Interestingly, anti-CD20 treatment markedly reduced the percentage of CD19⁺IL-10⁺ Breg cells and did not affect the percentage of CD4⁺FoxP3⁺ Treg cells in comparison to isotype control, demonstrating that the broad depletion of B-cell with anti-CD20 overall did not augment Treg cells and may have a negative effects on regulatory B-cell population (Fig. 5B). Thus, these data suggest the therapeutic effects of anti-IgD may involve the augmentation of Breg and Treg cells while depletion of Breg cells may attribute to the aggravated effects of anti-CD20 on chronic DNCB-induced CHS responses.

4. Discussion

A key pathological feature of allergic skin inflammation is an upregulation of Th2-driven cellular and humoral responses to allergens. Anti-IgD has been shown to deplete B cells and modulate both Th1/Th2 responses in vivo. To date, its effect on allergic skin inflammation is unknown. This study showed that therapeutic treatment with anti-IgD suppresses overt skin inflammation in both TMA-induced CHS (a predominantly Th2-driven response) and Th1-driven DNCB-induced CHS (a Th1-mediated response) murine models despite of a skew toward Th2-biased humoral response. In contrast, it demonstrates that treatment with anti-CD20 treatment exacerbates skin inflammation in the chronic DNCB-induced CHS model, despite the ability of anti-CD20 treatment to deplete B cells more extensively than anti-IgD treatment, suggesting a superior therapeutic mode of action of anti-IgD over anti-CD20 in the DNCB-induced CHS mouse model.

The emerging evidence from this study and previous studies suggest that the therapeutic effects of anti-IgD treatment on chronic TMA- and DNCB-induced CHS in mouse models may involve a number of key mechanisms of action that include: 1) the selective depletion of the ‘initiator’ CD19⁺IgM⁺IgD^{hi} B cells; 2) the augmentation of Breg and Treg cells; and 3)

the promotion of an anti-inflammatory Th2 response in vivo. In DNCB-induced CHS mouse model, the ‘initiator’ CD19⁺IgM⁺IgD^{hi} B cells have been shown to play an important part in initiating pro-inflammatory CHS responses [15,29]. Thus, depletion of these ‘initiator’ B cells by anti-IgD treatment may contribute to its therapeutic effects as observed in the preventive therapeutic treatments (Fig 1A). As an immune-modulator, the augmentation of Breg and Treg cells by anti-IgD treatment may play a significant role in dampening of the ongoing inflammatory responses [31-33]. In particular, IL-10⁺ Breg cells induced by anti-IgD play a non-redundant role in immune counter-regulation and are capable of suppressing inflammatory responses in both Th2-mediated allergies and Th1-mediated autoimmune conditions [23,35,37-39]. Thus, the augmentation of Breg cells by anti-IgD treatment may play a pivotal role in its therapeutic effects on both TMA- and DNCB-induced CHS responses as well as arthritic autoimmune inflammation in CIA mouse model [23]. Indeed, this notion is confirmed by a recent study demonstrating IL-10⁺ B cells and plasmablasts following therapeutic treatment with anti-IgD suppress C5a-mediated peritoneal inflammation and autoimmune skin inflammation in experimental epidermolysis bullosa acquisita mouse model [34]. Furthermore and consistent with a previous study [33], this study shows broad depletion of B cells with anti-CD20 treatment, which has been shown to depletes both mature B cells and Breg cells [33], exacerbates DNCB-induced CHS responses in vivo. This finding is consistent with clinical observations of occasion of worsening or new onset of psoriasis in some rheumatoid arthritis patients treated with anti-CD20 rituximab [40,41], which has been attributed to the depletion of Breg cells in inflammatory skin conditions. Collectively, these findings suggest the therapeutic modes of action by anti-IgD treatment may involve both B-cell depletion and immune-modulation via augmentation of Breg responses.

In this study, the therapeutic effects of anti-IgD treatment did not associate with a reduction in Th2-mediated allergen-specific IgM or IgE responses despite the deletion of mature B cells. Instead, the result showed that anti-IgD treatment enhances Th2-mediated antibody responses to repeated allergen challenges, as evidenced by an increase in allergen-specific IgG1 response and total IgE production. This finding of a Th2-favoured humoral response following anti-IgD treatment is consistent with previous studies [19,21,23,25]. Although, this finding seems to be counter-intuitive to the current dogma of the pathogenic roles of Th2-mediated immune responses in allergic inflammation there are both clinical and experimental evidence to suggest that a 'modified' Th2-mediated response that involves an IL-10-driven antibody response may be protective against allergic inflammations [42,43]. This notion is suggested by the finding of polyclonal IgE and IgG1 from such 'modified' Th2 responses can inhibit allergic inflammation by competing bindings with pathogenic allergen-specific IgE and IgGs to FcεRIα or FcRγ on mast cells or basophils and desensitizing mast cell and basophil activation by cross-linking inhibitory receptor FcγRIIB, activating receptor FcγRIIA and dectin-1 [44-46]. Thus, immuno-modulation by anti-IgD might have produced a 'modified' Th2 response via IL-10 production by B cells, T cells, FcεRI⁺ basophil and mast cells [19-25,34-36]. In addition, a skew toward such Th2-mediated responses may also contribute to the dampening down of Th1-driven inflammation in DNCB-induced CHS and thereby contribute further to the anti-inflammatory effects of anti-IgD treatment in these models [23,47].

