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Immune modulation of ovalbumin-induced lung injury in mice using β -glucosylceramide and a potential role of the liver

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ABSTRACT

CD1d-restricted natural killer T (NKT) cells are implicated in the pathogenesis of asthma. β -Glucosylceramide (GC), a naturally occurring lipid, was previously shown to alter NKT cell distribution in the liver. We hypothesized that GC can affect lung and liver NKT cell distribution and ameliorate asthma. Mice were sensitized by intra-peritoneal injection of ovalbumin (OVA) for 2 weeks followed by repeated intranasal OVA challenges to induce lung injury mimicking asthma. OVA induced asthma groups were either treated by intranasal instillation of normal saline, intranasal instillation of GC or inhaled budesonide. To investigate the role of the liver, hepatic fibrosis was induced using carbon tetrachloride prior to asthma induction. Allergen induced bronchoconstriction was measured prior to sacrifice. Isolated lymphocytes from lungs, livers and spleens were analyzed for OVA induced proliferation and flow cytometry. Liver and lung histology, serum aminotransferase and anti-OVA antibodies level were assessed. Treatment with GC significantly reduced OVA induced airway responsiveness ($p < 0.001$) similar to inhaled budesonide. GC significantly reduced the peri-bronchial and peri-vascular inflammatory infiltration mainly through an effect on T cells, as suggested by decreased T cell proliferation ($p = 0.009$). Liver CD4 and NKT cells significantly increased after GC treatment suggesting liver involvement. Inducing hepatic fibrosis blunted the propagation of asthma in spite of sufficient increase of serum anti-OVA titers. GC has an immunomodulatory effect on a murine model of experimental asthma. We also suggest that the liver acts as an immunomodulatory organ and might have a regulatory effect on pulmonary diseases.

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Introduction

Natural killer (NK) T cells are increasingly implicated in the regulation of different immune, allergic, antimicrobial, and anti-tumor responses (Yu and Porcelli 2005), including bronchial asthma (Lisbonne et al. 2003; Akbari et al. 2006, 2003). There is still controversy concerning the exact role of NKT cells in the pathogenesis of asthma. Increased number of lymphocytes was detected in the bronchoalveolar lavage (BAL) obtained from asthmatic patients

with a large fraction being CD4⁺ NKT cells (Akbari et al. 2006). Furthermore, up to 86% of CD3⁺ cells in the BAL of asthmatic patients were NKT cells. Similar to previous work in animal models (Hachem et al. 2005), these cells had a typical cytokine profile of Th-2 cells, expressing high levels of interleukin (IL)-4 and IL-13 but not interferon- γ (Akbari et al. 2006). Other recent works, however, failed to show a significant increase in NKT cells number in asthmatic patients (Vijayanand et al. 2007).

NKT cells are characterized by a narrow T cell antigen receptor repertoire, which recognizes glycolipid antigens in the context of a monomorphic CD1d antigen-presenting molecule (Taniguchi et al. 2003). NKT cells have the unique capacity of rapidly producing large amounts of both T helper Th1 and Th2 cytokines (Crowe et al. 2003). β -Glycosphingolipids have emerged as a family of potential ligands for NKT lymphocytes (Crowe et al. 2003; Bendelac et al. 2007). β -Glucosylceramide (GC) is a metabolic intermediate in the glycosphingolipids pathways that have been shown to alter immune responses in the setting of different autoimmune diseases as well as cancer (Radin and Inokuchi 1988). GC has also been

Abbreviations: α -sma, alpha smooth muscle actin; ALT, alanine aminotransferase; BAL, bronchoalveolar lavage; BUD, budesonide; CCL₄, carbon tetrachloride; Con-A, concavalin A; GC, beta glucosylceramide; H&E, hematoxylin and eosin stain; IL, interleukin; IN, intranasal; IP, intra-peritoneal; NKT, natural killer cells; NS, normal saline; OVA, ovalbumin; Penh, plethysmographic enhanced pause.

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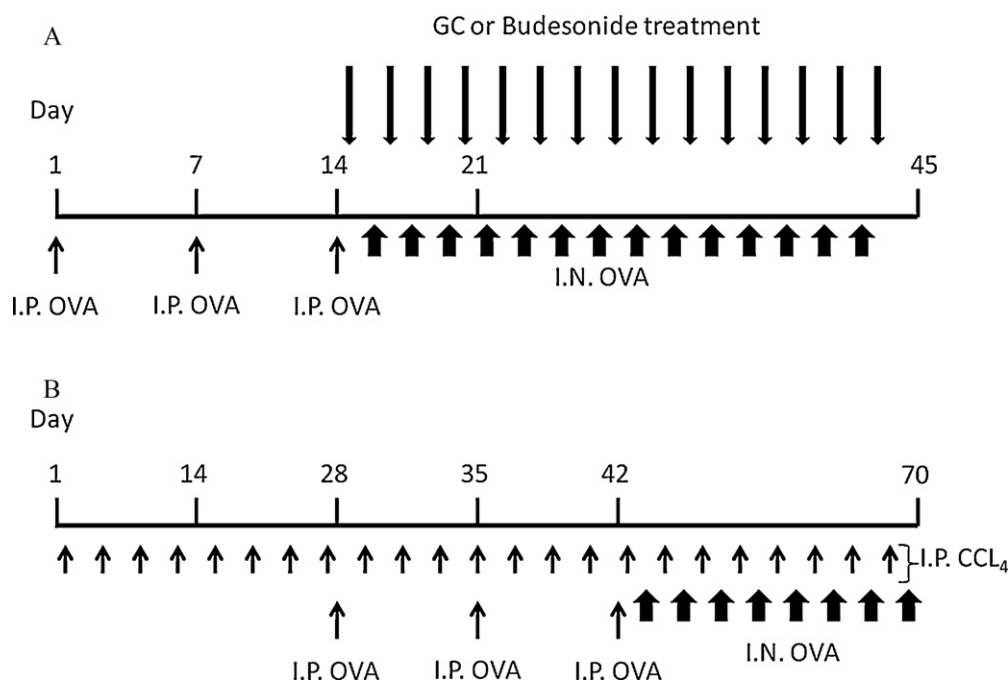


Fig. 1. Treatment regimen. (A and B) Model induction and treatment regimen of both the ovalbumin (ova) induced lung injury model (A) as well as the liver fibrosis and lung injury model (B). GC: glucosylceramide; IN: intranasal; IP: intra peritoneal; CCl₄: carbon tetrachloride.

shown to play a role in modulating NKT cell activation (Stanic et al. 2003).

The aim of this study was to determine the effect of GC treatment on ovalbumin (OVA) induced lung injury, in a model mimicking asthma (Horani et al. 2009). Furthermore, we also tracked changes in lymphocyte subsets in the lung, liver and spleen due to the known liver involvement in inherited deficiency of glucocerebrosidases (Elstein et al. 2002). We also investigated the impact of liver fibrosis as a model of increased hepatic lymphocyte recruitment (Muhanna et al. 2007, 2008), on the induction and development of ovalbumin induced asthma.

