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Montrouge, le 08/09/2008

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CD40 expression on antigen presenting cells and correlation with disease severity in atopic dermatitis

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CD40 expression on antigen presenting cells and correlation with disease severity in atopic dermatitis

Atopic dermatitis (AD) is a chronic inflammatory skin disease associated with cutaneous hyperreactivity to environmental stimuli, resulting in increased infiltration of inflammatory cells, IgE production and enhanced expression of various co-stimulatory molecules, Th2 cytokines and chemokines. Antigen presenting cells (APCs) are critical for AD disease pathogenesis and interaction between APCs and inflammatory T cells represents an important signal required for induction and maintenance of the inflammatory process. CD40 was shown to be upregulated on inflammatory cells in patients with atopic dermatitis; however the identity of these cells and their correlation with disease severity remained unknown. To address these questions, we determined CD40 expression in skin lesions and on peripheral blood cells from AD patients and identified a positive correlation between the numbers of CD40 positive cells and disease severity. Furthermore, we identified the nature of CD40 positive cells in skin lesions to include CD1a⁺ and CD11b⁺ APCs. Finally, a correlation between serum PARC and IgE levels and the numbers of CD40⁺ cells in AD skin lesions was found. Combined, these findings demonstrate that CD40 is upregulated on APCs, cell types previously shown to contribute to AD disease pathology, and suggest that therapeutic strategies targeting CD40 positive cells may provide benefit to AD patients.

Key words: CD40, atopic dermatitis, APCs, PARC, CD1a⁺

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CD40 is a member of the tumor necrosis factor (TNF) receptor superfamily which is expressed as a type I transmembrane protein on different types of hematopoietic cells. CD40 is upregulated on activated APCs, including B cells, dendritic cells, monocytes and macrophages [1]. The ligand for CD40, CD40L, also known as CD154, is preferentially expressed on activated T cells and platelets. The CD40-CD40L signaling pathway has important biological functions in the regulation of immune responses, including the stimulation of T and B-cells, monocytes, dendritic cells; T-dependent antibody production, isotype class-switching and upregulation of co-stimulatory molecules [2]. In atopic dermatitis, CD40/CD40L interactions play key roles in the migration of inflammatory infiltrates to inflamed tissues by inducing cell adhesion molecules such as $\alpha 4\beta 1$ integrin, cutaneous lymphocyte-associated antigen (CLA) and multiple vascular-cell adhesion molecules [3, 4]. In addition, CD40 ligation is associated with the production of antigen specific IgE antibodies following T-B cell interaction and the pathogenesis of atopic dermatitis [1, 5, 6]. AD is a chronic inflammatory skin disorder characterized by severe itching, age-dependent skin manifestations, fluctuating clinical course and elevated serum IgE levels.

Atopic dermatitis is considered a chronic inflammatory skin disease where, besides an acquired immune mechanism, the innate immune response and abnormal skin barrier functions are also involved. AD is associated with cutaneous hyper-reactivity to environmental triggers that are innocuous to normal non-atopic individuals [7, 18]. Previous studies conducted with primary AD patient skin samples identified the mononuclear infiltrates as consisting mostly of lymphocytes and monocytes localized to the dermis, and to a lesser extent to the epidermis [8, 9]. Others found these infiltrates to represent predominantly CD4⁺ T lymphocytes [10]. Uno and Hanifin described an increase in intraepidermal Langerhans cells, especially in lichenified lesions [11]. Similarly, large numbers of antigen presenting cells, including dendritic cells and macrophages [12], but also leukocytes and B cells, were identified in the cellular infiltrates in the skin of AD patients [13]. When analyzed in the context of other inflammatory diseases, leukocytes in AD skin lesions were shown to express CD40 [14]. However, the nature of the CD40 positive cells in AD skin lesions remained to be determined. Here we report CD40 expression on APCs within AD skin lesions and on peripheral blood cells of AD patients. APCs are critical during T cell activation and maintenance and a role for APCs in AD development has been proposed [4, 15]. Therefore, our findings indicate that CD40 may represent a

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therapeutic target to interfere with progression of AD. Finally, we identified a novel correlation between CD40 expression with disease progression and other markers of AD disease severity, including chemokines. Combined, these findings suggest that CD40 levels may represent a novel, independent biomarker indicative of AD disease stages.

