

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:	<i>Aspergillus oryzae</i>
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 _H 1 cells
DC2:	DCs driving the differentiation of T _H 2 cells
DC17:	DCs driving the differentiation of T _H 17 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 10⁷ 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; Invivo-Gen, San Diego, Calif), or peptidoglycan (PGN) from *Staphylococcus aureus* (10 µg/mL) for 24 hours at 37°C and 5% CO₂ (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

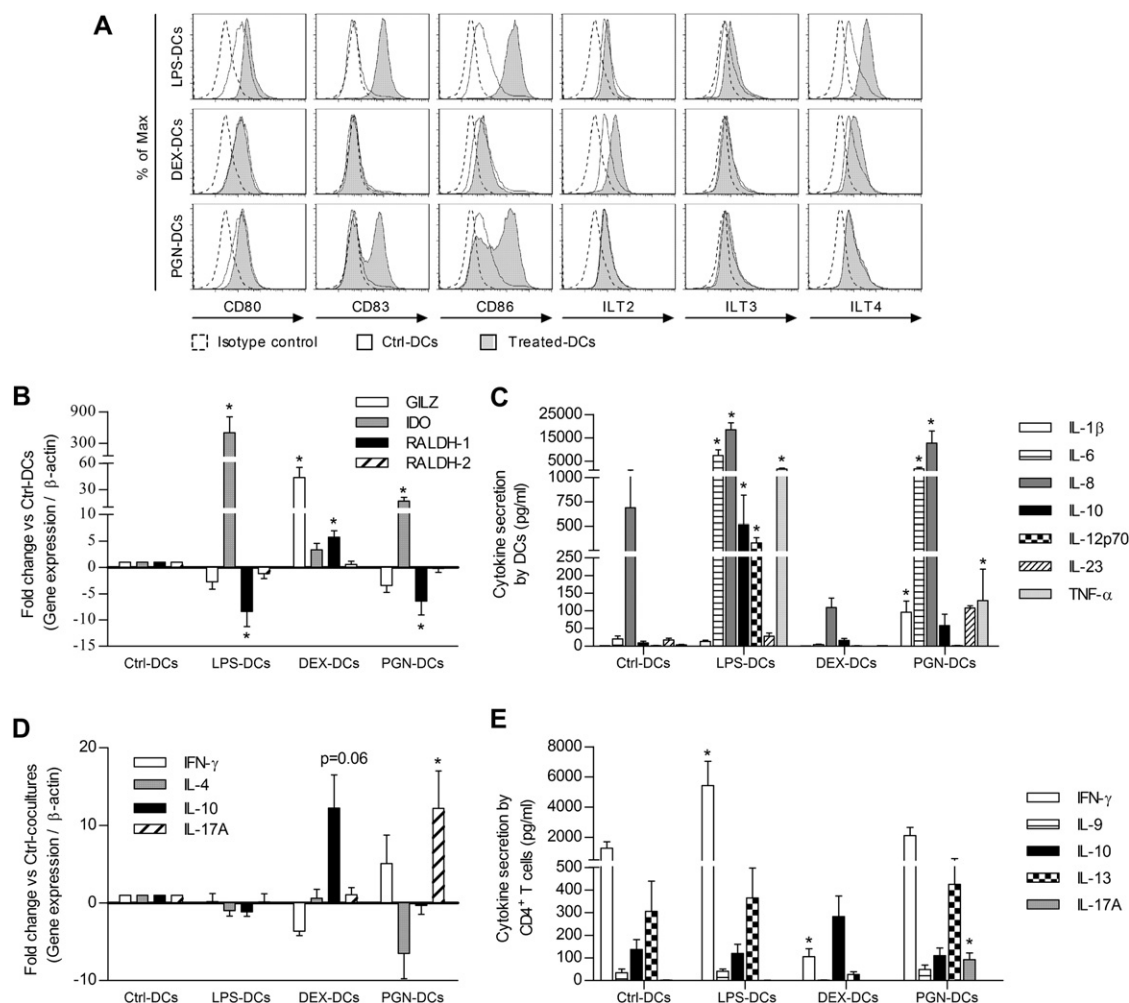


FIG 1. *In vitro* treatment of DCs with LPS, PGN, or DEX induces proinflammatory and tolerogenic DCs. **A**, Cell-surface phenotype was assessed by using FACS. **B**, Expression of tolerogenic genes was assessed by using qPCR. **C–E**, Cytokine production in DCs or cocultures with CD4⁺ T cells was analyzed by using ELISA, cytometric beads array (CBA), or qPCR. A representative donor is presented in Fig 1, A. Data are shown as means $\pm$ SEMs (n = 6) in Fig 1, B to E. *A P value of less than .05 was considered significant (Wilcoxon test).

contrast to PGN-DCs, which produced IL-1 β and IL-23 (Fig 1, C). Importantly, cocultures with naive CD4⁺ T cells confirmed a DC17 and DC1 polarization, respectively, because *IL17A* gene expression was enhanced in PGN-DC/CD4⁺ T-cell cocultures (Fig 1, D), whereas incubation with LPS-DCs induced IFN- γ secretion by T cells at day 5 (Fig 1, E). As we previously reported,⁸ treatment of DCs with DEX led to the generation of tolerogenic DCs, upregulating the expression of ILT2 and ILT4 molecules, as well as genes, such as the glucocorticoid-induced leucine zipper (*GILZ*), *IDO*, or retinaldehyde dehydrogenase 1 (*RALDH1*; Fig 1, A and B). CD4⁺ T cells cocultured with DEX-DCs were *bona fide* T_H1 cells because they upregulated IL-10 (Fig 1, D and E), were forkhead box protein 3 negative, and exhibited a suppressive activity in third-party experiments.⁸ All these comparisons were performed with a nonparametric Wilcoxon test and a P value for significance of less than .05. Altogether, these cellular assays confirmed that the effector DC1, DC17, and DCreg subsets can be derived *in vitro* from human moDCs.

Identification by using DiGE of molecular markers for effector and tolerogenic human DCs

We subsequently investigated potential differences in protein expression between control-DCs, LPS-DCs, DEX-DCs, and PGN-DCs generated from 6 independent donors. To this aim, we first relied on 2-dimensional DiGE for quantitative comparison of DC proteomes (see Fig E1). In these analyses a total of 1250 protein spots could be precisely quantified in human DCs, as shown in representative 2-dimensional patterns (Fig 2, A and B). Of those, 52 spots were differentially expressed under at least 1 condition, with an FDR P value of less than .05 and a minimum of 1.2-fold change in volume (multiple comparison test). As shown in Table E1 in this article's Online Repository at www.jacionline.org, 46 (88%) differentially expressed spots were identified by means of MS, corresponding to 38 nonredundant proteins. Identified proteins were further clustered based on similar expression profiles (see Table E1, A–D). To validate our findings, we selected some candidate markers for effector or tolerogenic DCs and assessed their expression by using Western blotting or