An unexpected finding in this study is the significant accumulation of mast cells in the dermal tissues of both TMA- and DNCB-induced CHS mice following treatment with anti-IgD. While early experimental evidence demonstrates the important role of mast cells in mediating early phases of acute inflammation in DNCB-induced CHS responses [48], there are emerging evidence suggesting mast cells may play an important role in dampening down

the late phase of chronic inflammation associated with acquired immune and innate responses to repeated challenges with allergenic stimuli such as DNCB or oxazolone, chronic exposure to ultraviolet light-B (UVB) or autoimmune diseases via production of IL-10 [49,50]. It is therefore possible that the accumulation of activated dermal mast cells following treatment with anti-IgD may limit local skin inflammation via IL-10 production by mast cells that inhibits rather than exacerbates chronic skin inflammation. Such accumulation of activated mast cells, and possibly other innate immune cells such as monocyte and basophils, could also be directly modulated by anti-IgD treatment via soluble IgD binding on the surface of these cells [22-25]. Further investigations into this cellular mechanistic aspect of anti-IgD are warranted.

In summary, this study demonstrates that the immune-modulation by anti-IgD treatment suppresses overt skin inflammation in chronic TMA- and DNCB-induced CHS murine models via depletion of 'initiator' CD19⁺IgD^{hi} B cells and promoting the immune responses toward an anti-inflammatory phenotypes. This finding highlights the therapeutic potential of targeting IgD B-cell receptor as a novel treatment for chronic human allergic skin inflammation.

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Figure legends

Fig. 1. Treatment with anti-IgD suppresses skin inflammation in chronic TMA-induced CHS mouse models. (A) Net increases in ear thickness. Means $\pm$ s.e.m. of 5 mice are from one of two independent experiments. Arrows indicate times of allergen challenges and anti-IgD treatments. Net increases in ear thickness were calculated by subtracting thickness of solvent treated control right ear from that of TMA-challenged left ear of each individual mouse. (B) H&E staining of ear tissue cross sections from 3 representative mice collected on day 13 of the experiment. $*P < 0.05$ for early anti-IgD and $\# P < 0.05$ for preventive anti-IgD versus isotype control.

Fig. 2. Anti-IgD and anti-CD20 early therapeutic treatments (4h) produce opposite effects on skin inflammation in chronic DNCB-induced CHS mouse model. (A) Net increases in ear thickness. Means $\pm$ s.e.m. of 5 mice are from one of two independent experiments. Arrows indicate times of allergen challenges and antibody treatments. Net increases in ear thickness

were calculated by subtracting thickness of solvent treated control right ear from that of TMA-challenged left ear of each individual mouse. (B) H&E staining of ear tissue cross sections from 3 representative mice collected on day 14 of the experiment. * $P < 0.05$ for anti-IgD versus isotype control, # $P < 0.05$ for anti-CD20 versus isotype control.

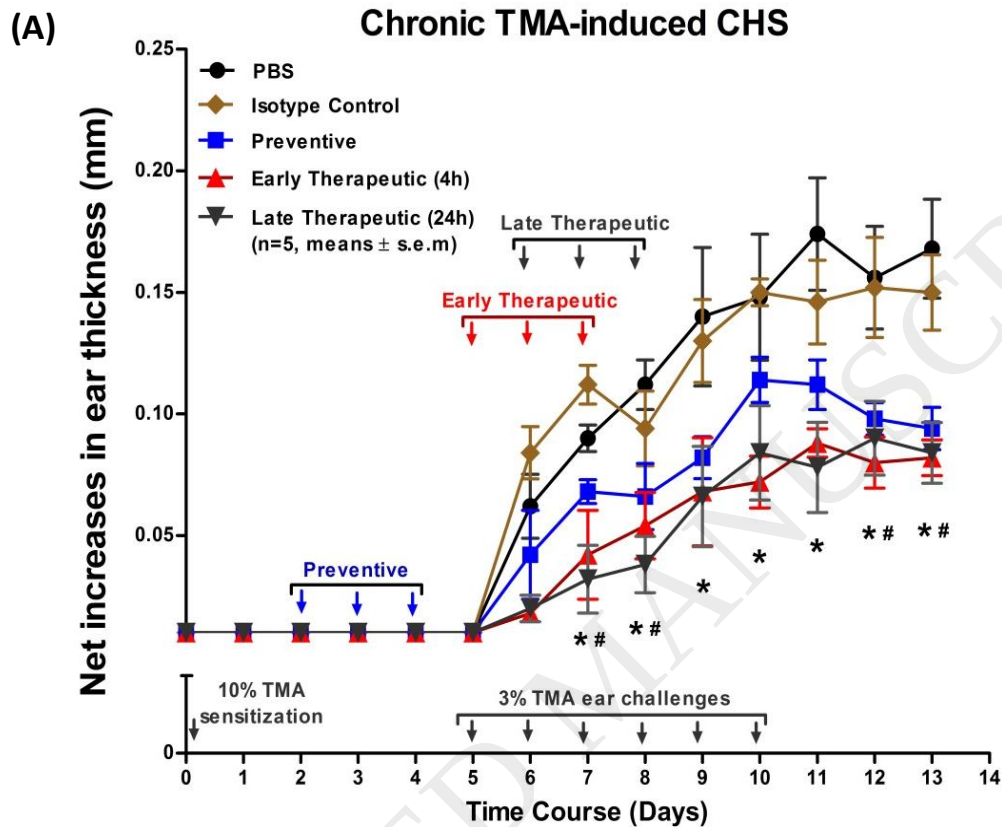
Fig. 3. The effects of anti-IgD and anti-CD20 early therapeutic treatments (4h) on immune cell infiltration in dermal tissues of chronic DNCB-induced CHS mouse model. (A) Toluidine blue and immunohistochemistry staining for infiltrating mast cells, GR1+ neutrophils, CD4+ and CD8+ T cells. (B) Cell counts per field of view (FoV) from 3 different FoV along ear tissue cross sections of 3 representative mice. Means $\pm$ s.e.m. are presented. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Fig. 4. The effects of anti-IgD treatment on allergen-specific antibody responses. (A) TMA-specific antibody responses in chronic TMA-induced CHS mouse model. (B) DNCB-specific antibody responses in DNCB-induced CHS mouse model. Mouse serum were collected at the end of experiment (day 13 or 14) and antibody levels were analysed by ELISA as described in the Materials and Method section. Data represents 5 mice per group from one experiment. Means $\pm$ s.e.m. are presented. * $P < 0.05$ and ** $P < 0.01$.

