

# Allergen-enhanced thrombomodulin (blood dendritic cell antigen 3, CD141) expression on dendritic cells is associated with a T<sub>H</sub>2-skewed immune response

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**Background:** Dendritic cells (DCs) are important in allergic diseases such as asthma, although little is known regarding the mechanisms by which DCs induce T<sub>H</sub>2-polarized responses in atopic individuals. It has been suggested that intrinsic properties of allergens can directly stimulate T<sub>H</sub>2 polarizing functions of DCs, but little is known of the underlying mechanisms.

**Objective:** To identify novel genes expressed by house dust mite (HDM) allergen-exposed DCs.

**Methods:** We screened for allergen-induced gene expression by microarray, and validated differentially expressed genes at the mRNA and protein levels.

**Results:** Thrombomodulin (CD141, blood dendritic cell antigen 3) expression by microarray was higher on HDM-stimulated DCs from atopic (relative to nonatopic) individuals. These findings were confirmed at both the mRNA and protein levels in an independent group. Purified thrombomodulin<sup>+</sup> DCs induced a strongly T<sub>H</sub>2-polarized cytokine response by allergen-specific T cells compared with DCs lacking thrombomodulin. *In vivo*, thrombomodulin<sup>+</sup> circulating DCs were significantly more frequent in subjects with HDM allergy and asthma, compared with control subjects. Furthermore, thrombomodulin expression in blood leukocytes was higher in children with acute asthma than at convalescence 6 weeks later.

**Conclusion:** Thrombomodulin expression on DCs may be involved in the pathogenesis of atopy and asthma. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

**Key words:** Dendritic cells, thrombomodulin, allergy

## Abbreviations used

BDCA3: Blood dendritic cell antigen 3/thrombomodulin  
DC: Dendritic cell  
FACS: Fluorescence-activated cell sorting  
HDM: House dust mite  
mDC1: Myeloid dendritic cell type 1  
mDC2: Myeloid dendritic cell type 2  
MFI: Mean fluorescence intensity  
SPT: Skin prick test

Allergic diseases are characterized by T<sub>H</sub>2-skewed immune responses to environmental allergens, leading to dysregulated production of IL-4, IL-5, and IL-13 that in turn triggers IgE production, tissue eosinophilia, and excessive mucous production. The mechanisms leading to T<sub>H</sub>2 polarization in human allergic disease are not completely understood, but are likely to involve both T-cell-dependent and dendritic cell (DC)-dependent factors.

*In vivo* models have shown that DCs play a key role both in T<sub>H</sub>2 priming and in the maintenance of allergic airway inflammation.<sup>1-5</sup> In human asthma, increased numbers of DCs are present within the human respiratory mucosa at baseline,<sup>6</sup> with rapid DC recruitment into the airway after inhalation of allergens.<sup>7,8</sup> DC function also differs in allergic disease, with investigators reporting a variety of alterations in cytokine, prostaglandin, and chemokine synthesis and costimulatory molecule expression by DCs from patients with allergy compared with healthy control subjects.<sup>9-16</sup> Although these studies have collectively provided evidence that DCs play a role in allergic disease, they have been less successful in identifying novel genes expressed by DCs that might be relevant to T<sub>H</sub>2 skewing and disease pathogenesis.

The ability of DCs to polarize T<sub>H</sub> responses is highly susceptible to exogenous factors, and a number of molecules have been identified that can convert DCs into T<sub>H</sub>2-polarizing antigen-presenting cells, including prostaglandin E<sub>2</sub><sup>17</sup> and thymic stromal lymphopoietin.<sup>18</sup> In addition, emerging evidence suggests that some allergens are not merely inert proteins, but may have important biological properties that alter DC function, independently of T cells. Molecules derived from pollen grains, including phyto-prostanes, can condition purified human DCs into T<sub>H</sub>2-polarizing antigen-presenting cells.<sup>19,20</sup> Allergens derived from the house dust mite (HDM) *Dermaphagoides pteronyssinus* induce differential changes in DCs from individuals with HDM allergy, but not

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healthy individuals,<sup>12,13</sup> and these allergen-conditioned DCs may preferentially induce a T<sub>H</sub>2-polarized immune response.<sup>21</sup>

We therefore reasoned that studying allergen-exposed DCs would provide a means to identify novel genes that are relevant to the pathogenesis of allergic disease. By using a microarray approach, we identified thrombomodulin (also known as CD141 and blood dendritic cell antigen 3 [BDCA3]) as a gene that was differentially induced in HDM-stimulated DCs from atopic relative to nonatopic subjects, and confirmed these findings by RT-PCR and flow cytometry. Fluorescence-activated cell sorting (FACS)-sorted thrombomodulin<sup>+</sup> DCs induced a more clearly T<sub>H</sub>2-polarized cytokine response by allergen-specific T cells than DCs lacking thrombomodulin that induced a mixed T<sub>H</sub>1/T<sub>H</sub>2 cytokine response. A subset of circulating myeloid DCs is also characterized by thrombomodulin expression, and we showed that these DCs were present at greater frequency in the peripheral blood of atopic subjects with asthma compared with healthy subjects. Thrombomodulin expression also increased during acute exacerbations of asthma in children. Although thrombomodulin has traditionally been associated with regulation of coagulation (see review<sup>22</sup>), our findings suggest for the first time that DC expression of thrombomodulin may have a significant role in the pathogenesis of allergic disease.

## METHODS

### Subjects

**Adult volunteers.** Adult volunteers (age range, 21–65 years) were recruited into the study and underwent blood sampling and skin prick testing to common allergens including HDM (*D pteronyssinus*), perennial ryegrass, southern grass mix, mold mix, feather (chicken, duck, and goose), cat pelt, dog hair dander, peanut, egg, and milk (Hollister-Stier Laboratories). All subjects also completed a questionnaire detailing symptoms of asthma, allergic rhinitis, and eczema. Adult subjects were classified as atopic if they were skin prick test (SPT)-positive (wheal size  $\geq 3$  mm) to 1 or more of the tested allergens, including a positive reaction to HDM (median wheal size, 9 mm; range, 3–15 mm) or nonatopic if they were SPT-negative to all allergens tested (wheal size 0 mm). Subjects SPT-positive to other allergens, but not HDM, were not studied.