Materials and methods

Patient criteria and tissue materials

The criteria of Rajka-Langeland [16, 17] were used for the diagnosis of AD. Thirty patients with AD and 10 healthy controls were enrolled in the study after providing informed consent. The investigational protocol was approved by the Ethics Committee of the Oregon Health Sciences University. AD patients ceased all atopic dermatitis-specific therapy, such as topical steroids and topical calcineurin inhibitors as well as antihistamines, at least one week before enrollment. Blood samples and skin biopsies were obtained from all subjects, including 10 healthy, 10 mild, 10 moderate and 10 severe AD based on Rajka-Langeland baseline assessment [16, 17]. Skin biopsies were obtained from lesional skin that was representative of the overall disease severity. For example, if a patient's Rajka-Langeland score was "mild", a biopsy was obtained from only a lesion with mild disease. Biopsies were taken from lesions with an acute phenotype; chronic lichenified lesions were not biopsied. For immunohistological analysis, 5-mm punch biopsies were generally obtained from most active eczematous skin lesion. The biopsies were embedded in OCT Tissue-Tek (Sakura Finetek USA, Torrance, CA), and stored at -80°C until further processing.

Immunohistochemical staining

Frozen tissue sections were prepared and stored as described above for the immunofluorescence staining procedure. To determine the distribution and the number of CD40+ cells in lesional skin sections, single immunostainings were performed at Phenopath Laboratories. Briefly, to prevent nonspecific protein binding, antibodies were mixed with 1% BSA in PBS (Invitrogen, Carlsbad, CA) and applied overnight to frozen sections in a humid chamber at 4°C . Mouse anti-human CD40, clone 5C3 BD (Pharmin-gen, San Jose, CA) was diluted 1:500. An IgG1 isotype monoclonal antibody (Pharmin-gen, San Jose, CA) was used as negative control. Envision+ Mouse HRP Polymer Detection System was used (DakoCytomation, Carpinteria, CA). After washing the slides with PBS with 0.05% Tween, DAB+ Substrate (DakoCytomation, Carpinteria, CA) one drop Chromogen per each 1.0 mL substrate buffer was used. The procedure was followed by Mayer's Hematoxylin for counter stain and 0.5% Ammonia Water for blue staining of nuclei. After two steps incubation with Ethanol 95% and 100% and Xylene, the slides were mounted under cover slips and were read in a blinded fashion at a 200 $\times$ magnification using light microscopy (Carl Zeiss MicroIm-aging, Inc., Thornwood, NY). Pictures from three to five fields per individual lesions were taken and the average number of CD40 positive cells was recorded and divided by numbers of the fields analyzed.

Immunofluorescence staining

Frozen tissues were cut into 5- μm -thick sections and mounted on capillary gap microscope slides. The cryostat sections were air-dried for 20 min, fixed in ice-cold acetone for 10 min and stained immediately. To determine the phenotype, distribution and the numbers of APC in lesional and healthy skin, single or multicolor immunostaining was performed. The monoclonal antibodies used in the study were mouse anti-human CD11b-FITC (BD Pharmingen, San Jose, CA), mouse anti-human-CD1a-Alexa Flour 647 and goat-anti-human-IgG- Alexa Flour 568, (Molecular Probes, Eugene, OR). Mouse anti-human CD40 antibody (Biolegend, San Diego, CA) was conjugated with Alexa Flour 568, (Molecular Probes, Eugene, OR) at Seattle Ge-netics by the antibody drug conjugate group. Double and triple immunofluorescence staining was performed as fol-lows: purified antibodies were mixed with 1% bovine se-rum albumin (BSA; Sigma-Aldrich, St. Louis, MO) in PBST (Invitrogen, Carlsbad CA) and applied to frozen sections in a humidified chamber at room temperature for 1 hr. After washing with PBST, the sections were incubated with directly labeled antibodies for 1 hr at room tempera-ture. After washing, slides were incubated with DAPI (0.5 $\mu\text{g}/\text{mL}$, Molecular Probes, Eugene, OR) to visualize the nucleus for 5 min, then slides were washed and mounted in Vectashield Mounting Medium (Vector Labo-ratories, Burlingame, Calif., USA). Biopsy specimens were read in a blinded fashion at 200 $\times$ magnification by two independent investigators using a fluorescence microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY) equipped with a filter for double or triple staining.

