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FUNCTIONAL AND PHENOTYPICAL IMPAIRMENT OF POLYMORPHONUCLEAR CELLS IN ATOPIC DERMATITIS: AN ADDITIONAL CAUSE FOR THE KNOWN SUSCEPTIBILITY TO INFECTIONS?

Authors

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Short title

Neutrophil PMN impairment in atopic dermatitis.

Key-words

Atopic dermatitis; human PMNs; intracellular killing; phagocytosis; infections; TLRs.

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ABSTRACT

Background: Atopic dermatitis (AD) patients present an high susceptibility to infections. Polymorphonuclear granulocytes' (PMN) phagocytic activity is mediated by the interactions between Toll-like receptors (TLRs) and Pathogen-Associated Molecular Patterns. **Objective:** to investigate functional activity and phenotype of PMN in AD patients. **Methods:** *in vitro* PMN phagocytosis and intracellular killing towards *Klebsiella pneumoniae* were evaluated in 24 AD patients; flow-cytometry was applied to analyze PMN phenotype. **Results:** PMNs from AD patients displayed both a reduced phagocytic activity and intracellular killing against *K. pneumoniae* than healthy subjects (HS). CD11b, CD66b, TLR2, TLR4 and TLR5 median fluorescence intensity (MFI) on PMN membrane were significantly higher in AD patients than in HS. **Conclusion:** PMN functional impairment in AD patients could represent an additional cause of skin infections, coupled with other known defects in the innate immune system. The increased MFI of adhesion molecules and TLRs is rather a consequence of the skin barrier increased permeability to bacterial molecules capable of stimulating immunological reactions.

INTRODUCTION

Atopic dermatitis (AD) is a multifactorial, inflammatory skin disease with a chronic or a relapsing course, affecting nearly 5% of the white population; it is characterized by an onset in childhood with intense itching and it is closely associated with other atopic diseases, namely allergic rhinitis and asthma [1]. AD can be divided into 2 distinct variants: the extrinsic, allergic variant, accompanied by high concentrations of total and allergen-specific serum IgE levels, and the intrinsic, nonallergic variant, with no detectable sensitization and low serum IgE levels [2].

Reduction in antimicrobial peptides [3], diminished recruitment of innate immune cells [polymorphonuclear granulocytes (PMNs), plasmacitoid dendritic cells (pDC) and natural killer cells (NK)] to the skin [4-5], epithelial barrier disruption [4], Th2 polarization [6] and TLR2 defects [7] are all explanations for AD patients' susceptibility to pathogens such as *Staphylococcus aureus* and Herpes simplex virus, which may occur with a particularly aggressive clinical course and lesions frequently spread across the entire tegument. Approximately 80-100% of AD patients are colonized on lesional and nonlesional skin by *S. aureus* [7]. The *S. aureus* role in the development of the atopic symptoms is to exacerbate chronic inflammation by releasing molecules, like peptidoglycans (PGN), that can stimulate the proinflammatory cytokine production of T cells, macrophages, eosinophils and keratinocytes [8]. PMNs constitute an essential part of the innate immune system and are the first among inflammatory cells to migrate towards the site of inflammation [9]: surprisingly, the absence of PMNs in lesional biopsies from AD patients is characteristic, even in the presence of infection [7]. A number of studies have pointed to a chemotactic defect of PMNs or monocytes in AD patients, although results are variable [10-12]. PMN functional activities, namely phagocytosis and capacity to produce reactive oxygen species, seem to be particularly impaired in AD patients with concomitant bacterial infections [13]. PMN phagocytosis and killing activity against pathogens is mediated by the interactions between Toll-like receptors (TLRs) expressed on the PMN surface and Pathogen-Associated Molecular Patterns (PAMPs) [14], which include cell wall constituents such as lipopolysaccharide (LPS), PGN, single or double-stranded RNA. Activation of TLRs facilitates phagocyte recruitment by upregulation of adhesion molecules and alteration of PMN trafficking, in order to localize neutrophils at the site of infection. PMNs also respond to TLR activation with the generation of reactive oxygen species [15], increased phagocytosis and secretion of cytokines.

Recently our group reported an impairment of phagocytic activity in Sézary syndrome, a cutaneous T cell lymphoma characterized by a Th2-cytokine expression profile: this defect was more evident in patients who experienced frequent infection episodes [16].