**Adolescent cohort.** Teenagers who provided blood samples as part of the 14-year assessment of a longitudinal birth cohort, as described previously,<sup>23</sup> were studied. These subjects were classified as atopic if they had a positive SPT to 1 or more of the tested allergens as described, including a HDM SPT wheal size of 3 mm or larger (median wheal size, 5 mm; range, 3–8 mm), or nonatopic if they were SPT-negative to all tested allergens (further details of the study participants can be found in this article's Table E1 in the Online Repository at [www.jacionline.org](http://www.jacionline.org)). Subjects SPT-positive to other allergens, but not HDM, were not studied. The definition of current asthma was based on a doctor's diagnosis of asthma and symptoms, such as wheeze, displayed during the previous year. Subjects refrained from taking antihistamines or inhaled corticosteroids for 24 hours before blood collection.

**Acute asthma cohort.** Children (mean age, 6.9 years; range, 2–15; see this article's Table E2 in the Online Repository at [www.jacionline.org](http://www.jacionline.org)) presenting to the emergency department with acute asthma were recruited into the study as described in detail elsewhere,<sup>24</sup> and were treated with  $\beta_2$ -agonists and oral prednisolone 1 mg/kg. Blood was collected within 18 hours of admission and approximately 6 weeks later at convalescence. *In vitro* studies from our laboratory have shown that dexamethasone does not alter thrombomodulin expression within PBMCs (data not shown). Viral infections were detected in 64% of patients (94% rhinovirus), and 80% of the cohort was atopic as defined in Table E2.

The adult studies were approved by the Ethics Committee at Sir Charles Gairdner Hospital, and the adolescent and acute asthma studies were approved by the Princess Margaret Hospital for Children Ethics Committee, Perth. All subjects or parents/guardians of subjects provided written informed consent.

### Cell separation and culture

PBMCs were prepared as previously described,<sup>25</sup> and CD14<sup>+</sup> monocytes were isolated by using immuno-magnetic beads (Miltenyi Biotec, North Ryde, Australia) according to the manufacturer's directions. Monocytes were cultured in RPMI containing 5% autologous plasma, 2-mercaptoethanol ( $4 \times 10^{-5}$  mol/L), IL-4 (20 ng/mL; ProSpec-Tany TechnoGene Ltd, Rehovot, Israel) and GM-CSF (100 ng/mL; GlaxoSmithKline, Berne, Switzerland) for 5 days to generate DCs. The HDM allergen extract (*D pteronyssinus*; CSL, Parkville, Australia) has been used extensively in studies from our laboratory<sup>25–27</sup> and has been characterized elsewhere.<sup>28</sup>

### Affymetrix microarray screen

Dendritic cells were stimulated for 16 hours with 10  $\mu$ g/mL HDM or 1 ng/mL LPS (Alexis, San Diego, Calif) and total RNA isolated. Two pools of RNA (5 subjects in each pool, with an equal amount of RNA from each subject) were prepared, 1 from adult nonatopic donors (wheal size, 0 mm to all the tested allergens) and 1 from adult HDM SPT-positive donors (wheal size  $\geq 9$  mm). Standard Affymetrix protocols were followed using U133 Plus 2.0 microarrays (Affymetrix, Santa Clara, Calif) and analyzed as described elsewhere.<sup>26</sup>

### Quantitative RT-PCR

Total RNA was isolated by using the RNeasy minispin kit (Qiagen, Doncaster, Australia) and DNase treated using Turbo DNase (Ambion, Scoresby, Australia) before reverse transcription with the Omniscript kit (Qiagen), according to the manufacturer's protocols. Quantitect thrombomodulin primers were obtained from Qiagen. Reverse-transcribed RNA samples were quantified by using QuantiTect SYBR Green (Qiagen) on the ABI PRISM 7900HT (Applied Biosystems, Scoresby, Australia). Melting curve analysis was used to assess the specificity of the assay, and relative standard curves were prepared from serially diluted RT-PCR products or plasmid standard curves and normalized to the reference gene UBE2D2.<sup>29</sup>

### T cell-polarizing capacity of thrombomodulin-expressing DCs

Using flow cytometry, monocyte-derived DCs were identified by typical forward and side scatter profiles and presence of HLA-DR and absence of CD14, and analyzed for their expression of CD141 (thrombomodulin). In some experiments, DCs were FACS-sorted on the basis of expression of CD141 by using a FACSaria flow cytometer (Becton Dickinson, North Ryde, Australia; purity was above 96% for CD141<sup>+</sup> DCs and 86% for CD141<sup>+</sup> DCs). To examine the T-cell-polarizing capacity of these sorted DCs, they were cultured at a ratio of 1:10 with PBMCs ( $2 \times 10^5$  cells/well) in RPMI-1640, 2-mercaptoethanol ( $4 \times 10^{-5}$  mol/L), and 5% autologous plasma. Cultures were performed in duplicate in round-bottom 96-well plates for 48 hours at 37°C with 5% CO<sub>2</sub>.

### Detection of cytokine protein

Protein levels of IL-5, IL-13, and IFN- $\gamma$  in culture supernatant were measured by in-house time-resolved fluorometry as described in detail elsewhere.<sup>27</sup>  $\delta$  values are presented with the response of the unstimulated cells subtracted from the response of stimulated cells. The limit of detection for each assay was 10 pg/mL.

### Flow cytometry

Circulating DC subsets were identified by staining with a cocktail of mAbs against CD1c (myeloid dendritic cell type 1 [mDC1]), CD141 (myeloid dendritic cell type 2 [mDC2]), CD303, CD14, and CD19 and isotype-matched control antibodies (Miltenyi Biotec) and analyzed by using a FACSCalibur flow cytometer (Becton Dickinson). The identification of DC subsets is described in detail in this article's Fig E1 in the Online Repository at [www.jacionline.org](http://www.jacionline.org). Numbers of mDC1 and mDC2 subsets, expressed as percentages of PBMCs, and mean fluorescence intensity (MFI) of CD141 expression were determined.