Methods

Animals

This study was approved by the animal ethics committee of the Hebrew University-Hadassah Medical School (MD-07-10298, MD-112.07-3). Male Balb/c mice, age 8–10 weeks, purchased from Harlan Laboratories Inc., were used for this study and received care according to the National Institutes of Health guidelines. Mice were purchased and housed in a barrier facility. Animals were sacrificed 24 h after the last administration of antigen. At the time of sacrifice, mice were weighed and anesthetized through the intra-peritoneal (IP) injection of 0.1 ml of ketamine:xylazine:acepromazine [4:1:1] per 30 g of body weight. Blood samples were collected from the inferior vena cava. Whole lungs, spleens and livers were harvested and weighed. Samples were harvested from both liver lobes to reduce sampling variability among experimental and control mice.

Animal model of experimental allergic bronchitis

Experimental allergic bronchitis mimicking asthma (Nials and Uddin 2008) was induced by intra-peritoneal (IP) injection of 10 µg ovalbumin (OVA) (Grade III; Sigma–Aldrich) in 3 mg aluminum hydroxide (Al(OH)₃) on days 0, 7 and 14. Animals were thereafter challenged with intranasal instillation of OVA 100 µg in 50 µl

saline, 3 times a week for 4 weeks starting on day 14 to mimic a chronic disease. The OVA model of asthma prolonged therefore a total of 6 weeks (Fig. 1A).

Animal model of liver fibrosis

Hepatic fibrosis was induced by IP injection of carbon tetrachloride (CCl₄; Sigma, C-5331) diluted to 10% with corn oil. CCl₄ was administered at a dose of 5 µl/g body twice weekly, for a total of 10 weeks; starting 4 weeks prior to asthma induction (Fig. 1B).

Experiment design

Following the OVA induction of asthma in three groups (10 animals each); mice were either treated by intranasal instillation of normal saline (OVA/NS), inhaled Budesonide (OVA/Bud), or by intranasal instillation of GC (OVA/GC). A fourth group served as a healthy naive control group.

To investigate the effect of liver injury on the propagation of asthma, we induced liver fibrosis using CCl₄ in 2 animal groups (10 animals each). One of the groups underwent OVA sensitization while the other was used as a negative control. The asthma-OVA model, spanning 6 weeks, was induced after 4 weeks of liver fibrosis induction.

Treatments

All treatments (saline, GC and budesonide) were given every other day; starting on day 14, after asthma was induced. Intranasal treatments and OVA instillations were not administered on the same day. Intranasal GC, 50 µg in 50 µl per animal, was dissolved in ethanol and emulsified in normal saline. Intranasal instillations of 50 µl normal saline were used in untreated control animals. Budesonide was administered using an inhalation cage, at a dose of 2 mg per cage.

Bronchial allergen challenge assessment

Bronchial reaction to allergen inhalation was performed under continuous airflow conditions using a non-invasive method. Bronchial allergen challenge assessment was performed after the last allergen challenge. Mice were placed in a whole-body plethysmograph connected to a pneumotach, which was connected to a 10 ml bottle at its other end. The pneumotach was connected to a pre-amplifier. Analogue signals from the amplifier were converted to digital signals by an AD card. Software (System XA, model SFT 1810, Buxco Electronics) was used to analyze 10 breath signals and calculate the plethysmographic enhanced pause (Penh) value. Penh value is a unit-less indicator of changes in airway resistance that correlates with specific airway resistance. It reflects the time of pressure decay to 36% of total box pressure during expiration (Hamelmann et al. 1997; Lomask 2006). The Penh value was calculated before and 5 min after allergen challenge. Airway hyper-responsiveness was expressed as the percent change of the Penh value. The Penh value was used as a non-invasive screening method to assess for changes in airway hyper-responsiveness in the studied animals.

Splenocyte isolation

Spleens were harvested at the time of sacrifice and fractionated through a 70- μ m nylon cell strainer. Red blood cells were lysed by the addition of lysis buffer containing NH_4Cl , KHCO_3 and Na-EDTA. Splenocytes were washed, suspended in RPMI 1640 medium and stored at 4°C until FACS analysis (Safadi et al. 2004).

Lymphocyte isolation from the liver and the lung

Livers and lungs were homogenized and incubated at 37°C for 30 min. Next, the digested liver or lung cell suspension was centrifuged to remove parenchymal cells and cell clumps. The supernatant was then centrifuged to obtain a pellet of cells depleted of parenchymal cells to a final volume of 1 ml. Lymphocytes were then isolated from this cell suspension by 24% metrizamide gradient cell separation (Safadi et al. 2004).

Lung histology and morphometry

Lungs were removed and fixed by inflation with paraformaldehyde at a pressure of 20 cm H_2O . The middle third was embedded in paraffin and histology slides were obtained randomly by longitudinal cut into three sections. Slides were stained with eosin–hematoxylin. Other slides were stained with Masson's Trichrome for assessment of sub-epithelial fibrosis. The sections were reviewed by an experienced pathologist who was blind to the treatment groups.

We devised a severity score to objectively evaluate histological changes in the lungs. Sections were scored on a scale of 1–4, such that: (1) implied normal histology; (2) minimal peribronchial lymphocytic infiltration without smooth muscle hypertrophy; (3) increased peribronchial lymphocytic infiltration with smooth muscle hypertrophy and grade 4 indicated extensive lymphocytic infiltration, smooth muscle hypertrophy and obliteration of normal lung structure. Sections were scored by a pathologist who was blind to study groups.

Histological assessment of liver injury

The posterior one-third of the liver was fixed in 10% formalin for 24 h and then paraffin-embedded in an automated tissue processor. Seven-millimeter liver sections were cut of each sample. Sections (15 μ m) were stained with 0.1% Sirius Red F3B in

saturated picric acid (both from Sigma). Sections also underwent hematoxylin–eosin staining. The slides were reviewed by an experienced pathologist who was blind to the treatment groups (Safadi et al. 2004).

Flow cytometry

Lymphocytes were analyzed by a fluorescence-activated cell sorter (FACScalibur, Becton Dickinson, Immunofluorescence systems, Mountain View, CA) (Safadi et al. 2004). Lymphocytes derived from splenocytes were identified and gated based on their size (forward light scatter, FSC) and granularity (side light scatter, SSC). We carried out immunophenotypical analysis using monoclonal antibodies to CD45 and CD3 as pan lymphocytes marker and T cells, respectively. Splenocytes were stained with monoclonal anti-mouse CD4-FITC and CD8-PE and were detected by FL-1 PMT and FL-2 PMT, respectively using log amplification. NK and NKT cells were identified by staining with NK1.1/CD3⁽⁻⁾ and NK1.1/CD3⁽⁺⁾ monoclonal antibodies, respectively. For each assay, unstained cells, and anti-mouse-IgG negative controls were used. The results were analyzed by the FACS-equipped CellQuest® software.

