Biomatrix for upper and lower airway biomarkers in patients with allergic asthma

To the Editor:

Although many biomarkers have been proposed for asthma monitoring, only the noninvasively accessible fraction of exhaled nitric oxide has been established into clinical routine, whereas implementation into guidelines is pending.[1] The upper and lower airways are considered a morphologic and functional unit and share air transport, physical barrier, mucociliary clearance, and immune interface as common features.[2] Furthermore, not only do allergic rhinitis and asthma share mechanisms of allergic inflammation, but also, transition of allergic rhinitis into asthma can represent a steady continuum depending on endotype. Therefore nasal secretions

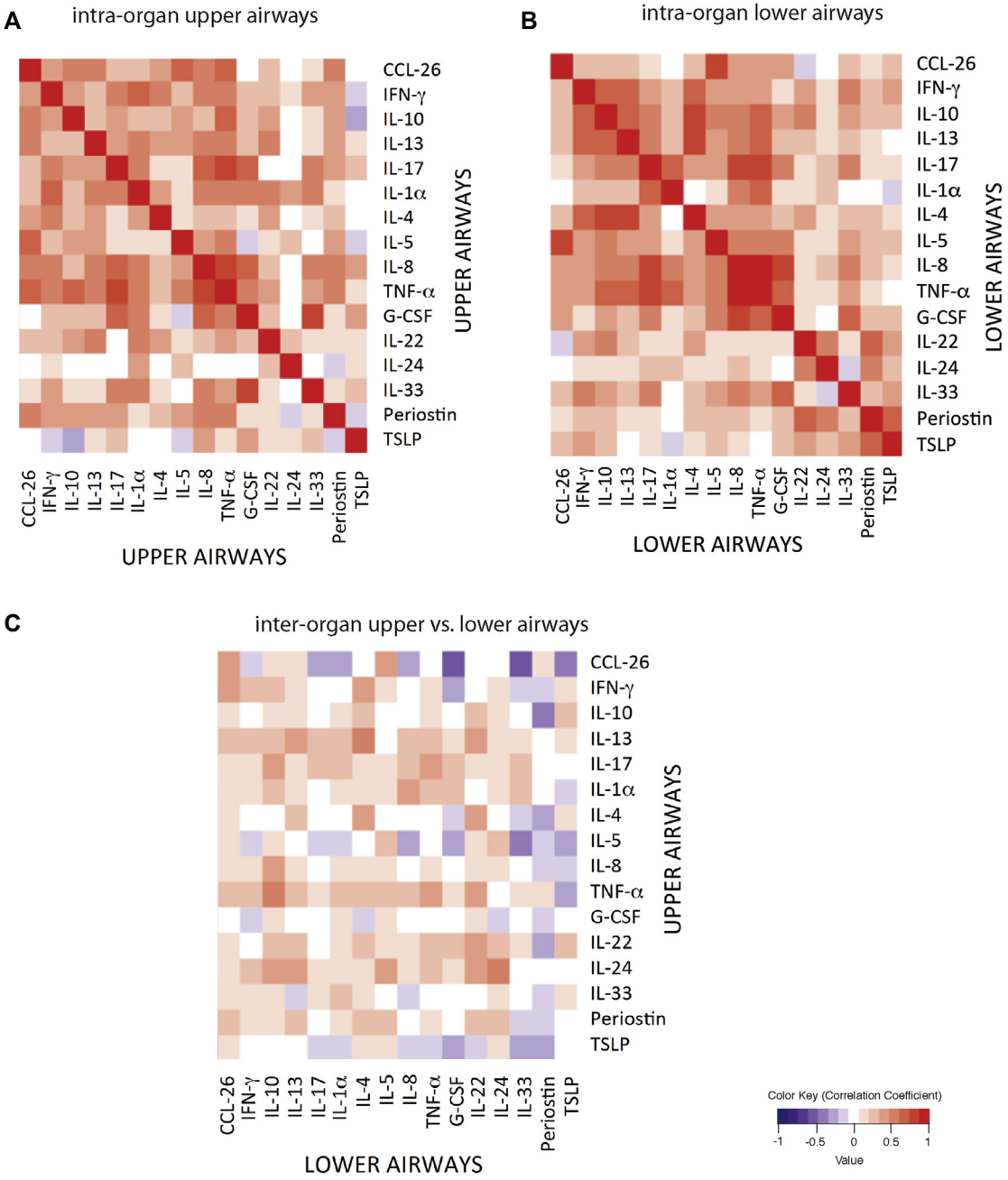

FIG 1. Biomarker grids reflecting correlations between upper and lower airways cytokine levels. Intraorgan correlations for upper **(A)** and lower **(B)** airways and interorgan correlations **(C)** were depicted by using respective correlation coefficients *(r)* from negative (dark blue) to positive (red) values. Intraorgan correlations seemed to be predominantly strong, whereas interorgan relations were most visible by using nasal and sputum IL-24 measurements. *G-CSF*, Granulocyte colony-stimulating factor; *TSLP*, thymic stromal lymphopoietin.