In order to better define a possible additional cause of the frequent infections in AD patients, we focused our attention on the phenotype and functional activity of PMNs in patients suffering from severe AD comparing it with those of a sex- and age-matched control group. In addition, we aimed at investigating the TLR role in mediating PMN dysfunctions in AD.

MATERIALS AND METHODS

Study design

The study design, performed according to the principles of good clinical practice and to the Declaration of Helsinki Principles, included peripheral blood analysis with PMN phenotypical and functional determinations. The cohort was made up of 24 patients (11 men and 13 women, average age: 49.3 years, range: 21-56) seen at Section of Dermatology, Department of Biomedical Sciences and Human Oncology, University of Turin, Italy, over a period of 18 months. For ethical reasons, the trial was limited to adult patients who gave their written informed consent. The control population was made up of 15 healthy subjects (HS), correlated by age and sex with the patients examined and found negative to the test for bacterial and viral infections in progress. None of the HS presented a personal or family history of atopy. The PMN number/mm³ at test time varied between 1.768 and 9310 in AD patients, with median values (4.557/mm³) similar to those of HS (4.483/mm³). The study protocol was approved by the Turin University Ethical Committee.

PMN functional activity

Bacteria. A clinical strain of *Klebsiella pneumoniae* was cultured on MacConkey Agar (Oxoid S.p.A., Garbagnate Milanese, Milan, Italy). Young colonies (18–24 h) were picked up to approximately 3–4 McFarland standard and inoculated into cryovials containing both cryopreservative fluid and porous beads to allow bacteria to adhere (Microbank, Biomérieux; Rome, Italy). After inoculation, cryovials were kept at –80°C for extended storage [17-19].

PMNs. PMNs from AD patients and HS were separated from lithium heparinized venous blood using Ficoll–Paque (Pharmacia S.p.A., Milan, Italy) and incubated at 37°C in a shaking water bath before the addition of *K.pneumoniae* (10⁷ CFU/ml). The PMN viability before and after each experiment was greater than 95%.

Phagocytosis and intracellular killing assays. The phagocytosis of radiolabelled klebsiellae [³H-uracil (specific activity: 1.27 TBq/mmol; NEN Products, Milan, Italy)] and the intracellular *K. pneumoniae* killing by PMNs were investigated by incubating the bacteria and the phagocytes (bacterium:PMN ratio was 10:1) at 37 °C for periods of 30, 60 or 90 minutes. Phagocytosis and intracellular killing were assessed by the methods previously described [17-19]. Briefly, aliquots of 1 ml of *K.pneumoniae* in RPMI 1640 with 10% FCS were added to PMNs (10⁶ cells) and incubated at 37°C in a shaking water bath. After incubation for a period of 30, 60 or 90 minutes, the PMNs were twice centrifuged at 1,200 rpm for 5 minutes to remove free bacteria. 100 µl of suspended cells were then placed in scintillation fluid (Atomlight, NEN) and counted by liquid scintillation spectrophotometry. Radioactivity was expressed as the counts per min/sample. The percentage of phagocytosis at a given sampling time was calculated as follows: percent phagocytosis = [(cpm in PMNs pellet)/(cpm in total bacterial pellet)] × 100. In order to measure PMN antimicrobial activity, an aliquot of the cells containing bacteria was lysed by adding sterile water, and a viable count of intracellular *K. pneumoniae* was performed (time zero). The cells were then incubated further, and at intervals (30, 60, 90 minutes), the viable counts of the surviving intracellular bacteria were measured in the same way. The PMN killing values were expressed as survival index (SI), which was calculated by adding the number of surviving bacteria at time zero to the number of survivors at time *x*, and dividing by the number of survivors at time zero. According to this formula, if

bacterial killing was 100% effective, the SI would be 1 whereas a value > 2 indicates absence of killing.