Alpha smooth muscle actin (α SMA) immunoblot analysis

Immunoblot analysis of α SMA in liver extracts was performed, using anti-SMA mouse monoclonal antibody (DAKO, cat# M0851) and peroxidase-conjugated goat anti-mouse IgG (P.A.R.I.S., Compiègne, France). It was correlated to β -actin assessment of the same tested samples, as previously described (Safadi et al. 2004). A sample of 5 animals from each group was used for western blotting.

T cell proliferation

Splenocytes harvested from the treatment groups were cultured in RPMI 1640 medium in the presence or absence of OVA (10 and 100 μ g/ml) for 72 h, followed by an 18-h pulse with 1 μ Ci ^3H -thymidine (ICN, Costa Mesa, CA). Incorporated radioactivity was measured on a flatbed micro-beta-counter (Wallac, Gaithersburg, MD). As a positive control for T cell proliferative capacity, cells were stimulated with concanavalin A (Con-A) at 1 μ g/ml and proliferation was calculated as counts per minute (CPM). T-cell proliferation post-OVA stimulation was divided by that obtained from non-stimulated lymphocytes. The increased value was used to calculate the percentage of proliferation reduction post-feeding (Safadi et al. 2005). Data was presented as mean CPM $\pm$ SEM.

Anti-OVA serum antibody levels

Serum was separated from blood collected at the time of sacrifice. ELISA plates (Nalge NUNC, Naperville, IL) were coated with 100 μ l/well of 5 μ g/ml OVA in 0.01 M carbonate buffer, pH 9.5, and incubated overnight. Serum samples diluted to 1:1000, 1:5000 and 1:25,000 were prepared and incubated for one hour at room temperature. As controls, non-immunized (normal mouse sera) and OVA-immunized mouse sera were used at concentrations identical to the samples. Plates were then incubated for 1 h with 100 μ l/well horseradish peroxidase-conjugated goat anti-mouse IgG (Roche Molecular Biochemicals, Indianapolis, IN) diluted to 0.2 μ g/ml and reacted with 100 μ l of A/B substrate (Pharmingen) mixture. Colorimetric analysis was performed on an ELISA reader (Bio-Tek Instruments) at a wavelength of 630 nm according to the manufacturer instructions (Safadi et al. 2005).

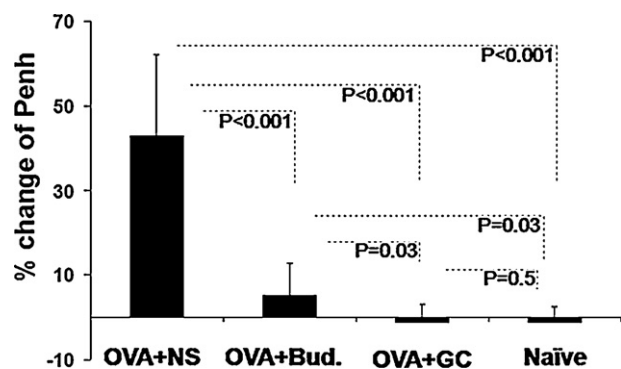


Fig. 2. Airway reactivity expressed as % change of Penh (enhanced pause). Both glucosylceramide (GC) and budesonide (Bud) treatments significantly ($p < 0.001$) decreased the % change of Penh. The change seen after treatment with GC was more pronounced than that seen with budesonide ($p = 0.03$). OVA: ovalbumin; NS: normal saline.

ALT levels as an indicator of liver injury

Serum was separated from blood collected at the time of sacrifice. Serum alanine aminotransferase (ALT) level was measured by an automatic analyzer. Determination of serum ALT was performed using an automated enzymatic assay by the Vistros Chemistry Systems 950.

Statistical analysis

Data is given as mean $\pm$ SEM unless otherwise specified. The comparison of FACS results between different animal groups, lymphocyte subsets and serum aminotransferase levels were analyzed for significance with ANOVA and Student's *t*-test. An analysis of variance with Dunnett's test for multiple comparisons was used to determine the statistical significance of differences from the values obtained for non-sensitized animals after OVA-challenges. A two-way analysis of variance was used with Tukey's multiple range tests to determine the statistical significance of differences among strains after OVA challenges. p -values < 0.05 were considered statistically significant.

Results

Effect of GC on OVA induced asthma model

Treatment with GC, following asthma induction, significantly decreased the % change of Penh (enhanced pause)

Airway reactivity was measured using a whole-body plethysmograph and expressed as % change of Penh $\pm$ STD (Fig. 2). Penh significantly increased from $-1.1 \pm 3.7\%$ change from baseline in the naïve state, to $43 \pm 19.1\%$ ($p < 0.001$) following asthma induction and treatment with normal saline. Treatment with GC, following asthma induction, significantly decreased the % change of Penh to $-1.2 \pm 4.4\%$ ($p < 0.001$). Likewise, treatment with budesonide decreased the % change of Penh to $5.3 \pm 7.5\%$ ($p < 0.001$). GC treatment showed better improvement as compared to budesonide treatment ($p = 0.03$). Budesonide did not eliminate completely asthma induction when compared to naïves ($p = 0.03$). GC, on the other hand, suppressed the induction of asthma in such a way that the Penh score was similar to that seen in naïve animals ($p = 0.5$).

Animals treated with GC showed no obvious differences in behavior or weight as compared to the other study groups. Furthermore, treating animals with GC prior to the last dose of IP OVA (data not presented) produced a similar improvement in the Penh value.

GC and budesonide attenuated the lung inflammation

OVA induced lung injury was associated with abundant inflammatory infiltrations, as evident in lung sections stained with H&E (Fig. 3A, arrows). Prominent peri-bronchial and peri-vascular infiltrations were noted after OVA induced lung injury (Fig. 3A) as compared to the naïve group (Fig. 3D). This inflammatory infiltration was clearly attenuated by budesonide treatment as evident in (Fig. 3B) and to a further extent after treatment with GC (Fig. 2C).

To try to quantify the degree of inflammation, we devised a simple scoring system as explained in *methods* and presented as mean $\pm$ SEM. Inflammatory scoring, was significantly reduced in the OVA challenged group treated with GC as compared to the untreated OVA group (1.17 ± 0.2 and 2.9 ± 0.3 , respectively, $p = 0.002$). Therefore, GC achieved a similar inflammatory alleviation as seen in the budesonide group (1.2 ± 0.2).

