Inflamm. res. 54 (2005) 514–521
1023–3830/05/120514-8
DOI 10.1007/s00011-005-1387-0

Inflammation Research

Deficiency of regulatory B cells increases allergic airway inflammation

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Received 10 June 2005; returned for revision 13 September 2005; accepted by M. Parnham 14 September 2005

Abstract. *Objective:* To investigate the effect of the X-linked immunodeficiency (Xid) B cell defect on the response to the cockroach allergen in mice.
Methods: Two cockroach allergen immunization and challenge protocols were employed to sensitize CBA/J wild-type and CBA/CaHN-btk(-/-)xid/J (Xid) mice. Blood and tissue samples were collected 24 and 48 hrs after the last intratracheal antigen challenge and were analyzed for several parameters of allergic inflammation.
Results: Nearly equivalent amounts of serum IgE were detected in Xid and CBA/J mice after short-term antigen challenge despite the B cell deficiency in Xid mice. A decreased concentration of IgE was detected in CBA/J mice after repeated allergen challenges but not in the Xid mice. Correlating with the discrepancy in serum IgE levels, higher levels of IL-13, IL-5, IL-10 and CCL5 were measured in whole lung homogenates from allergen-challenged Xid mice compared to CBA/J mice. In addition, draining lymph node cells from Xid mice expressed elevated levels of IL-4, IL-5, IL-10 and IFNγ mRNA compared to cells from CBA/J mice after *in vitro* culture with cockroach antigen. An increase in lung inflammation, interstitial eosinophilia and mucus production was also observed in allergen-challenged Xid mice. CD95L expression increased on B-1a cells following allergen challenge, which was accompanied by an increase in lung $CD4^+$ Th cell apoptosis in wild-type CBA/J mice. In contrast, Xid mice did not have an increase in $CD4^+$ T cell apoptosis following allergen challenge.
Conclusions: These data suggest a regulatory role for B-1a cells in reducing cytokine production, pulmonary inflammation, and $CD4^+$ T cell survival during cockroach allergen-induced airway inflammation.

Key words: Allergy – B cells – T cells – Inflammation – Apoptosis

Introduction

Asthma is a chronic disease of the lung caused by exacerbated immune response toward environmental allergens. Much previous investigation has established that $CD4^+$ T helper lymphocytes play a pivotal role in the establishment and maintenance of asthma. [1–3] These T helper cells produce Th2-associated cytokines including: IL-4, IL-5 and IL-13, which have pluripotent roles in promoting allergic reactions. [4, 5] In the setting of asthma, IL-4 stimulates differentiation of Th2 cells and proliferation of B cells, drives antigen-specific antibody production by B cells toward IgG1 and IgE isotypes, and inhibits production of the Th1-associated cytokine IL-12. [6–10] IgE binds to basophils and mast cells through surface Fcε receptors, which when stimulated by antigen, cause the release of basophilic granules containing histamines and leukotrienes, that in turn stimulate the contraction of airway smooth muscle. [11–13] In asthma, IL-5 stimulates the production and maturation of eosinophils, which travel to the airways and release granular material, leading to mucus production and eventual airway remodeling in severe cases. [14] IL-13 shares some common activities with IL-4 through a common receptor, but has some non-redundant roles including being a major contributor to fibroblast stimulation and increased pulmonary fibrosis. [15–17] In addition to the Th2 cytokines, $CD4^+$ T cells are a major source of chemokines that direct trafficking and activation of lymphocytes and

Abbreviations: CRAg, cockroach antigen; B-1a cells, CD5+ B cells; Xid, X-linked immunodeficiency; CD95L, CD95 ligand or Fas ligand; Btk -/- Bruton's tyrosine kinase deficient.
Funding source: U. S. Government NIH Grant# AI36302

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leukocytes into inflamed airways. [5, 18] Thus, the $CD4^+$ Th cell is an attractive target for understanding and regulating asthmatic responses.

A major, naturally occurring mechanism of $CD4^+$ T cell regulation during inflammation is activation-induced cell death or apoptosis. Apoptosis is an important mechanism in the regulation of lung inflammation in asthma as indicated by an increase in chronicity when apoptosis is reduced. [19] Although histological analysis has not yet established an increase in total $CD4^+$ T cell apoptosis in asthmatic patients, careful examination has revealed that T cells expressing IFN-γ are more apoptotic in asthmatics than in healthy subjects. [20] Although T cell apoptosis has been identified in asthmatics, the effector cells involved and the mechanisms employed to induce death are not completely understood.

It has recently been demonstrated that B cells, especially the $CD5^+/CD19^+$ B-1a cell subset, increase expression of surface Fas ligand (CD95L) in response to the Th2-inducing antigens of schistosome eggs. [21, 22] The B-1a cell subset is present in the pleural and peritoneal cavities, as well as the spleen but is not commonly found in lymph nodes. [23, 24] B-1a cells tend to recognize T-independent antigens such as lipids and carbohydrate moieties on glycoproteins and glycolipids, which are found in most common allergens. [25] Additionally, peritoneal B-1a cells have been shown to produce IL-10 upon antigenic stimulation. [26–28] These properties suggested a potential role for B-1a cells as a regulatory cell population at mucosal surfaces including the airways of the lung.

