A regulatory dendritic cell signature correlates with the clinical efficacy of allergen-specific sublingual immunotherapy

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Background: Given their pivotal role in the polarization of T-cell responses, molecular changes at the level of dendritic cells (DCs) could represent an early signature indicative of the subsequent orientation of adaptive immune responses during immunotherapy.
Objective: We sought to investigate whether markers of effector and regulatory DCs are affected during allergen immunotherapy in relationship with clinical benefit.
Methods: Differential gel electrophoresis and label-free mass spectrometry approaches were used to compare whole proteomes from human monocyte-derived DCs differentiated toward either regulatory or effector functions. The expression of those markers was assessed by using quantitative PCR in PBMCs from 79 patients with grass pollen allergy enrolled in a double-blind, placebo-controlled clinical study evaluating the efficacy of sublingual tablets in an allergen exposure chamber over a 4-month period.
Results: We identified several markers associated with DC1 and/or DC17 effector DCs, including CD71, FSCN1, IRF4, NMES1, MX1, TRAF1. A substantial phenotypic heterogeneity was observed among various types of tolerogenic DCs, with ANXA1, Complement component 1 (C1Q), CATC, GILZ, F13A, FKBP5, Stabilin-1 (STAB1), and TPP1 molecules established as shared or restricted regulatory DC markers. The expression of 2 of those DCs markers, C1Q and STAB1, was increased in PBMCs from clinical responders in contrast to that seen in nonresponders or placebo-treated patients.
Conclusion: C1Q and STAB1 represent candidate biomarkers of early efficacy of allergen immunotherapy as the hallmark of a regulatory innate immune response predictive of clinical tolerance. (J Allergy Clin Immunol 2012;129:1020-30.)

Key words: *Biomarker, dendritic cell, efficacy, proteomics, sublingual immunotherapy, tolerance*

Dendritic cells (DCs) are specialized antigen-presenting cells (APCs) with a unique capacity to integrate a variety of incoming signals to orchestrate adaptive immune responses. Bidirectional interactions between DCs and T cells eventually lead to either effector or tolerogenic responses, which are crucial to establish appropriate defense mechanisms while precluding uncontrolled inflammation.[1] Depending on the type of pathogen/danger signal encountered and the costimulatory molecules engaged, DCs are at the inception of immune polarization, with a capacity to support the differentiation of either effector T_H1, T_H2, T_H17, or suppressive/regulatory $CD4^+$ T cells.[2-5]

There is currently a great interest in characterizing molecular markers associated with polarized DCs (respectively termed DC1, DC2, DC17 and DCreg [DCs driving the differentiation of T_H1, T_H2, T_H17 and regulatory T {Treg} cells, respectively]), with the assumption that the latter could represent an early signature within the innate immune system indicative of the subsequent orientation of adaptive immune responses.[6] Such markers might have obvious applications to monitor the success of immunotherapy protocols because specific variations of innate immune responses were recently reported to be predictive of long-term adaptive responses induced after yellow fever or flu vaccination in human subjects.[6,7]

Herein we undertook to identify novel markers specific for subsets of polarized DCs that could be used to monitor the efficacy of allergen immunotherapy (AIT). Specifically, starting from monocyte-derived dendritic cells (moDCs), we generated various subtypes of effector and regulatory human DCs *in vitro* and compared their whole-cell proteomes by using 2 complementary quantitative proteomic strategies: differential gel electrophoresis (DiGE) and label-free mass spectrometry (MS). Among the markers identified for DC1, DC17, and DCreg subsets, we report that complement component 1 (C1Q) and the receptor stabilin-1 (STAB1) are associated with tolerogenic DCs and that their induction in PBMCs is indicative of clinical responses induced by AIT.

METHODS

Clinical samples from VO56.07A pollen chamber study

After an initial screening visit, 89 eligible patients were randomized 1:1 to receive either a grass pollen or placebo tablet through the sublingual route.

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Abbreviations used

AIT: Allergen immunotherapy
ANR: Active nonresponder
ANXA1: Annexin-1
APC: Antigen-presenting cell
AR: Active group, responder patients
ARTSS: Average Rhinoconjunctivitis Total Symptom Score
ASP: *Aspergillus oryzae*
C1Q: Complement component 1
CATC: Cathepsin C
CBA: Cytometric beads array
CD71: Transferrin receptor protein 1
DC: Dendritic cell
DC1: DCs driving the differentiation of T_H1 cells
DC2: DCs driving the differentiation of T_H2 cells
DC17: DCs driving the differentiation of T_H17 cells
DCreg: DCs driving the differentiation of regulatory T cells
DEX: Dexamethasone
DiGE: Differential gel electrophoresis
F13A: Factor 13A
FDR: False discovery rate
FKBP5: FK506 binding protein 5
FSCN1: Fascin 1
GILZ: Glucocorticoid-induced leucine zipper
IDO: Indoleamine 2,3-dehydrogenase
ILT: Immunoglobulin-like transcript
IRF4: Interferon regulatory factor 4
moDC: Monocyte-derived dendritic cell
MS: Mass spectrometry
MX1: Myxovirus resistance 1
NMES1: Normal mucosa of esophagus-specific gene 1 protein
qPCR: Quantitative PCR
PGN: Peptidoglycan
PNR: Placebo nonresponder
PR: Placebo responder
RALDH: Retinaldehyde dehydrogenase
RAPA: Rapamycin
ROC: Receiver operating characteristic
STAB1: Stabilin-1
TPP1: Tripeptidyl peptidase 1
TRAF1: TNF receptor-associated factor 1
Treg: Regulatory T
VitD3: 1,25 dihydroxyvitamin D3