Fig. 5. The effects of anti-IgD and anti-CD20 early therapeutic treatment (4h) on mature B cells, Breg and Treg cell numbers in chronic DNCB-induced CHS mouse model. (A) Representative dot plots of splenic mature B220+IgD+ and CD19+IgD+ B cells. Graphs show the percentage of mature B220+IgD+, CD19+IgD+ B cells and total B cells from one of two independent experiments. (B) Representative dot plots of splenic mature CD4+Foxp3+ T cells and CD19+IL-10+ B cells. Graphs show the percentage of mature CD4+Foxp3+ T cells and CD19+IL-10+ B cells from one of two independent experiments. Spleen cells were collected at the end of each experiment and immediately stained with

fluorescent-labelled antibodies for flow cytometry. Data represent the percentage of positive cells within the splenic cell population. Means $\pm$ s.e.m. are presented. * $P<0.05$ and ** $P<0.01$.

Figure 1



(B) Chronic TMA-induced CHS

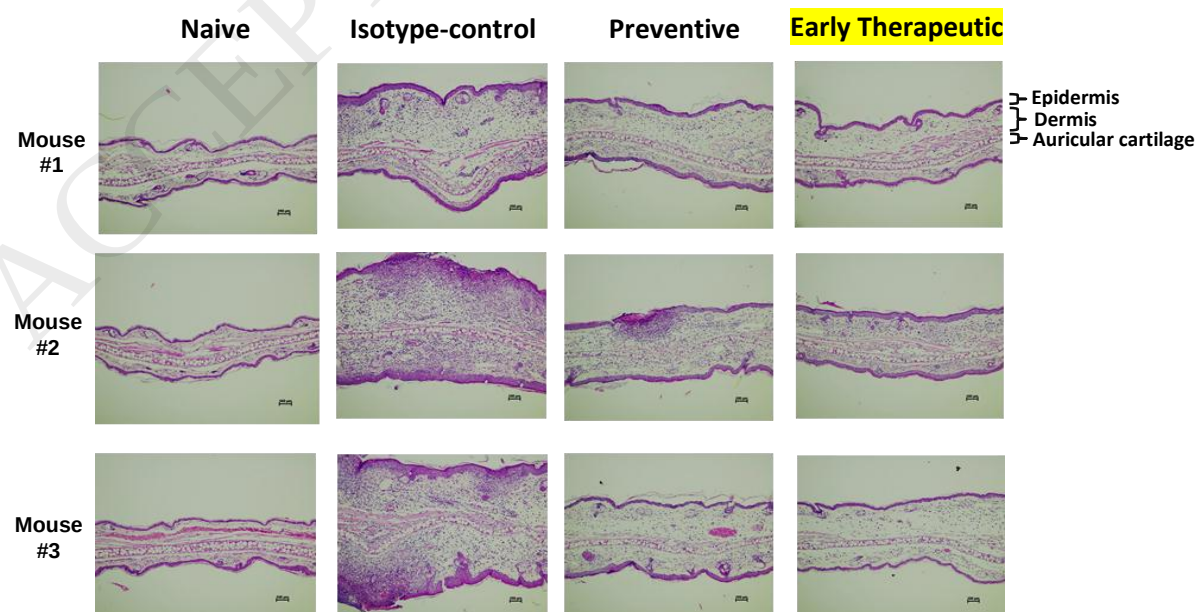
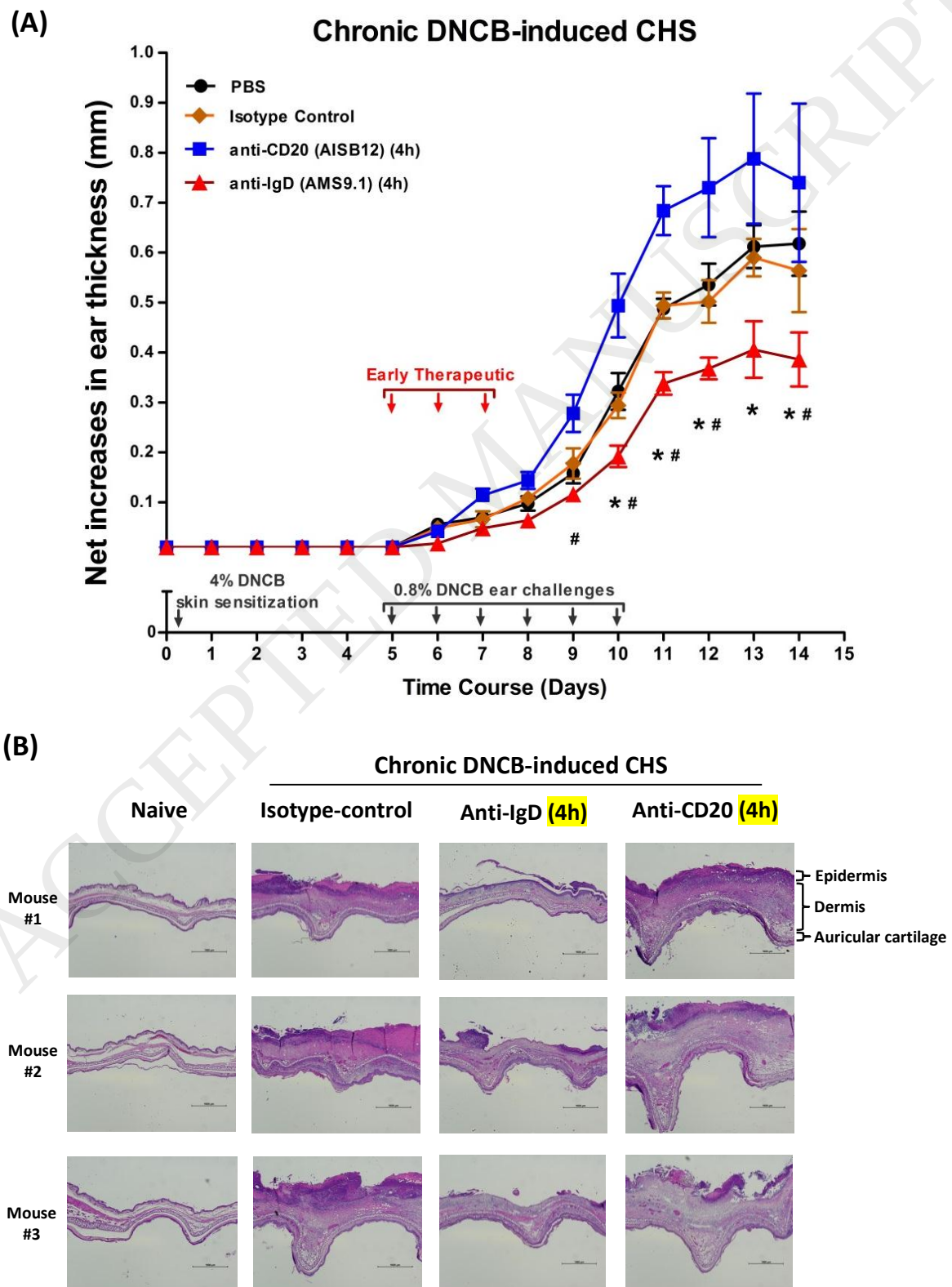


