

BRIEF COMMUNICATION

The inhibitory receptor CD300a is up-regulated by hypoxia and GM-CSF in human peripheral blood eosinophils

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Abstract

Background: Eosinophils are involved in several inflammatory processes including allergic inflammation. It has been shown that eosinophil functions may be regulated by activating or inhibitory receptors. Hypoxia is a feature of inflamed tissues and has recently been shown to regulate eosinophil viability and pro-angiogenic potential. In this study, the effect of hypoxia and GM-CSF on the inhibitory receptor CD300a in human peripheral blood eosinophils was investigated.

Methods: CD300a expression on eosinophils was analyzed by flow cytometry and evaluated by immuno-fluorescence; mRNA levels were evaluated by RT-PCR.

Results: An increase in the expression of CD300a was observed in hypoxic eosinophils compared to the normoxic ones. GM-CSF strongly induced CD300a increase also after 3 h in culture. In addition, hypoxia augmented mRNA levels of CD300a. Inhibition of hypoxia-inducible factor (HIF)-1 abolished the hypoxia-/GM-CSF-induced CD300a increase.

Conclusion: CD300a expression is up-regulated by hypoxia, and GM-CSF where HIF-1 might play an important role. These results are important for the understanding of eosinophils behavior in inflamed tissue and suggest a new effect on their function in allergic inflammation. Taken together our data point out CD300a as a novel target for the treatment of allergy.

Eosinophil infiltration characterizes a variety of allergic inflamed tissues where their reactivity is orchestrated by an interplay between stimulatory and inhibitory signals of various types. We previously found that eosinophil functions are regulated by receptors known to be expressed mainly by T cells and NK cells such as the inhibitory receptors CD300a and Siglec7 from the IgG-like superfamily and stimulatory receptors such as CD48 and 2B4 belonging to the CD2 family (1, 2).

In allergic inflamed tissue, high levels of granulocyte-macrophage colony-stimulating factor (GM-CSF) are present that recruit, sustain, and activate the eosinophils. Hypoxia, due to an inadequate blood supply and high consumption of oxygen by the infiltrated cells, is also often evident. Inflammatory cells, such as the eosinophils, must be able to maintain viability and function under hypoxic conditions. Hypoxia causes an alteration in gene expression due to the up-regulation of a number of transcription factors, among them the hypoxia-inducible factor (HIF)-1 that functions as a master regulator of oxygen homeostasis. Recently we demonstrated that eosinophils respond to hypoxia by HIF-1 up-regulation and by increasing their viability and

pro-angiogenic potential (3). Indeed, eosinophil prolonged survival and activation are 'hallmarks' of allergic diseases. In the present study, the effect of hypoxia on CD300a expression was investigated on human peripheral blood eosinophils cultured in normoxic or hypoxic conditions with or without GM-CSF. The inhibitory receptor CD300a, containing four immunoreceptor tyrosine-based inhibitory motifs (ITIM) (4, 5), is of particular interest to us as a possible immunopharmacological tool to down-regulate allergic responses. In fact, CD300a was demonstrated to effectively down-regulate eosinophil functions in vitro and to inhibit inflammation in a model of eosinophilic allergic murine asthma (6).

Material and methods

Eosinophil purification

Eosinophils were purified as previously described (3) from the peripheral blood of untreated, mildly atopic volunteers (blood eosinophil levels 5–10%), who were asymptomatic and therefore not taking any drug for their condition, and on the

day of blood donating, any other drug. Written informed consent was obtained according to the guidelines of the Hadassah-Hebrew University Human Experimentation Helsinki Committee. Eosinophils collected at a purity of >98% (Kimura staining), with a viability of >98% (trypan blue staining) were re-suspended (5×10^6 or 10^6 cells/ml) in culture medium consisting of RPMI 1640 supplemented with L-glutamine (300 mg/l), 10% heat-inactivated FCS, and penicillin–streptomycin solution (100 u/ml) (Biological Industries, Beit Haemek, Israel).