Flow Cytometry

Peripheral blood leukocytes (PBL) were analyzed by six-colour immunofluorescence using a FACSCantoII cytometer (Becton Dickinson, San José, CA, USA) equipped with a 488 and a 633 laser. At least 3×10^4 leukocytes were collected for each antibody combination and isotype-matched negative controls conjugated to each fluorochrome were used. The following mouse anti-human antibodies were used: CD11b APC (clone D12) and CD45 PerCP-Cy5.5 (clone 2D1) from BD Biosciences, San Jose, CA, USA, CD62L PE (clone DREG56) from Invitrogen Ltd, Paisley, UK and CD66b FITC (clone 80H3) from Beckman Coulter, Brea, CA, USA, TLR2/CD282 APC (clone TL2.1), TLR4/CD284 Alexa Fluor 647 (clone HTA125) and TLR5 PE (clone 85B152.5) from IMGENEX, San Diego, CA, USA. TLR9 and TLR8/CD288 cytoplasmic expression was analyzed using whole blood samples. Cells were intracellularly stained with mouse anti-human TLR8 PE (clone 44C143) from IMGENEX, and rat anti-human TLR9/CD289 PE (clone eB72-1665) from eBioscience; San Diego, CA according to the manufacturer's protocol, using Fix&Perm reagents (Invitrogen Ltd, Paisley, UK). Antigen fluorescence intensity was expressed as median fluorescence intensity (MFI).

Statistical analysis

Statistical analyses were performed using the Graphpad Prism version 3.00 for Windows (Graphpad Software, San Diego, Calif., USA). The Mann–Whitney U-test was used to compare AD patient's and HS's data. $P < 0.05$ was considered statistically significant.

RESULTS

All 24 patients enrolled in this study reported an AD diagnosis.

A high IgE level (>100 kU/L) was found in 19/24 patients, therefore considered as extrinsic AD, while the remaining 5 patients were regarded as intrinsic AD. Eight out of 24 patients were erythrodermic, in the remaining 16 the eczematous lesions involved more than 40% of skin surface. No patient had active infection, but medical history documented in most of them (16/24) frequent episodes of skin infections, mainly consisting of staphylococcal or Herpes simplex infections. Two patients referred a previous Herpes Zoster episode. At the test time, 8 patients were in treatment with systemic prednisone at a dosage ranging between 10 and 25 mg/daily per os; the remaining 16 only with topical steroids (8 patients), tacrolimus (4 patients) or emollients (4 patients). Thereafter, due to disease extension, 10 patients underwent a systemic cyclosporine treatment at a dosage of 3 mg/kg/daily for three months with a complete resolution of cutaneous lesions. In 8 patients a short course of e.v. metilprednisolone was started at an initial dosage of 40 mg daily, then tapered in a few weeks and followed by UVB phototherapy. Three achieved a complete response and 5 a partial response. In the remaining 6 patients a topical tacrolimus treatment coupled with emollients allowed the partial control of the disease.

PMN functional activity against *K.pneumoniae* in AD patients and healthy subjects

In all the experiments the viability of PMNs remained unchanged throughout. Each phagocytosis and killing test was performed in triplicate, data were expressed as median and range. The pattern of phagocytosis and intracellular killing against *K. pneumoniae* by PMNs harvested both from HS and AD patients is shown in Table 1a and b. According to our results, PMNs from AD patients displayed a reduced phagocytic activity when compared to HS.

Indeed, PMNs harvested from HS phagocytized *K. pneumoniae* at a percentage ranging from 19.1 to 15.5% over a period of 90 minutes of incubation while the percentage of bacteria phagocytized

by the PMNs from AD patients was already significantly reduced after 30 and 60 minutes (15.6%, $p = 0.0133$ and 12.5%, $p = 0.0040$) (Tab 1). The reduced phagocytic activity was also accompanied by a decreased PMN efficiency in killing the ingested bacteria, with a survival index (SI) of 1.69 compared to 1.43 of the controls ($p < 0.0001$) after 30 minutes, and a total absence of killing ($SI > 2$) after 60 and 90 minutes, compared to 1.74 and > 2 of the controls.

The results were quite homogeneous and phagocytosis and intracellular killing defect were documented in each single patient, regardless to disease extension and severity. More in detail, looking at the phagocytosis results of single patients, only 2/21 showed a value of 17,8 at 60' which was reduced but near to the normal (median 19.7 range 16.8-21.4), the remaining being under 13. As to the killing results, the higher value detected was 1,9 at 60' in 3 patients, significantly lower than normal (median > 2 ; range 1.8- > 2), the remaining being always > 2 . We are therefore unable to correlate this defect with the clinical characteristics of patients' subgroups.

Cytometric detection of activation markers and Toll-Like Receptors on PMNs

Flow-cytometry did not reveal differences in the percentage values of CD11b, CD62L and CD66b, expressed by more than 95% of AD granulocytes, as in HS. On the contrary, CD11b (median: 1142,0 range: 660,0-3984,0) and CD66b (median: 1454,0 range: 735,0-3979) MFI was found to be significantly higher than in the controls (median 528,0 range 227,0-1583,0; $p=0,0007$), while the CD62L value was lower in AD patients than in the controls (median: 1748,0 range:1036,0-5348,0 vs median: 2012,0 range: 1370,2-2548,5) though not statistically significant (Fig. 1,2).