Figure 2



ACCEPTED MANUSCRIPT

Figure 3

(A)

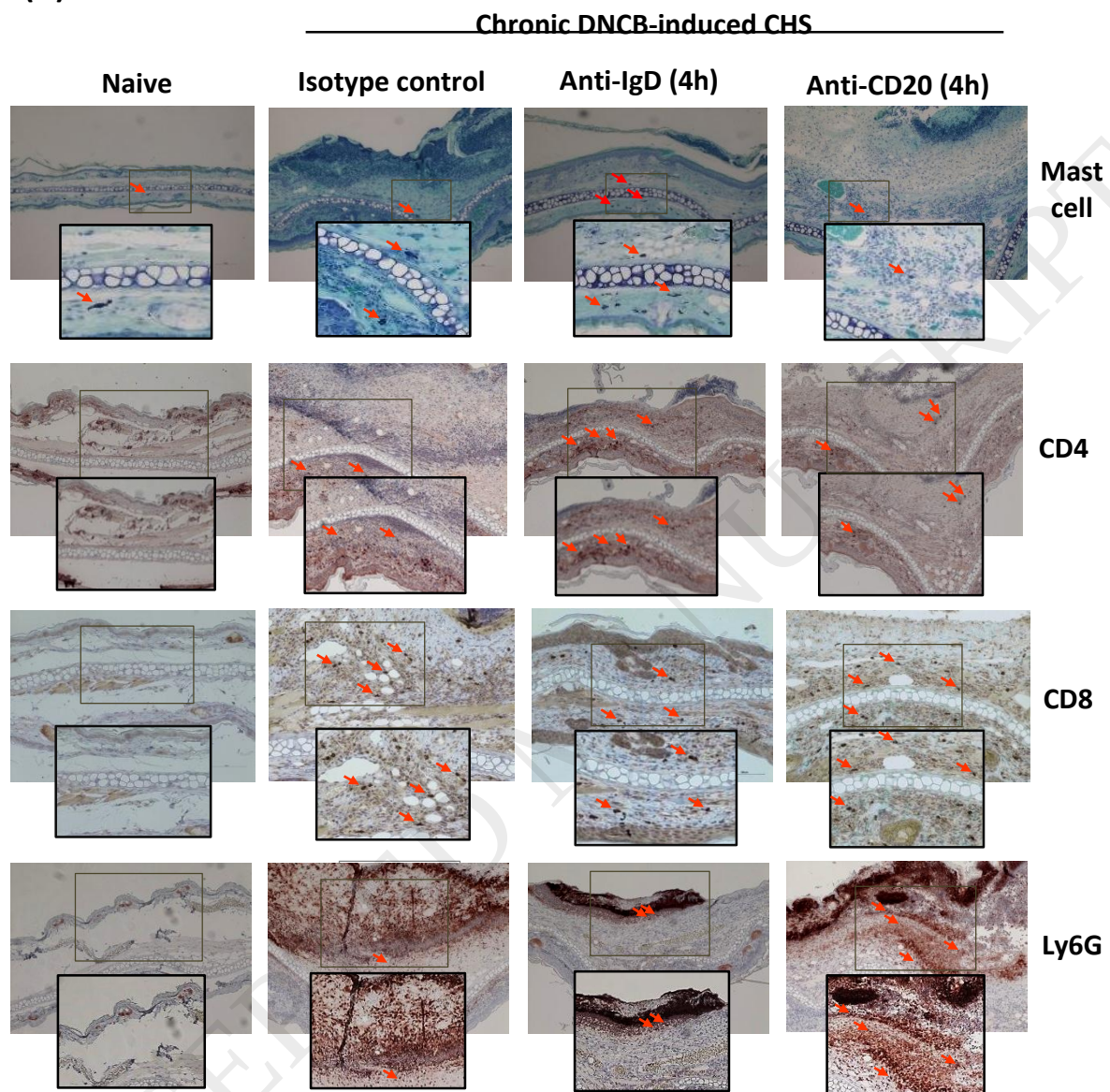