Eosinophil culture in hypoxic or normoxic condition

Eosinophils were cultured in 96-well u-shaped plates (Nunc, Rochester, NY, USA) in 200 μ l of medium alone or supplemented with 20 ng/ml of GM-CSF, IL-3, or IL-5 (Pepro-Tech, Rocky Hill, NJ, USA) according to the experiment. For experiments on HIF-1-binding inhibition, cells were cultured at the indicated times with or without 10 nM echinomycin (Calbiochem, Darmstadt, Germany). For experiments with the different inhibitors, cells were cultured at the indicated times with or without 10 μ M PD98059 (Calbiochem), brefeldinA or cycloheximide (Sigma Aldrich, Rehovot, Israel). For experiments in hypoxia, plates were placed in a closed humidified chamber at 37°C, with a continuous flow of gas mixture of 95% N₂ and 5% CO₂. The oxygen percentage in the medium was monitored with a dissolved oxygen meter before each time point, checked and found to be $\leq 3\%$ (MettlerToledo, Schwerzenbach, Switzerland). At this time point, eosinophils were added to the wells. All the assays were performed on eosinophils cultured for a further 1 h, to permit full re-equilibration of the oxygen in the chamber (<3%). For experiments in normoxia, plates were placed at 37°C in a humidified incubator with 5% CO₂ and air.

Flow cytometry analysis

For flow cytometry analysis, eosinophils (10^6 cells/ml) were incubated for 10 min on ice in Hanks balanced salt solution supplemented with 5% goat serum and 0.1% bovine serum albumin (Biological Industries, Kibbutz Beit Haemek, Israel). Eosinophils were then incubated with Abs recognizing CD300a (mAbs from clone #12) kindly provided by Prof. O. Mandelboim (Department of Immunology, The Hebrew University of Jerusalem, Jerusalem, Israel) (5), CD48 (e-bioscience, San Diego, CA, USA), or IgG1 isotype control all at a final concentration of 5 μ g/ml followed by 40-min incubation on ice with goat anti-mouse FITC (1 : 500; ICN Pharmaceuticals, Costa Mesa, CA, USA).

After staining, the cells were analyzed on a Becton Dickinson FACSCalibur System (Becton Dickinson, San Jose, CA, USA). For each staining, at least 10 000 events were collected, and data analysis was performed using CellQuest software (BD, Mansfield, MA, USA).

RT-PCR

Total RNA (15 μ g) from fresh eosinophils was extracted using TriReagent (Sigma), and reverse transcription was performed using SuperScript First-Strand kit (Invitrogen, Carlsbad, CA,

USA). The cDNA was amplified using primers for human VEGF (sense: 5'-CGC CGA CCA AGG AAA ACT C-3', antisense: 5'-GGG TAC TGG ATG TCA GGT CTG C-3', 569 bp), CD300a (sense: 5'-AGCATCCAGGAGGAAA CTGA-3', antisense: 5'-GAGGCCTCTGAGCAGCTATC-3'), or CD48 (sense: 5'-GGCTCTGGAATTGCTACT-3', antisense: 5'-CTGTTCTGGAGCTCCTTTG-3'). Beta-actin was used as control. Thirty-five cycle-PCR products were visualized by Gel-red (Biotium, Hayward, CA, USA)-stained gel electrophoresis. Quantitative analysis was performed calculating the OD of the band relatively to the OD of the corresponding actin band.

Immunofluorescence

For immunofluorescence (IF), 10^5 eosinophils per sample were blocked in 5% goat serum in Hanks balanced salt solution supplemented with 0.1% bovine serum albumin for 10 minutes on ice. Cells were then incubated with anti-CD300a antibody or IgG1 isotype control (5 μ g/ml) for 1 h on ice and, after washing, with goat anti-mouse Alexa fluor 647 (1:500; Molecular Probes, Eugene, OR, USA) for 40 min on ice. Stained cells were fixed with 4% PFA and transferred to a glass slide precoated with poly-L-Lysine (0.01%; Sigma). Analysis was performed with a FluoView FV1000 confocal microscope (Olympus) at 10 $\times$ and 60 $\times$ magnifications.

Promoter analysis

Promoter region was identified using UCSC genome browser as 1000 nt upstream to the transcription start site (TSS). Promoter analysis was performed using Genomatix software (Munich/Germany; <http://www.genomatix.de/>), and a total of 169 promoters binding sites were found. The HIF-1 promoter was located at position-927 from the TSS.