The effect of GC in modulating ovalbumin induced lung injury was not mediated through a humoral response

Semi-quantitative ELISA was performed to study the serum level of anti-OVA antibodies at different dilutions. Serum from each animal ($n = 10$) was studied at several dilution and an average was calculated for the entire study group at that dilution. Arbitrary primary ELISA optical density (OD) readings were used to assess anti-OVA titers in a semi-quantitative manner (Fig. 4). Increased serum anti-OVA titers as compared to the naïve animals, expressed as increased OD readings, express successful induction of asthma. The differences between the study groups were better demonstrated at a serum dilution of 1:5000. Serum anti-OVA titers at a dilution of 1:5000 were 0.1 ± 0.01 OD in the presence of medium only (no serum) and 0.16 ± 0.02 in the presence of serum from naïve animals. Anti-OVA serum titers rose significantly to 0.33 ± 0.015 in the untreated asthma group and to 0.3 ± 0.02 in the GC treated asthma group ($p < 0.0001$). In contrast to GC, a significant fall in serum anti-OVA levels to 0.2 ± 0.04 OD was observed in the budesonide treatment group as compared to the untreated group ($p = 0.01$). These changes suggest that the improvements seen in pulmonary function with GC and budesonide were mediated by different mechanisms. The effect of GC in modulating the asthmatic response in this model was probably not mediated through the humoral response, as opposed to budesonide.

GC suppressed T cell proliferation

In order to study the effect of GC on the cellular response, T-cells proliferation was measured in the different treatment groups before and after OVA stimulation (Fig. 5). Con-A was used as a positive control to confirm that splenocytes from all groups have proliferative potential. Cells were cultured in either medium alone, medium with Con-A or medium with OVA. Neither GC nor budesonide were added to the culture media (Safadi et al. 2005). Spleen T-cells from all study groups ($N = 10$ per group) showed low and similar proliferative profiles when cultured in medium only (Fig. 5, right panel). T cell proliferation readings when cultured in medium were 1936 ± 160 cpm in naïves, 1933 ± 261 in the untreated asthma group, 2359 ± 218 in the budesonide treatment study group, and 2472 ± 146 in GC treatment study group.

Non-specific stimulation with Con-A also increased the proliferation of cells from all groups without significant differences in-between groups (Fig. 5, middle panel): 3998 ± 923 , 4907 ± 708 , 6440 ± 1273 and 5800 ± 1305 cpm, for the naïve, untreated OVA, budesonide and GC groups, respectively. Specific stimulation with OVA, however, caused increased proliferation in all cells originally coming from the OVA induced asthmatic groups but not from the naïve group (Fig. 5, left panel). T cell proliferation following OVA stimulation was 2094 ± 285 cpm in naïves, which significantly increased to 5842 ± 253 cpm in the untreated asthma group ($p < 0.0001$), 6102 ± 659 in the budesonide treat-

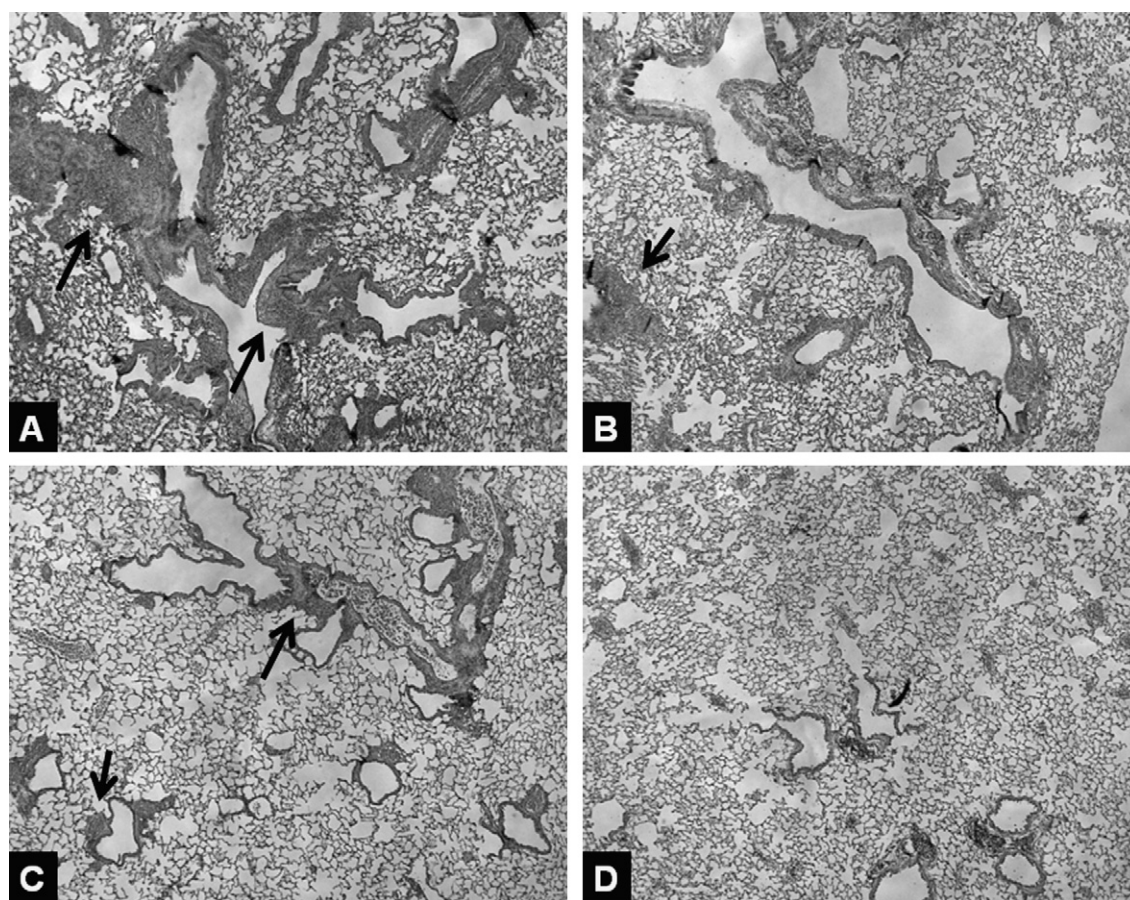


Fig. 3. H&E stain of lung tissue (10 $\times$). Prominent perivascular and peribronchial infiltration are seen after OVA induced lung injury (A). These infiltrations were attenuated in the budesonide (B) and in the glucosylceramide treatment (C) groups almost comparable to the naive group (D).

ment group ($p=0.0001$) and 4046 ± 579 cpm in the GC treatment group ($p=0.006$), respectively. Despite the aforementioned, GC treatment was associated with significantly less OVA mediated T cell proliferation when compared to the budesonide treatment group ($p=0.02$) and to the untreated OVA induced asthma group ($p=0.009$). Although budesonide caused improvement in

bronchial hyper-reactivity, it did not affect the high proliferation of T-cells.

OVA challenge induced changes in lymphocyte cell distribution in the spleen and liver

Flow cytometry analysis (FACS) was performed on splenocytes and lymphocytes extracted from the livers and lungs of the different study groups. We extended our analysis to include the liver and the spleen, due to the known liver involvement in inherited deficiency of glucocerebrosidases (Elstein et al. 2002). The results of the different lymphocyte subsets are presented in Fig. 6 as a % of all CD45+ cells.