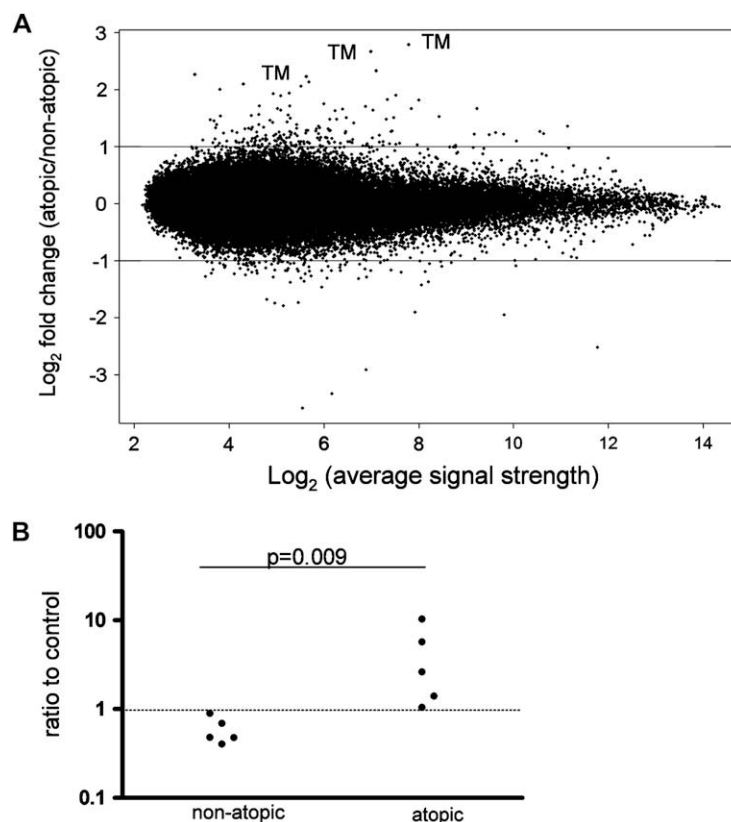


**FIG 1.** Microarray analysis of HDM-stimulated DCs. **A**, Microarrays were performed on pooled RNA samples and are expressed as a microarray plot; the *vertical axis* displays the stimulated (HDM stimulated/unstimulated) fold change of atopics to nonatopics, and the *horizontal axis* displays the average signal. **B**, Thrombomodulin (TM) mRNA expression levels after HDM stimulation from the same donors used in the microarray are presented as a ratio to control. Note the log scale.

### Statistical analysis

All statistical analyses were performed by using the statistical package SPSS (SPSS Inc, Chicago, Ill). Differences within groups were analyzed by using a Wilcoxon matched pairs signed-rank test for paired responses, and comparisons between groups were made by using a Mann-Whitney *U* test for unpaired responses. Group data are presented as medians and interquartile ranges unless otherwise stated.

## RESULTS

### Differential expression gene expression by allergen-exposed DCs

Initially we used a microarray approach to screen for genes that were differentially expressed by DCs from atopic versus non-atopic adults. Monocyte-derived DCs were stimulated with HDM, and pooled RNA from HDM-atopic individuals was compared with pooled RNA from nonatopic individuals (randomly selected from the adult volunteer population). A pooling strategy was adopted to reduce variance caused by intersubject variation in gene expression.<sup>26</sup> Using a threshold of 2-fold difference in gene expression, the microarray screen identified 156 gene probes that appeared to be more highly expressed in DCs from atopic subjects than in DCs from nonatopic subjects (Fig 1, A). Thrombomodulin showed the highest degree of differential expression, with 3 transcripts for this gene identified within the 8 most highly expressed genes. For this reason, and because thrombomodulin had not previously been associated with allergic disease, we elected to focus on this gene. When individual RNA samples from these subjects

were examined using quantitative real-time PCR (Fig 1, B), all HDM-stimulated DCs from atopic subjects showed increased thrombomodulin expression compared with control (although the size of the response varied), whereas all HDM-stimulated DCs from nonatopic subjects showed decreased thrombomodulin expression compared with control ( $P = .043$ ). Accordingly, after HDM stimulation there was a significant difference in the level of thrombomodulin expression between atopic and nonatopic DCs.

### HDM allergen, but not LPS, enhances thrombomodulin mRNA expression in an independent group

It was important to confirm these findings in a separate, independent group of atopic and nonatopic adults. After 6 hours of HDM stimulation, thrombomodulin mRNA was higher in monocyte-derived DCs from atopic individuals than DCs from nonatopic individuals (Fig 2). In contrast, when DCs were stimulated with LPS for 6 hours, there was no change in thrombomodulin expression in either the atopic or nonatopic subjects (Fig 2).

### Surface expression of thrombomodulin on monocyte-derived DCs

We next quantified thrombomodulin on the cell surface by flow cytometry. Expression on DCs was heterogeneous, with both thrombomodulin-positive and thrombomodulin-negative cells

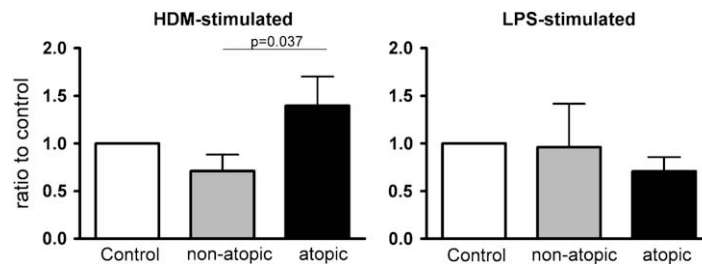


**FIG 2.** Thrombomodulin mRNA expression in HDM and LPS-stimulated DCs. DCs from an independent set of atopic ( $n = 7$ ) and nonatopic ( $n = 8$ ) individuals were stimulated with HDM or LPS for 6 hours, RNA extracted, and qRT-PCR performed. Expression levels were normalized to *UBE2D2* and are presented as the ratio to control (mean  $\pm$  SE).