Statistical analysis

The control and experimental groups were compared by paired *t*-test for the evaluation of significance (**P* < 0.05; ***P* < 0.05). The data are expressed as mean $\pm$ standard error of measurement (SEM) of at least three independent experiments performed in triplicate. The KyPlot analysis tool-pack was used to perform the statistical analysis.

Results and discussion

We first evaluated the influence of the survival cytokines interleukin (IL)-3, interleukin-5, and GM-CSF on the receptor expression. Flow cytometry analysis shows that after 18 h, GM-CSF and IL-3 increased CD300a cell surface expression, while IL-5 had no effect (Fig. 1A). Short-term exposure (3 and 6 h) of eosinophils to GM-CSF also significantly increased CD300a expression (Fig. 1B). In an attempt to better understand this phenomenon, the protein synthesis inhibitor cycloheximide was used. Cycloheximide had no effect on the short-term regulation of CD300a (Fig. 1C). Also, Brefeldin A, a membrane transport inhibitor, did not

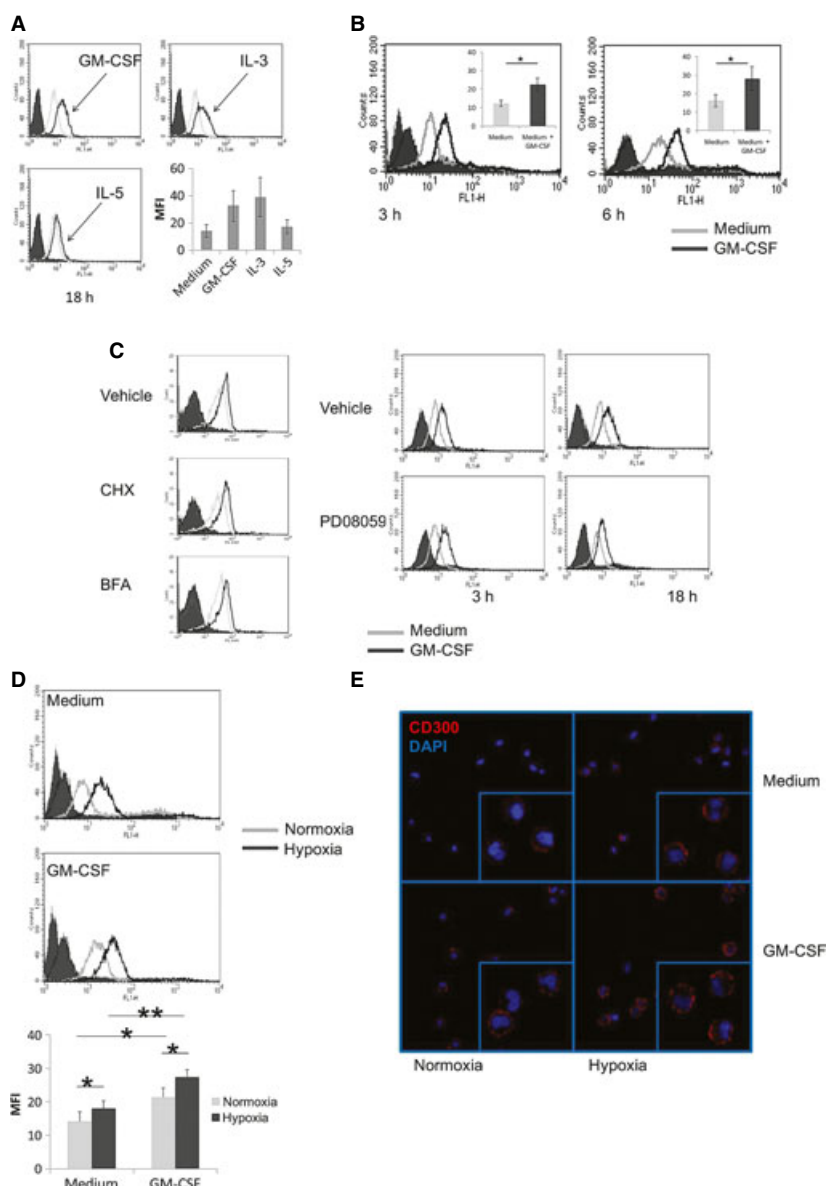


Figure 1 GM-CSF and hypoxia regulate CD300a expression. Flow cytometry evaluation of CD300a expression on human peripheral blood eosinophils: (A) eosinophils cultured for 18 h in medium alone (gray histograms) or with GM-CSF, or IL-3 or IL-5 (20 ng/ml; black histograms; $n = 3$); (B) eosinophils cultured for 3 and 6 h in medium alone or with GM-CSF (20 ng/ml; $n = 3$); (C) in the left panel eosinophils cultured for 3 h without or with GM-CSF in medium with vehicle or with cycloheximide (CHX; 1 μ g/ml) or brefeldinA (BFA; 1 μ g/ml; $n = 3$). In the right panel, eosinophils cultured for 3 or 18 h in