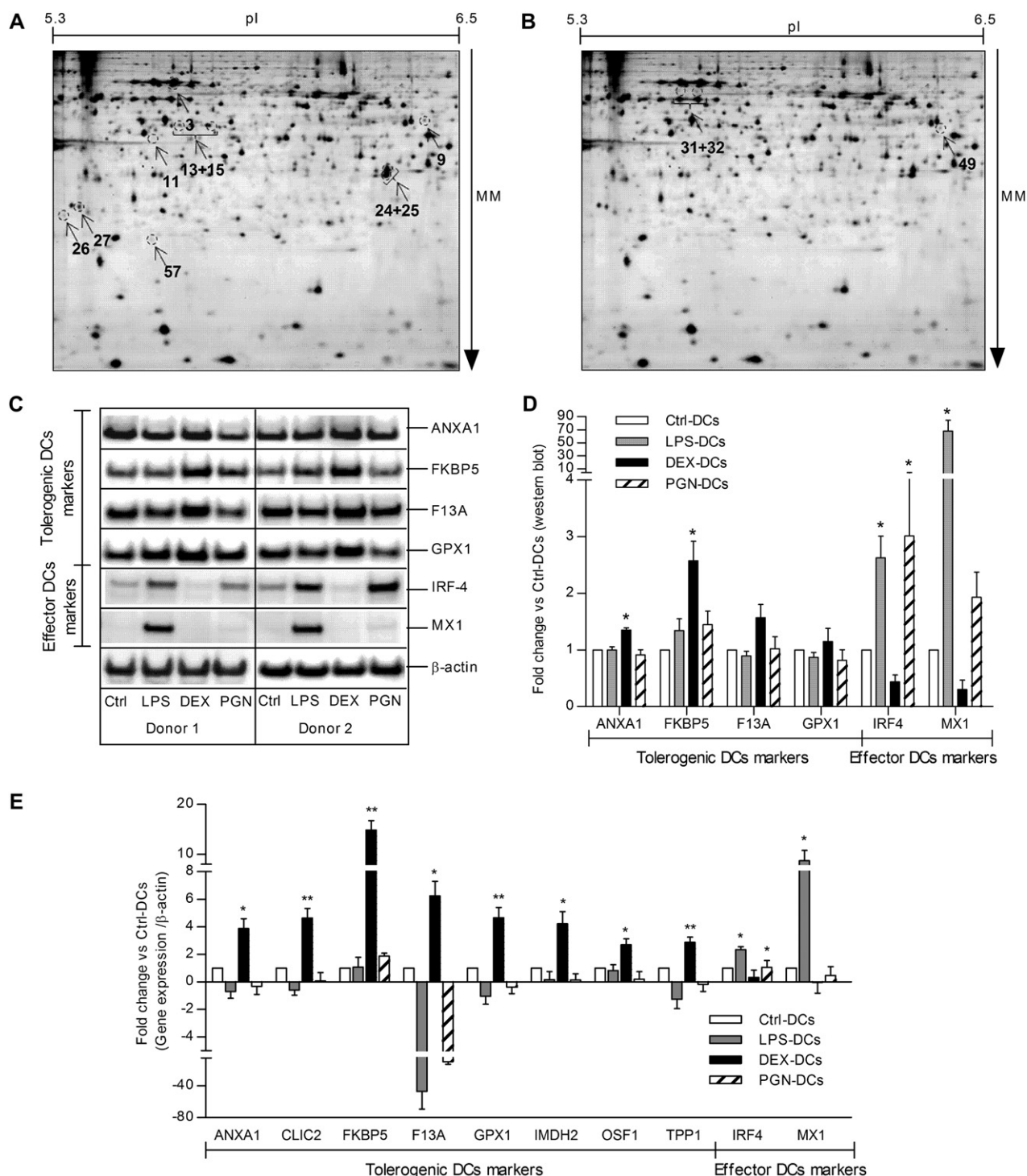


FIG 2. Markers of regulatory and effector DCs identified by using 2-dimensional DiGE. **A** and **B**, Representative gels with localization of differentially overexpressed proteins (Fig 2, A: regulatory DC proteins, spot no. 3 = F13A; 9 = IMDH2, 11 = TPP1, 13/15 = FKBP5, 24/25 = ANXA1, 26 = OSF1, 27 = CLIC2, and 57 = GPX1; Fig 2, B: effector DC proteins, no. 31/32 = MX1, 49 = IRF4). **C** and **D**, Western blot analyses of target proteins. **E**, Validation of gene expression by using qPCR. Two representative donors are presented in Fig 2, C. Means $\pm$ SEMs ($n = 6$) are presented in Fig 2, D and E. P values of less than .05 (*) or .01 (**) were considered significant (Wilcoxon test).

qPCR. Representative protein and gene expression data are shown in Fig 2, C to E. Whereas the IRF4 protein was significantly induced under both effector conditions, MX1 was only strongly

overexpressed in LPS-DCs. Two markers of regulatory DCs were confirmed to be statistically overexpressed in DEX-DCs at the protein level (ANXA1 and FKBP5), whereas the validity of

the other molecules as markers was confirmed at the mRNA level (Fig 2, E). The known function of each of those potential markers of regulatory or effector DCs in immunity/tolerance is summarized in Table E2 in this article's Online Repository at www.jacionline.org.

Identification by means of label-free MS of molecular markers for effector and tolerogenic human DCs

To overcome some limitations of DiGE (eg, overlooking of molecules with extreme isoelectric points, molecular masses, and hydrophobicity), we initiated label-free MS-based approaches and further compared protein expression profiles between treated DCs (see Fig E1). As shown in Fig 3, A, an analysis of DC peptides resulted in the detection of 33,500 isotope patterns (ie, features characterized by specific retention times and mass over charge [m/z] ratios), which were further quantified with the Progenesis LC-MS software. Up to 945 features were detected as significantly differentially expressed under at least 1 DC treatment condition (with an FDR *P* value of less than .01 and a fold increase of minimum 1.5 [multiple comparison test]). As a representative example, Fig 3, B, shows a higher abundance of the m/z 865.70 molecular ion in DEX-DCs in comparison with control-DCs, LPS-DCs, and PGN-DCs (with an abundance of 16,300 vs 8,700, 5,800, and 6,095, respectively). Differentially regulated peptides were subsequently fragmented in MS/MS mode, leading to the identification of proteins further matched to sequence databases. Up to 354 features were identified, representing a total of 190 nonredundant proteins (≥ 1 peptide, data not shown). To increase the stringency and accuracy of protein quantification, only proteins identified with 2 or more peptides were included in the final analysis, representing a total of 60 differentially expressed proteins. Fifty-three of those proteins were significantly upregulated or downregulated in effector DCs, whereas 7 proteins were specifically upregulated in tolerogenic DCs, as summarized in Table E3 in this article's Online Repository at www.jacionline.org. Also included in this list are 3 proteins identified with 1 peptide (ie, ITAM, MX1, and CLIC2) which were previously shown to be regulated in DCs by DiGE (see Table E1 and Fig 2), as well as PGRP1 and C1QB exhibiting a greater than 90- and 2.5-fold increase in PGN-DC and DEX-DC, respectively. Proteins were subsequently clustered based on abundance within each type of polarized DC (see Table E3). Interestingly, the 2 proteomic approaches confirmed the upregulation of FSCN1 and MX1, as well as the downregulation of ITAM in effector DCs. Two proteins (ie, ICAM1 and TRAF1) previously shown by others to be increased in effector DCs^{9,10} were also detected, thus validating our label-free MS approach. Furthermore, we reported 9 proteins with levels consistently increased in tolerogenic DEX-DCs when compared with those seen in control-DCs, LPS-DCs, and PGN-DCs (see Table E3, A). It is noteworthy that 4 of those proteins (ANXA1, CLIC2, F13A, and FKBP5) had been identified in our 2-dimensional DiGE analysis, whereas the other proteins (C1QB, C1QC, CATC, MRC1, and STAB1) were only detected when using the LC-MS approach. Most interestingly, C1QC-specific peptides (eg, m/z 964.46 molecular ion; Table E3, A) were increased by up to a 5-fold in DEX-DCs. We next performed validation experiments by using both Western blotting and qPCR (Fig 3, C-E). Three protein markers for effector DCs (CD71, NMES1, and TRAF1) were confirmed to be strongly upregulated

in both LPS-DCs and PGN-DCs (Fig 3, C and D). Importantly, Western blot analyses confirmed significantly higher levels of C1Q, CATC, MRC1, and STAB1 in DEX-DCs (by 12-, 1.5-, 1.4-, and 2.2-fold, respectively) in comparison with levels seen in effector DCs. Moreover, as shown in Fig 3, E, the expression of genes encoding *C1Q*, *CATC*, *MRC1*, *STAB1* or *CD71*, *FSCN1*, *NMES1*, and *TRAF1* was significantly increased in DEX-DCs or effector DCs, with up to a 12-fold increase in *C1QA*, *C1QB*, and *STAB1* mRNAs. The known function of each of those molecules associated with tolerogenic or effector DCs in immunity/tolerance is summarized in Table E2. Altogether, experiments conducted using 2 distinct analytic methods led to the identification of several candidate markers for effector DCs (eg, CD71, FSCN1, NMES1, and TRAF1), as well as regulatory DCs (including C1QA, C1QB, C1QC, CATC, MRC1, and STAB1).