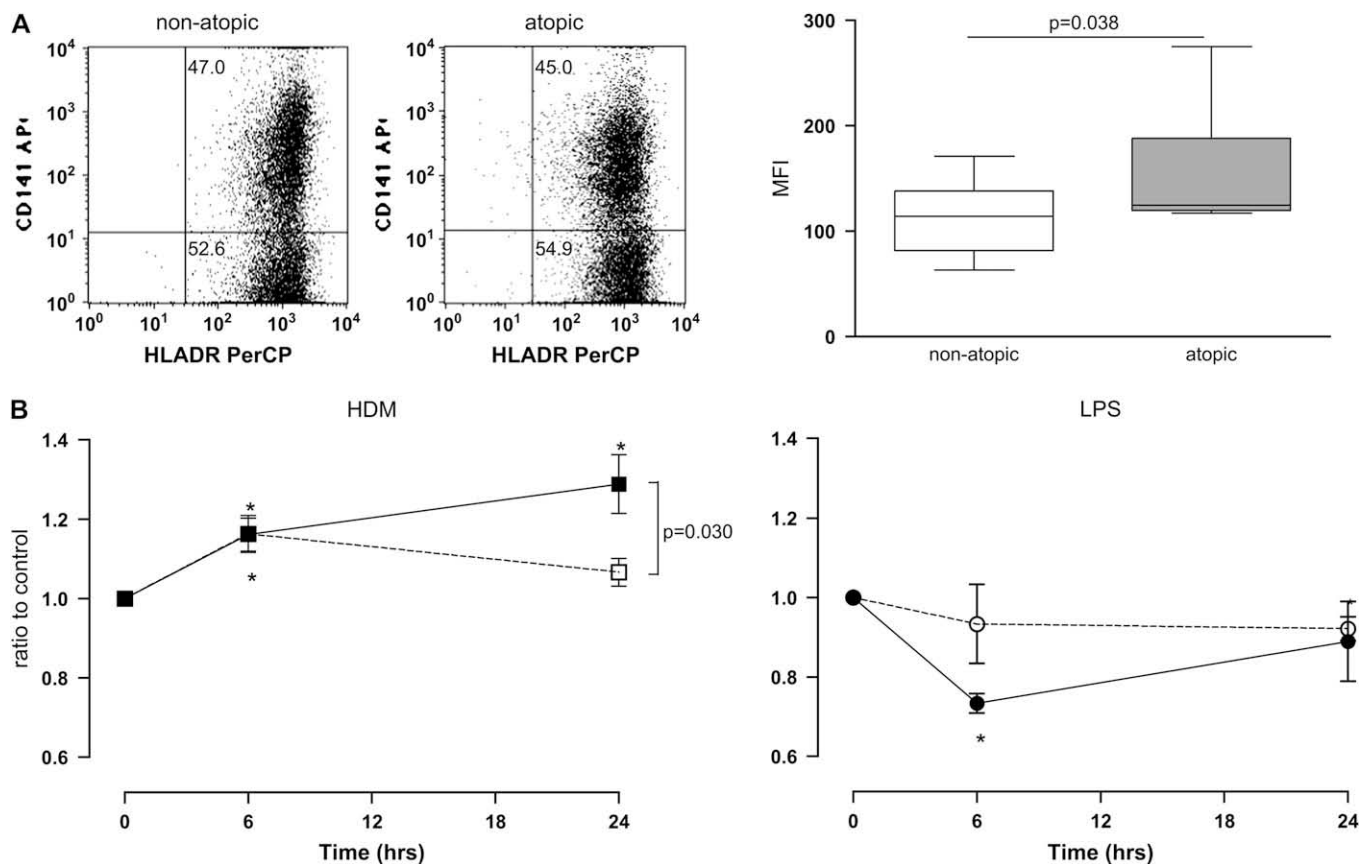


**FIG 3.** Thrombomodulin protein expression on HDM and LPS-stimulated DCs. Thrombomodulin (CD141) expression levels were measured on DCs from atopic ( $n = 8$ ) and nonatopic ( $n = 9$ ) individuals. **A**, Representative plot showing the percentage of CD141 positive DC and MFI on unstimulated DCs. **B**, MFI expressed as ratio to control (equal to 1.0, mean  $\pm$  SE) of atopic (closed symbols, closed line) or nonatopic (open symbols, dashed line) DCs after stimulation. \* $P < .022$  versus control. PerCP, Peridinin chlorophyll protein.

identified. MFI was measured to assess the level of thrombomodulin expression on the thrombomodulin-positive DCs, and it was found that constitutive expression of thrombomodulin was higher on DCs from atopic individuals than on DCs from nonatopic subjects ( $P = .038$ ; Fig 3, A). After exposure to HDM allergen, thrombomodulin expression initially increased on DCs from both atopic ( $P = .012$ ) and nonatopic individuals ( $P = .011$ ; Fig 3, B), but by 24 hours, only DCs from atopic subjects showed sustained thrombomodulin upregulation ( $P = .017$ ). Thrombomodulin at 24 hours was significantly higher on DCs from atopic subjects than on DCs from nonatopic subjects ( $P = .030$ ; Fig 3, B). Thrombomodulin

expression on DCs from nonatopic subjects was similar to unstimulated DC levels at 24 hours. In contrast to HDM, LPS induced down-regulation of thrombomodulin expression in the atopic group at 6 hours ( $P = .012$ ) and in the nonatopic group at 24 hours ( $P = .021$ ). These findings confirmed the qRT-PCR data (Fig 2).