No statistically significant difference was observed in the percentage expression of TLR2, 4, 5, 8 or 9 in the PMNs between AD patients and HS (percentage of expression ranging from 80 to 95%), while MFI value was found to be significantly higher in patients with AD for TLR2 ($p=0.0004$), TLR4 ($p=0.0046$) and TLR5 ($p=0.0152$). It was also higher for TLR8 and TLR9 but not to a statistically significant degree (Fig 3,4).

As TLR2 is known to be also expressed on monocyte's surface, its presence was also evaluated on monocytes using a different gate in dot-plot FSC vs SSC: TLR2 was found on more than 97 % of monocytes both in AD patients and controls. Median MFI was 500 (range 311-795) in HS and 482,5 (range 303-702) in AD patients (Mann-Whitney test n.s.) (Fig 3).

DISCUSSION

Many defects in the innate immune system account for the increase in skin infections in atopics: skin barrier abnormalities, partially explained by the observation of loss-of-function mutations of the filaggrin gene, low levels of antimicrobial peptides, depletion of pDCs, which have been observed in lesional skin of atopics, and Th2-dominated immune response that weaken anti-microbial immunity. Phagocytes are the immune system's key defenders against bacterial infection, their primary function being to ingest and destroy invading pathogens, subjecting them to an array of both oxygen-independent and oxygen-dependent killing mechanisms. These include the release of antimicrobial and proteolytic peptides into the phagosomal space, along with the production of reactive oxygen species by the NADPH oxidase complex that assembles in the membrane [20].

In the present study we have focused our attention on PMN functional activity in AD patients, using a radioactive technique that allowed us to document an impairment of phagocytic activity against *K. pneumoniae*, a pathogen which can cause severe problems in immunosuppressed subjects. If compared to flow cytometric analysis, widely used to rapidly screen PMNs, this technique has the advantage to accurately reflect PMN *in vivo* behaviour. The impairment of PMN phagocytic activity was accompanied by a reduced PMN efficiency to kill ingested bacteria compared to that of healthy subjects. The data obtained seem to indicate that the decreased activity

of PMNs isolated from AD patients are at least partly related to an impairment of the oxidative pathways required for the efficient destruction of microorganisms.

In literature, only few papers centre on PMN functional abnormalities in AD patients: in particular, a study by Forte et al. on 19 young subjects with moderate to severe AD demonstrated a reduction in chemotactic response and phagocytic activity of neutrophils and monocytes [12] analyzing the phagocytosis of zymosan particles. Other reports describe a phagocytosis impairment and a reduced capacity to produce reactive oxygen species only in patients with concomitant bacterial infections [10-11, 13], also a β glucuronidase [21] and leukotriene B4 [22] release defect have been described. PMN functional abnormalities have also been documented in lymphoproliferative disorders such as multiple myeloma [23], B-cell chronic lymphocytic leukaemia patients with frequent infections [24] and cutaneous T cell lymphoma [16,25]. A phagocyte activation with increased MFI values of the adhesion molecules CD66b and CD11b was found in association with PMN functional impairment: this phenomenon could be rather a consequence [26] than a cause of frequent infections. In fact, it may be hypothesized that the increased permeability of the skin barrier to microbial components facilitates the penetration of bacterial molecules capable of stimulating immunological reactions, including the over-expression and hyperactivity of the TLRs. The CD11b upregulation has already been described by Yamada et al. [27] on eosinophils from atopic patients and could lead to PMN hyperadhesivity and interfere with PMN recruitment at the infection site. CD11b upregulation on granulocytes is known to be induced by a variety of soluble mediators as IL-8/CXCL8 and Eotaxin [28, 29]: the mechanisms underlying this phenomenon were not investigated in the present paper and further studies pointed to document an abnormal presence of chemokines upregulating CD11b and CD66b in AD patients are needed.