Assessment of candidate marker expression in distinct subtypes of tolerogenic DCs

We further investigated the expression of the most promising tolerogenic markers in various types of regulatory DCs obtained under distinct cell-culture conditions. To generate a variety of tolerogenic DCs, we either treated moDCs with proteases from *Aspergillus oryzae* for 24 hours⁸ or cultured monocytes during the differentiation step with either DEX, IL-10, RAPA, VitD3 or TGF- β for 7 days, as reported by others.¹¹⁻¹⁵ The detailed phenotype and functional characterization of those cells is shown in Fig E2 and described in the Results section in this article's Online Repository at www.jacionline.org. The expression of candidate markers identified either through our quantitative proteomic studies (listed in Table E2) or from the literature (ie, GILZ, IDO, RALDH1, and RALDH2)¹⁶⁻¹⁸ was assessed in the 6 types of tolerogenic DCs by using qPCR and Western blotting (Fig 4). Of those experiments, we could define 5 subgroups of candidate markers for regulatory DCs. ANXA1, CATC, and GILZ are expressed either as a protein or as mRNA in all tolerogenic DC types and thus represent panregulatory DC markers. A second group of markers encompasses C1Q and TPP1, which are associated with most tolerogenic DCs, with the exception of ASP-DCs and TGF- β -DCs. A third group comprising CLIC2, FKBP5, GPX1, IMDH2, and OSF1 proteins were upregulated in both DEX_{24h}/diff-DCs and RAPA-DCs, thus representing a family of immunosuppressant-induced proteins. Furthermore, F13A, MRC1, and STAB1 proteins were consistently and jointly upregulated in IL-10-DCs and DEX_{24h}/diff-DCs. Lastly, the overexpression of *IDO*, *RALDH1*, and *RALDH2* genes was restricted to ASP-DCs and DEX_{24h}/diff-DCs, which is in agreement with our previous report.⁸ All these comparisons were performed with a nonparametric Friedman test and a *P* value for significance of less than .05. Collectively, our data document a substantial phenotypic heterogeneity among known tolerogenic DCs and also highlight the broad relevance of ANXA1, C1Q, CATC, GILZ, and TPP1 molecules as shared regulatory DC markers.

Assessment of markers for effector/tolerogenic DCs in PBMCs from patients undergoing AIT

In light of the known effect of AIT on adaptive immune responses,¹⁹⁻²¹ we investigated a potential shift from effector to tolerogenic DCs during treatment. To test this hypothesis, we assessed the expression of genes encoding our candidate markers in

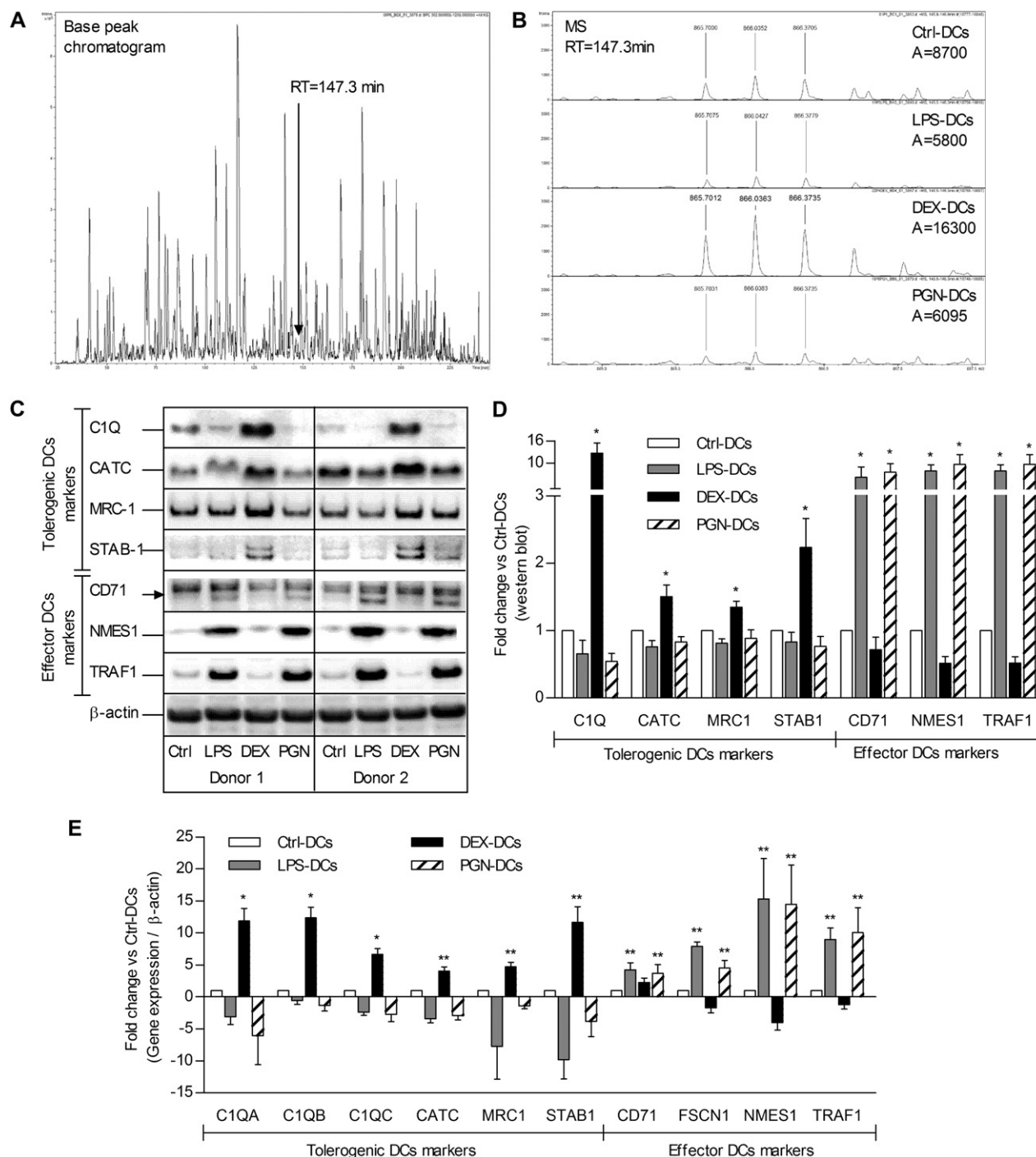
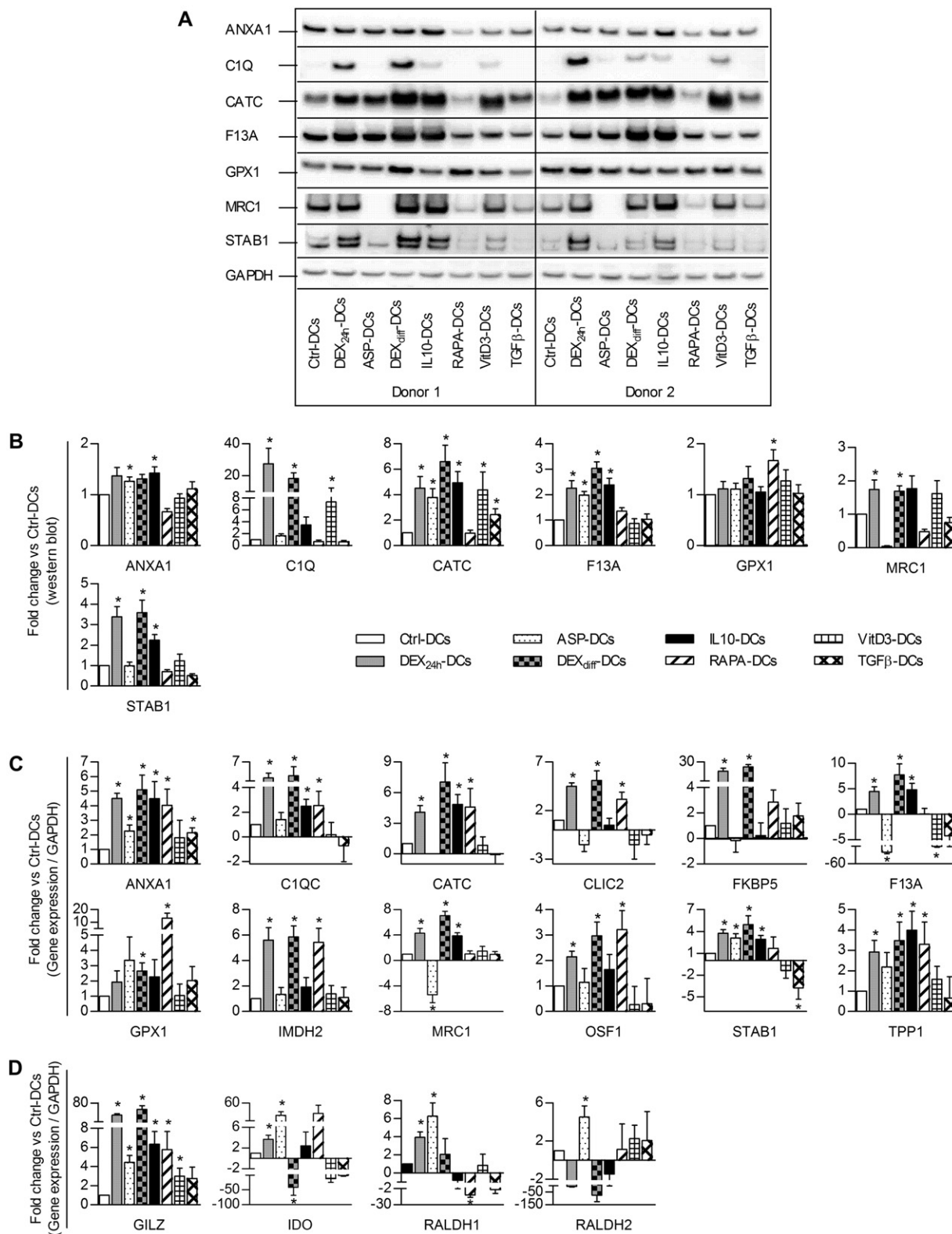


