

Effect of a bronchial provocation test with house-dust mite on blood eosinophilia, eosinophil cationic protein, soluble interleukin-2 receptor, and interleukin-6 in asthmatic children

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Eighteen children with perennial asthma and allergy to house-dust mite (HDM) underwent a bronchial challenge with HDM. Before and 24 h after the test, a venous blood sample was taken to determine levels of eosinophils, eosinophil cationic protein (ECP), soluble interleukin-2 receptor (IL-2R), and interleukin-6 (IL-6). A histamine challenge was performed before and 24 h after the HDM challenge. All subjects showed an immediate asthmatic reaction (IAR). A definite late asthmatic reaction (LAR) was observed in 15 children, a probable LAR in two, and no LAR in one. Because of persistent bronchial obstruction ($FEV_1 < 70\%$), eight children were unable to perform a histamine challenge 24 h after the allergen challenge. These were the children with the lowest prechallenge provocation dose (PD_{20}) of histamine. In the other 10 children, the mean PD_{20} histamine decreased after the HDM challenge (mean PD_{20} before was 0.56 mg/ml; after challenge it was 0.14 mg/ml; $P = 0.007$). After the HDM challenge, an increase was detected in the mean values of blood eosinophils (mean before was $446/\text{mm}^3$; mean after was $733/\text{mm}^3$; $P = 0.002$), ECP (mean before was $26.3 \mu\text{g/l}$; mean after was $34.3 \mu\text{g/l}$; $P < 0.040$), and IL-2R (mean before was 116.35 U/ml ; mean after was 128.52 U/ml ; $P < 0.040$). On the other hand, IL-6 remained unchanged after the HDM challenge (mean before was 9.47 pg/l ; mean after was 9.70 pg/l ; $P = 0.360$). Furthermore, as compared with a group of normal, age-matched children ($n = 18$), asthmatic children were found to have higher prechallenge levels of ECP (mean: $10.3 \mu\text{g/l}$ compared with $26.3 \mu\text{g/l}$) ($P < 0.001$) and IL-2R (mean: 80.30 U/ml compared with 116.35 U/ml) ($P = 0.009$), but not of IL-6 (mean: 11.34 pg/l compared with 9.47 pg/l) ($P = 0.436$).

A correlation was found between the duration of asthma and the severity of the LAR expressed as area under the curve (AUCLAR) ($r = 0.50$; $P < 0.040$). Furthermore, a correlation was detected between the level of total IgE and the level of ECP ($r = 0.51$; $P < 0.030$). The decrease in FEV_1 during the LAR tended to correlate with the increase of IL-2R ($r = 0.48$; $P = 0.050$). This tendency was not found with the increase of eosinophils, nor with the increase of ECP. We conclude that both lymphocytes and eosinophils are activated by an allergen challenge, but that only the activation of lymphocytes tends to correlate with the LAR, suggesting that lymphocytes are also closely involved in the pathogenesis of the allergen-induced LAR.

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Asthma is considered to be an inflammatory disease of the airways in which various cell types, including mast cells, macrophages, eosinophils, and lymphocytes, take part (4, 15). The physiopathologic processes of naturally occurring bronchial asthma closely resemble those of the late asthmatic reaction (LAR) induced by a bronchial allergen challenge (23). In contrast to the immediate asthmatic reaction (IAR), the LAR is considered to be an inflammatory event. It is now becoming clearer that lymphocytes and eosinophils are important cells in the pathogenesis of the LAR, although other cell types seem also to take part in the LAR (11). Activated lymphocytes and eosinophils are found in bronchial biopsies of subjects with stable atopic asthma (1). Furthermore, T cells and their soluble products regulate eosinophilia in allergic and nonallergic asthma (22). Therefore, further knowledge of the interaction between lymphocytes and eosinophils seems to be of crucial importance in understanding the mechanisms underlying the development of the LAR and of naturally occurring asthma.

In the present report, we studied the changes in the blood of eosinophils, eosinophil cationic protein (ECP), soluble interleukin-2 receptor (IL-2R), and interleukin-6 (IL-6) induced by an allergen challenge, correlating these changes with each other and with clinical indicators. Furthermore, we determined ECP, IL-2R, and IL-6 in a group of normal children.