To test the hypothesis that B-1a cells contributed to the regulation of $CD4^+$ T cells in asthma, cockroach allergen-induced (CRAg) models of asthma were performed in B-1a cell deficient, CBA/CaHN(btk -/-)Xid/J mice and compared to wild-type CBA/J mice. Increased serum IgE levels and airway inflammation were observed in allergen-challenged Xid mice. Lung cytokine levels were elevated in Xid mice and antigen-stimulated lymph node cells from Xid mice produced more Th1 and Th2 cytokines compared to wild-type mice. Decreased T cell death was also observed in allergen-challenged Xid mice. Thus, the B cell population missing from Xid mice appeared to play an important role in regulation of allergen-sensitized T cells and control of airway inflammation.

Material and methods

Mice and Reagents

Age-matched, female CBA/J and CBA/CaHN-btk(-/-)xid/J (Xid) mice were purchased from Jackson Labs (Bar Harbor, ME) and housed under pathogen free conditions in the University Laboratory Animal Management facility at the University of Michigan Medical School. Mice were fed and drank ad libitum and were tested (negative) for the presence of many standard mouse pathogens. To ensure mutant status of mice, peritoneal lavage cells and collagenase dispersed lung cells were stained with anti-mouse CD19-FITC and anti-mouse CD5-PE antibodies (Pharmingen, San Diego, CA) and analyzed by two-color flow cytometry. All Xid mice completely lacked CD5+/CD19+ double positive B cells and had an approximately 50% reduction in CD5-/CD19+ B cells. [29] There were no detectable differences in growth, behavior or health between the two groups of mice throughout the course of the experiments. Skin test-grade cockroach antigen (CRAg) extracts were purchased from Hollister Stier (Spokane, WA) and tested negative for bacterial lipopolysaccharides by limulus amoebocyte assay. Antibodies for flow cytometry and other reagents were purchased from BD/Pharmingen (San Diego, CA) and Sigma/Aldrich (St. Louis, MO).

Cockroach Allergen Sensitization and Challenge

Mice (8–10 weeks old) were injected i.p and s.c. with 0.1 ml of cockroach allergen (CRAg, 20,000 protein nitrogen units/ml) emulsified 1:1 in Incomplete Freund's adjuvant (Sigma) on day 0. In the standard challenge model, mice received an intranasal challenge with 15 µl of undiluted CRAg on day 14, followed by intratracheal injections of 40 µl CRAg on days 21 and 23. In the chronic challenge model, mice received intranasal challenges on days 14, 18, 22, 26 and 30, followed by intratracheal challenges on days 34 and 38. In both models, mice were tested for methacholine-elicited airway hyperreactivity (see description below) at 16–20 hours after the final i.t. challenge, unless otherwise indicated, then immediately sacrificed for immunological and histological analysis.

Serum IgE Detection

Blood was obtained by retro-orbital eye bleed of anesthetized mice at the time of sacrifice and allowed to clot at 4°C for 1hr prior to removal of serum. ELISA plates were coated with polyclonal anti-Ig (BD Pharmingen), washed and incubated with serum for 1 hr. Monoclonal anti-IgE-biotin conjugated antibody and streptavidin-horseradish peroxidase were used for detection of serum IgE. Data represent the combined results of six experiments.

Measurement of Lung Cytokine Levels by ELISA

The left lobe was removed from chronically challenged CBA/J and Xid mice 16–20 hours after the final i.t. challenge and snap frozen. Lung tissue was homogenized in 1 ml PBS buffer containing Triton X-100 and protease inhibitors, and debris removed by high speed centrifugation. Samples were loaded onto ELISA plates precoated with anti-murine cytokine/chemokine antibodies (R&D Systems, Minneapolis, MN). Sandwich ELISA was performed following manufacturer's instructions and recombinant cytokines were used to generate a standard curve for each assay. Data are from a representative experiment of three performed.

Real-time PCR for Cytokine Expression in Cultured LN Cells

Mediastinal lymph nodes were removed from chronically challenged CBA/J and Xid mice at 16–20 hours post-i.t. challenge. Cells were dispersed through a mesh screen, counted and plated in triplicate ($5x10^6$ cells/ml) in the presence or absence of 200 PNU/ml of CRAg. Cells were harvested 16 hrs after initiation of culture, placed in Trizol reagent (Invitrogen, Carlsbad, CA), and snap frozen. Total RNA was purified following manufacturers instructions and mRNA was reverse transcribed using oligo-dT and MMLV-RT from Invitrogen. Real-time PCR was performed using an ABI Prism 7700 Sequence Detector and reagents from PE Biosystems (San Diego, CA). Individual samples were normalized to an internal GAPDH amplification control, then mRNA expression was compared between media control and CRAg-stimulated samples for each lymph node cell population. Data are expressed as the mean fold increase in mRNA expression ± standard error for the triplicate samples of a representative experiment of the three performed.

Histological Analysis of Peribronchiolar Eosinophilia

Right lung lobes were removed from chronically challenged CBA/J and Xid mice 16–20 hrs after the final i.t. challenge and inflated with 10% neutral buffered formalin. Lungs were embedded in paraffin, sectioned

and stained with either hematoxylin and eosin or periodate acid Schiff reagent. Eosinophils were quantified from H&E stained slides by counting inflammed fields adjacent to large airways at 1000X magnification for each mouse. Data presented are the number of eosinophils / 20HPF for individual mice (n = 5 mice/ strain). Sections from three experiments were counted with consistent results.