Figure 3

(B)

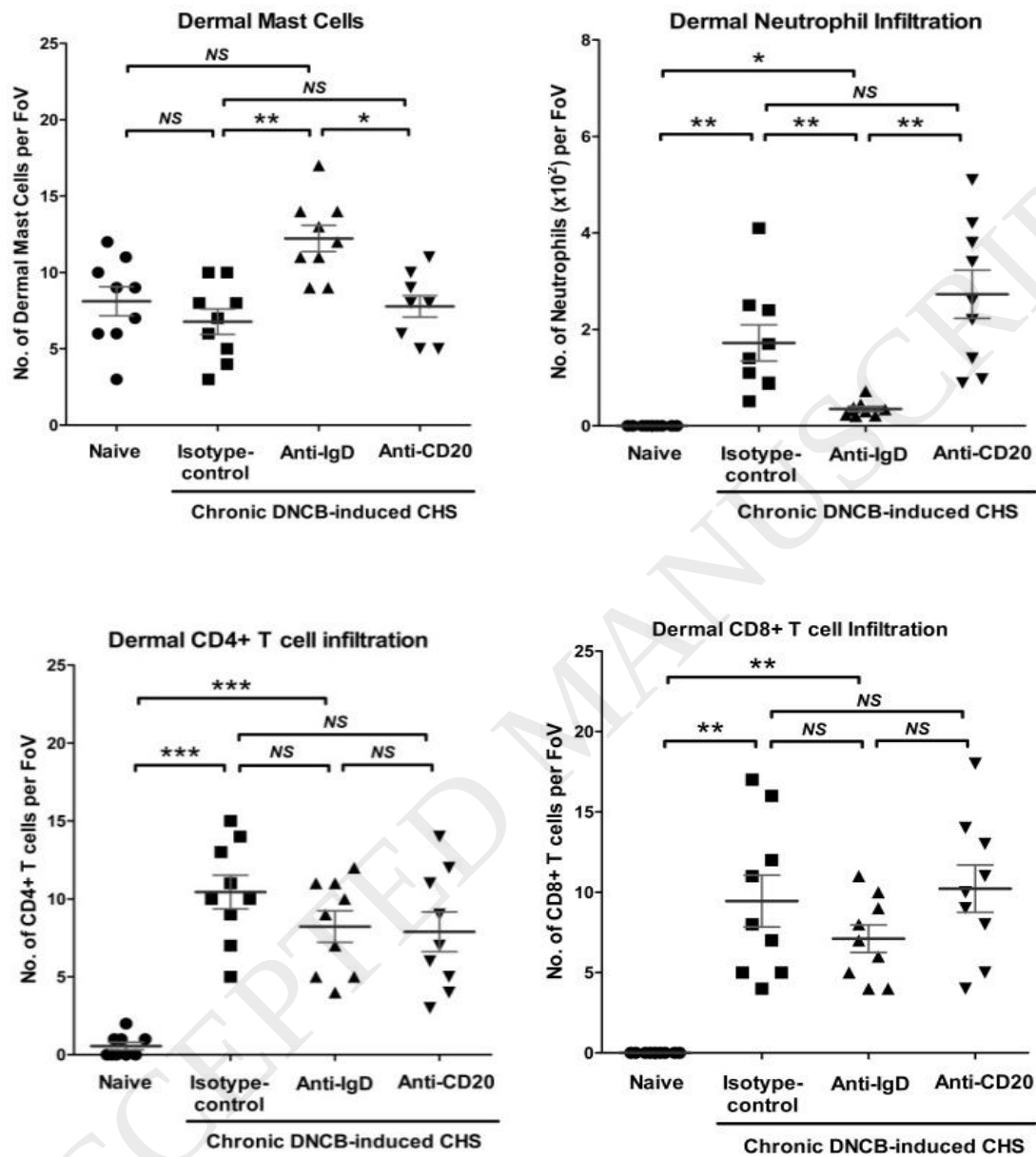
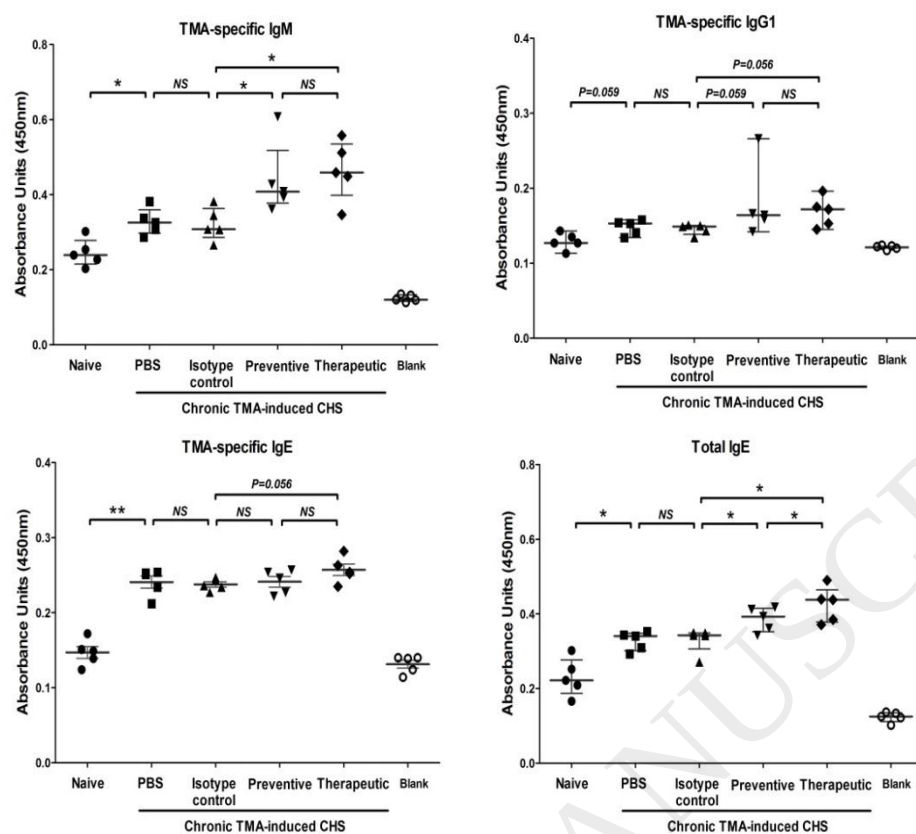