Subjects and methods

Subject selection

Eighteen asthmatic children were selected for this study. All suffered from moderate to severe perennial asthma, and all were sensitized to house-dust mite (*Dermatophagoides pteronyssinus*) (HDM), as proved by positive prick tests and positive specific IgE (CAP, Pharmacia, Brussels, Belgium). All children regularly performed lung-function tests. To be included in this study, all subjects had to have an FEV₁ of more than 70%. Furthermore, all had to be able to dispense with antiasthmatic drugs before challenges with histamine and with HDM. The characteristics of the subjects are shown in Table 1.

For this study, we also selected 18 normal, age-matched children (mean age 11 ± 1.8 years, range 8–15). By history, all were found to be free of atopic or chronic respiratory disease, all had negative specific IgE against HDM, and all had a negative family history of atopy or chronic respiratory disease. In these children, ECP, IL-2R, and IL-6 were determined without bronchial provocation tests.

Study design

All patients were challenged with HDM. The day before and the day after the HDM challenge, a histamine challenge was performed and a venous blood sample was taken. Levels of blood eosinophilia, ECP, IL-2R, and IL-6 were determined.

Provocation tests

Two standardized histamine challenges and one HDM challenge were performed according to a protocol earlier described (18). Before the provocation tests, all children had stopped inhaled corticosteroids or disodium cromoglycate for 48 h and had inhaled beta-agonists for 24 h. Between challenges, only inhaled beta-agonists were allowed. The last dose of beta-agonists was inhaled at least 12 h before the HDM or histamine challenge. None of the patients used systemic corticosteroids, theophylline, or antihistamines. Challenges were started if the subject was in a stable condition and if the subject's resting FEV₁ and vital capacity (VC) exceeded 70% of predicted normal. Furthermore, all subjects had to be free of respiratory infection for at least 3 weeks. After the first histamine challenge, performed between 0830 and 0930, lung function was recorded hourly by peak-flow measurement, until 1800 to detect any spontaneous decrease (control day for the LAR), as it has been shown that peak-flow measurement seems to be a reasonable substitute for FEV₁ measurement (9).

Table 1. Characteristics of the 18 asthmatic children

Age (years)	mean ($\pm$ SD): 11 ($\pm$ 2.2) range: 7.4–15.3
Duration of asthma (years)	mean ($\pm$ SD): 7.9 ($\pm$ 2.6) range: 2.5–14
Total IgE (kU/l)	mean ($\pm$ SD): 474 ($\pm$ 369.3) range: 38–1580
Specific IgE against HDM (kU/l)	mean ($\pm$ SD): 63 ($\pm$ 34.7) range: 5–100
Skin test with HDM (mm)	mean ($\pm$ SD): 7.1 ($\pm$ 2.9) range: 3–14
PD ₂₀ histamine (mg/ml) (before HDM challenge)	mean ($\pm$ SD): 0.44 ($\pm$ 0.463) range: 0.08–1.56
FEV ₁ decrease during IAR (% maximum decrease)	mean ($\pm$ SD): 40 ($\pm$ 13.6) range: 22–65
PD ₂₀ of IAR (AU/ml)	mean ($\pm$ SD): 1018 ($\pm$ 1026.2) range: 196–4348
FEV ₁ decrease during LAR (% maximum decrease)	mean ($\pm$ SD): 41.8 ($\pm$ 18.55) range: 4–79
Area under the curve (LAR) (% min)	mean ($\pm$ SD): 10953 ($\pm$ 6398.4) range: 43–25267

Histamine provocation test

After establishing base-line values for VC and FEV₁, the subjects inhaled through a mouthpiece, and with nose occluded, solutions delivered by a Bird Mark 7 respirator (Bird Corporation, Palm Springs, CA, USA) with nebulizer (particle size 0.5–5 µm according to the manufacturer) at an approximate mean flow rate of 0.9 l/s. Before each challenge, saline was inhaled for 1 min. For evaluation of non-specific hyperresponsiveness, progressive twofold increases in concentration of histamine hydrochloride in saline were inhaled for 1 min, starting with a dilution of 0.06 mg/ml. VC and FEV₁ were measured immediately and then 10 min after inhalation. Provocation was stopped when FEV₁ decreased to at least 20% of the initial value of the best value recorded during the provocation procedure. In order to compare degrees of hyperresponsiveness, we determined provocation dose 20 (PD₂₀) of histamine. This was based on the first decrease of FEV₁ of more than 20%; the concentration of histamine causing a 20% decrease of FEV₁ was calculated. According to this challenge procedure, bronchial hyperresponsiveness was defined as a PD₂₀ histamine less than 5.88 mg/ml.