Measurement of Airway Hyperreactivity

Cockroach allergen immunized mice were anesthetized with pentabarbitol in order to paralyze the diaphragm and eliminate background noise due to normal breathing activity. Mice were then directly connected to a plethysmograph by intratracheal insertion of tubing connected to a timed air pump. Expansion of the lungs is measured as the change in air pressure in a plexiglass chamber surrounding the mouse compared to the amount of air pumped into the lung. A baseline measurement of airway resistance is taken (range 1 to 1.5 arbitrary units) prior to intravenous tail-vein injection of a 250 µg/kg dose of methacholine. This dosage of methacholine induces little to no increase in airway resistance in naïve mice. Within 1–2 minutes of methacholine injection, a peak value of airway resistance is reached in allergic mice, that returns to baseline within 5 minutes. The values reported are the ratio of peak / baseline airway resistance for each mouse and statistics are based on the number of data points shown on the graph.

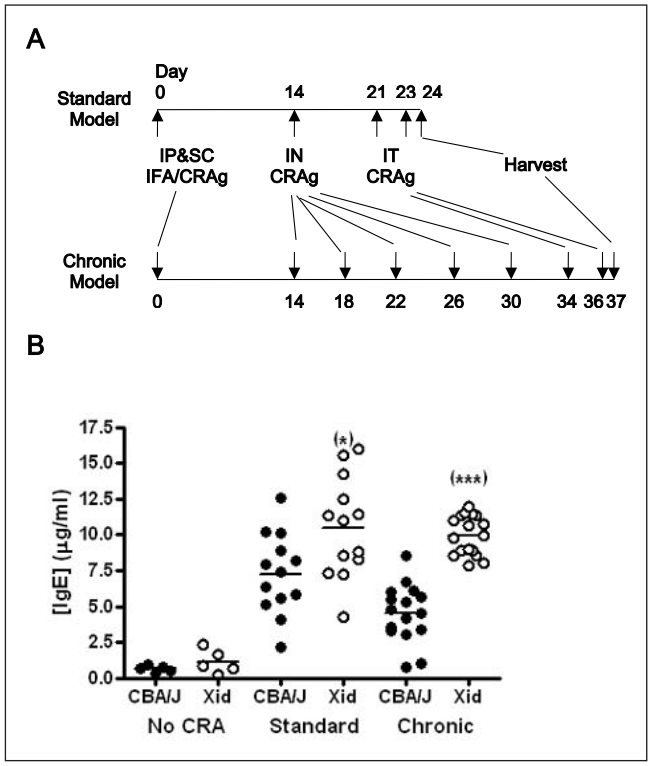

Fig. 1. (**A**): Serum IgE levels remain elevated in Xid mice after chronic allergen challenge. Female, 8–10 wk old CBA/J and CBA/HN *btk-/-* <xid>/J (Xid) mice were sensitized by injection with 0.1 ml of CRAg: IFA emulsion i. p. and s. c. on day 0. Starting at day 14, all mice received an intranasal challenge with 15 µl of undiluted CRAg. Mice in the 'chronic' model received an additional four i.n. challenges at four day intervals. Two intratracheal challenges of 40 µl were given to the mice in both models on the indicated days. Mice were sacrificed at time intervals ranging from 24 to 72 hrs after the second i. t. challenge as indicated in each figure. (**B**): Age matched, naïve and CRAg-sensitized mice were anesthetized and eye bled 24 hrs after the second i. t. allergen challenge. Serum was prepared and tested for the presence of IgE by sandwich ELISA. Dots represent individual mice in six experiments. Mean serum IgE concentrations are shown by the bars and significant differences in Xid compared to CBA/J mice in the same model are indicated by asterisk (* $P < 0.05$, *** $P < 0.001$). The mean serum IgE level was reduced in CBA/J mice in the chronic compared to the standard model ($P < 0.01$).