Lung NKT cells (NK+CD3+) significantly decreased following asthma induction from $15.4 \pm 4.7\%$ in the untreated naïve group to $7.9 \pm 3.8\%$ in the untreated OVA-induced asthma group ($p=0.01$). This decrease was also seen when comparing the naïve animals to both treatment groups: $5 \pm 2.5\%$ in the budesonide treatment group ($p<0.001$) and to $6.9 \pm 2\%$ in the GC treatment mice ($p<0.001$), respectively. There was no significant difference in lung NKT cells among the 3 OVA induced groups (Fig. 6A). Similarly to what was seen in the lungs, NKT cells extracted from the spleen significantly decreased from $4.6 \pm 1.5\%$ in naïve animals to $2.9 \pm 0.6\%$ ($p=0.01$) in the untreated OVA group, to $3 \pm 0.6\%$ ($p=0.01$) in the budesonide treatment group and $2.2 \pm 0.9\%$ ($p=0.02$) in the GC treatment group (Fig. 6B). Again, no significant differences were seen among the different OVA induced groups.

Liver NKT cells, on the other hand, showed no change among the different groups, except for a significant increase in the GC treatment group (Fig. 6C). Liver NKT cells were $7 \pm 3.6\%$ in naïve study group and remained unchanged in the untreated OVA ($5.6 \pm 4.7\%$)

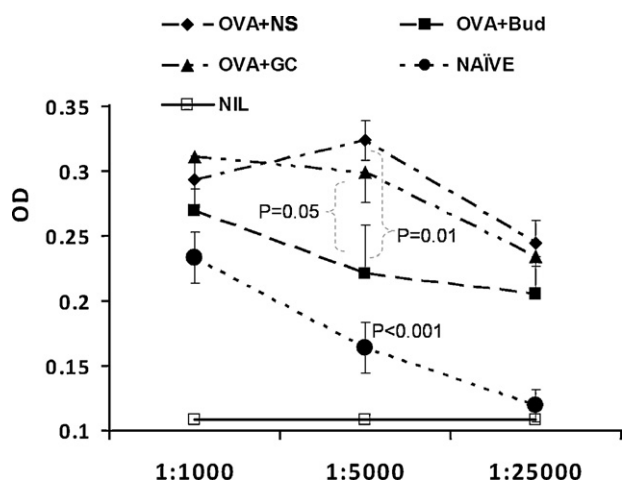


Fig. 4. Semi-quantitative ELISA analysis of anti-OVA antibodies. Serum anti-ovalbumin (OVA) titers were measured by ELISA using arbitrary OD units and were presented as means $\pm$ SD ($n=10$). A different dose response in serum anti-OVA levels was seen after budesonide (OVA+Bud) treatment as compared to the untreated group ($p=0.01$). The beneficial effect of glucosylceramide (OVA+GC) did not affect the production of anti-OVA antibodies when compared to the untreated OVA group.

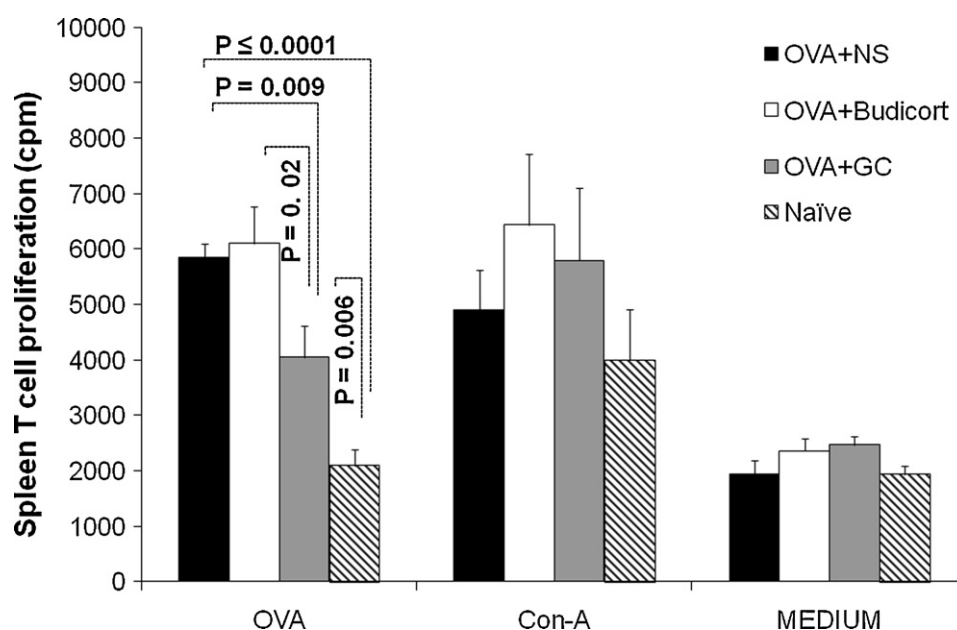


Fig. 5. T-cell proliferation post-OVA stimulation. Specific stimulation with ovalbumin (OVA), caused increased proliferation in all cells originally coming from OVA treated donors but not from naïve group (left panel). Glucosylceramide (GC) treatment caused a significant decrease in T cell proliferation compared to budesonide (Bud) ($p = 0.02$ and $p = 0.009$ for GC vs. budesonide treatment and GC vs. OVA + NS, respectively).