Flow cytometry

Peripheral blood samples were processed within 24 hours following collection. Wright-stained smears were pre-pared; complete blood cell counts were performed on a Beckman-Coulter ActT Diff2 cell counter. 250,000 to 500,000 leukocytes were aliquoted into a round-bottom polystyrene tube in a volume of 50 μL , and incubated with an optimized cocktail of fluorescently-conjugated mono-clonal antibodies for 15 minutes at room temperature in dark conditions. The antibodies used included: CD 19-Pacific Blue (Dako, Carpinteria, CA, clone HD37), CD45RO-FITC, (Beckman-Coulter (BC), Hialeah, FL, clone UCHL1), CD40-PE-CY5 (BD, clone 5C3), CD4-PE-CY7 (BC, clone SFC112T4D11) and CD8-APC-CY7 (BD, clone SK1). Following antibody incubation, 1.5 mL of lyse/fix reagent (ammonium chloride/ultrapure formalde-hyde) was added directly to the tube and incubated for an additional 15 minutes at room temperature in the dark, to lyse the red blood cells. Following centrifugation, the cells were washed once in 3 mL of solution of phosphate buff-ered saline (PBS)/1% bovine serum albumin (BSA)/0.01% sodium azide. Following a second centrifugation, the cells were resuspended in approximately 250 μL of PBS/BSA/azide. Cells were analyzed on the same day they were processed, using a BD LSRII flow cytometer. Typi-cally, 100,000 to 150,000 viable leukocytes were acquired per specimen, and the flow cytometry data were analyzed using BD's FACSDiva software, version 4.1.2.

Chemokine analysis

Serum sample analysis of IgE, MDC, PARC and TARC were conducted by Pierce's SearchLight Multiplex Sample

Analysis service utilizing the SearchLight Multiplex Array System (Pierce Biotechnology, Cambridge, MA). This system is a multiplex sandwich ELISA (enzyme-linked immunosorbent assay) in a planar, plate-based array format, for the quantitative measurement of secreted proteins in serum. Briefly, each well of the microplate is pre-spotted with analyte-specific antibodies. These antibodies captured specific proteins in the standards and samples added to the plate. After unbound proteins are washed away, the biotinylated detecting antibodies are added and bind to a second site on the target proteins. After washing away excess detecting antibody, streptavidin-horseradish peroxidase (SA-HRP) was added. The HRP enzyme was subsequently allowed to react with the substrate, SuperSignal ELISA Femto Chemiluminescent Substrate to produce a luminescent signal that was detected using the SearchLight CCD Imaging and Analysis system. The amount of signal produced was proportional to the amount of each protein in the original standard or sample. Customized Array Vision software utilized a weighted four parameter curve fit to back-calculated unknowns. The results were then sent back to Seattle Genetics Inc. Graphing the results and statistical analysis was conducted using the linear regression analysis provided in the Graphpad Prism Software Package version 4.01 (Graphpad, San Diego, CA).

Results

CD40 expression on CD1a and CD11b positive cells in severe AD lesions

We analyzed normal control and skin biopsies from AD patients using immunohistochemical and hematoxylin and eosin staining procedures. Compared to skin sections taken from healthy control individuals, atopic dermatitis patients displayed increased epidermal thickness, hyperplasia, immune cell infiltrates and spongiosis (*figure 1A and B*). In