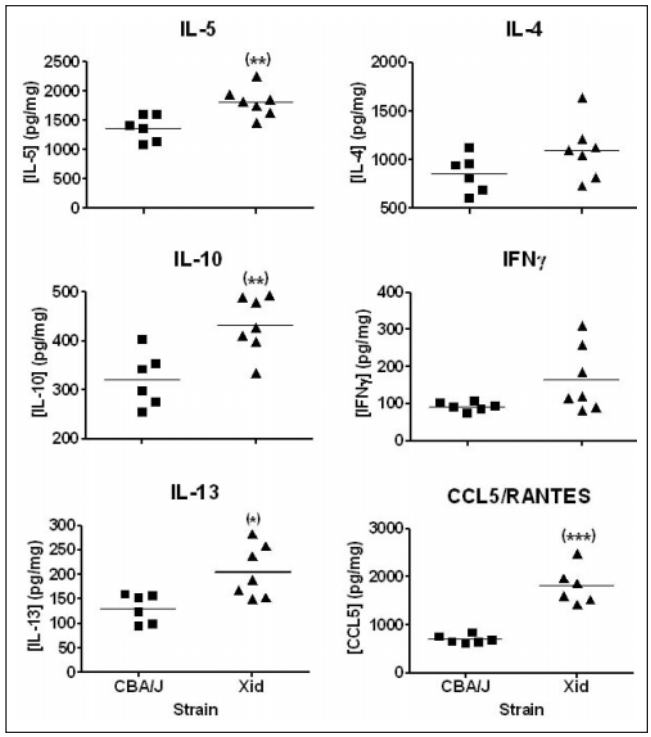

Fig. 2. Increased levels of Th2 cytokines and RANTES in the lungs of chronically challenged Xid mice. Mice from the chronic model of CRAg-induced asthma were sacrificed 24 hrs after the second i. t. challenge. The left lobe of the lung was removed and snap frozen, later homogenized, and analyzed for the presence of the indicated cytokines and CCL5 (RANTES) by sandwich ELISA. Data were normalized to total protein content in each sample. Dots represent cytokine levels from individual mice in a single representative experiment of three performed. Means are shown by the bar and significance by asterisk (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Detection of CD95L Expression on Lung Lymphocyte Subsets

Right lobes of mouse lungs were removed at the indicated times post i.t. challenge, minced and digested with Type IV collagenase to produce single cell suspensions. Cells were fixed with 1 % paraformaldehyde/ PBS overnight, washed and resuspended in flow staining buffer (PBS/ 0.2 % BSA/0.1 %NaAzide). One million cells were stained with Fc-Block followed by staining with anti-mouse CD95L-biotin. After being washed, cells were either stained with anti-mouse CD4-PE, CD8-FITC and streptavidin-CyChrome, or with anti-mouse CD5-PE, CD19-FITC and streptavidin-CyChrome. Analysis of CD95L expression was performed by three-color flow cytometry on a Beckman Coulter EPICS XL flow cytometer (Brea, CA). Data are presented as the ratio of mean fluorescence intensity ± standard error (n = 5 mice/group) of each cell population compared to the average staining on the CD4 population at 24hr post challenge. The data are from one representative experiment of the three performed.

Measurement of Lung Th Cell Apoptosis

Lymphocytes were isolated by the method described for CD95L detection from lungs of CBA/J and Xid mice that received the standard CRAg

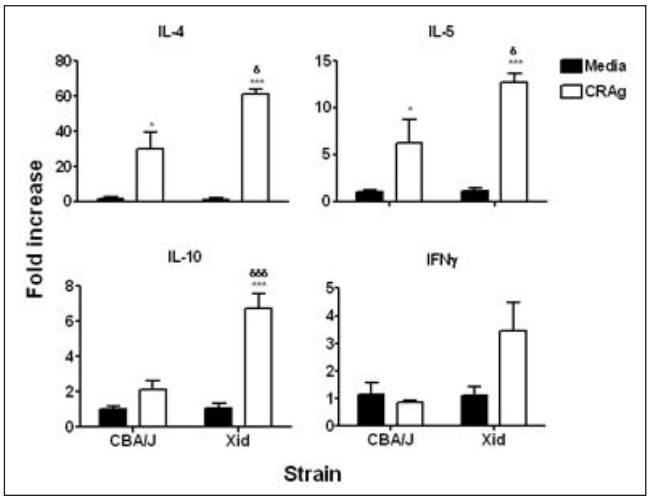

Fig. 3. Increased cytokine mRNA expression in antigen-stimulated lymph node cells from Xid mice. Mediastinal lymph nodes were harvested from mice treated with the chronic model 24 hrs after the second i. t. challenge. Single cell suspensions were prepared and cultured in the presence or absence of CRAg for an additional 16 hrs. Purified RNA was reverse transcribed and analyzed for cytokine expression by real-time PCR. Data for each sample were normalized to GAPDH expression within the sample to control for differences in cell number. Bars indicate the fold increase in cytokine mRNA expression following CRAg stimulation compared to the average media control value for that strain. Error bars indicate the standard error of replicate samples (n = 4/group) and results were reproducible in three experiments.

challenge at the indicated time points after the second i. t. challenge. Unfixed cells (1×10^6) were stained with FcBlock in staining buffer, followed by anti-CD4-PE antibodies for 30 min at 4°C. Cells were then washed with AnnexinV staining buffer (Saline/HEPES/$CaCl_2$) and stained with AnnexinV-FITC (Pharmingen) and propidium iodide (Sigma) following manufacturer's instructions. Three-color analysis was performed on the EPICS XL flow cytometer. Dead cells were removed from analysis on the basis of PI^+ staining and marker position for $AnnV^+$ cells was set using live-gated, lung $CD4^+$ Th cells that were not stained with AnnV. Data are the mean percentage of $AnnV^+$ cells within the live $CD4^+$ cell population ± standard error, and are the combined results from 6 experiments with the indicated number of mice / time point.

Statistical Analysis

Data were plotted and statististically analyzed using the GraphPad Prism 4.0 (GraphPad Software, San Diego, CA). Where multiple groups or time points were present, analysis of variance was performed and significant differences were confirmed by individual Student's t tests. All other analyses were performed using Student's t test. Data were considered significant when $P < 0.05$ and were reproducible in replicate experiments.

Results

Lack of serum IgE downregulation in chronically challenged Xid mice.