FIG 3. Markers of regulatory and effector DCs identified by using label-free MS. **A**, Peptide separation obtained along the retention time (RT) and leading to the quantification of 33,500 isotope patterns. **B**, NanoLC-MS quantitation of molecular ion at m/z 865.70. **C** and **D**, Western blot analyses of target proteins. **E**, Validation of gene expression by means of qPCR. Two representative donors are presented in Fig 3, C. Means $\pm$ SEMs of 6 independent experiments are presented in Fig 3, D and E. *P* values of less than .05 (*) or .01 (**) were considered significant (Wilcoxon test).

PBMCs collected from 79 patients enrolled in a double-blind, placebo-controlled clinical study recently conducted in an allergen challenge chamber to evaluate a sublingual grass pollen allergy vaccine.²² In this study clinical improvement was monitored individually, confirming a relative mean improvement in ARTSS of 29.3% after 4 months of treatment in the active versus the placebo

group ($P < .0003$).²² Because PBMCs contain less than 0.5% to 1% DCs, we first selected a shortlist of 17 candidate markers based on gene expression data reported in various blood cell populations (bioGPS database) to eliminate those expressed at high levels by either T, B, natural killer, polynuclear, or endothelial cells. All selected genes encoding regulatory (ie, *ANXA1*,



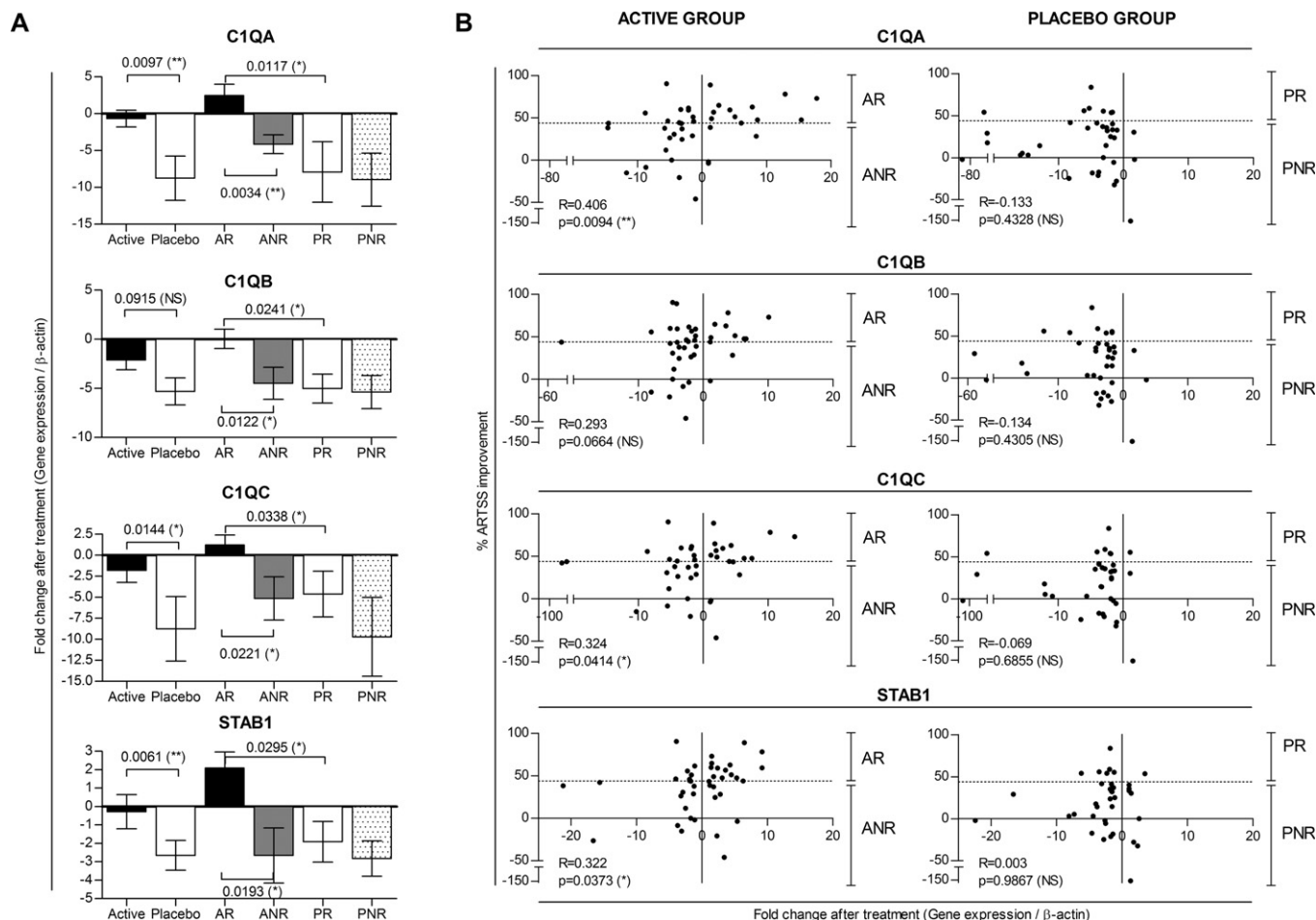


FIG 5. Induction of *C1Q* and *STAB1* genes in PBMCs from patients with grass pollen allergy receiving AIT correlates with clinical efficacy. **A**, mRNA expression of *C1QA*, *C1QB*, *C1QC*, and *STAB1* in PBMCs from 41 patients in the active group in comparison with the placebo group ($n = 38$) or in clinical responders versus nonresponders (percentage of ARTSS improvement $\geq 43.9\%$ and $< 43.9\%$, respectively). P values of less than .05 were considered significant (Mann-Whitney test). **B**, Spearman correlation of mRNA expression with percentages of ARTSS improvement in patients from the active and placebo groups after 4 months of AIT (ARs, $n = 21$; ANRs, $n = 20$; PRs, $n = 7$; and PNRs, $n = 31$).