Challenges were performed before treatment and after 1 week and 1, 2, and 4 months of treatment. Because patients were challenged before treatment, individual clinical responses were evaluated by calculating percentages of improvement in Average Rhinoconjunctivitis Total Symptom Scores (ARTSSs) between baseline and after 4 months of treatment. The median percentage ARTSS improvement in the active group (corresponding to at least a 43.9% decrease of ARTSS after treatment) was considered a threshold to identify clinical responders. Subjects with a percentage of ARTSS improvement greater than or equal to this threshold were considered responders, and those with improvement lower than the threshold were considered nonresponders. Immunologic results were described for 4 subgroups, including active responders (ARs; n = 21), active nonresponders (ANRs; n = 20), placebo responders (PRs; n = 7), and placebo nonresponders (PNRs; n = 31). Whole blood was collected in 79 patients before and after treatment for serum measurements and cellular assays. PBMCs were purified from blood samples and frozen. At the end of the study, samples were thawed, maintained for 48 hours in culture, washed, and used for quantitative PCR (qPCR) analysis to measure the mRNA expression of candidate markers. All samples were coded and analyzed in a blind manner by the operators.

MoDC polarization

MoDCs were generated from PBMCs from healthy volunteers, and 10^7 DCs were plated in the presence of either medium, dexamethasone (DEX; 1 μg/mL; Sigma, St Louis, Mo), LPS from *Escherichia coli* (1 μg/mL; InvivoGen, San Diego, Calif), or peptidoglycan (PGN) from *Staphylococcus aureus* (10 μg/mL) for 24 hours at 37°C and 5% CO_2 (see Fig E1, Model A, in this article's Online Repository at www.jacionline.org). For tolerogenic DC models (see Fig E1, Model B), cells were cultured for 24 hours with either DEX or proteases from *Aspergillus oryzae* (ASP, 20 μg/mL; Sigma)[8] or incubated during the differentiation step with either DEX, IL-10 (10 ng/mL; R&D Systems, Minneapolis, Minn), TGF-β (20 ng/mL; R&D Systems), rapamycin (RAPA, 10 nmol/L; Sigma), or 1,25 dihydroxyvitamin D3 (VitD3, 10 nmol/L; Sigma). Drugs were added to cultures at day 1, with fresh medium provided every other day. Treated DCs were stimulated with LPS (1 μg/mL) for 24 hours to monitor a potential anti-inflammatory effect.

DC/T-cell coculture experiments

Treated DCs were cultured with allogeneic naive $CD4^+$ T cells at a 1:10 DC/T-cell ratio for 5 days. Naive $CD4^+$ T cells were isolated from PBMCs by means of negative selection with the MACS naive CD4 isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany). Such naive T cells were confirmed to have a purity greater than 95% based on CD3, CD4, and CD45RA expression evaluated by means of flow cytometry. Supernatants were analyzed for cytokine release, as described in the Methods section in this article's Online Repository at www.jacionline.org.

Statistical analysis

Data are expressed as means ± SEMs. Statistical differences between groups were assessed by using 2-tailed nonparametric tests (Wilcoxon and Mann-Whitney tests for paired or independent data, respectively, and the Friedman test for multiple comparisons. Treatments were compared with controls, and *P* values of less than .05 or .01 were considered significant. Correlation analyses were performed by using the nonparametric Spearman test, and receiver operating characteristic (ROC) analyses were assessed by using an empiric model. Statistical and graphic analyses were performed with Prism5 software (GraphPad Software, Inc, La Jolla, Calif). Significant differences in protein expression changes in DiGE analysis and in peptide abundance in label-free MS experiments were assessed by using multiple comparison tests and a false discovery rate (FDR)–adjusted *P* value threshold of .05 and .01, respectively. Statistics on proteomic data were performed with 2 software programs from Nonlinear Dynamics (Newcastle upon Tyne, United Kingdom) called Samespots or Progenesis LC-MS.

For detailed information on the characterization of effector and regulatory DCs, RNA isolation, qPCR, Western blotting, and proteomic studies (DiGE and label-free MS), please refer to the Methods section in this article's Online Repository.

RESULTS

Establishment of human effector DC1, DC17, and tolerogenic DC subsets

After a screening of 50 biological and pharmacologic agents, we selected 3 molecules capable of inducing either effector or tolerogenic DCs from moDCs. The bacterial LPS was the most potent inducer of the effector DC1 subset, whereas the PGN from the *Staphylococcus aureus* wall was the best inducer of the DC17 subset. As shown in Fig 1, *A*, LPS-DCs and PGN-DCs upregulated the expression of costimulatory but not inhibitory molecules, with the exception of the immunoglobulin-like transcript (ILT) 4, which was induced by LPS treatment. Such treated DCs also upregulated indoleamine 2,3-dioxygenase *(IDO)* gene expression and secreted high amounts of IL-6 and IL-8 (Fig 1, *B* and *C*). LPS-DCs secreted IL-12p70 and TNF-α in

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