inhibit membrane CD300a up-regulation. ERK1/2 phosphorylation is a feature of eosinophil activation, and it was found to be induced by GM-CSF. The specific ERK1/2 inhibitor PD98059 partially inhibited the enhancing effect of GM-CSF on CD300a at 18 h, while no effect was observed on the short-term increase of the receptor (3 h; Fig. 1C). It can be concluded from this evidence taken together that in eosinophils

medium alone or with GM-CSF (20 ng/ml) with or without PD98059 (10 μ M) (vehicle and medium histograms were superimposable, and therefore only the medium line is indicated for sake of clarity; $n = 3$); (D) eosinophils cultured for 18 h in normoxia or hypoxia with or without GM-CSF (20 ng/ml), and quantitative analysis performed on five experiments. (E) Immunofluorescence staining for CD300 in eosinophils cultured for 18 h in normoxia or in hypoxia with or without GM-CSF; representative field (×60 magnification) and digital magnification (×4) in the inset.

while the overnight increase of CD300a would imply de novo synthesis probably through ERK1/2 phosphorylation, the short-term increase would rather involve a rapid mechanism of receptor transport to the membrane. In previous works, it has also been shown that GM-CSF leads to the rapid up-regulation of CD300a expression on neutrophils membrane in an event which does not involve new protein synthesis (7). The