### Blood DCs expressing thrombomodulin are more frequent in allergic asthma

A minor subset of circulating myeloid DCs, referred to as mDC2s, express thrombomodulin,<sup>30</sup> in contrast with the major

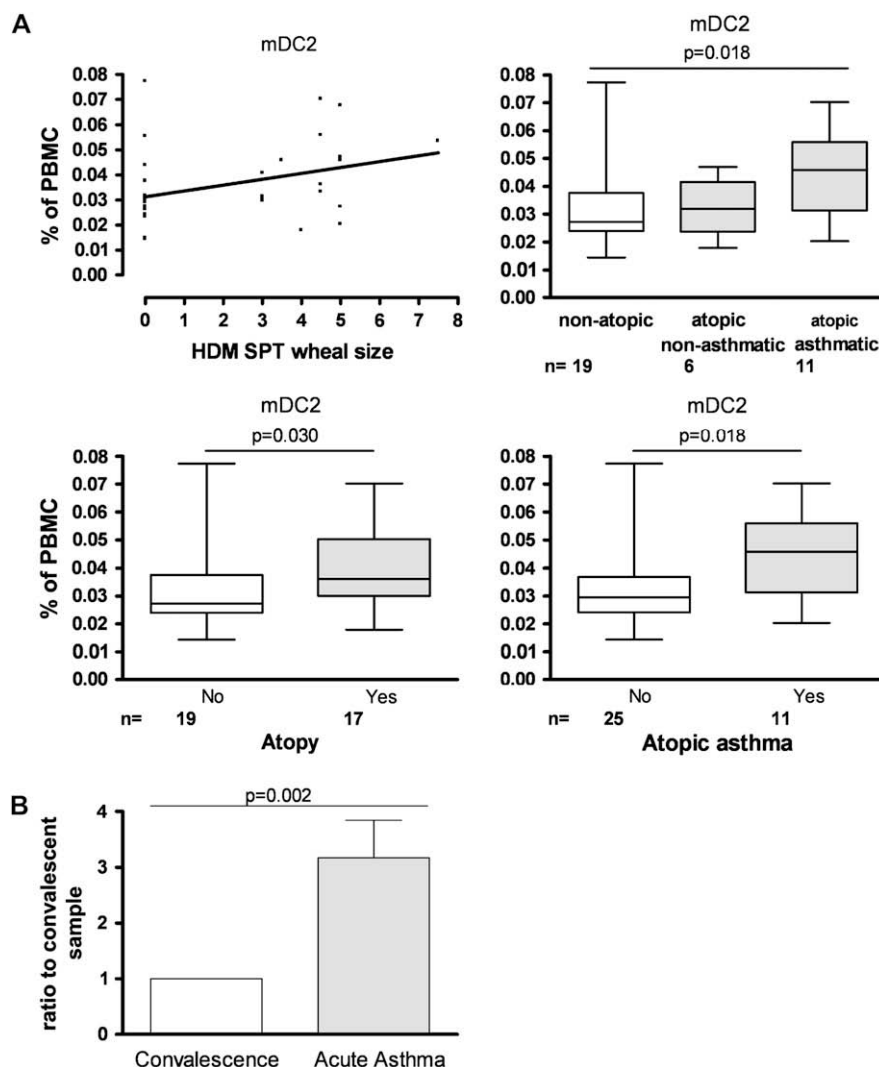


**FIG 4.** Percentage of mDC2s in peripheral blood and thrombomodulin expression during an acute asthmatic attack. **A**, *top left*, Correlation between the percentage of mDC2s and HDM SPT wheal size with the line of best fit ( $n = 36$ ); *top right*, The percentage of mDC2s from nonatopic subjects without asthma ( $n = 19$ ), atopic subjects without asthma ( $n = 6$ ), and subjects with atopic asthma ( $n = 11$ ). Comparisons are also made between subjects with ( $n = 17$ ) and without ( $n = 19$ ) atopy and with ( $n = 11$ ) and without ( $n = 25$ ) atopic asthma. **B**, Thrombomodulin mRNA expression measured during an acute asthmatic attack and presented as a ratio to the convalescent sample (mean  $\pm$  SE;  $n = 25$ ).

subset of myeloid DCs (mDC1s) that express CD1c. We thus examined the frequency of these DC subsets in peripheral blood from a cohort of adolescents, and a representative FACS plot is shown in Fig E1. Initial analysis (Fig 4, A) showed a positive correlation between the frequency of mDC2s and the extent of HDM allergy as determined by SPT wheal size (Spearman  $\rho r^2 = 0.383$ ;  $P = .023$ ;  $n = 36$ ). Accordingly, when the subjects were split based on atopy and asthma, mDC2s were more prevalent in the peripheral blood of subjects with allergy and asthma compared with nonatopic subjects without asthma ( $P = .018$ ; Fig 4, A). Both HDM atopy ( $P = .030$ ) and atopic asthma ( $P = .018$ ) were significantly associated with increased mDC2 numbers in peripheral blood. Furthermore, thrombomodulin MFI on these cells was significantly higher in subjects who were HDM atopic or had atopic asthma, compared with nonatopic control subjects or control subjects without asthma ( $P = .022$  and  $P = .038$ , respectively; data not shown). In contrast, the frequencies of

mDC1s (see Fig E2 in the Online Repository at [www.jacionline.org](http://www.jacionline.org)) were similar across all 3 groups.

Thrombomodulin expression was also examined in children during an acute asthma exacerbation. Thrombomodulin mRNA was measured in unseparated PBMCs without *in vitro* stimulation. Thrombomodulin expression was significantly higher in acute asthma than at convalescence ( $P = .002$ ; Fig 4, B). The limited numbers of cells available from these children meant that we were not able to confirm these findings at the protein level or ascertain how much of this thrombomodulin was expressed on DCs or other cells.

### Thrombomodulin expression distinguishes DCs with differing effects on $T_H1$ and $T_H2$ cytokine production

We next determined whether thrombomodulin expression on DCs was functionally important, particularly whether it was