CD62L down-regulation has already been described both in elderly and long-term steroid-treated patients and seems to induce an adhesion defect which enhances the susceptibility to infections [30]. As microbial ligands activate the innate immune system response by binding to TLRs and activation of TLR may also lead to an excess of inflammation, we decided to complete our study with the TLR expression analysis on PMN membrane. TLRs are transmembrane proteins which differ from each other in ligand specificities, expression patterns and target genes they induce [31,32]. At least 11 TLRs have been identified in humans, expressed predominantly but not exclusively in antigen presenting cells such as macrophages, neutrophils (PMNs) and dendritic cells [33, 34]. Polymorphisms of TLR2 have been described in patients with severe AD but it is still under debate whether they are of clinical relevance [35-38].

So far, the only report in literature concerning TLR expression on PMN membrane in AD is that of Sümegi A et al. [39], describing an up-regulation of the CD14/TLRs system on PMN and monocytes, with TLR2 and TLR4 overexpression. According to this observation, we found an increased MFI of TLR2, which recognizes a wide spectrum of Gram-positive and Gram-negative bacteria including *S. aureus*, of TLR4, which recognizes Gram-negative bacteria through its ligand LPS, of TLR5 and also of TLR8 and TLR9, although not statistically significant.

In conclusion, focusing on an adult population affected by severe AD with lesions involving more than 40% of skin surface, our data evidence an impairment of PMN functional activities when compared to healthy subjects, displaying both reduced phagocytic activity and intracellular bacterial killing. At the moment we are unable to correlate this impairment with disease activity and previous treatments: a larger series, also including patients with limited disease, is needed in order to better clarify whether phenotypical and functional abnormalities are confined to a subgroup of patients with severe disease; actually, in our series an impairment of PMN functional activity was found in all patients. These data, in addition to the already known keratinocyte membrane disorders, could represent a major contributory cause of infection: a systematic screening of AD patients could

help in identifying a subset of patients particularly susceptible to infections and therefore worthy of closer follow-up or targeted treatment.

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Table 1. Functional activity of PMNs from healthy subjects (HS) and atopic dermatitis (AD) patients: **a.** *K. pneumoniae* percentage of phagocytosis (median; range) - **b.** Intracellular killing against *K. pneumoniae* (median; range).

a. PHAGOCYTOSIS (%)			
	30 min	60 min	90 min
HS	19.1 (16.2-20.8)	19.7 (16.8-21.4)	15.5 (14.1-17.0)
AD patients	15.6 (12.5-21.3)	12.5 (8.8-17.8)	11.5 (9.0-17.7)
Mann Whitney test	p =0.0133	p = 0.0040	p = 0.0030

b. INTRACELLULAR KILLING (Survival Index)			
	30 min	60 min	90 min
HS	1.43 (1.3-1.5) (57%*)	1.74 (1.4-2.0) (26%*)	> 2
AD patients	1.7 (1.5-1.8) (30%*)	> 2 (1.8->2)	> 2
Mann Whitney test	p < 0.0001	p = 0.0002	n.s.

* % percentage of initial bacterial population killed by PMNs

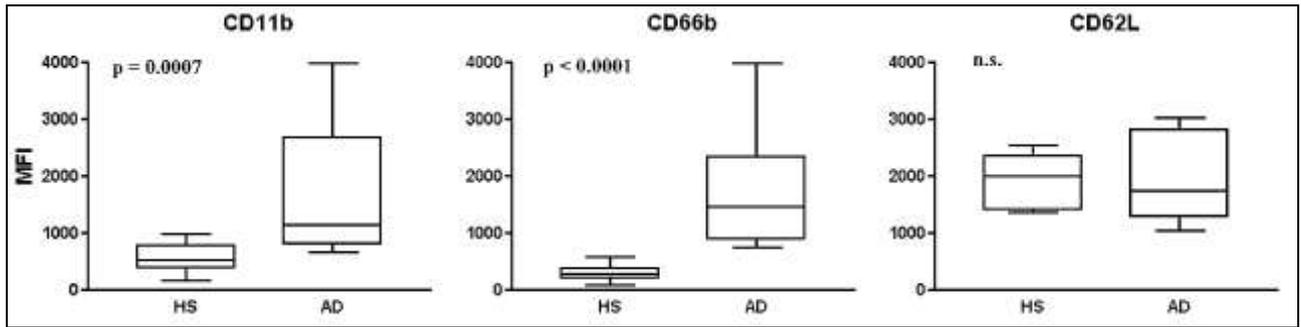


Figure 1. CD11b, CD66b and CD62L median fluorescence intensity (MFI) of granulocytes in healthy subjects (HS) and atopic dermatitis (AD) patients. Data are shown as box plots. Box plots show range, median, 25th and 75th percentile.