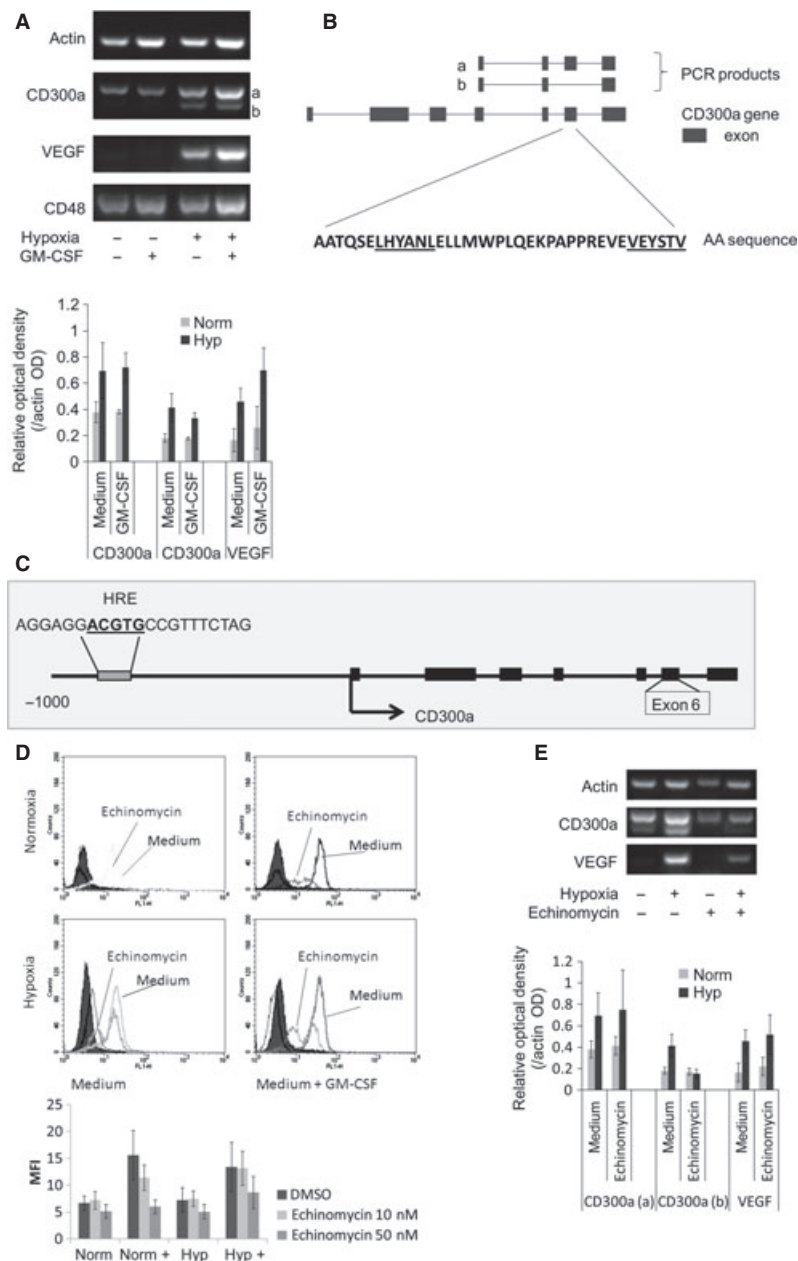


Figure 2 Effect of hypoxia on CD300a expression and transcription in eosinophils. (A) RT-PCR analysis for CD300a, CD48, VEGF and actin on mRNA from eosinophils cultured for 18 h in normoxia or hypoxia with or without GM-CSF (20 ng/ml; $n = 3$). A representative PCR is shown and the quantitative analysis resulting from three different experiments. (B) Diagram of the two transcripts obtained for CD300a with the detailed amino acid sequence corresponding to the spliced exon with the immunoreceptor tyrosine-based inhibitory motifs (ITIM) underlined. (C) Schematic view of

the CD300a gene with the upstream region including the hypoxia responsive element (HRE) site. (D) Eosinophils cultured for 18 h in normoxia or hypoxia in medium alone or with GM-CSF (20 ng/ml) with or without echinomycin (50 nM; $n = 3$). A quantitative analysis from five different experiments is shown in the chart. (E) RT-PCR analysis for CD300a, VEGF and actin on mRNA from eosinophils cultured for 18 h in normoxia or hypoxia with or without echinomycin (20 nM); a representative PCR is shown and the quantitative analysis resulting from three different experiments.

notion that an intracellular pool of CD300 might be maintained for fast translocation suggests the importance of this receptor for the prompt modulation of eosinophil function. However, the mechanism has yet to be elucidated.

We then evaluated the effect of hypoxia in the presence or absence of GM-CSF on CD300a expression. Hypoxia increased CD300a expression after 18 h culture (MFI: normoxia 14.08 ± 2.94 ; hypoxia 17.98 ± 2.34 ; $P = 0.045$;

$n = 5$). The addition of GM-CSF caused a further significant up-regulation in CD300a (normoxia $P = 0.0027$; hypoxia $P = 0.0005$; Fig. 1D, E). Interestingly, the expression of other eosinophil receptors, such as Siglec-7, CD48, and 2B4 was not significantly influenced by either GM-CSF or hypoxia (not shown).

To further study the effect of hypoxia, RT-PCR for CD300a genes was performed on mRNA from eosinophils cultured in normoxia or hypoxia with or without GM-CSF. Hypoxia increased mRNA levels of CD300a (Fig. 2A band a) and VEGF (positive control) but not CD48 (used as negative control). Surprisingly, in the CD300a product, an additional PCR product, shorter than the expected one, appeared under hypoxic conditions (Fig. 2A band b). Notably, GM-CSF had no effect on the two CD300a products. The two PCR products were sequenced and according to a BLAST analysis both corresponded to CD300a. The shorter one resulted as a spliced transcript missing the exon-containing sequences of two of the four CD300a ITIMs (1, 5) (Fig. 2B).