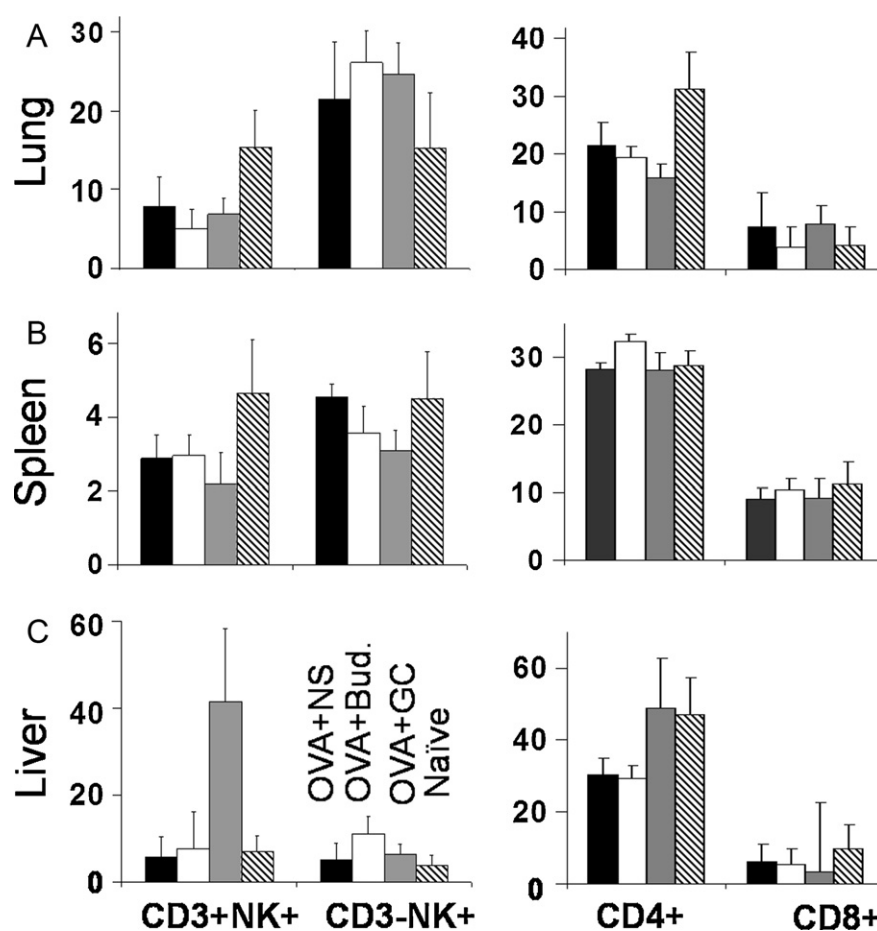


Fig. 6. Flow cytometry analysis of intrahepatic, splenic and lung lymphocytes. Lymphocytes were isolated from each organ and then stained as described in "Methods". Results of lymphocyte subsets are presented as % of all CD45+ cells. Lung NKT cells (NK+CD3+) significantly decreased following asthma induction and were similar in all 3 ovalbumin (OVA) groups. Similar changes were seen in the spleen. Liver NKT cells were similar in all groups except for a significant increase in the glucosylceramide (GC) treatment group. GC treatment was also associated with increased liver CD4+ content as compared to untreated and budesonide (Bud) treated animals with asthma ($p = 0.02$).

and budesonide treatment groups ($7.7 \pm 8.6\%$). GC treatment however, caused a significant increase in liver NKT cell numbers to $41.516.7\%$ ($p < 0.001$).

When studying lung CD4+ cells distribution (Fig. 6A), we noticed a significant decrease in CD4+ cell numbers following OVA induction ($21.5 \pm 5.8\%$) as compared to the naïve group ($31.2 \pm 3.4\%$), $p < 0.001$. No significant changes were seen between the treated and untreated OVA induced groups ($21.5 \pm 5.8\%$ in untreated asthma, $19.3 \pm 3.4\%$ in the budesonide treatment group and $15.9 \pm 3.2\%$ in GC treatment group). Changes in CD4+ cell distribution were perceived in the spleen (Fig. 6B) and liver (Fig. 6C) as well. Spleen CD4+ cells increased significantly only after budesonide treatment ($32.3 \pm 1.6\%$) as compared to the untreated OVA challenged group ($28.3 \pm 1.7\%$), $p < 0.001$. There was no significant changes in-between the rest of the study groups.

Following OVA induction, liver CD4+ cells significantly decreased from $47 \pm 6.9\%$ seen in the naïve group to $30.4 \pm 5\%$ in the untreated OVA challenged group ($p = 0.001$) and to $29.3 \pm 4.4\%$ in the budesonide treatment group. GC treatment, on the other hand, significantly ($p = 0.02$) increased liver CD4+ content to $49 \pm 19.2\%$ (similar to naïves) as compared to the untreated OVA challenged and budesonide treatment groups. No remarkable CD8 alterations were seen in any of the studied organs (lungs, liver or spleen lymphocytes).

OVA challenge induced changes in NK cell distribution

Changes were also perceived when looking at other immune cell subsets. Lung NK cells (NK+CD3–) increased following OVA induction from $15.3 \pm 7\%$ in naïves to $21.5 \pm 7.3\%$ ($p = 0.08$) in the untreated OVA challenged study group (Fig. 6A). Both treatments caused a significant rise in lung NK cells compared to the naïve animals ($26.2 \pm 4\%$ in the budesonide group ($p < 0.001$) and $24.7 \pm 3.9\%$ in the GC group ($p = 0.01$), respectively). Spleen NK cells, on the other hand, remained unchanged in the untreated OVA challenged group as compared to the naïve group ($4.6 \pm 0.3\%$ and $4.5 \pm 1.3\%$, respectively) (Fig. 6B) but significantly decreased following either treatment ($3.6 \pm 0.7\%$, ($p = 0.01$) in the budesonide and $3.0 \pm 0.6\%$ ($p = 0.001$), in the GC treatment groups, respectively). Liver NK cells showed a significant increase only following budesonide treatment ($11 \pm 4.1\%$, $p = 0.01$). Liver NK cell numbers were similar among the other study groups with no significant changes seen when comparing either the untreated OVA challenged ($5.0 \pm 3.8\%$) or the GC ($6.3 \pm 2.4\%$) study groups to the naïve group ($3.7 \pm 2.4\%$) (Fig. 6C).

In summary, following asthma induction, a significant decrease in lung CD4+ T cells to similar levels was seen in the OVA/NS, OVA/GC and OVA/bud groups. GC treatment however, significantly increased liver CD4+ and NKT cells as compared to the decreased values seen in the untreated and budesonide treated animals. The overall changes seen suggested a possible involvement of the liver in the modulation of lung injury. To further evaluate the latter, we induced lung injury using ovalbumin in animals with established liver disease (Fig. 1B) to increase liver adhesion of lymphocytes (Muhanna et al. 2007, 2008).

OVA challenge can cause mild liver injury and increase in ALT levels

Serum ALT levels increased from 27.2 ± 7.6 u/ml in naïve animals to 77.6 ± 16.1 u/ml ($p = 0.01$) in the untreated OVA induced asthma group, and to 61.9 ± 26.3 u/ml in the budesonide treatment asthma group ($p = 0.04$) while remained unchanged (32.3 ± 10.1 u/ml) following GC treatment. This pattern suggests liver involvement in the OVA induced lung injury model, which was blocked by GC.