The decrease of the PD₂₀ histamine induced by the allergen challenge was calculated by dividing the PD₂₀ histamine before the allergen challenge by the PD₂₀ histamine after the allergen challenge.

Bronchial provocation test with house-dust mite

For HDM provocation, progressive increases in concentration (5, 50, 500, and 5000 AU/ml) (AU = allergy unit) of a commercial allergen extract of HDM (*D. pteronyssinus*, Haarlem Allergen Laboratories, HAL, Haarlem, the Netherlands) were inhaled for 1 min, starting at a dilution of 5 AU/ml. Bronchial responses were measured by serial monitoring of FEV₁ and VC by a dry spirometer, immediately and 10 min after each inhalation procedure. If the decrease in FEV₁ was less than 20% (as compared with the postbuffer control) after inhalation of 5000 AU/ml, no further challenge was given, and the subject was considered to have no IAR. In order to compare degrees of specific hyperreactivity to HDM between the bronchial challenges, we determined PD₂₀ HDM by the same method used for the histamine reactivity. After completion of the procedure for measuring the IAR, the FEV₁ was recorded at 1, 4, 6, and 8 h, or until a significant LAR occurred. An LAR was definitely present when the FEV₁ decreased to at least 20% of the postbuffer value in the observation period starting 4–8 h after the beginning of the allergen challenge. An LAR was definitely not present when the fall in FEV₁ as described was less

than 5%. An LAR was probably present when the fall in FEV₁ was 5–20%. The severity of the LAR was shown by the greatest FEV₁ decrease during the period 1–8 h after the HDM inhalation and by calculating the area under the curve (AUCLAR); that is, the area enclosed by the curve plotted as the change in FEV₁ 1–8 h after the HDM challenge. At 8 h, a persistent symptomatic LAR was reversed by 2.5 mg nebulized fenoterol.

Determination of levels of ECP, IL-2R, and IL-6

ECP in serum was measured by a double antibody radioimmunoassay kindly provided by Pharmacia, Brussels, Belgium (21). The range of the standard curve was 2–200 µg/l. Serum was centrifuged at 1350 g for 10 min after coagulation of the whole blood for 1 h at room temperature. The serum was frozen and stored at –20 °C until analysis. The concentration of ECP was expressed in µg/l.

IL-2R was determined by means of a sandwich enzyme-linked immunosorbent assay (ELISA) with two monoclonal antibodies recognizing different epitopes of IL-2R, kindly provided by Eurogenetics (Tessenderloo, Belgium). The concentration of IL-2R was expressed as U/ml. The intra-assay precision was less than 8% at 50 U/ml and less than 7% at 500 U/ml. The minimum detectable concentration of IL-2R was 12.5 U/ml.

IL-6 was determined by an ELISA method kindly provided by Eurogenetics (Tessenderloo, Belgium). The concentration of IL-6 was expressed as picogram per milliliter (pg/ml). The intra-assay precision was less than 9% at 5 pg/ml and less than 4% at 500 pg/ml. The minimum detectable concentration of IL-6 was 4 pg/ml.

The increase of ECP, IL-2R, and IL-6 was calculated by dividing the value obtained after the allergen challenge by the value before the allergen challenge. The same was done to obtain the increase in blood eosinophils.

Statistics

Data of the asthmatic children before and after the allergen challenge were analyzed by Wilcoxon's signed-rank test. Correlations were studied by Spearman's correlation test. Data of asthmatic and normal children were compared by the Mann-Whitney rank sum test. A *P* value of less than 0.05 was considered to be statistically significant.