Hypoxia-inducible factor-1, a heterodimer of HIF-1 α and HIF-1 β , binds to the hypoxia responsive element (HRE) present in the promoter regions of hypoxia responsive genes, such as VEGF. Taking into consideration the responsiveness of the CD300a gene to hypoxia, HREs presence in the promoter was investigated using a genomix promoter analysis on the first 1000 bases upstream of the transcription initiation site of the CD300a gene (Fig. 2C). Having identified different HREs, the involvement of HIF-1 in CD300a up-regulation under hypoxia/GM-CSF was investigated by testing the effect of the HIF-1 inhibitor echinomycin. Echinomycin partially inhibited CD300a increase (Fig. 2D). Particularly this inhibition was observed for the GM-CSF-driven increase in normoxia suggesting the involvement of HIF-1 in the GM-CSF-mediated regulation of CD300a. Notably, the short-term increase of CD300a was not affected by echinomycin (not shown). Importantly, in the presence of echinomycin, the shorter CD300a PCR product was significantly decreased (Fig. 2E).

References

- Munitz A, Bachelet I, Eliashar R, Moretta A, Moretta L, Levi-Schaffer F. The inhibitory receptor IRp60 (CD300a) suppresses the effects of IL-5, GM-CSF, and eotaxin on human peripheral blood eosinophils. *Blood* 2006;**107**:1996–2003.
- Munitz A, Bachelet I, Eliashar R, Khodoun M, Finkelman FD, Rothenberg ME et al. CD48 is an allergen and IL-3-induced activation molecule on eosinophils. *J Immunol* 2006;**177**:77–83.
- Nissim Ben Efraim AH, Eliashar R, Levi-Schaffer F. Hypoxia modulates human eosinophil function. *Clin Mol Allergy* 2010;**8**:10.
- Clark GJ, Ju X, Tate C, Hart DN. The CD300 family of molecules are evolutionarily significant regulators of leukocyte functions. *Trends Immunol* 2009;**30**:209–217.
- Lankry D, Simic H, Klieger Y, Levi-Schaffer F, Jonjic S, Mandelboim O. Expression and function of CD300 in NK cells. *J Immunol* 2010;**185**:2877–2886.
- Munitz A, Bachelet I, Levi-Schaffer F. Reversal of airway inflammation and remodeling in asthma by a bispecific antibody fragment linking CCR3 to CD300a. *J Allergy Clin Immunol* 2006;**118**:1082–1089.
- Alvarez Y, Tang X, Coligan JE, Borrego F. The CD300a (IRp60) inhibitory receptor is rapidly up-regulated on human neutrophils in response to inflammatory stimuli and modulates CD32a (Fc γ RIIa) mediated signaling. *Mol Immunol* 2008;**45**:253–258.
- Del Rey MJ, Izquierdo E, Usategui A, Gonzalo E, Blanco FJ, Acquardo F et al. The transcriptional response of normal and rheumatoid arthritis synovial fibroblasts to hypoxia. *Arthritis Rheum* 2010;**62**:3584–3594.
- Bosco MC, Puppo M, Santangelo C, Anfoso L, Pfeffer U, Fardin P et al. Hypoxia modifies the transcriptome of primary human monocytes: modulation of novel immune-related genes and identification of CC-chemokine ligand 20 as a new hypoxia-inducible gene. *J Immunol* 2006;**177**:1941–1955.

We have previously shown a cooperation between GM-CSF and hypoxia in the up-regulation of HIF-1 (3). In light of our novel results, we suggest a role for the interplay between hypoxia and GM-CSF in the up-regulation of CD300a.

Notably, other studies have shown that hypoxia induced up-regulation of CD300a mRNA level in synovial fibroblasts and monocytes. In these studies, protein expression level was not characterized (8, 9). Here, we provide the first evidences for CD300a regulation in hypoxic eosinophils at both the mRNA and protein level. Whether different eosinophil receptors can undergo a regulation similar to the one described for CD300a, and whether CD300a regulation takes place in a similar fashion on eosinophils *in vivo*, has yet to be further investigated.

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Author contributions

A.H.N.B.E. designed the research, performed all experiments, analyzed data, and wrote the manuscript; L.K. designed the primers and critically revised the manuscript; M.B.Z. contributed to the conception and the interpretation of the data and critically revised the manuscript; and F.L.S. designed the research, received the grant supports, provided overall supervision, analyzed the data and edited the manuscript.

Conflicts of interest

The authors have no financial relationships to disclose.

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