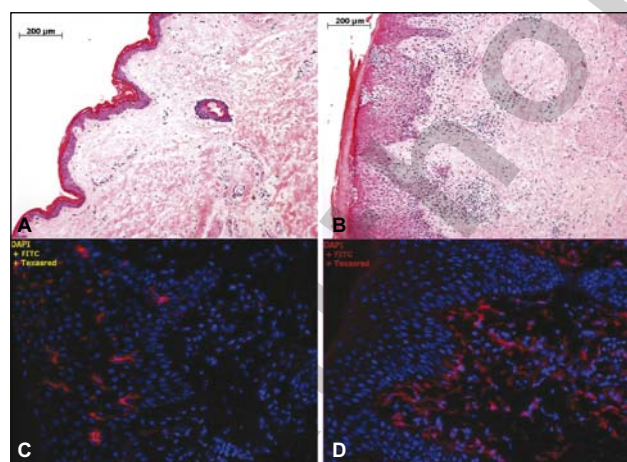


Figure 1. APCs are present and activated in AD skin lesions. **A)** H&E staining of a skin lesion derived from healthy control. **B)** H&E staining of a skin lesion derived from a severe AD lesion. Immunofluorescence staining of a severe AD lesion skin with **C)** mouse-anti human-CD1a antibody (red label), **D)** mouse-anti human-CD11b antibody (red label), DAPI staining of nuclei is indicated with blue color in all immunofluorescence slides. All pictures were taken with 200× magnification using a Zeiss fluorescence microscope.

contrast to healthy control tissues, which contained only few resident T cells (data not shown), presence of CD1a positive Langerhans (*figure 1C*) and CD11b positive macrophages (*figure 1D*) was observed in sections from severe AD patients. Confirming previous reports [11], we found Langerhans cells to localize to the epidermis region, while macrophages were mainly in the dermis region, in close association with T cells (*figure 1C and D*, respectively). To determine the identity of cells expressing CD40, we conducted immunofluorescence labeling studies with antibodies detecting CD40 and Langerhans cells (CD1a, *figure 2A*) or macrophages (CD11b, *figure 2C*). CD40 was present on both, CD1a+ Langerhans cells (*figure 2B*) and CD11b+ macrophages (*figure 2D*). As expected, CD40 staining alone and in the presence of DAPI, staining the nuclei of cells, demonstrated cytoplasmic and membranous staining of CD40 in cells within AD patient lesions (*figure 2E and F*, respectively). To investigate the activation status of APCs, we additionally stained sections with an anti-CD83, a marker of cell activation. In contrast to sections from healthy individuals, which were mostly negative for CD83, lesions from atopic dermatitis patients showed a strong positive staining in both dermis and epidermis (data not

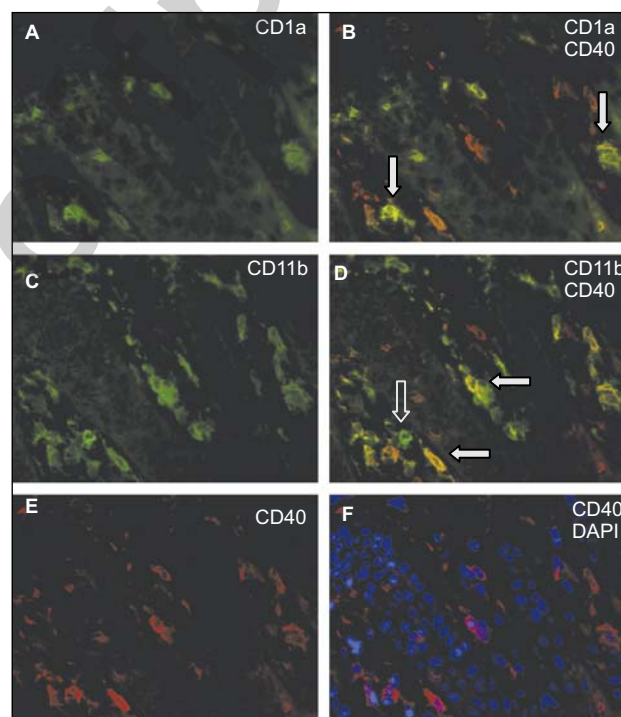


Figure 2. CD40 is expressed on APCs. Immunofluorescence staining of severe AD lesion skin with **A)** mouse-anti human-CD1a antibody (green label), **B)** the merged images, mouse-anti human-CD1a (green label), mouse anti-CD40 antibody (red label), CD1a CD40 double positive (yellow) shown with filled arrows **C)** mouse-anti human-CD11b antibody (green label), **D)** the merged images, mouse-anti human-CD11b antibody (green label), mouse anti-CD40 antibody (red label), CD11b CD40 double positive (yellow) shown with filled arrows. CD11b⁺ CD40⁻ is shown with unfilled arrows **E)** mouse-anti human-CD40 antibody (red label), **F)** the merged images, mouse-anti human-CD40 antibody (red label) DAPI staining of nuclei (blue color). All pictures were taken with 400× magnification using a Zeiss fluorescence microscope.

shown). Combined, our findings confirmed the morphological changes in AD lesions described by others and identified APCs as CD40 positive cells. These findings support the notion that APCs may play important roles in AD pathogenesis.