CATC, *C1QA*, *C1QB*, *C1QC*, *CLIC2*, *F13A*, *GILZ*, *IDO*, *MRC1*, *RALDH1*, and *STAB1*) or effector (ie, *CD71*, *FSCN1*, *MX1*, *NMES1*, and *TRAF1*) markers were first assessed by using qPCR in a subgroup of 20 patients. Our data indicated no significant changes in most of the tolerogenic DC markers (see Fig E3) and in none of the effector DC markers during AIT (see Fig E4). Importantly, a strong increase in the expression of *C1Q* (subunits A, B and C) and *STAB1* was detected in PBMCs from patients in the active group but not in the placebo group. Parallel experiments conducted on sorted leukocytes from peripheral blood of 3 healthy donors confirmed that monocytes (and likely monocyte-derived tolerogenic APCs) are the most prominent source of *C1Q* and *STAB1* gene expression in the blood (see Fig E5). On the basis of these results, the expression of these candidate regulatory markers was tested in the whole cohort ($n = 79$ patients). The expression of 4 genes encoding effector DC markers (*CD71*, *FSCN1*, *MX1*, and *TRAF1*) was also monitored in these patients as a control. Altogether, our data confirmed a statistically significant regulation of *C1QA*, *C1QC*, and *STAB1* (and a comparable trend for *C1QB*) genes in PBMCs from patients in the active

group when compared with the placebo group (with P values of .0097, .0144, and .0061, respectively; Mann-Whitney test; Fig 5, A). Even more interestingly, *C1QA*, *C1QB*, *C1QC*, and *STAB1* were mostly upregulated in those patients with a confirmed clinical response to the treatment in contrast to ANRs, in whom a downregulation was observed (with P values of .0034, .0122, .0221, and 0.0193, respectively; Mann-Whitney test). Differences in levels of expression were also documented between ARs and PRs (with P values of .012, .024, .034, and .030; Mann-Whitney test), confirming that *C1Q* and *STAB1* are selectively induced by the treatment. When plotted against percentages of ARTSS improvement for each patient, *C1Q* and *STAB1* mRNA expression levels were significantly correlated with clinical benefit in patients from the active group (with Spearman correlations of 0.41 [$P = .009$] for *C1QA* and 0.32 [$P = .037$] for *STAB1*; Fig 5, B), whereas no such correlation was observed in placebo-treated patients.

The pertinence of these potential biomarkers of efficacy was further assessed by using an ROC analysis. Areas under the ROC curves were 0.77, 0.73, 0.71, and 0.71 for *C1QA*, *C1QB*, *C1QC*,

and *STAB1*, respectively, confirming that these markers are useful to discriminate clinical responders from nonresponders (with *P* values of .0033, .0118, .0210, and .0187, respectively).

Lastly, no significant difference was detected in the expression of effector genes between the active and placebo groups (see Fig E6, A), and no correlation was observed with symptom improvement (see Fig E6, B), indicating that the clinical efficacy of AIT is not associated with significant changes in effector DC markers. Collectively, our data establish *C1Q* and *STAB1* as markers associated with clinical tolerance induced by AIT, thereby indicating that short-term efficacy is linked to a regulatory immune response.

DISCUSSION

A broadly accepted paradigm to explain the clinical efficacy of AIT is a modulation of specific CD4⁺ T-cell responses from a T_H2 toward a T_H1 Treg cell pattern associated with a downregulation of IgE secretion by B cells and a decrease in mast cell/basophil activation.¹⁹⁻²¹ In this regard the capacity of DCs to initiate and orient effector and Treg cell responses implies that these cells contribute to both allergic inflammation and its resolution.²³ Specifically, there is now ample evidence that DCs play a key role in allergic sensitization through their capacity to induce and maintain allergen-specific T_H2 responses.⁵ In contrast, tolerogenic DCs have been detected at mucosal surfaces, such as within the oral mucosa, and have proved critical to establish clinical tolerance after sublingual AIT through the induction of T_H1 and Treg cell responses in draining cervical lymph nodes.²⁴⁻²⁸ Collectively, these observations support the hypothesis that the polarization of immune responses could be assessed at the level of DCs with immediate application to the follow-up of AIT.

In this context we proceeded to identify markers associated with moDCs polarized toward either DC1, DC17, or DCreg subsets, with a final goal to investigate whether the balance between effector and tolerogenic DCs is impacted during AIT. As a first step, we implemented 2 quantitative proteomic strategies to compare whole proteomes from effector versus tolerogenic DCs. As a result, we identified NMES1 as a new marker and confirmed the pertinence of other effector DC markers (ie, FSCN1, IRF4, TRAF1, or MX1). In parallel, 12 molecules (ANXA1, C1Q, CATC, CLIC2, F13A, FKBP5, GPX1, IMDH2, MRC1, OSF1, STAB1, and TPP1) were found to be overexpressed in various types of tolerogenic DCs. Interestingly, patterns of expression of regulatory markers revealed a substantial phenotypic heterogeneity among tolerogenic DCs, with only 3 panregulatory DC markers (ie, ANXA1, CATC, and GILZ). Several markers (eg, CLIC2, GPX1, OSF1, and TPP1) had never been described in the context of tolerance, whereas the expression of others had previously been reported in APCs located in tolerogenic environments. For example, F13A⁺ APCs, characterized by CD11b, MRC1, and STAB1 expression, were described among dermal DCs producing retinoic acid and inducing forkhead box protein 3-positive Treg cells, indicating a clear tolerogenic phenotype.²⁹⁻³¹ Our results are also consistent with the observation that tolerogenic DCs/macrophages contributing to fetal implantation in the human decidua coexpress MRC1, F13A, and STAB1, as well as other known tolerogenic markers, such as ILT2, ILT4, and IL-10.^{32,33} Interestingly, several recent studies also highlight the importance of C1Q, CATC, and F13A in fetomaternal tolerance. Thus, although our markers have been defined by using *in vitro*-generated MoDCs, those combined studies suggest that

such markers have a broad relevance in immunoregulation.³⁴⁻³⁶ Moreover, a direct tolerogenic effect of C1Q on DCs was even suggested in that the differentiation of DCs in the presence of this complement component gives rise to cells with a low expression of costimulatory molecules and a blunted capacity to secrete cytokines.³⁷

To investigate whether markers of effector or tolerogenic DCs are affected during AIT, we took advantage of a randomized, double-blind, placebo-controlled sublingual AIT study conducted in an allergen chamber in a cohort of patients with grass pollen allergy.²² Importantly, because patients underwent allergen challenges before and after AIT, we could investigate the potential correlation between the changes in DC markers and clinical benefit at an individual level. Although no functional assays are available to confirm the presence of human regulatory DCs *in vivo*, *C1Q* and *STAB1* mRNA levels increased significantly in PBMCs from patients with grass pollen allergy receiving the active tablet and exhibiting a clinical response in contrast to levels seen in ANRs or patients receiving placebo. As a control, no difference between groups was detected regarding the expression of effector DC markers. It is noteworthy that the upregulation of *C1Q* and *STAB1* was observed in 50% to 60% of patients with AR. The lack of expression of such markers in PBMCs from some responders can be explained by either a contribution of the placebo effect in clinical responses or alternatively by the involvement of other immune mechanisms distinct from DCreg responses in tolerance induction.