OVA induced lung injury was also performed in animals with established liver disease. Serum ALT levels increased from 23 ± 2.4 u/ml in naïve animals to 81.8 ± 19.4 u/ml after OVA induced lung injury ($p = 0.0002$), to 236 ± 73 u/ml in animals with only liver

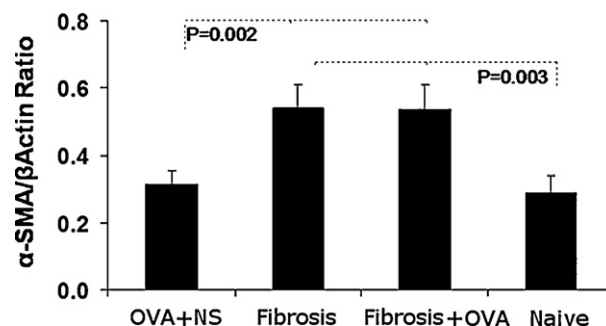


Fig. 7. Western blot densitometry. Alpha smooth muscle actin (α-SMA) expression from liver protein extracts was similar in both fibrosis groups when compared to β-actin. Calculated α-SMA/β-actin ratios are shown. OVA: ovalbumin; NS: normal saline.

fibrosis ($p = 0.0002$) and to 283.1 ± 94.6 u/ml in the liver fibrosis and lung injury group ($p = 0.0003$). ALT serum levels in both groups with liver fibrosis were significantly higher ($p = 0.01$) than that seen in the untreated OVA group without fibrosis. No significant difference was seen in-between both liver fibrosis groups.

Ovalbumin induced lung injury was not associated with increased liver fibrosis

Fibrosis severity was also determined by western blot assessment of α-SMA levels from liver protein extracts. α-SMA expression was similarly prominent in both fibrotic groups (with and without OVA induction) when compared to β-actin. Measurements of band densities (Fig. 7) and calculated α-SMA/β-actin ratio confirmed this pattern. α-SMA/β-actin ratio was 0.29 ± 0.05 in the naïve group with similar values in the OVA induced asthma group 0.32 ± 0.04 . This ratio was significantly increased to 0.54 ± 0.07 in the liver fibrosis group and 0.54 ± 0.08 in the liver fibrosis with OVA induction group ($p = 0.002$ compared to naïves and $p = 0.003$ compared to OVA induced asthma).

Furthermore, despite the mildly increased ALT levels, Sirius Red stains of liver sections revealed no collagen deposition in either the naïve group or the OVA induced lung injury groups.

Liver injury, associated with increased lymphocyte adhesion, blunted ovalbumin induced airway reactivity

Airway reactivity was measured in all animal groups using a whole-body plethysmograph and was presented as % change of Penh. Airway reactivity significantly increased ($p = 0.0005$) from $-1.4 \pm 3.2\%$ change of Penh in naïves to $16.9 \pm 8.3\%$ change of Penh in OVA induced asthma (without fibrosis). Interestingly, in the liver fibrosis group that is lacking OVA induction, there was a significant increase in airway reactivity to $5 \pm 5\%$ change of Penh ($p = 0.02$) but still lower than that seen in the OVA induction without fibrosis group. Moreover, in the liver fibrosis group that underwent OVA induction, airway reactivity although increased significantly to $7 \pm 2.7\%$ change of Penh compared to the naïve group ($p = 0.001$), however it still was significantly lower than the Penh score reached in the asthma group without liver fibrosis ($p = 0.02$). These changes also correlated with the severity of airway lymphocyte infiltration as was seen in the H&E lung sections. Compared to normal lung, OVA induction without liver fibrosis was associated with massive inflammatory infiltrates and thickening of airway walls. In contrast, airway inflammation was barely seen in the CCl₄ fibrosis groups either with or without OVA induction (Fig. 8). Assessment of serum anti-OVA antibodies confirmed successful sensitization to ovalbumin. Therefore, failed OVA induction, cannot account for the appreciated differences between the different groups. At a serum dilution of 1:5000, anti-OVA titers were 0.1 ± 0.01 OD in the presence of medium only (no serum) and 0.5 ± 0.07 in serum from naïve

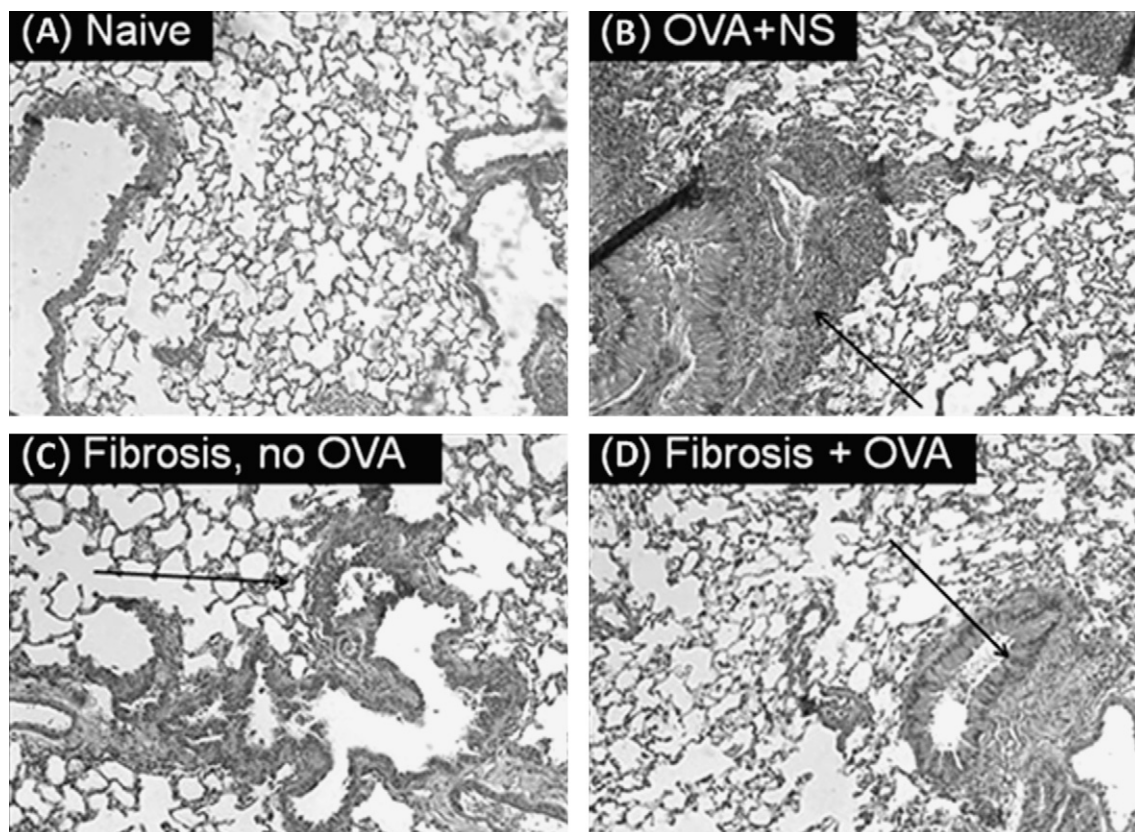


Fig. 8. H&E stain of lung sections. Compared to normal lung histology (A), OVA induction without liver fibrosis (B) contributed to massive inflammatory infiltrates and thickening of airway walls (see arrows). Airway inflammation was barely seen in CCl4 fibrosis groups either without (C) or with OVA induction (D).

animals. Following OVA induction, serum anti-OVA titers rose significantly to 1.3 ± 0.068 and to 1.38 ± 0.04 , respectively, in both lung injury groups, with and without liver fibrosis ($p < 0.0001$).