Correlation between CD40 expression and AD disease severity

To determine the relationship between CD40 levels and disease progression, we quantified CD40 positive cells in skin lesions from patients with varying grades of disease severity by using immuno-histochemical methods. We noticed an almost complete absence of CD40 positive cells in skin sections of healthy controls (*figure 3A*). In contrast, there was a gradual increase in CD40 positive cells in the skin of mild, moderate and severe atopic dermatitis patients (*figure 3B, C, D*). Statistical analysis using linear regression revealed that the numbers of CD40 positive cells correlated with the disease severity in AD patients ($r^2 = 0.4163$ $p = 0.0002$) (*figure 3F*). We also found that the average number of CD40 positive cells in AD skin lesions to gradually increase with disease stage and was significantly higher relative to healthy control samples

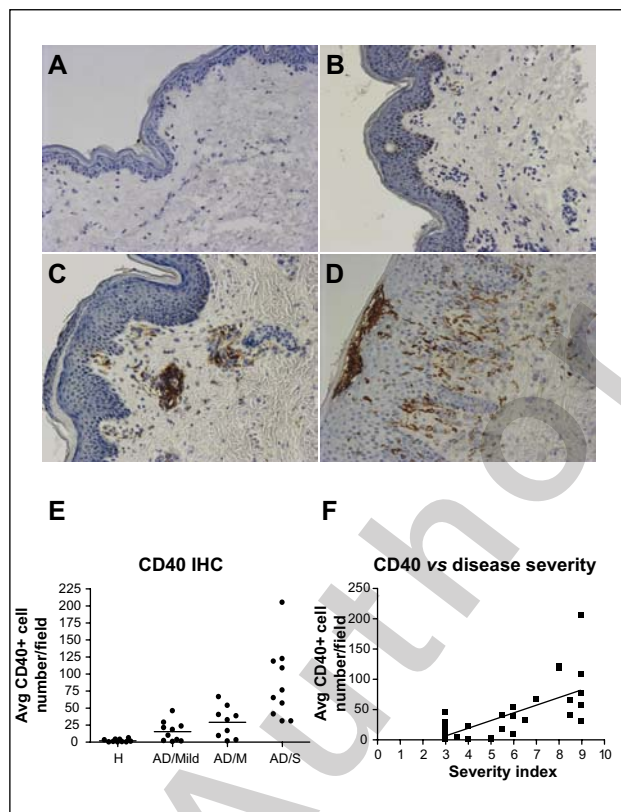


Figure 3. CD40 expression is present and increases with disease severity. Immunohistochemical analysis of CD40 expression of skin lesion derived from **A**) healthy control, **B**) patient with mild disease activity, **C**) patient with moderate disease activity, **D**) Patient with severe disease activity. **E**) Average CD40 positive cell number n for healthy (H) =10, for patients with mild disease activity (AD/Mild) =10, moderate disease activity (AD/M) =10, severe disease activity (AD/S) =10. **F**) Correlation of average CD40 positive cell number per field with disease severity index.

(*figure 3E*). We detected only low levels of CD40 positive B-cells in AD skin lesions (0-3 cell per field), which represent only a minor fraction of the total number of CD40 positive cells (15 to > 150 cells per field, *figure 3E*). These results support the notion that the relative amounts of CD40 positive cells in AD tissues may have utility as an independent marker to determine disease severity.

The numbers of circulating CD40⁺ B cells correlate with disease severity

In addition to determining the levels of CD40 expression on APCs in skin lesions, we investigated CD40 expression on circulating monocytes and B cells in AD patient samples. We found that CD40 expression on monocytes was minimal and no differences between controls and AD samples were detected (data not shown). When analyzing the relationship between the percentage of circulating CD40 positive cells and disease severity, we found a moderate but statistically significant correlation between the two datasets ($r^2 = 0.3420$ $p = 0.0007$) (*figure 4A*). Since B cells play important roles in IgE secretion, we examined the serum IgE levels in AD patients. In agreement with previous findings [19, 20] we found a statistically significant correlation between disease severity and IgE serum concentrations ($r^2 = 0.3506$ $p = 0.0006$) (*figure 4B*). Finally, there was a significant correlation between CD40+ infiltrating cells in lesional skin and the serum IgE levels ($r^2 = 0.5307$ $p < 0.0001$, *figure 4C*). The differences in the cell types expressing CD40 in the peripheral blood vs. skin lesions can be explained by the fact that Langerhans cells and macrophages represent tissue-specific or differentiated cell types, derived from DC and monocytes, respectively. Combined, these findings demonstrate that CD40 expression on leukocytes in skin lesions and circulating B cells of AD patients correlates with disease severity and serum IgE levels.