Results

The children selected for this trial suffered from moderate to severe asthma, and all were allergic to HDM (Table 1). They all regularly (daily to weekly)

took beta-agonists, while 4/18 and 12/18 had prophylactic treatment with disodium cromoglycate and beclomethasone dipropionate, respectively (two children used only beta-agonists). With this treatment, all children were virtually free of symptoms for several months, and showed normal basal lung-function indicators (FEV_1 and VC). All children showed marked bronchial hyperresponsiveness with a mean PD_{20} histamine of 0.44 mg/ml, determined the day before the allergen challenge (Table 1). After inhalation of HDM, all subjects demonstrated an IAR. A definite LAR was observed in 15 children. An LAR was probably present in two children showing a decrease in FEV_1 of 16 and 19%, respectively, while one child definitely had no LAR (that is, a decrease in FEV_1 of 4%). A correlation was found between the duration of asthma and the AUCLAR ($r = 0.50$; $P < 0.040$).

The PD_{20} HDM of the IAR ranged from 196 AU/ml to 4348 AU/ml (mean: 1018 AU/ml). A correlation was detected between the PD_{20} HDM of the IAR and PD_{20} histamine determined the day before the allergen challenge ($r = 0.64$; $P < 0.003$). An inverse correlation existed between the PD_{20} HDM and the level of specific IgE against HDM ($r = -0.53$; $P < 0.030$), and between the PD_{20} HDM and the wheal reaction of the skin test with HDM ($r = -0.52$; $P < 0.030$). Furthermore, a correlation was detected between the level of total IgE and the level of ECP ($r = 0.51$; $P < 0.030$).

Although some children experienced a severe LAR, demonstrating a decrease in FEV_1 of more than 50%, all were able to dispense with intervention treatment during the LAR period, which was 4–8 h after HDM inhalation procedure. After this period the children who still showed an FEV_1 of less than 70% received nebulized fenoterol. The day after the HDM challenge, eight children were unable to perform a histamine challenge because their basal FEV_1 was still below 70% of predicted normal. The eight children who were unable to perform a histamine challenge the day after the allergen challenge were those who had the lowest PD_{20} histamine the day before challenge; that is, the children with the highest degree of bronchial responsiveness. In the other 10 children, a statistically significant decrease of the mean PD_{20} histamine was found (mean PD_{20} before was 0.56 mg/ml; after, it was 0.14 mg/ml; $P = 0.007$) the day after the HDM challenge. In the children who did not develop a definite LAR, it was found that only the child showing no LAR (that is, an FEV_1 decrease of 4% during the LAR period) had no decrease of the PD_{20} histamine (0.09 mg/ml before and after). The two children showing a probable LAR showed a decrease in PD_{20} histamine; the child whose fall in FEV_1 was 16% had a decrease in PD_{20} histamine from 0.83 mg/ml to 0.20 mg, and

Table 2. Values of ECP, IL-2R, and IL-6 in normal and asthmatic children (prechallenge values in asthmatic children)

	Normal children (n=18)	Asthmatic children (n=18)	P value
ECP ($\mu\text{g/l}$)	10.3 $\pm$ 5.65	26.3 $\pm$ 17.05	<0.001
IL-2R (U/ml)	80.30 $\pm$ 29.89	116.35 $\pm$ 38.04	0.009
IL-6 (pg/ml)	11.34 $\pm$ 11.33	9.47 $\pm$ 3.61	0.436

Table 3. Blood eosinophils, ECP, IL-2R, and IL-6 before and 24 h after the house-dust mite challenge in asthmatic children (mean $\pm$ SD)

	Before	After	P value
Eosinophils (mm^3)	446 $\pm$ 197.8	733 $\pm$ 301.7	0.002
ECP ($\mu\text{g/l}$)	26.3 $\pm$ 17.05	34.3 $\pm$ 20.85	<0.040
IL-2R (U/ml)	116.35 $\pm$ 38.04	128.52 $\pm$ 39.00	<0.040
IL-6 (pg/ml)	9.47 $\pm$ 3.61	9.70 $\pm$ 2.92	0.360

the child whose fall in FEV_1 was 19% had a decrease in PD_{20} histamine from 0.11 mg/ml to 0.07 mg/ml.

As compared with a group of 18 normal, age-matched children, the asthmatic children were found to have higher prechallenge levels of ECP ($P < 0.001$) and IL-2R ($P = 0.009$), but not of IL-6 ($P = 0.436$) (Table 2).