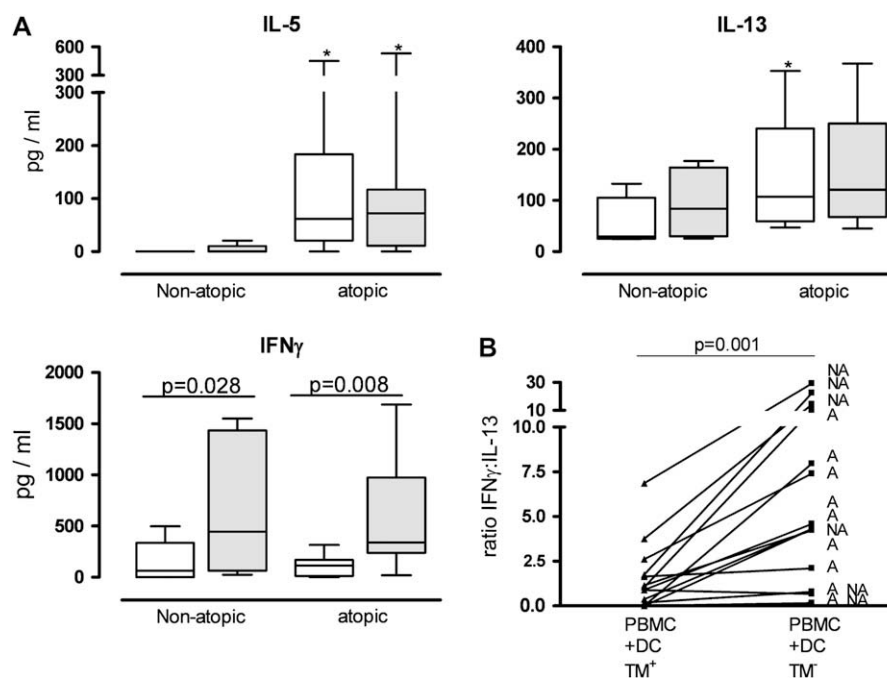


**FIG 5.** HDM-stimulated cytokine production after coculture with thrombomodulin (TM)<sup>+</sup> DCs or thrombomodulin<sup>-</sup> DCs. **A**, TM<sup>+</sup> (white bars) and TM<sup>-</sup> (shaded bars) sorted DCs were cocultured with autologous PBMCs and stimulated with HDM (n = 9 atopic and n = 6 nonatopic). Significant differences between the TM<sup>+</sup> and TM<sup>-</sup> sorted DCs are indicated by horizontal bars, whereas \* indicates  $P < .04$  between atopic and nonatopic donors. **B**, The ratio of IFN- $\gamma$ :IL-13 is shown for each atopic (A) or nonatopic (NA) donor.

associated with altered T<sub>H</sub>1/T<sub>H</sub>2 cytokine production by allergen-specific memory T cells. It was necessary for these experiments to use monocyte-derived DCs, rather than blood mDC2s, because the frequency of mDC2s in peripheral blood is very low, and it is not possible to isolate these cells in sufficient numbers to perform these experiments. Monocyte-derived DCs were cocultured with autologous PBMCs (as a source of responder T cells) in the presence or absence of HDM allergen. Forerunner studies (Yerkovich ST, Roponen M, Smith ME, McKenna K, Bosco A, Subrata LS, et al, unpublished data, October 2008) have shown that supplementation of PBMCs with autologous DCs enhances T-cell cytokine production compared with PBMCs cultured without additional DCs (data not shown). When DCs were sorted into thrombomodulin<sup>+</sup> and thrombomodulin<sup>-</sup> populations, the thrombomodulin<sup>-</sup> DCs elicited a strong IFN- $\gamma$  response, whereas the thrombomodulin<sup>+</sup> DCs elicited a significantly weaker IFN- $\gamma$  response in both atopic and nonatopic donors (Fig 5, A). Although the atopic group showed higher T<sub>H</sub>2 cytokine levels compared with the nonatopic group, neither IL-5 nor IL-13 responses varied in relation to DC expression of thrombomodulin in either group. The ratio of IFN- $\gamma$  to IL-13 in cocultures with thrombomodulin<sup>-</sup> DCs was significantly higher than cocultures with thrombomodulin<sup>+</sup> DCs (Fig 5, B;  $P = .001$  combined,  $P = .008$  atopic group,  $P = .075$  nonatopic group). Thus, thrombomodulin<sup>+</sup> DCs elicited a more clearly T<sub>H</sub>2-skewed response, whereas thrombomodulin<sup>-</sup> DCs produced a mixed T<sub>H</sub>1/T<sub>H</sub>2 response.

## DISCUSSION

Although it is well recognized that DCs are important in human allergic diseases such as asthma, little is known regarding the phenotypic features that distinguish DCs from atopic versus

nonatopic individuals, nor the mechanisms by which DCs induce or reinforce T<sub>H</sub>2 polarization in response to common aeroallergens. Moreover, DCs are exquisitely sensitive to environmental stimuli including aeroallergens themselves, some of which can modulate DC phenotype and function.<sup>12,13,21</sup> Little is known concerning how this occurs, particularly in DCs from atopic subjects with asthma who appear clinically most sensitive to aeroallergens. We reasoned that microarray analysis of allergen-exposed DCs would offer the opportunity to uncover novel allergy-induced genes associated with the response to aeroallergen exposure and the downstream consequences of such responses in relation to disease expression. Using this approach, we demonstrate herein that upregulation of thrombomodulin (CD141, BDCA3) expression after HDM exposure distinguishes DCs from atopic compared with nonatopic individuals (Figs 1-3). Importantly, it was shown that allergen and LPS have opposing effects on thrombomodulin expression. Thrombomodulin<sup>+</sup> DCs induced a more strongly T<sub>H</sub>2-polarized cytokine response by allergen-specific T cells *in vitro*, compared with DCs lacking thrombomodulin. The *in vivo* relevance of these findings was confirmed by showing that thrombomodulin<sup>+</sup> DCs are present at a higher frequency in the circulation of those with HDM atopy and asthma, compared with healthy subjects, and by the observation that thrombomodulin mRNA expression is enhanced during an acute asthma exacerbation and wanes during convalescence.

This is the first report showing that an allergen itself can alter thrombomodulin expression on DCs. Thrombomodulin was initially identified on endothelial cells, where it acts as a receptor for thrombin, initiating the anticoagulation pathway.<sup>22</sup> There is a large amount of literature regarding thrombomodulin's traditional role as an anticoagulant, and the data presented here suggest that when thrombomodulin is expressed on DCs, it may have an additional and unexpected role in the context of allergic disease.