Antigen-reactive IgE contributes to the pathophysiology of asthma through binding to Fcε receptors on the surface of mast cells and basophils. The Xid mutation causes decreased levels of B cells, particularly of the B-1a cell subset, and impaired antibody responses to some antigens. Therefore, it was of interest to measure levels of serum IgE after CRAg challenge in CBA/J and Xid mice. Using a 'standard' induction model (Figure 1a) with intraperitoneal and subcutaneous injection of CRAg emulsified in incomplete Freund's adjuvant, followed by a single intranasal challenge and two intratracheal challenges, it was found that serum IgE was induced in both strains of mice by CRAg treatment, and that Xid mice had an elevated amount of serum IgE compared to CBA/J mice (Figure 1b). To confirm this result, a more chronic model (Figure 1a), in which mice received four additional intranasal doses of CRAg at four-day intervals was utilized. As shown in Figure 1b, serum IgE levels were diminished following repeated allergen challenge in CBA/J mice but were unchanged in Xid mice given repeated CRAg stimulation.

Elevated cytokine levels in lungs of allergen-challenged Xid mice.

The increased production of IgE suggested that dysregulation of T cell-derived cytokine production may have occurred. Cytokine protein levels in whole lung homogenates were assessed by ELISA 24 hrs after the final CRAg-challenge. As shown in Figure 2, elevated levels of IL-13, IL-5, IL-10 and CCL5 were detected in the lungs of Xid mice after repeated allergen challenge. A similar trend was detected for IL-4 and IFN-γ in the lung, although the increase was not statistically significant. In contrast, the levels of eotaxin and MIP-1α in the lung were similar in CBA/J and Xid mice (data not shown).

Since elevated levels of several cytokines were detected in the lungs of Xid mice following repeated allergen challenge, it was important to determine whether or not the difference was due in part to an increased capacity of T lymphocytes to produce cytokines in Xid mice. Isolated mediastinal lymph node cells from chronically allergen-challenged CBA/J and Xid mice were cultured in the presence or absence of CRAg for 16 hrs. The data in Figure 3 indicated significant cytokine mRNA increases for IL-4 and IL-5 in CBA/J lymph node cell cultures. A more dramatic increase in IL-4 and IL-5 mRNA production was observed in lymph node cells from Xid mice in response to CRAg. Additionally, elevated mRNA levels of IL-10 and IFNγ were detected in the cultures from Xid mice but not from CBA/J mice. IL-13 mRNA expression was increased equally in both strains of mice in the cultures containing CRAg (data not shown). Taken together, the cytokine data indicate that B-1a cells may play a role in regulating both Th1 and Th2 cells during chronic allergen challenge.

Allergen-challenged Xid mice have increased airway inflammation.

Airway inflammation, mucus hypersecretion and peribronchial eosinophilia are hallmarks of the Th2 cytokine-mediated inflammation observed in asthma. As shown in Figure 4 (upper panels) an overall increase in pulmonary inflammation was observed in Xid mice compared to CBA/J mice after chronic