One limit in our study is that we have yet to identify *bona fide* markers for DCs supporting the differentiation of T_H2 cells. This DC2 subset is involved in the induction of allergic inflammation, and numbers of these cells are predicted to decrease during successful immunotherapy. Unexpectedly, in contrast to previous reports,^{38,39} we did not detect in the present study any upregulation of CD4⁺ Treg cells and more generally any change in the balance of T_H1/T_H2/T_H17/Treg cells in the peripheral blood of patients exhibiting clinical benefit (unpublished data). This apparent discrepancy might be explained by the fact that allergen-specific regulatory CD4⁺ T cells induced during immunotherapy are only transiently found in peripheral blood before migrating to mucosal surfaces,³⁹ whereas regulatory DCs might persist as a smoking gun in the periphery for a longer time period. Also, a longer course of AIT is likely needed to deplete the pools of specific memory CD4⁺ T cells, as indeed has been reported for long-lived IgE-producing B cells.¹⁹⁻²¹ In contrast, our data suggest that AIT might affect the orientation of innate responses in a shorter time frame, which, as a consequence, would be more predictive of the onset of efficacy of sublingual AIT documented as early as after 1 month of treatment in this trial.²²

It is noteworthy that few studies have investigated the possible effect of AIT on APCs. An increase in IFN- α production after Toll-like receptor 9 stimulation of peripheral blood plasmacytoid DCs has been reported in patients receiving subcutaneous AIT.⁴⁰ Other studies documented changes in the phenotype of blood monocytes after venom immunotherapy, with an early induction 5 days after the first injection of tolerogenic molecules, such as ILT3 and ILT4, as well as the secretion of IL-10.^{41,42} Lastly, changes in the expression of function-associated surface molecules on DCs were also described in the course of AIT in patients with allergies to hymenoptera venom or house dust mites.⁴³ However, none of these studies established any firm link between changes in blood monocyte/DC phenotype and clinical benefit.

Mechanisms involved in the regulation of DC polarization in peripheral blood after mucosal administration of the allergen remain to be investigated. In this regard a possible role of local Treg cells⁴⁴ or activated mast cells⁴⁵ in modulating DC phenotype and function, likely through cytokine production after allergen exposure, should be considered.

Altogether, the induction of C1Q and STAB1, 2 proteins expressed by various types of tolerogenic DCs, correlates with clinical tolerance induced by AIT, suggesting a critical role of regulatory immune responses. The characterization of such biomarkers for short-term efficacy easily detected in peripheral blood opens new avenues for the follow-up of patients and the development of new allergy vaccines.

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Clinical implications: Two proteins, C1Q and STAB1, expressed by various types of regulatory DCs and easily detected in PBMCs by using qPCR can be used to distinguish clinical responders from nonresponders during allergen-specific immunotherapy.

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ONLINE REPOSITORY

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

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SUPPLEMENTARY METHODS

VO56.07A pollen chamber study

This clinical trial assessed the efficacy and onset of action of 5-grass pollen tablets (Stallergenes SA)^{E1} administered sublingually every day under controlled allergen exposure conditions provided in a challenge chamber^{E2}. The study was a randomized, double-blind, parallel-group, placebo-controlled, single-center trial, conducted outside of the pollen season. All patients provided a written informed consent before study entry. The study is registered at clinicalTrials.org under the number NCT00619827. It was performed in accordance with the Declaration of Helsinki and good clinical practice, following approval by an ethic committee and Austrian health authorities.

Characterization of effector and regulatory DCs

For immuno-fluorescence staining, cells were incubated with the following mAbs: FITC anti-CD14, FITC anti-CD80, PE anti-CD86, PC5 anti-CD83 (Beckman coulter), FITC anti-ILT2, PE anti-ILT4, PC5 anti-ILT3 (R&D systems) or PE anti-CD11c (Miltenyi Biotec) and analyzed by flow cytometry. IFN- γ , IL-1 β , IL-6, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-23, IL-17A and TNF- α were measured in supernatants using CBA kits (BD Biosciences). IL-23 concentration was measured by ELISA (ebiosciences).

Differential gel electrophoresis analysis of DC types

Polarized DCs were washed 3 times with cold PBS and cell pellets were lysed in buffer A containing 8.3 M urea, 2 M thiourea, 4% CHAPS, 50 mM DTT and 24 mM spermine (all obtained from Sigma). After centrifugation (16 000 g, 1 h, 20°C), supernatants were recovered and stored at -80°C. Proteins were then quantified using a Bradford assay (Biorad) and fractionated over a 4-12% gradient precast gel (NuPAGE, Invitrogen) to control protein quality.

43 For 2D-DiGE analysis, proteins were precipitated using the PerfectFocus kit (G-Biosciences),
44 resuspended in DiGE labeling buffer containing 8.3 M urea, 2 M thiourea, 4% CHAPS and 30
45 mM Tris pH 8.8, labeled with Cy2/3/5 dyes (CyDye DiGE Fluors, GE Healthcare) and
46 separated on 24 cm Immobiline pH 5.3-6.5 Drystrip gels by isoelectrofocusing (IEF) using the
47 Ettan IPGphor system (GE Healthcare). For analytical and preparative experiments, IEF was
48 done for a total of 132 and 145 kVh, respectively. Strips were then equilibrated in reduction
49 and alkylation buffers (containing 6 M urea, 50 mM Tris pH 8.8, 30% glycerol, 2% SDS and
50 either 1% DTT or 5% iodoacetamide) before loading onto 11% SDS-polyacrylamide gels for
51 separation according to molecular mass using an Ettan DALT Six electrophoresis system (GE
52 Healthcare). DiGE gels were scanned using an Ettan DiGE imager (GE Healthcare) according
53 to the manufacturer's instructions. Differentially expressed spots were determined by image
54 analysis with SameSpots software (Nonlinear Dynamics) and selected for automatic spot
55 picking (FDR p-value ≤ 0.05 and fold change ≥ 1.2).

56 Preparative gels stained with SYPRO Ruby (Invitrogen) were used for automatic spot picking
57 (EXQuest spot cutter, Biorad) of differentially expressed protein spots. Gel plugs were then
58 washed with 200 μ l of 100 mM NH_4HCO_3 /50% acetonitrile (ACN) for 45 min at 37°C and
59 then dehydrated in ACN. Each spot was digested with trypsin (10 ng/ μ l of 40 mM
60 NH_4HCO_3 /10% ACN, Sigma) at 37°C overnight and subsequently 6 μ l of ACN was added to
61 the mixture prior to ultrasonication for 45 min at 30°C.

62 NanoLC-MS/MS analysis was accomplished using the Ultimate 3000 RS nano LC system
63 (Dionex) coupled to an ESI-Qq-TOF MS (Maxis, Bruker Daltonics). H_2O /ACN/FA (100/0/0.1
64 volume ratios, respectively) was used as solvent A and H_2O /ACN/FA (20/80/0.1 volume
65 ratios, respectively) as solvent B. Tryptic peptides diluted in 0.1% FA were injected and
66 trapped on an Acclaim PepMap100 column (100 μ m x 2 cm, C_{18} , 5 μ m, 100 Å, Dionex) with
67 a flow rate of 12 μ l/min (2% ACN, 0.1% FA). Separation was performed using an Acclaim

68 PepMap RSLC column (75 μm x 15cm, C_{18} , 2 μm , 100 $\text{\AA}$, Dionex) with a flow rate of 450
69 nl/min and a linear gradient (5-45% B for 45 min, 45-95% B for 1 min and 95% B for 15
70 min). Database search was carried out using a Mascot server (Matrix Science, version 2.3)
71 against the Swiss-Prot and NCBI nr databases. Data were searched against the *Homo sapiens*
72 and then the mammalian databases with precursor mass tolerance of 15 ppm and fragment
73 mass deviation of 0.05 Da. The search included cysteine carbamidomethylation as a fixed
74 modification and methionine oxidation as a variable modification. Up to two missed
75 cleavages were allowed for protease digestion. All identifications were based on the
76 sequencing of more than one peptide and only proteins with a Mascot score with $p \leq 0.05$
77 were considered for identification. Protein scores were derived from individual ion scores.