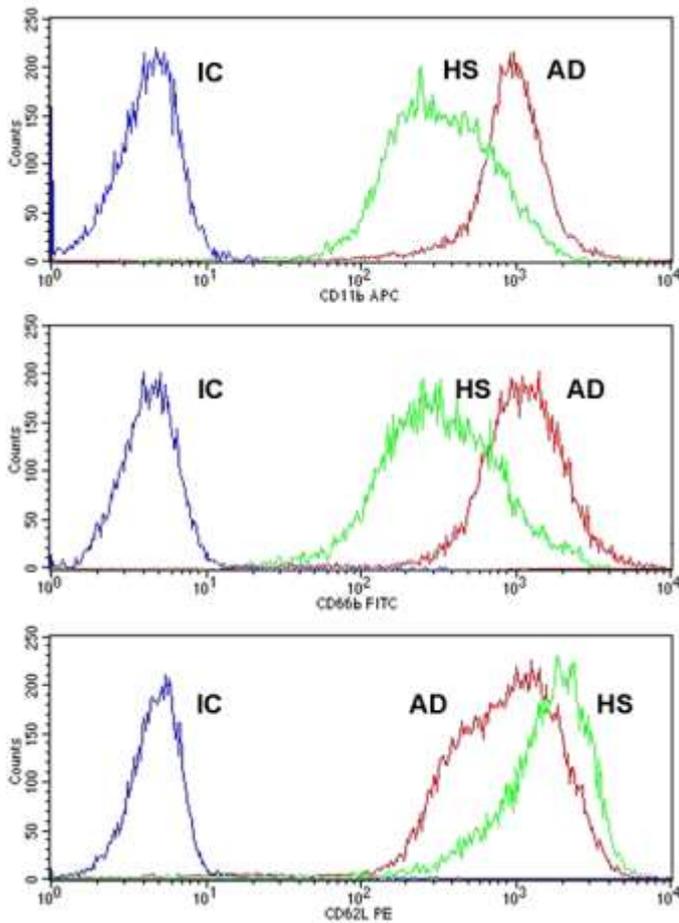


Figure 2. CD11b, CD66b and CD62L representative histogram (black line: isotype control; light gray line: HS; dark gray line: AD patient).