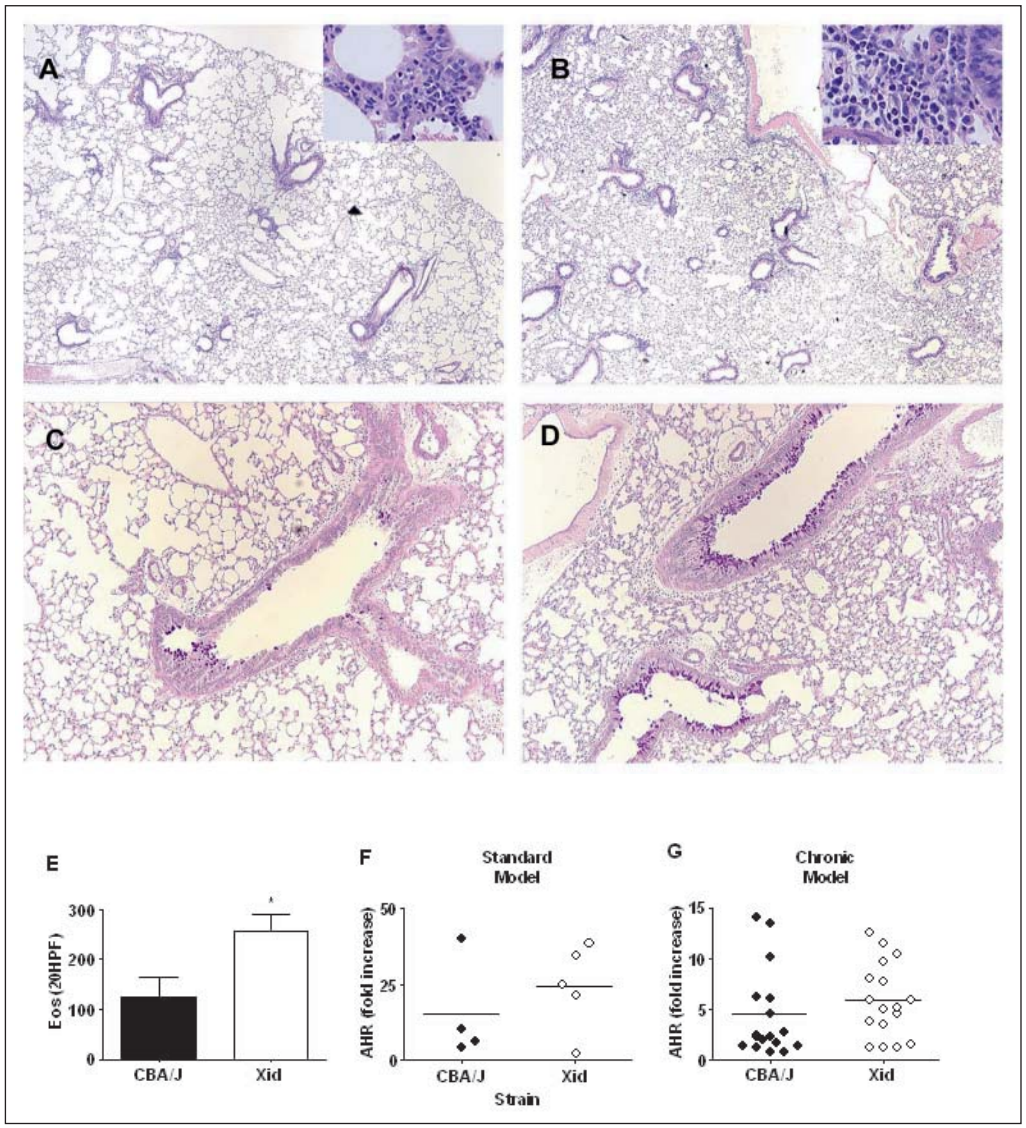

Fig. 4. Lung inflammation is increased in Xid mice without an increase in airway hyperreactivity. Lungs were removed 24 hrs after the second i.t. challenge from chronically sensitized CBA/J (**A&C**) and Xid (**B&D**) mice, paraffin embedded, sectioned and stained with hematoxylin and eosin **(A&B)** or with periodate acid Schiff reagent (**C&D**). Insets show areas of leukocytic inflammation surrounding airways in the H&E sections with higher numbers of eosinophils in the Xid sample (**B**). Mucus secretion is detectable by the red staining in the PAS stained sections. **(E)** Eosinophils were counted within areas of airway inflammation and plotted as the mean ± standard error (20 high powered fields/mouse, 5 mice/group). **(F&G)** Airway hyperreactivity was measured on anesthetized mice and is plotted as the increase in airway constriction following methacholine challenge compared to baseline levels. Each dot indicates an individual mouse from a representative experiment of three performed.

CRAg challenge. Increased inflammation was accompanied by a higher frequency of periodate acid Schiff-staining, mucus-secreting airway epithelial cells in Xid mice (Figure 4 middle panels). Within the inflammatory lesions, a two-fold increase in the number of eosinophils was observed in Xid mice compared to CBA/J mice (Figure 4-E). Despite the alterations seen in systemic IgE, lung cytokines and eosinophilia, airway hyperreactivity in response to methacholine challenge was not significantly increased in Xid mice (Figure 4-F & G).

Fas ligand expression is induced on lung CD5+ B cells following allergen challenge.

Upregulation of CD95L expression on splenic B cells, particularly those of the B-1a subset, has been previously observed during Th2-type granulomatous response. These CD95L-bearing B-1a cells were shown to regulate $CD4^+$ T cell apoptosis and granuloma formation. To determine whether B-1a cell-mediated apoptosis of $CD4^+$ T cells was a plausible mechanism of regulation in the CRAg model, surface CD95L expression was measured on lung lymphocyte populations. Control CBA/J mice were treated with the standard induction protocol. As shown in Figure 5a, CD95L expression on pulmonary $CD5^+$ B-1a cells was elevated at 24 hrs post i.t. challenge compared to other lung lymphocyte cell types. At 48 hrs post-challenge, CD95L expression was 2–3 fold higher on B-1a cells than on other lymphocyte cell types in the lung. Less dramatic, yet statistically significant increases in CD95L expression were observed on the $CD4^+$ T and $CD5^-$ B cell subsets but not on the $CD8^+$ T cell subset at 48 hrs post CRAg challenge.

Impaired CD4+ T cell apoptosis in allergen-challenged Xid mice.

To determine the relative contribution of B-1a cells to $CD4^+$ T cell apoptosis in the CRAg model, lung lymphocytes from allergen sensitized and challenged Xid mice and CBA/J mice were harvested and stained for CD4 and the early apoptosis marker, Annexin V (Figure 5b). Lung $CD4^+$ T cell apoptosis increased two-fold from 24 to 48 hrs post-allergen challenge