78 ***Label-free MS analysis of DC types***

79 100 μg of proteins were mixed with urea-containing buffers in the filter unit (Amicon Ultra,
80 Ultracel-10 kDa membrane, Millipore), reduced with 20 mM DTT, alkylated with 50 mM
81 iodoacetamide, digested with Lys-C (37°C, 5 h, enzyme/substrate ratio of 1/50, Sigma) and
82 with trypsin (37°C, overnight, enzyme/substrate ratio of 1/50) as described elsewhere (filter-
83 aided sample preparation, FASP^{E3}). After digestion, peptides were desalted using RPC18
84 Dynal magnetic beads (Invitrogen), acidified with formic acid and analyzed by nanoLC-MS
85 or nanoLC-MS/MS.

86 NanoLC-MS analysis was accomplished using the Ultimate 3000 RS nano LC system
87 (Dionex) coupled to an ESI-Qq-TOF MS (Maxis, Bruker Daltonics). $\text{H}_2\text{O}/\text{ACN}/\text{FA}$
88 (100/0/0.15 volume ratios respectively) was used as solvent A and $\text{H}_2\text{O}/\text{ACN}/\text{FA}$ (20/80/0.15
89 volume ratios respectively) as solvent B. 1.5 to 2 μg of tryptic peptides were injected (36 $\mu\text{l} \pm$
90 3 μl) and trapped for 10 min on an Acclaim PepMap100 column (100 μm x 2 cm, C_{18} , 5 μm ,
91 100 $\text{\AA}$, Dionex) with a flow rate of 12 $\mu\text{l}/\text{min}$ (2% ACN, 0.15% FA). Separation was then
92 performed using an Acclaim PepMap RSLC column (75 μm x 50cm, C_{18} , 2 μm , 100 $\text{\AA}$,

93 Dionex) with a flow rate of 270 nl/min, two linear gradient segments (5-25% B for 180 min,
94 25-45% B for 50 min) and holding at 95% B for a further 10 min before returning to 5% B for
95 20 min. In MS mode, full scan MS spectra were acquired from m/z 280 to 1500 (1 MS
96 spectrum of 0.8 s) during 270 min. Ion intensities recorded in LC-MS data were analyzed
97 using Progenesis LC-MS v3.1 software (Nonlinear Dynamics) to provide reliable
98 measurements of peptide abundance across samples. Internal calibration was performed by
99 enabling the lock mass option in MS mode (minimum intensity > 200 and mass $\pm$ 0.015 Da).
100 Parameters used for peptide detection were peptide intensity > 300, peptide abundance > 2000
101 and $2^+ \leq$ peptide charge $\leq 6^+$. Data were then normalized by the “normalize to all features”
102 method and comparison between the four groups (obtained from Ctrl-, LPS-, DEX- and PGN-
103 DCs respectively) was performed to choose which peptides were statistically differentially
104 represented (FDR p-value $\leq$ 0.01 and fold change $\geq$ 1.5). Targeted nanoLC-MS/MS were
105 accomplished by means of an inclusion mass list in the MS instrument method. Inclusion lists
106 were generated from differentially expressed peptides and imported into MS acquisition
107 software (mass $\pm$ 0.02 Da and retention time $\pm$ 3 min). LC-MS/MS data were analyzed using
108 an in-house Mascot server (Matrix Science, version 2.3) against the Swiss-Prot database,
109 taxonomy *Homo sapiens*, assuming tryptic or semi-tryptic digestion. Identification parameters
110 were identical to those described for 2D-DiGE analysis. Peptide identifications were accepted
111 if established with a greater than 95 % probability, as specified by Mascot software. For
112 accurate mass measurements, the lock mass option was enabled in MS and MS/MS modes.

113 ***Western blot analysis***

114 NuPAGE-western blotting was carried out according to standard procedures (NuPAGE
115 technical guide, Invitrogen). Samples were separated on 4 to 12% MES, 3 to 8% Tris acetate
116 or 10 to 20% Tris glycine NuPAGE, depending upon the molecular mass of target proteins.
117 The following primary antibodies were used for immunoblotting analyses: anti-ANXA1 (Cat.

no. 3299, 1/1000), anti-GAPDH (Cat. no. 14C10, 1/1000), anti-GPX1 (Cat. no. 3286, 1/1000), anti-IRF4 (Cat. no. 4964, 1/1000) and anti-TRAF1 (45D3, 1/1000) from Cell Signaling Technology, anti- β -actin (Cat. no. MS-1295, 1/2000) and anti-F13A (Cat. no. RB-1464, 1/1000) from Neomarkers (Labvision), anti-FKBP5 (Cat. no. H00002289-M02, 1/250) and anti-MX1 (Cat. no. H00004599-B01P, 1/500) from Abnova, anti-CD71 (Cat. no. TA307375, 1/1000) from Origene (Rockville, MD), anti-CATC (Cat. no. sc-74590, 1/500), anti-NMES1 (Cat. no. sc-138479, 1/500) and anti-STAB1 (Cat. no. sc-98788, 1/500) from Santa Cruz (Santa Cruz, CA), anti-MRC1 (Cat. no. 18704-1-AP, 1/1000) from Proteintech group, anti-C1Q (Cat. no. ab71089, 1/1000) from Abcam. The rabbit polyclonal serum raised against GILZ was previously described^{E4}. Peroxidase-conjugated goat anti-mouse and anti-rabbit secondary antibodies were both obtained from Jackson ImmunoResearch Laboratories, and the chemiluminescence detection kit was from Pierce. Western blot signals were acquired with a CCD camera (Fusion FX7, Vilber-Lourmat) and band volume was quantified using the Bio-1D software (Vilber-Lourmat). β -actin or GAPDH were used as loading controls.

RNA isolation and Quantitative real-time PCR analysis

Total RNA was extracted from treated DCs or PBMCs using RNeasy mini kits and the Qiacube robot (Qiagen) and cDNAs were obtained using TaqMan reverse transcription reagents (Applied Biosystems) as per the manufacturer's instructions. Messenger RNA expression was evaluated by quantitative PCR on a 7900HT real-time PCR system (Applied Biosystems) with predesigned Taqman gene expression assays and reagents, according to the manufacturer's instructions. Expression of the following genes was assessed in DCs or PBMCs: GILZ (Hs00608272_m1), IDO (Hs00158032_m1), RALDH1 (Hs00167445_m1), RALDH2 (Hs00180254_m1), ANXA1 (Hs00167549_m1), CLIC2 (Hs01574555_m1), FKBP5 (Hs01561001_m1), F13A (Hs00173388_m1), GPX1 (Hs00829989_gH), IMDH2 (Hs00168418_m1), OSF1 (Hs00273458_m1), TPP1 (Hs00166099_m1), C1QA

143 (Hs00381122_m1), C1QB (Hs00608019_m1), C1QC (Hs00757779_m1), CATC
 144 (Hs00175188_m1), STAB1 (Hs01109068_m1), MRC1 (Hs00267207_m1), CD71
 145 (Hs00951083_m1), FSCN1 (Hs00979631_g1), IRF4 (Hs01056533_m1), MX1
 146 (Hs00895608_m1), NMES1 (Hs00260902_m1), TRAF1 (Hs01090170_m1). Expression of
 147 the following genes was assessed in T cells: IFN- γ (Hs00989291_m1), IL-4
 148 (Hs00174122_m1), IL-10 (Hs00961622_m1) and IL-17A (Hs00174383_m1).
 149 Data were interpreted for each target gene in comparison with endogenous β -actin
 150 (Hs99999903_m1) or GAPDH (Hs03929097_g1) as controls. The relative amount of target
 151 genes in each sample was calculated in comparison with the calibrator sample (untreated
 152 sample or PBMC before treatment) using the $\Delta\Delta C_t$ method. The magnitude of gene induction
 153 was calculated using the formula $2^{-\Delta\Delta C_t} = 2^{-(\Delta C_t \text{ for stimulated cells} - \Delta C_t \text{ for unstimulated cells})}$ or $2^{-\Delta\Delta C_t} = 2^{-(\Delta C_t \text{ PBMC}$
 154 $\text{after treatment} - \Delta C_t \text{ PBMC before treatment})}$.
 155