The day after the HDM challenge, a significant increase was detected in mean values of blood eosinophils ($P = 0.002$), ECP ($P < 0.040$), and IL-2R ($P < 0.040$) (Table 3, Figs. 1 and 2). In contrast, IL-6 remained unchanged after the allergen challenge ($P = 0.360$) (Table 3). The severity of the LAR, expressed as maximum decrease in FEV_1 , tended to correlate with the increase of IL-2R ($r = 0.48$;

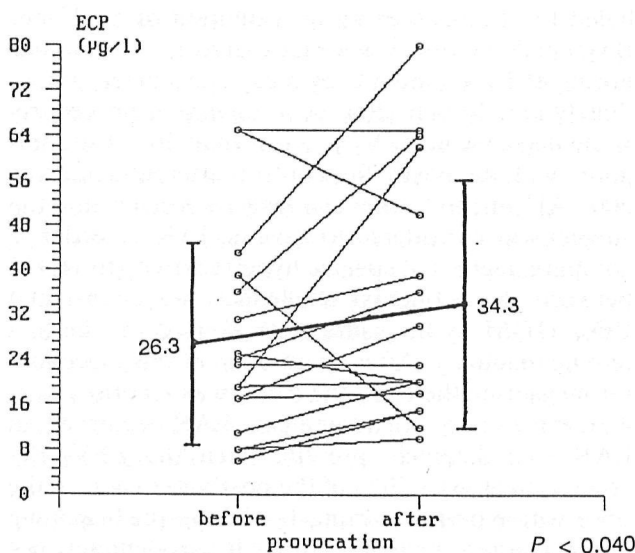


Fig. 1. ECP ($\mu\text{g/l}$) before and 24 h after allergen challenge.

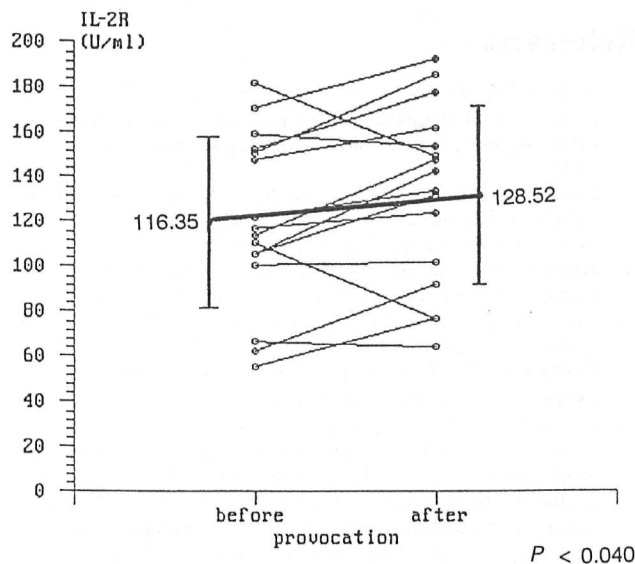


Fig. 2. IL-2R (U/ml) before and 24 h after allergen challenge.

$P = 0.050$). This tendency was not found in the increase of eosinophils, or in the increase of ECP.

Discussion

In this study on 18 children suffering from moderate to severe asthma, 15 showed a definite LAR, two showed a probable LAR, and only one did not show an LAR after HDM inhalation. Therefore, it seems reasonable to accept that 17 out of the 18 children exhibited bronchial inflammatory reactions after antigen challenge, clinically expressed as a definite or probable LAR and as a decrease in PD_{20} histamine the day after the allergen challenge. This agrees with studies indicating that the development of the LAR may occur as a continuum from no LAR to moderate and severe ones (7, 13). The present study demonstrates a relationship between the duration of asthma and the severity of the LAR, suggesting that prolonged bronchial hyperreactivity, manifested by currently occurring asthmatic symptoms, primes the airways to develop more severe inflammatory reactions after acute exposure to the allergen. Furthermore, an inverse correlation was found between the degree of IgE-mediated, specific sensitivity to HDM (expressed by the level of specific IgE and the degree of positivity of the skin test) and the PD_{20} IAR. This suggests that the extent of the immediate sensitivity of the airways to the allergen is dependent upon the level of IgE, which causes the acute mast cell degranulation initiating the IAR.

Furthermore, a correlation was found between the PD_{20} IAR and the PD_{20} histamine determined the day before the allergen challenge, suggesting that the immediate sensitivity of the bronchi to the allergen is dependent upon the degree of nonspecific bron-

chial responsiveness. This agrees with observations indicating that heightened airway responsiveness is associated with increased symptoms of asthma and an increased need for treatment (14).