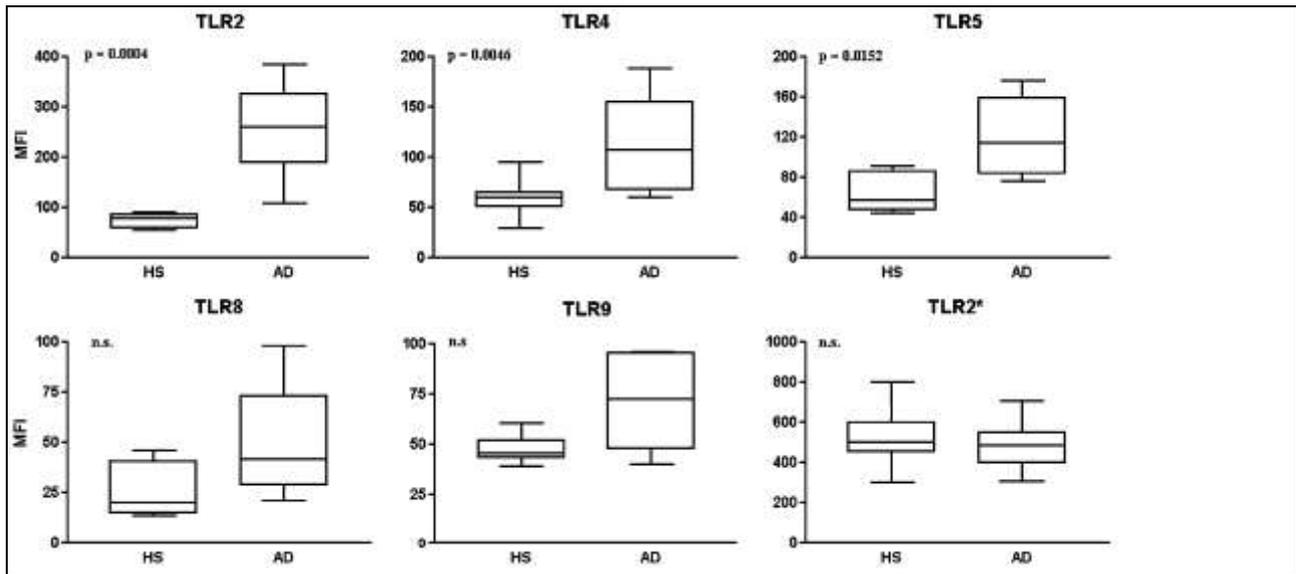


Figure 3. TLR 2, 4, 5, 8, 9 median fluorescence intensity (MFI) of granulocytes in healthy subjects (HS) and atopic dermatitis (AD) patients. * TLR 2 median fluorescence intensity (MFI) of monocytes in HS and AD patients. Data are shown as box plots.

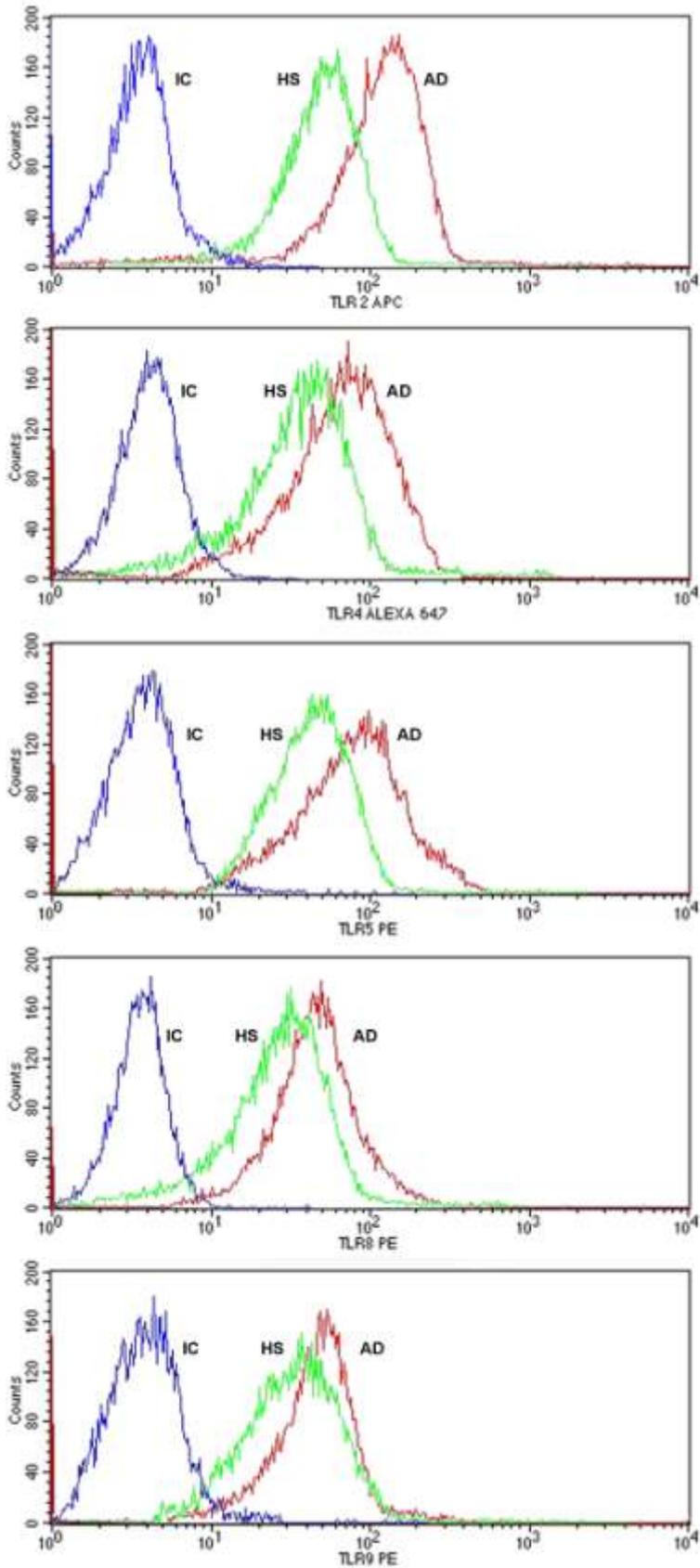


Figure 4. TLR5 representative histogram (black line: isotype control; light gray line: HS; dark gray line: AD patient).