SUPPLEMENTARY RESULTS

Characterization of the various subtypes of tolerogenic DCs

Surface phenotyping of various tolerogenic DCs confirmed that ASP-DCs exhibit a CD11c^{low} phenotype, in contrast to all other DC types which are rather CD11c^{high} (**Figure E2A**). CD14 expression allows to cluster DCs in 3 groups, with DEX_{24h} and TGF- β -DCs being CD14^{neg}, VitD3 and RAPA-DCs being CD14^{int} and DEX_{diff} and IL-10-DCs as CD14^{high} cells. The differential expression of ILT2, ILT3 and ILT4 further allows to distinguish DEX_{24h} and TGF- β -DCs, VitD3-DCs and RAPA-DCs, and DEX_{diff}-DCs and IL-10-DCs, respectively ($p < 0.05$, Friedman test). To confirm the anti-inflammatory profile of such DCs, we stimulated them with LPS during 24 h and assessed the expression of costimulatory molecules and cytokine secretion. As shown in **Figure E2B**, all tolerogenic DC subtypes exhibited a blunted LPS-induced upregulation of costimulatory molecules. LPS-induced cytokine secretion was also inhibited in all regulatory DC types, with the exception of IL-6, IL-8 and TNF- α secretion left uninhibited after LPS stimulation in VitD3-DCs (**Figure E2C**).

SUPPLEMENTARY REFERENCES

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SUPPLEMENTARY TABLE LEGENDS

Table E1: Proteins identified through the 2D-DiGE approach with a FDR p-value ≤ 0.05 .

Proteins identified with a multiple comparison test, a FDR p-value ≤ 0.05 and at least a 1.2-fold change in volume are listed, corresponding to a total of 38 differentially-expressed proteins. Max. fold represents the ratio of the average volumes of the highest vs. lowest conditions.

Table E2: Overview of candidate markers for regulatory and effector DCs identified through proteomic approaches.

The known function described in the literature for candidate markers for tolerogenic or effector DCs is summarized.

Table E3: Proteins identified through the label-free MS approach with a FDR p-value ≤ 0.01 .

Proteins identified with two or more peptides are listed corresponding to a total of 60 differentially-expressed proteins (multiple comparison test, FDR p-value ≤ 0.01). Included as well in this list are 3 proteins (*i.e.* ITAM, MX1 and CLIC2, 1 peptide) previously shown to be regulated in effector and tolerogenic DCs, respectively (**Table S2**), as well as PGRP1 exhibiting a > 90 -fold increase in PGN-DCs and C1QB increased in DEX-DCs. Max. fold represents the ratio of the average abundance in DCs obtained under conditions providing the highest vs. lowest values.

SUPPLEMENTARY FIGURE LEGENDS

Figure E1: Design of the study. Total proteins extracted from treated DCs (Ctrl-, LPS-, DEX- and PGN-DCs) were submitted to both 2D-DiGE and label-free MS quantification (FASP: filter-aided sample preparation). Differentially expressed protein spots or peptides were subsequently identified after MS/MS analysis. Candidate markers of tolerogenic DCs were validated by western blotting (WB) and/or qPCR in both Ctrl-, LPS-, DEX- and PGN-DCs (model A), in 6 distinct tolerogenic DC types (model B) and in clinical samples (PBMCs) obtained from grass pollen allergic patients undergoing sublingual immunotherapy (model C). In model A, WB were performed from whole cell lysates used in proteomic experiments, with additional treated DC samples collected from 8 donors for qPCR validation. In model B, DC samples were used for both WB and qPCR validations. For samples from the clinical study, PBMCs (n=158) were collected from 79 patients before and after 4 months of immunotherapy and candidate markers gene expression was assessed by qPCR.

Figure E2: Characterization of various subtypes of tolerogenic DCs. (A) Cell surface phenotypes were assessed by FACS. (B) Percentages of inhibition of LPS-induced expression of costimulatory molecules and (C) cytokine secretion in treated DCs. 100% represent a complete inhibition of the expression/secretion of the molecule. A representative donor out of 4 is presented in A and mean $\pm$ SEM (n= 4) in B/C. *p-value $\leq$ 0.05, **p-value $\leq$ 0.01 were considered significant (Friedman test).

Figure E3: mRNA expression of markers of regulatory DCs in PBMCs from 20 patients undergoing allergen immunotherapy. Markers of tolerogenic DCs that did not exhibit significant changes between placebo and active groups, nor between responder and non

responder groups are represented (Mann-Whitney test, p -value ≤ 0.05). Data are expressed as relative amounts of mRNA in PBMCs after 4 months of treatment in comparison with PBMCs before immunotherapy. Data are normalized to amounts of β -actin and shown for each patient and as a mean $\pm$ SEM for each group. (AR: active responders, ANR: active non responders, PR: placebo responders, PNR: placebo non responders).

Figure E4: mRNA expression of markers of effector DCs in PBMCs from 20 patients undergoing allergen immunotherapy. No significant change in markers of effector DCs was detected between placebo and active groups, nor between clinical responders and non responders (Mann-Whitney test, p -value ≤ 0.05). Data are expressed as relative amounts of mRNA in PBMCs after 4 months of treatment in comparison with PBMCs before immunotherapy. Data are normalized to amounts of β -actin and shown for each patient and as a mean $\pm$ SEM for each group. (AR: active responders, ANR: active non responders, PR: placebo responders, PNR: placebo non-responders).

Figure E5. *CIQA* and *STAB1* genes are preferentially expressed in peripheral blood monocytes / monocytic cells. Populations of either granulocytes (CD15⁺), monocytes / monocytic cells (CD14⁺), T (CD3⁺CD4⁺ and CD3⁺CD8⁺) or B lymphocytes (CD19⁺), myeloid (CD11c⁺) or plasmacytoid (CD123⁺) DCs were purified from PMBCs obtained from 3 healthy donors using a MoFlo (Dako) cell sorter. Cells within each subset were more than 99% pure, as confirmed by flow cytometry reanalysis. Total RNA was isolated and *CIQA* and *STAB1* gene expression was evaluated by qPCR. Data are expressed as relative amounts of mRNA in each cell subset in comparison with total PBMCs. Data are normalized to amounts of β -actin and shown as mean $\pm$ SEM (n=3).

Figure E6: Expression of markers of effector DCs in PBMCs from 79 patients undergoing allergen immunotherapy. (A) mRNA expression of *CD71*, *FSCN1*, *MX1* and *TRAF1* in PBMCs from patients in the active group (n=41) in comparison to the placebo group (n=38) or in responders (% ARTSS improvement ≥ 43.9) versus non responders (% ARTSS improvement < 43.9) (Mann-Whitney test, p-value ≤ 0.05). Data are expressed as relative amounts of mRNA in PBMCs after 4 months of treatment in comparison with PBMCs before immunotherapy. Data are normalized to amounts of β -actin and shown as a mean $\pm$ SEM for each group. **(B)** Correlation of mRNA expression with improvement in clinical symptoms in the active and placebo groups at the level of individual patients. R represents the Spearman correlation coefficient. A score of 1 represents a perfect correlation, whereas a score between 0 and 1 indicates that the two variables increase or decrease together. (AR: active responders, ANR: active non responders, PR: placebo responders, PNR: placebo non responders).