Figure 4

(A)



(B)

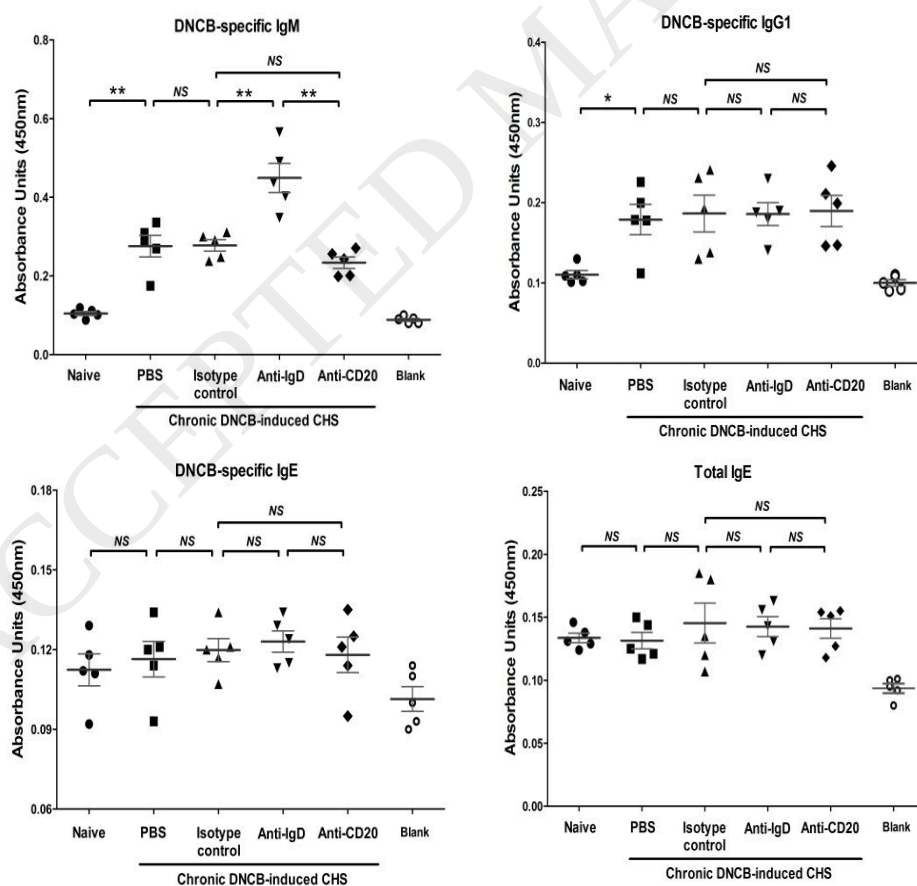
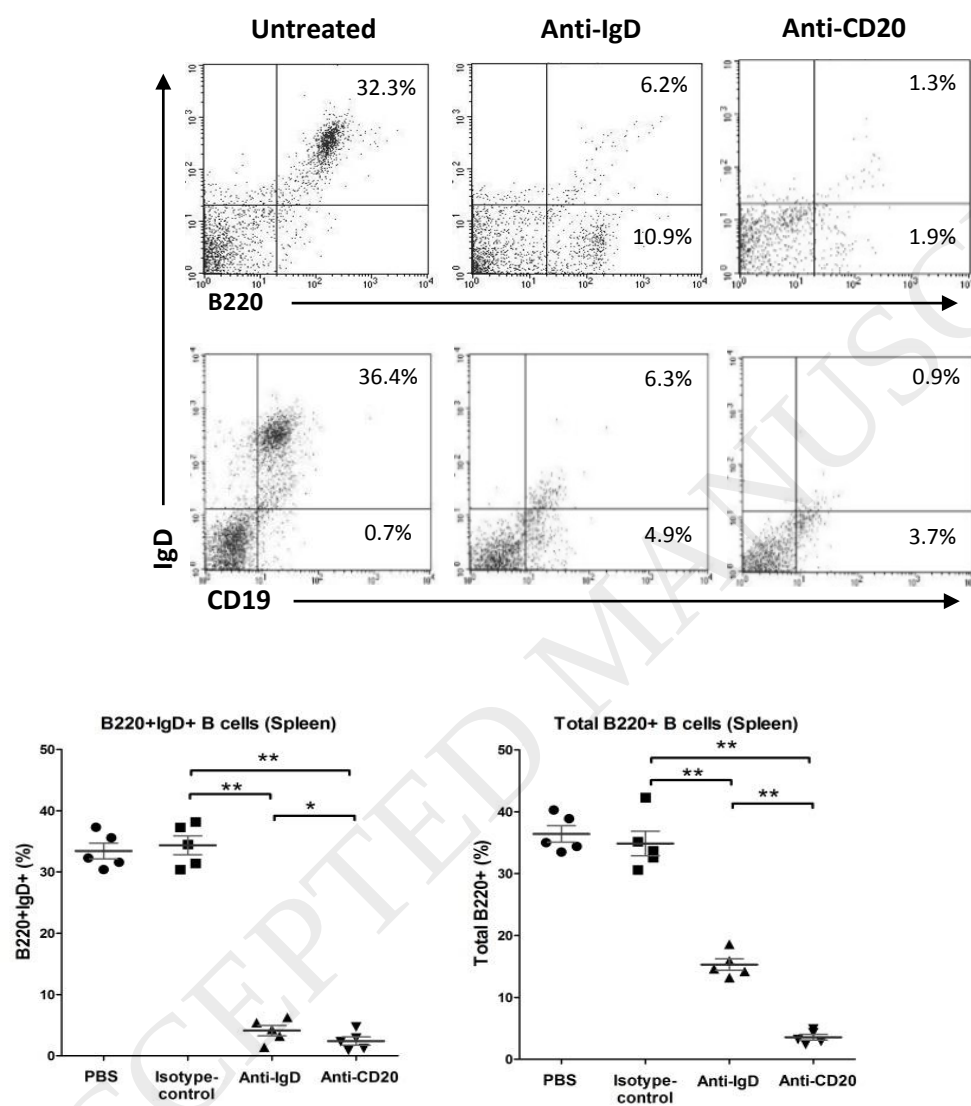


Figure 5

(A)