It is known that DCs can express thrombomodulin, and this marker has recently been used to differentiate a minor subset of myeloid DCs in human peripheral blood known as mDC2s that are distinct from the more numerous CD1c<sup>+</sup> mDC1 subset.<sup>30</sup> Thrombomodulin<sup>+</sup> mDC2s appear to localize preferentially to tissues such as the lung, where they outnumber mDC1 cells in human lung digests<sup>31</sup> and bronchoalveolar lavage fluid.<sup>32</sup> This is especially pertinent because we found an increased frequency of thrombomodulin<sup>+</sup> mDC2s in peripheral blood of adolescents with stable atopic asthma and in HDM atopic subjects compared with healthy control subjects (Fig 4). Our results are also in line with a recent report in which thrombomodulin<sup>+</sup> DCs were found to be more frequent in sputum from subjects with asthma compared with healthy control subjects.<sup>15</sup>

A role for thrombomodulin in asthma can also be inferred from our observation that thrombomodulin mRNA rises during acute asthma exacerbations in children, and falls with convalescence. Viral infections are the most common triggers of asthma exacerbations.<sup>33</sup> How viral infection might be linked to enhanced thrombomodulin gene transcription is not clear at present, although a nonspecific effect of an acute phase response seems unlikely. Proinflammatory cytokines such as IL-1 and TNF- $\alpha$  actually reduce thrombomodulin in a nuclear factor- $\kappa$ B-dependent manner,<sup>34,35</sup> whereas LPS inhibits thrombomodulin expression on monocytes/macrophages.<sup>34,36</sup> In contrast, the T<sub>H</sub>2 cytokines IL-4 and IL-13 can negate the reduction in thrombomodulin expression induced by LPS.<sup>37</sup> It is possible that increased trafficking of thrombomodulin<sup>+</sup> mDC2s to the lung in asthma sets up a positive feedback loop in which T-cell-derived IL-4/IL-13 further increases and sustains the allergen-induced thrombomodulin expression on DCs, leading to increased airway inflammation and disease expression.

When we sorted DCs on the basis of thrombomodulin expression and used these DCs to present allergen to autologous T cells, this had a major impact on the pattern of T<sub>H</sub>1/T<sub>H</sub>2 cytokine production (Fig 5). Thrombomodulin<sup>+</sup> DCs induced a more strongly T<sub>H</sub>2-polarized recall response by allergen-specific T cells, compared with the responses elicited by thrombomodulin-negative DCs that were characterized by strong IFN- $\gamma$  production and a mixed T<sub>H</sub>1/T<sub>H</sub>2 response to allergen. It therefore appears likely that thrombomodulin per se is responsible for the altered cytokine production, although the lack of blocking antibodies directed against thrombomodulin made it difficult to examine this further. Another study<sup>38</sup> generated DCs in the presence of IL-10 and described a subpopulation of these DCs that expressed BDCA3 (thrombomodulin). IL-10-exposed DCs exhibited reduced allosstimulatory capacity *in vitro* compared with control DCs, but in contrast with our work, no differences were noted in relation to the secretion of T<sub>H</sub>1/T<sub>H</sub>2 cytokines. Although Velten et al<sup>38</sup> were using a very different experimental system, their work does support the notion that thrombomodulin-expressing DCs can exhibit distinct functional properties. The mechanisms by which thrombomodulin expressed on DCs might influence T<sub>H</sub>1 and T<sub>H</sub>2 cytokine production are not clear. It seems unlikely that thrombomodulin represents a marker of nonspecific DC activation, because allergen and LPS have opposite effects on thrombomodulin expression. Moreover, both HLA-DR and CD86 were expressed to a similar extent on thrombomodulin-positive and thrombomodulin-negative DCs (data not shown), and we found no evidence in the microarray and PCR validation experiments that other costimulatory molecules were differentially regulated

in allergen-exposed DCs from atopic individuals compared with nonatopic individuals (data not shown). To our knowledge, there are no reports of thrombomodulin modulating the interaction between antigen-presenting cells and T cells. There is *in vivo* and *in vitro* evidence that IFN- $\gamma$  can inhibit thrombomodulin expression,<sup>39,40</sup> although whether the reverse can occur is not clear.

This interaction between thrombomodulin and IFN- $\gamma$  is intriguing and aligns our study within the context of the hygiene hypothesis, in which increased endotoxin exposure has been correlated to reduced allergic sensitization and enhanced IFN- $\gamma$  production.<sup>41</sup> We and others<sup>34,36</sup> have shown that LPS reduces thrombomodulin expression on DCs, whereas HDM enhances it, and that thrombomodulin-expressing DCs favor a T<sub>H</sub>2-skewed immune response. Therefore it is feasible that increased endotoxin exposure may reduce thrombomodulin expression leading to enhanced IFN- $\gamma$  production and reduced allergic sensitization.

An increasing body of evidence points to the involvement of the coagulation pathway in asthma and allergy.<sup>42</sup> Increased concentrations<sup>43</sup> and activity<sup>44</sup> of thrombin are present in asthma, and soluble thrombin acting through the protease-activated receptors may reduce phytohemagglutinin-stimulated production of IL-12<sup>45</sup> and IFN- $\gamma$ <sup>46</sup> while increasing production of the T<sub>H</sub>2 cytokine IL-4.<sup>46</sup> Although these findings point to a role for coagulation proteins in T<sub>H</sub>1 and T<sub>H</sub>2 cytokine regulation, we think it unlikely that the capacity of thrombomodulin<sup>+</sup> DCs to alter T-cell cytokine production (Fig 5) can be directly attributed to thrombin because thrombomodulin and protease-activated receptors compete for the same binding site on thrombin (see review<sup>47</sup>).

In conclusion, thrombomodulin expression on DCs appears to have a novel role in the allergic setting, and further research will ascertain the mechanisms by which allergen increases thrombomodulin expression on DCs in atopic individuals and induces a T<sub>H</sub>2-polarized response. Because thrombomodulin is a natural receptor for thrombin (a serine protease), the protease activity of HDM allergens may be important, tempting us to speculate that only allergens with protease activity will upregulate thrombomodulin. Future studies will aim to identify how allergens induce thrombomodulin expression on DCs and to what extent different allergens are capable of doing this. Nonetheless, this study highlights an important and previously unidentified role for thrombomodulin in the pathogenesis of human allergic disease.