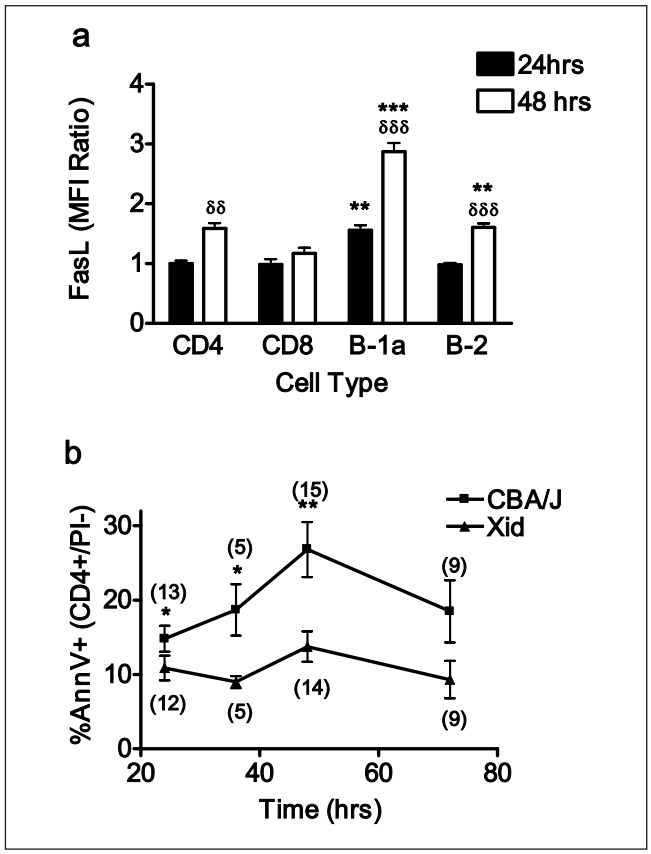

Fig. 5. Cell surface CD95L expression is increased on lung B-1a cells after allergen challenge and Xid mice have impaired $CD4^+$ T cell apoptosis. (**A**) Cells were collagenase dispersed from the lungs of CBA/J mice treated with the standard model at the indicated times after the second i.t. challenge. Paraformaldehyde fixed cells were stained with CD95L-biotin followed by cell surface marker staining and streptavidin-CyChrome. Three-color flow cytometric analysis was performed to determine surface CD95L expression on the indicated cell populations. A ratio of mean fluorescence intensity (MFI) values was determined by comparison to the average staining on the $CD4^+$ cell population at the 24 hr time point. Data are the mean ± standard error for 5 mice/group. (*) Significance compared to the $CD4^+$ group at the same time point, (§) significance compared to 24 hr time point within the same cell population. (**B**) Cells were dispersed from lungs of CBA/J and Xid mice at the indicated time points after the second i.t. challenge in mice treated with the standard model of CRAg-induced asthma. Unfixed cells were stained with CD4-PE followed by staining with Annexin-V-FITC and propidium iodide. Live gated Th ($CD4^+/PI^-$) cells were analyzed for apoptosis by Annexin-V staining. Data are the mean percent apoptotic cells ± standard error compiled from six experiments, number in parentheses indicates total number of mice per condition. Apoptosis in unimmunized CBA/J and Xid mice was 10.0 ± 2.1 and 5.8 ± 0.5 % respectively (n = 5 mice/group).

in CBA/J mice. No increase in lung $CD4^+$ T cell apoptosis was observed in Xid mice during the same time period. The percentage of lung T cells undergoing apoptosis in age-matched, allergen-naïve, CBA/J and Xid mice was 10.0 ± 2.1 and 5.8 ± 0.5, respectively.

Discussion

The current study supports the hypothesis that $CD5^+/CD19^+$ B-1a cells play a regulatory role in the CRAg murine model of asthma. There was a significant increase in serum IgE in B-1a cell deficient, Xid mice compared to CBA/J control mice indicating: 1) that the Xid mutation did not impair the ability of B cells to produce IgE antibody in response to CRAg sensitization, and 2) that a mechanism which normally controls IgE production or clearance was absent from Xid mice. Previous studies of immune response in Xid mice have shown that approximately 2/3 of the total B cells are absent from Xid mice. [29] Serum IgM and IgG3 titers were severely reduced in naïve New Zealand Black mice carrying the xid defect, while serum IgG1, IgG2b and IgA titers were similar to controls. [30] Antibody responses to thymus-independent antigens were severely compromised in Xid mice and response to T-dependent antigens were altered with a general reduction in antigen-specific IgM, IgG2a and IgG3. [31–34] However, the observed increase in IgE in this study is consistent with previous findings in parasite infection models in which Balb.Xid mice produced greater amounts of antigen-specific IgE than control Balb/c mice. [35, 36]

Levels of several Th2 cytokines were elevated in the lungs of Xid mice compared to control mice following chronic sensitization, suggesting that regulation of pulmonary cytokine levels during chronic asthmatic inflammation may be a normal function of B-1a cells. CRAg stimulation of mediastinal lymph node cells from Xid mice resulted in increased mRNA expression of IL-4, IL-5, IL-10 and IFN-γ. The increased level of IL-4 during the chronic inflammatory response may have been responsible for the sustained high level of serum IgE observed after repeated allergen challenge. Another possible explanation of the increased presence of serum IgE following cockroach allergen challenge is an impairment in mast cell uptake. Bruton's tyrosine kinase is critically involved in signal transduction following Fcε receptor ligation. [37–39] However, no previous reports of decreased numbers of mast cells or impairment in IgE uptake have been reported in Xid mice.