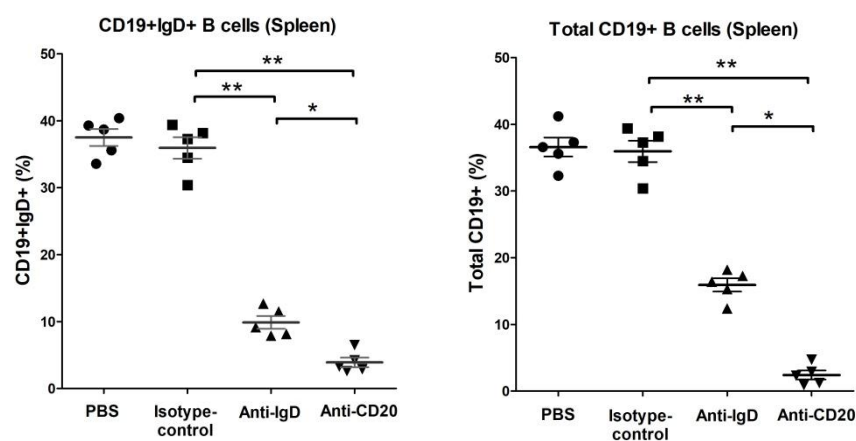
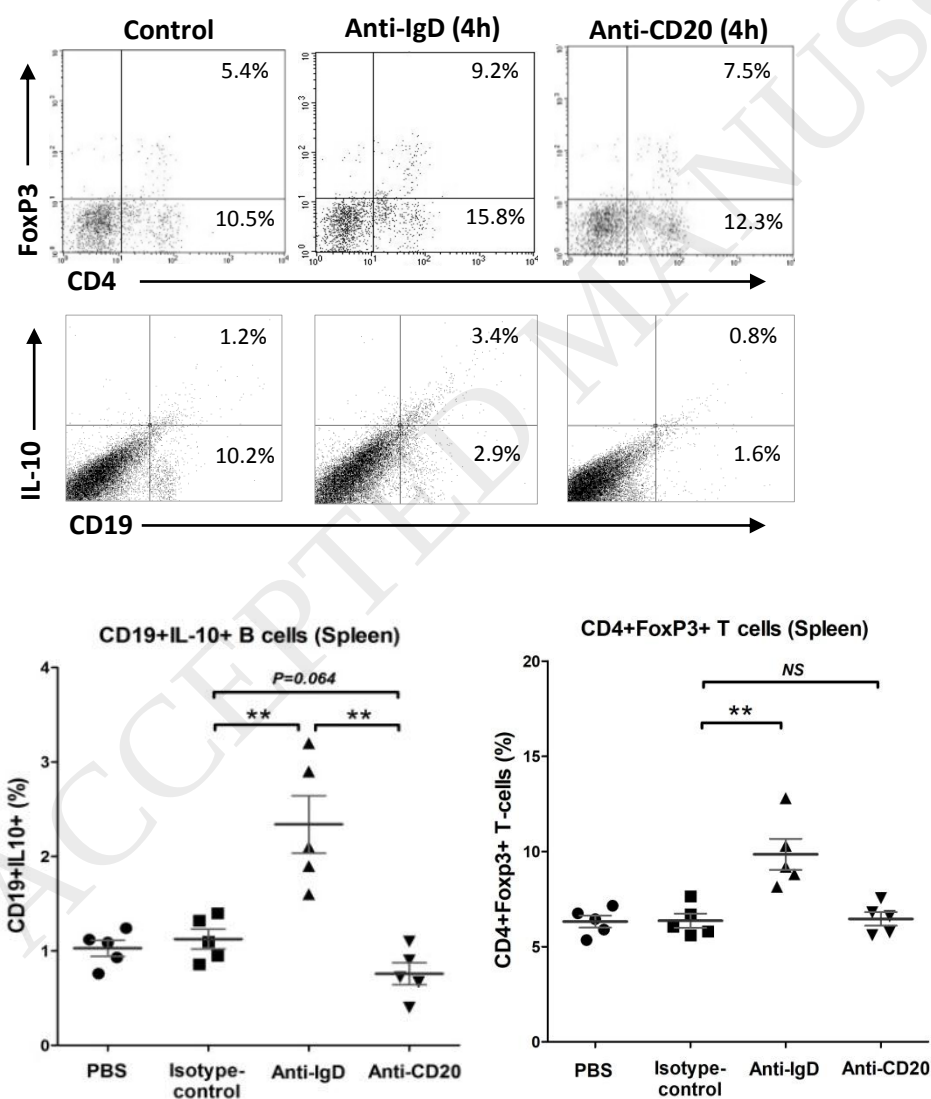