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**Clinical implications: This study highlights an important and previously unidentified role for thrombomodulin expression on DCs in the pathogenesis of human allergic disease.**

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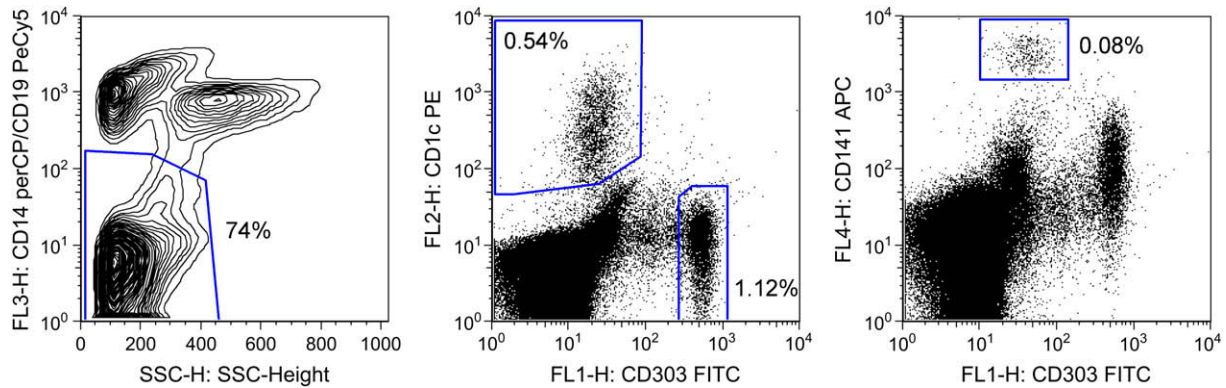


**FIG E1.** Representative FACS staining and gating strategy. **A**, Peripheral blood DC subsets were identified after exclusion of erythrocytes and debris, as well as CD19<sup>+</sup> B cells (which also express CD1c) and CD14<sup>+</sup> monocytes (which also express CD141). DCs were characterized as positive for CD1c (mDC1) (**B**), CD303 (plasmacytoid DC, data not shown), or highly positive for CD141 (mDC2) (**C**). Numbers indicate the percentage relative to total PBMCs. A minimum of 235,000 PBMCs (mean, 490,000 ± 80,000) were acquired to obtain sufficient numbers of DC subsets for accurate enumeration. *SSC-H*, Side scatter; *PerCP*, peridinin chlorophyll protein; *PE*, phycoerythrin; *FITC*, fluorescein isothiocyanate.

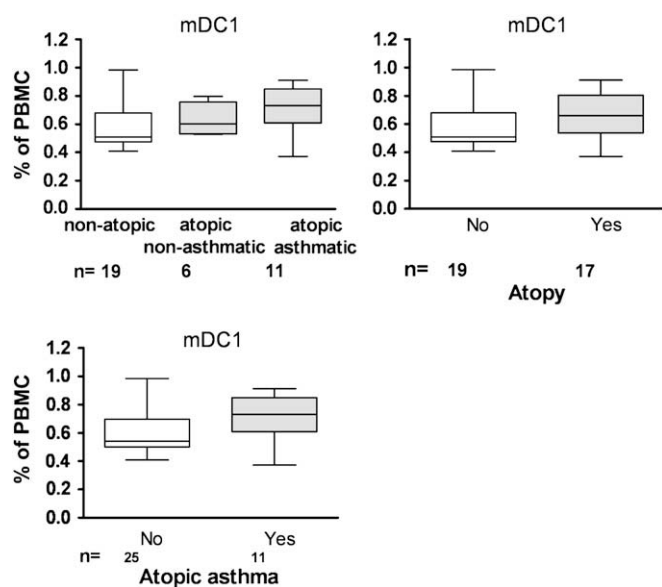


**FIG E2.** Percentage of myeloid mDC1s in peripheral blood. The percentages of mDC1s from nonatopic subjects without asthma ( $n = 19$ ), atopic subjects without asthma ( $n = 6$ ), and subjects with atopic asthma ( $n = 11$ ) are shown. Comparisons are also made between subjects with ( $n = 17$ ) and without ( $n = 19$ ) atopy and with ( $n = 11$ ) and without ( $n = 25$ ) atopic asthma.

**TABLE E1.** Characteristics of the adolescent cohort

|                                | Nonatopic | HDM atopic<br>nonasthmatic | HDM atopic<br>asthmatic |
|--------------------------------|-----------|----------------------------|-------------------------|
| n                              | 19        | 6                          | 11                      |
| Sex, male/female               | 5/14      | 4/2                        | 5/6                     |
| Sensitization,* n              |           |                            |                         |
| <i>D pteronyssinus</i>         | 0         | 6                          | 11                      |
| Rye grass                      | 0         | 1                          | 3                       |
| Grass mix                      | 0         | 0                          | 2                       |
| Total serum IgE (KU/L), median | 24.2      | 56.9                       | 489.2                   |

\*Sensitization was based on SPT wheal diameter  $\geq 3$  mm. Numbers of subjects sensitized to HDM and grass pollens (allergens with the highest reactivity) are shown.

**TABLE E2.** Characteristics of the acute asthma cohort

|                                               | Study cohort                  |
|-----------------------------------------------|-------------------------------|
| n                                             | 25                            |
| Age (y), mean $\pm$ SEM, range                | 6.88 $\pm$ 0.72, range 2-15   |
| Sex, male/female                              | 15/10                         |
| Atopic/nonatopic,* n                          | 20/5                          |
| Viral infection detected, yes/no/unknown      | 16/4/5                        |
| Type of viruses detected, rhinovirus/RSV      | 15/1                          |
| Exacerbation severity,† mean $\pm$ SEM, range | 11.58 $\pm$ 0.25, range 10-14 |

RSV, Respiratory syncytial virus.

\*Atopic status was determined by SPT of wheal size  $\geq 3$  mm to at least 1 of the following allergens: house dust mite (*Dermatophagoides pteronyssinus* and *D. farinae*), rye, mixed grass, egg white, cat and dog dander, cow's milk, and fungi (*Alternaria alternans* and *Aspergillus fumigatus*), total IgE levels of  $\geq$  age-specific cut-offs<sup>E1</sup> (median, 227.7; range, 30.3-3267.8) and HDM-specific serum IgE levels of  $\geq 0.35$  kU/L (median, 17.3; range, 0.35-125.13).

†The severity of the acute exacerbation was determined using a previously validated National Institutes of Health scoring system.<sup>E2</sup>

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