Discussion

The current study demonstrates that GC, a glycolipid known to manipulate NKT cells (Stanic et al. 2003), affected lymphocyte subsets and alleviated bronchial reactivity in a murine model mimicking asthma. Cellular inflammation seen in lung sections was significantly ameliorated after GC treatment, to a similar extent to the improvement seen after budesonide treatment. Unlike budesonide, GC therapy caused inhibition of *in vitro* OVA mediated T-cell proliferation. This latter observation suggests that GC has a direct effect on lymphocytes, which at least in part explains the alleviation of asthma associated inflammation. However, the beneficial effect on OVA induced bronchial reactivity was not solely caused by direct inhibition of T cell proliferation but also by significant changes in lymphocyte distribution.

Airflow obstruction in asthma is associated with airway inflammation that is enriched with eosinophils and CD4⁺ T lymphocytes (Busse and Lemanske 2001). The role of CD4⁺ T cells in the pathogenesis of asthma is well documented (Bosse and Rola-Pleszczynski 2007), though little is known about the role of other lymphocytic subtypes. CD8⁺ T cells for instance, have been suggested to play a more prominent role in allergic asthma in children (Machura et al. 2008). Furthermore, to the best of our knowledge, the effect other organs might have on the severity and modulation of lung injury has not been documented before. In the current study, we have seen major changes in lymphocyte subsets not only within the lungs, but also in the liver and to a lesser extent in the spleen. The location and timing of antigen-specific T cell division might have a signifi-

cant impact on airway inflammation (Hutchison et al. 2009). Extra pulmonary immune organs can also be a target for migration and allocation of antigen-specific T cell division at different phases of the disease.

Following ovalbumin induced lung injury; a significant decrease in lung CD4⁺ T cells was seen among all 3 OVA induced groups to a similar level. GC treatment significantly increased liver CD4⁺ and NKT cells content as compared to the decreased values seen in untreated and budesonide treated animals. These lymphocytes might have been either trapped in the liver or migrated to it as a result of exposure to intranasal GC.

NKT cells also play an essential pathogenic role in asthma (Akbari et al. 2006). Activation of NKT cells can either induce or prevent allergen induced airway hyper-reactivity (Hachem et al. 2005; Stock et al. 2004; Kim et al. 2004). The current study shows a significant decrease in NKT numbers in the lungs and spleen following asthma induction. Liver NKT cells on the other hand, increased significantly after intranasal treatment with GC. Again, exposure to intranasal GC might have induced NKT cell shift to the liver from the lung and possibly other immune organs. Lung NK cells increased following OVA induction, but were not affected by either treatment. The CD4⁺ pattern was similar to that seen with NKT cells, except for less involvement of the spleen. The overall pattern of changes seen, suggest that lung injury as well as treatment directed against this injury, is not necessarily confined to the lung, but might involve other distant organ that can modulate the severity of the disease. GCs effect on the innate immune response seems to be different than the anti-humoral effect of budesonide. Anti-OVA antibodies further confirm that budesonide alleviates OVA induced asthma via an effect on the humoral arm of the immune system, compared to GC that had little effect on antibody production.

Of interest are the changes seen in the intrahepatic distribution of CD4+ and NKT+ cells in response to different treatments. This latter observation suggests that the liver might have a potential role in the immune regulation of lung injury. This is further strengthened by the relative hepatotoxic effect seen after inducing asthma using intranasal OVA instillation. The increase in ALT levels following OVA induction can alternatively be due to an inherent hepatotoxic effect of the OVA itself. However this latter observation has not been reported in other animal model using ovalbumin. Furthermore, this possible OVA induced hepatotoxicity is less relevant in our model as it affected all treated and non-treated asthma groups.

To evaluate the possible role of the liver in OVA-induced lung injury, we induced hepatic fibrosis using CCl₄ in animals prior to asthma induction. CCl₄ induced hepatic fibrosis is known to increase hepatic lymphocyte recruitment and adhesion (Muhanna et al. 2007, 2008). CCl₄ induced fibrosis resulted in less airway reactivity as well as less lung lymphocytic infiltration compared to animals that underwent asthma induction but had no previous liver disease. This latter observation can be explained by lymphocytes accumulating in the fibrotic liver, thus made less available to cause severe disease in the lungs.

CCl₄ induced liver fibrosis may exert multiple effects including increased levels of profibrotic cytokines such as transforming growth factor beta1 (Galicia-Moreno et al. 2009). However, the decrease in asthma reactivity observed in our model is probably not mediated by this increase in cytokines since many have been linked to increased asthma activity (Bosse et al. 2010; Camoretti-Mercado and Solway 2005; Boxall et al. 2006). Moreover, this decreased airway reactivity is less likely to be caused by failed asthma induction, since animals with liver disease that also underwent OVA induction had high serum anti-OVA antibody levels similar to the level seen in the control asthma group.

Over the last decade, an increasing number of reports have suggested that chronic HCV infection is also associated with both direct and indirect effects on pulmonary tissue. These data are limited by sample size but provide intriguing information that should urge investigators to pursue further trials (Moorman et al. 2005). The only prospective study (Kanazawa et al. 2003a) to address the association between HCV and COPD was carried out by investigators from Japan, who randomly enrolled 30 HCV-positive and 29 HCV-negative patients with COPD. The authors suggested that the airway disease may be related to underlying chronic inflammation with possible effects mediated by HCV-specific T lymphocytes. Several studies have also documented an accelerated decline in lung function in asthmatic patients with chronic HCV infection. It was associated with impaired responses to inhaled β_2 -adrenoceptor agonists and corticosteroids and increased responses to inhaled anticholinergic agents (Kanazawa et al. 2003b). Moreover, declines in pulmonary function over time were reversible with successful control of viral replication by IFN treatment (Kanazawa and Yoshikawa 2004). The authors suggested that chronic HCV infection might induce CD8+ T lymphocytes that cause asthma with a COPD-like inflammation. The few studies analyzing BAL fluid from groups with HCV infection have found varying results, with significant increases in neutrophils alone (Idilman et al. 2002), lymphocytes and neutrophils (Yamaguchi et al. 1997), or lymphocytes and eosinophils (Kubo et al. 1996). Increases in the numbers of CD2+, CD3+, CD4+, and human leukocyte antigen-DR+ T lymphocytes were also noted; these were, however, small studies that were done in asymptomatic patients with no clinical or radiologic evidence of respiratory disease.

In summary, we suggest that GC has a potential role in alleviating lung injury. We have seen changes in the distribution of NKT and CD4+ cells in the lungs after treatment with GC. These changes paralleled changes in the intra-hepatic cell distribution. We have

also shown that liver fibrosis prevented the induction of asthma. This latter observation is probably due to increased liver trapping of lymphocytes, which are thus made less available to the lungs.

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