The number of CD40+ cells in AD skin lesions correlates with serum PARC levels and other markers of AD disease stage

Serum MDC, TARC and PARC levels were shown to be increased in AD patients [5, 6, 21-26], however, a correlation between these chemokines and AD disease stage has not been reported. To address this question, we determined the serum levels of these chemokines and found statistically significant correlations between the serum levels of MDC and PARC and disease severity ($r^2 = 0.3699$ $p = 0.0004$), ($r^2 = 0.4505$ $p < 0.0001$), respectively (*figure 5A and B*). MDC and TARC are being produced by a variety of hematopoietic lineages, including B-cells, following co-stimulation of CD40 and IL-4 [6]. Therefore we examined whether the levels of these chemokines correlate with the numbers of CD40+ circulating B cells in AD patients. Our analysis revealed an absence of correlations between % CD40 positive B cells and serum MDC, TARC and PARC levels (data not shown).

Discussion

In this report, we demonstrate that CD40 positive cells in skin lesions of AD patients represent APCs, including CD1b+dendritic cells, CD11b+ macrophages. In addition,

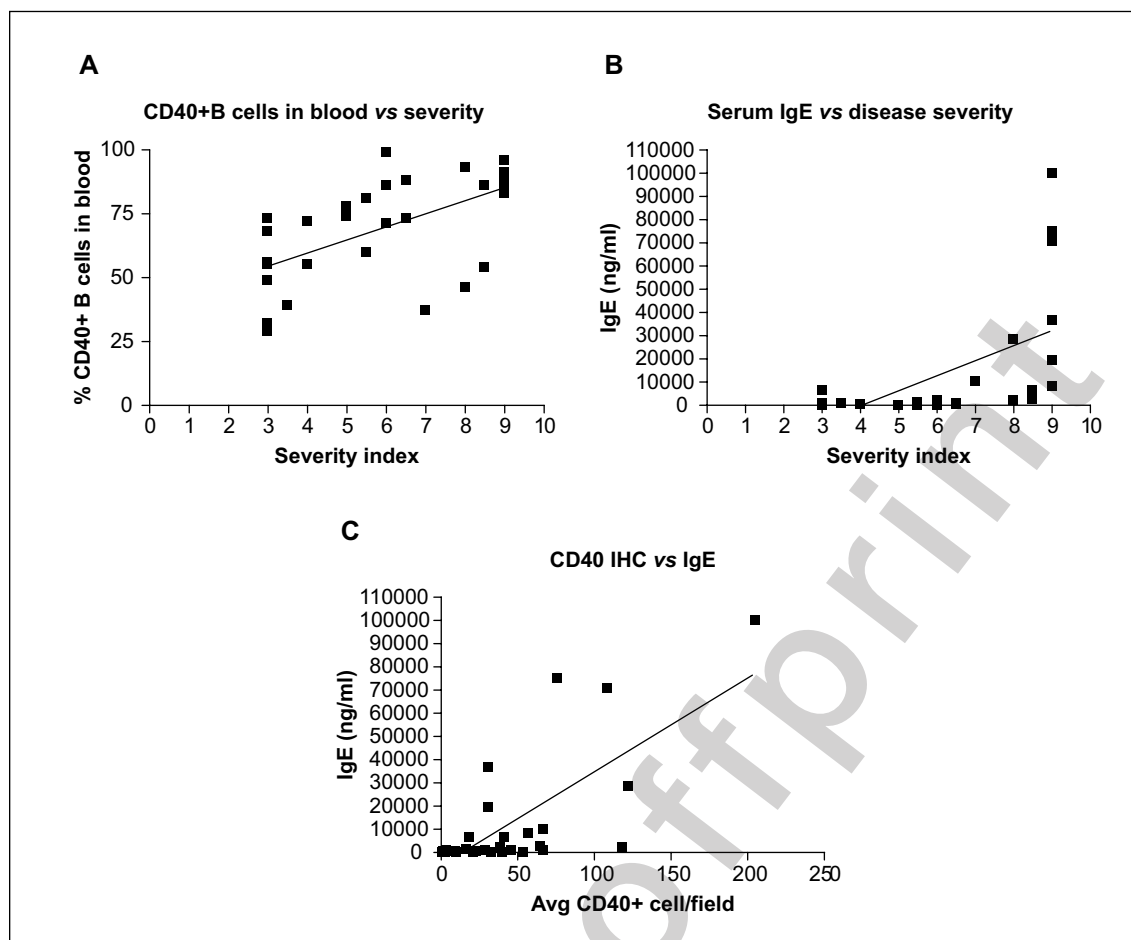


Figure 4. Circulating CD40 positive B cells increases with the disease severity and serum IgE levels. **A)** Correlation of percent CD40 positive B cells in circulation with disease severity index. Patients with mild disease activity (AD/Mild) =10, moderate disease activity (AD/M) =10 and severe disease activity (AD/S) =10 were enumerated via FACS analysis. **B)** Correlation of serum IgE levels with disease severity index. Patients with mild disease activity (AD/Mild) =10, moderate disease activity (AD/M) =10 and severe disease activity (AD/S) =10 were analyzed for IgE levels via ELISA SearchLight Multiplex Array System. **C)** Correlation of serum IgE levels with percent CD40 positive B cells in circulation of patients with mild, moderate and severe disease activity. **D.** Correlation of serum IgE levels with percent CD40 positive cells in skin lesions of patients with mild, moderate and severe disease activity.