In accordance with other studies, we found that asymptomatic asthmatic children (that is, asthmatic children who were free of any symptom for at least 3 weeks) have higher prechallenge levels of ECP and IL-2R than normal children (16, 17). This finding suggests subclinical activation of eosinophils and lymphocytes in asthmatic children during symptom-free intervals. This could mean that there are persistent, continuous inflammatory reactions in the airways, showing the chronicity of childhood asthma and perhaps the constant need for antiinflammatory treatment during symptom-free periods.

We found an increase in blood eosinophils 24 h after allergen challenge. This was already reported by several authors, and it was related to the development of the LAR (2, 8, 10). However, we found no correlation between the increase in blood eosinophils and the magnitude of the LAR. The level of ECP increased after the allergen provocation test. The increase of ECP was not related to the severity of the LAR, suggesting that the severity of the LAR is independent of the degree of eosinophilic activation. Venge & Dahl have shown that patients who develop an LAR demonstrate higher levels of prechallenge ECP than those who experience only an IAR (19). Since virtually all the subjects of the present study showed an LAR, it was impossible to confirm these data. However, in another study, which used multiple ECP determinations after an antigen challenge, a uniform pattern of the ECP level after an antigen challenge was detected. It was found that the level of ECP showed a rapid increase during the IAR, followed by a rapid decrease with the lowest values 3–5 h after allergen challenge. Thereafter a slow increase of the ECP concentration was seen, resulting in an increase at 24 h after the allergen challenge (20). The same study also found that the maximum levels of ECP occurring during the IAR did not correlate with the appearance or severity of the LAR, a finding which agrees with the present study. Furthermore, we found a correlation between the level of total IgE and the prechallenge level of ECP, suggesting that activation of eosinophils might be IgE-dependent, or that there is a common source of activation and production.

Activation of T cells has been correlated with symptoms and severity of asthma. Corrigan et al. found that patients with acute severe asthma had significant increases of lymphocyte surface proteins, including surface IL-2R, which are associated with T-cell activation, as compared with control subjects (5, 6). In a recent study, levels of soluble IL-2R were found to be higher in asthmatic patients than in

healthy controls. However, no difference was found among different subgroups of asthmatic patients (acute asthma, mild asthma, and chronic asthma) (3). In a study of a child who died from asthma, the soluble IL-2R was found to be progressively rising just before death, and it was suggested that IL-2R may provide an index of risk of death in asthmatic subjects (24). In the present study, the level of IL-2R was increased by the antigen challenge. To our knowledge, this is the first report showing an *in vivo* increase of the blood level of IL-2R caused by intrabronchial administration of an antigen in an asthmatic subject. Furthermore, the increase of IL-2R tended to correlate with the decrease in FEV₁ during the LAR, suggesting that T cells are involved in the inflammatory processes of the LAR.

In the present study, we determined IL-6 because a potential role of IL-6 in the development of the LAR was suggested (12). However, we found no change in the serum level of IL-6 before and after the antigen challenge. On the other hand, a significant increase in IL-6 was demonstrated in alveolar macrophage supernatants of patients showing an LAR, as compared with patients showing no LAR (12). Therefore, it seems reasonable to assume that the determination of IL-6 in the blood is too imprecise and that the study of IL-6 participation in the LAR should be performed by investigating local processes through cells (i.e., macrophages) or fluid from bronchial biopsies or bronchial lavages.

In conclusion, the present study shows that asthmatic children have higher prechallenge levels of ECP and IL-2R than normal children, indicating continuous activation of eosinophils and lymphocytes (inflammation) during symptom-free periods in childhood asthma. Furthermore, it seems that the number and degree of activation (ECP level) of eosinophils are increased by an antigen challenge, but that these changes are not correlated with the severity of the LAR. On the other hand, the present study shows that lymphocytes are activated by an allergen challenge (i.e., IL-2R) and that the degree of activation tends to correlate with the severity of the allergen-evoked inflammatory reactions (i.e., LAR), suggesting that lymphocytes are closely involved in the processes of the LAR. However, since, in the present study, only two serum measurements have been done in one patient, it is impossible to argue that any one cell type/mediator is more involved than another.

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