The increase in CRAg-induced IFN-γ from Xid mediastinal lymph node cells was noteworthy, since IFN-γ production was not induced in LN cells from control mice. This finding suggested that antigen-reactive, Th1-type cells were allowed to persist in Xid mice, and correlated well with findings from other groups. Splenocytes from schistosome-infected Balb.Xid mice produced more IFN-γ and IL-4 following specific antigenic challenge *in vitro.* [36] Balb.Xid mice were also able to resist infection with *Leishmania major* and produced significantly elevated levels of IFN-γ from lymph node cells following leishmanial antigen stimulation compared to Balb/c control mice. [40] Similarly, resistance to Chagas' disease was reported in Balb.Xid mice along with elevated levels of splenic IL-2, IL-4 and IFN-γ during early stages of infection, and increased susceptibility following anti-IFN-γ treatment of Balb.Xid mice. [41] In each of the above studies, a decreased level of IL-10 was detected from the antigen-stimulated cells, suggesting that the lack of regulation of other cytokines was due to the decreased levels of IL-10 in Xid mice. Peritoneal B-1a cells have been shown to produce IL-10 in response to carbohydrate antigens in the schistosome model. [26] However, in the current study, IL-10 levels were elevated in the lungs of mice receiving chronic CRAg challenges and IL-10 expression was increased in the mediastinal lymph node cultures from Xid mice, arguing

against a role for IL-10-mediated down regulation of local cytokine production. Yet, it remains possible that B-1a cell-derived IL-10, that is normally present at the cell-cell interface during cognate B:T cell interactions, plays a regulatory role on allergen-sensitized T cells.

Increased lung inflammation, peribronchial eosinophilia and mucus production by airway epithelial cells were observed in Xid mice following chronic allergen challenge. Notably, the proinflammatory chemokine, CCL5 (RANTES), was highly prevalent in the lungs of all CBA.Xid mice regardless of antigenic stimulus or sensitization protocol. CCL5 has been previously shown to direct recruitment of eosinophils to the lungs in asthma models. [42] The other eosinophil chemotactic chemokines, CCL11 (Eotaxin) and CCL3 (MIP-1α), were not elevated in Xid mice implicating CCL5 as a key mediator regulating lung inflammation in this study.

Despite the increases in serum IgE, lung and lymph node cytokine expression, and peribronchial eosinophilia, no significant increase in airway hyperreactivity was detected in Xid mice. This finding may relate to a previously established defect in mast cell activation in Xid mice. [37–39] However, the increased lung inflammation most likely led to other detrimental responses, including increased mucus production within the lungs of Xid mice. It remains to be determined whether other methods of blocking B-1a cell regulatory function would result in exacerbation of both mucus production and airway hyperreactivity in the chronic CRAg model.

In addition to the above-mentioned role of IL-10 in Th cell regulation, another plausible mechanism of Th cell regulation by B-1a cells is induction of apoptosis mediated by CD95L. The intensity of surface CD95L expression was higher on the lung B-1a cell subset isolated from allergen-challenged mice than on the lung $CD5^-/CD19^+$ B cell subset or the $CD4^+$ or $CD8^+$ T cell subsets. Further, CD95L expression increased on the B-1a cell subset from 24 to 48 hrs post-allergen challenge to a greater extent than the increase observed on $CD4^+$ T cells or $CD5^-/CD19^+$ B cells. These findings correlated with a recent study in the schistosome granuloma model in which CD95L was constitutively present on splenic B-1a cells, was upregulated by infection and by *in vitro* antigen challenge, and in which the B-1a cells were demonstrated to be potent effector cells mediating Th cell apoptosis. [22] Lung $CD4^+$ T cells in wild-type CBA/J mice reacted with an increase in apoptosis 36 hours after airway allergen challenge. The apoptotic response peaked at 48 hours post challenge but remained elevated at 72 hours. In contrast, lung $CD4^+$ T cells in CRAg- challenged Xid mice did not display an increase in apoptosis at any time point tested. The Xid mutation results in a systemic loss of B-1a cells, but only a partial loss of function by follicular B cells. [43, 44] Thus, the decline in Th cell apoptosis in Xid mice implicates the B-1a cell subset as an important effector of pulmonary Th cell death during CRAg-induced asthma. This is the first report of defective T helper cell apoptosis in B-1a cell deficient Xid mice.

Since the initial report of mitogen-inducible CD95L expression on murine B cells, several lines of evidence have supported a functional role of 'regulatory' B cells *in vivo*. [45] Studies of experimental autoimmune encephalomyelitis (EAE), inflammatory bowel disease and schistosome infection in B cell deficient mice have resulted in impairment of T cell regulation and increased disease severity. [21, 46–48] Further, transfer of LPS-stimulated, CD95L-bearing, purified B cells into NOD mice resulted in inhibition of spontaneous diabetes incidence. [49] Transfer of purified B cells from male mice into female recipients resulted in CD95L-dependent tolerance to subsequent male-female skin grafts in a transplantation model. [50] The current study further supports the regulatory function of B cells, particularly the B-1a cell subset, during chronic inflammation.

The presence of a regulatory function of B cells has been established in several clinically relevant mouse models, including the CRAg-induced asthma model. Further, several correlations have been drawn between the expression of CD95L on human B cell lymphomas and autoreactive B cells and escape from immune surveillance. [51–53] It remains to be determined whether alterations in regulatory B cell function result in increased T cell activity and exacerbated disease severity in asthmatic patients. The current study suggests that differences in antibody production, lung inflammation, lung cytokine levels, or Th cell apoptosis may be markers of impaired B-1a cell regulatory function.

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