we found significant correlations between AD disease severity and CD40 positive cells in skin lesions and in the circulation of AD patients. Combined with previous reports demonstrating the important roles of APCs in AD disease pathogenesis, our findings suggest that CD40 may represent a therapeutic target and may have utility as diagnostic marker to determine disease stages in AD.

Previous reports have shown that a variety of inflammatory cells, including T cells and platelets, produce MDC, TARC and PARC and serum levels of all three chemokines were shown to correlate with AD disease severity [5, 6, 21-26]. It was also reported that human B cells produce TARC and MDC following co-stimulation with CD40 and IL-4 [6]. The present study demonstrates that both the number of CD40-positive B cells in the circulation and the relative amounts of CD40+ cells in skin lesions correlate with AD disease stages. While the numbers of circulatory CD40-positive B cells failed to correlate with the serum chemokine levels (PARC, TARC and MDC), CD40 expression correlated with the PARC serum level. The significance of the correlation between chemokine levels and the numbers

of CD40 expressing cells remains to be determined. Our results suggest that, beside B cells, which are involved in IgE production, other CD40-positive antigen presenting cells, including macrophages and Langerhans cells, may play important roles in the development of skin lesions after the onset of inflammation.

In humans, CD40 is expressed on APCs, such as Langerhans cells, dendritic cells, macrophages and B-cells, while the CD40 ligand (CD154) is present on activated T cells. Antigens, such as dust mite and LPS, become engulfed and are presented by Langerhans cells located within the epidermis of the skin [4]. Subsequent to antigen stimulation, Langerhans cells can trigger an inflammatory response by producing chemokines such as PARC and pro-inflammatory cytokines including TNF- α , resulting in the up-regulation of CD40 on APCs [4]. PARC is known to stimulate CD4-positive T cells to produce the type 2 helper (Th2) cytokine IL-4 [22]. Th2 cytokines regulate a wide range of events associated with chronic allergic inflammation, typically associated with AD. Chemokines such as eotaxin, RANTES, monocyte chemoattractant protein MCP-3,