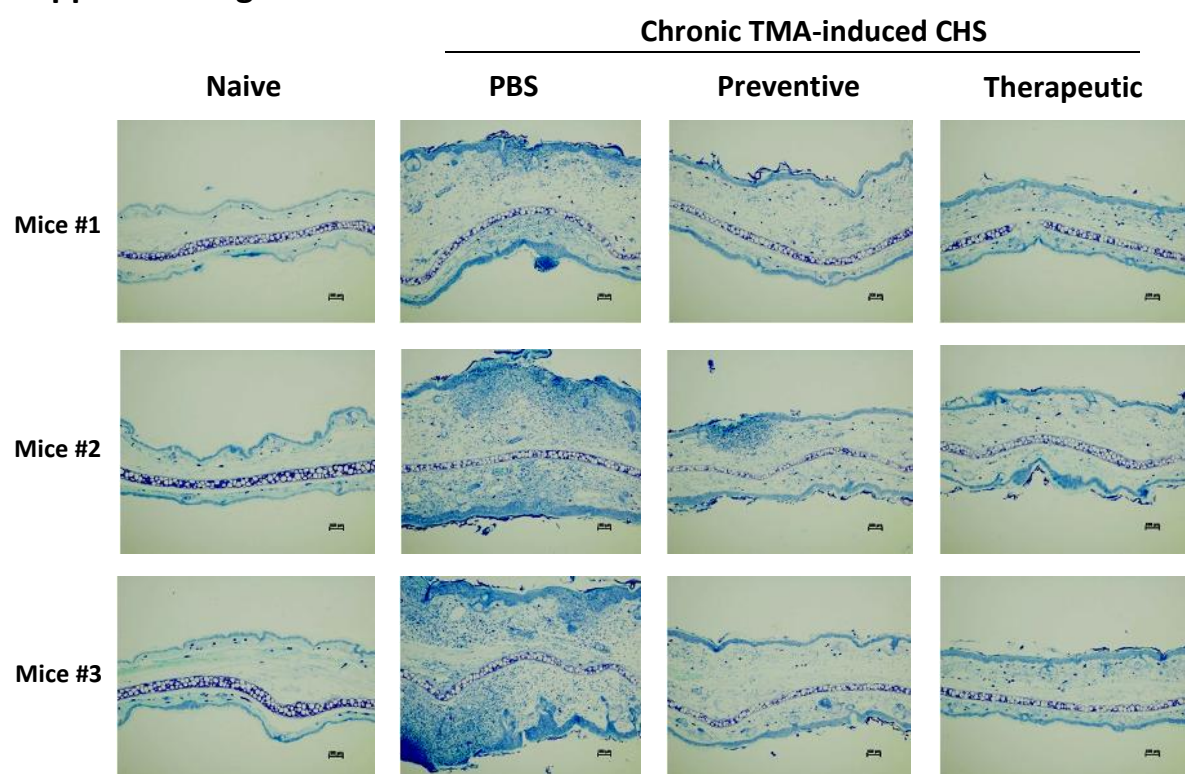


Figure 5

(B)



Supplement Fig 1



Supplement Fig 2