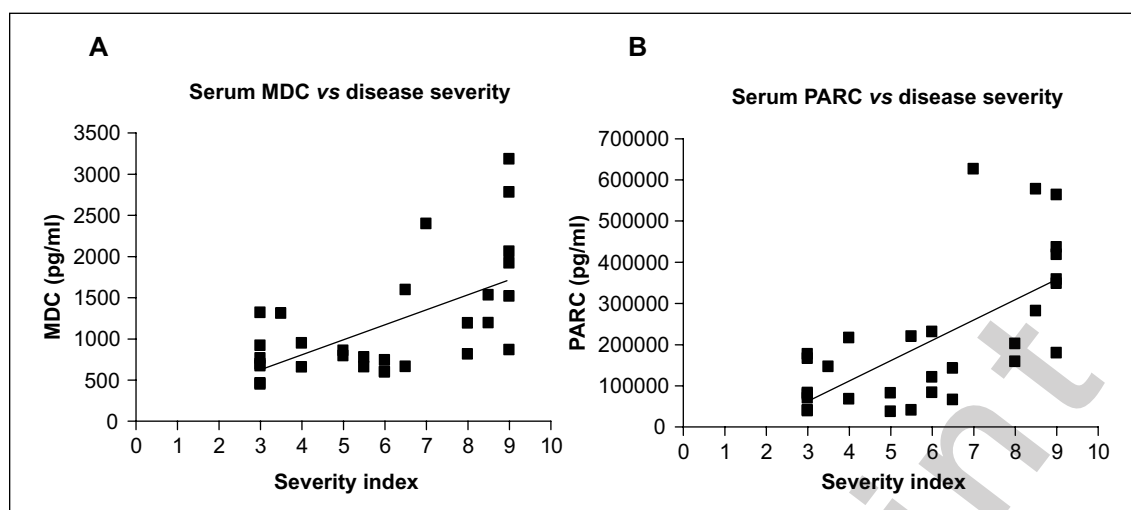


Figure 5. CD40+ cells in AD skin lesion correlates with the serum PARC levels. Correlation of chemokine levels with disease severity. Serum of patients with mild disease activity (AD/Mild) =10, moderate disease activity (AD/M) =10 and severe disease activity (AD/S) =10 were analyzed for A) MDC, B) TARC, and C) PARC levels. The chemokine levels were determined using a sandwich ELISA SearchLight Multiplex Array System. D) Correlation of PARC levels with average CD40 positive cell numbers per field taken from patients with mild disease activity (AD/Mild) =10, moderate disease activity (AD/M) =10 and severe disease activity (AD/S) =10.

MCP-4 and CC chemokines were shown to induce accumulation of inflammatory cells in AD [27-30], including pulmonary and activation-regulated chemokines (PARC/CCL18), thymus and activation-regulated chemokines (TARC/CCL17) and macrophage-derived chemokines (MDC/CCL22). These chemokines were shown to be significantly increased in the sera of patients with atopic dermatitis, and a correlation with disease severity has been described [5, 6, 21-26]. On B-cells, signaling between CD40 and CD40L results in IgE production [14]. Superantigens produced by commensal bacteria have been implied to stimulate B cell proliferation, somatic hypermutation, class switching to immunoglobulin (Ig) E and the production of allergen-specific IgE in mucosal B cells in AD [31]. IgE binding to Fc ϵ R expressed either on mast cells, eosinophils or basophils, can trigger histamine production and release of inflammatory molecules by these cells, contributing to the inflammatory cascades. IgE antibodies engender chronic inflammation and the persistent sensitization to conventional allergens of mast cells and antigen-presenting cells in atopic dermatitis. In our analysis, we identified correlations between CD40+ peripheral B-cells and disease severity, supporting the notion of an important role for B-cells in AD pathogenesis.

Based on our findings, a model can be developed wherein CD40 expressing APCs are part of a misguided immune response during chronic inflammatory conditions such as are present in AD. In this model, APCs play a central role in orchestrating an inflammatory response, which makes them candidate targets for therapeutic intervention. Given the important roles of the CD40 signaling pathways in inflammatory cells present in AD lesions, it is tempting to speculate that interference with CD40 signaling may affect disease progression and may provide benefit to AD patients. Novel, targeted therapies for moderate to severe AD are greatly needed and CD40 represents an attractive target, as its blockade may interrupt several pathogenic processes operative in AD inflammation, such as antigen presenta-

tion, chemokine secretion, and T cell activation. Co-stimulatory proteins have been targeted individually by therapeutic compounds in clinical trials and several strategies have shown promising initial responses [32]. Better understanding of the molecular and cellular events leading to AD may ultimately contribute to a better understanding and improved diagnosis and therapy of this skin disease. ■

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