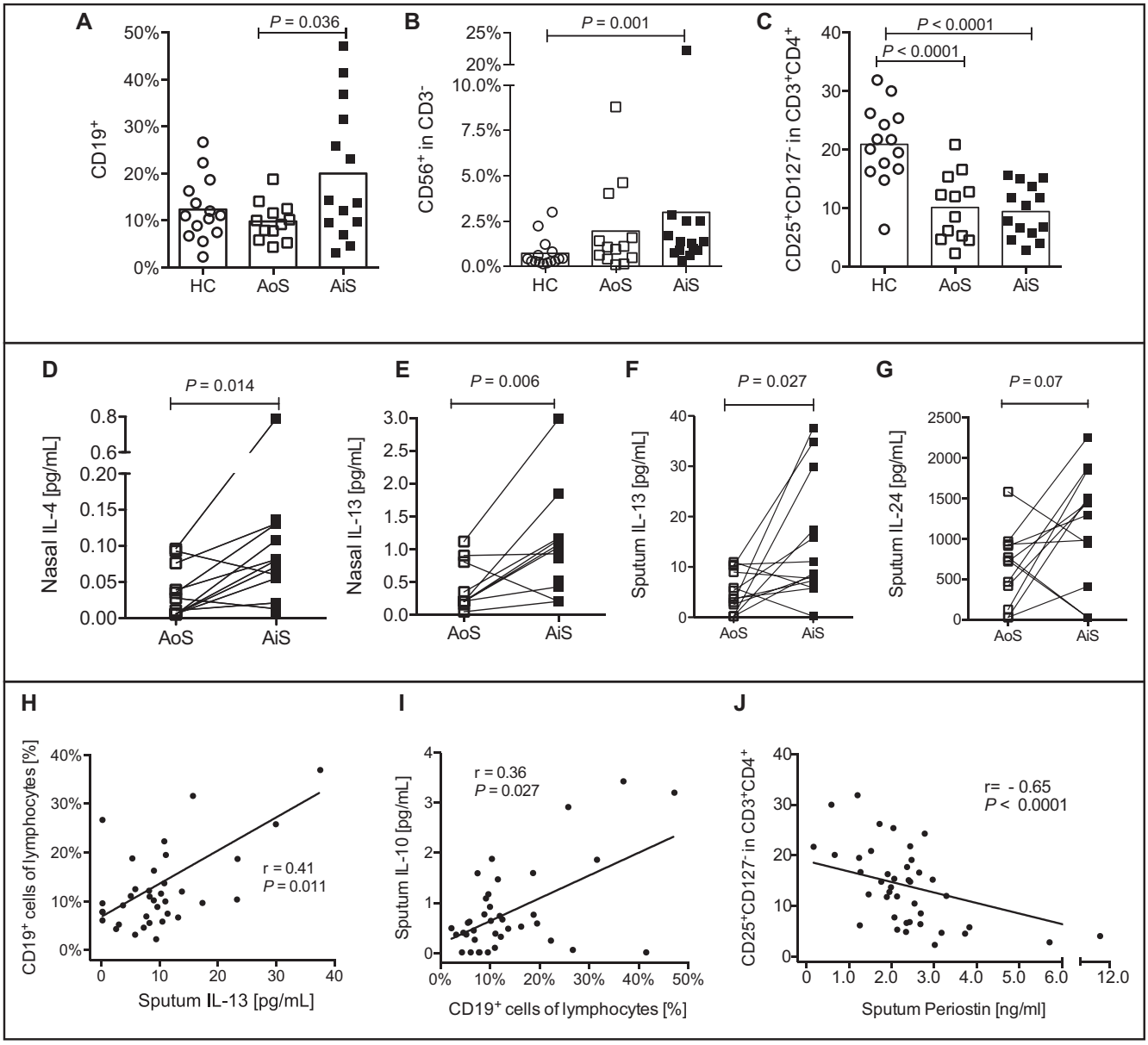

FIG 2. Airway cytokines and cell populations. **A-C,** Although numbers of B lymphocytes and natural killer cells were upregulated in asthmatic patients during the grass pollen season, in Treg cells there was only a difference between patients and healthy control subjects. **D-G,** Type 2 cytokines were differentially regulated in asthmatic patients out of the grass pollen season, as well as in the grass pollen season. **H-J,** Positive correlations were visible for B-cell counts and sputum IL-13 and IL-10 levels, respectively, whereas a strong negative correlation was seen for sputum periostin levels and Treg cell counts. *AiS*, Asthmatic patients in the grass pollen season; *AoS*, asthmatic patients out of the grass pollen season; *HC*, healthy control subjects.

of the upper airways can prove useful as noninvasive and easily accessible proxies mirroring lower airway inflammation.[3] The objective of the current study was to visualize the relationship of elementary protein biomarkers of the upper against the lower airways to identify potential nasal proxy candidates for the lower airways.

Nasal lining fluids and induced sputum were collected to find practical solutions to assess airway inflammation by using noninvasive methods because sputum opens another window to assess lower airway inflammation.[4] We included 15 patients with seasonal allergic asthma and 16 healthy participants, all of them nonsmokers, into the study (see Table E1 in this article's Online Repository at www.jacionline.org) and analyzed sputum supernatants and nasal secretions by using multiarray technology (see the Methods section in this article's Online Repository at www.jacionline.org). Furthermore, we assessed clinical scores as follows: Rhinoconjunctivitis Quality of Life Questionnaire (RQLQ), Asthma Control Score (Global Initiative for Asthma [GINA]), and Perceived Stress Questionnaire (PSQ). Standard sputum differential cell counts (see Table E2 in this article's Online Repository at www.jacionline.org) and additional

sputum flow cytometry (fluorescence-activated cell sorting) were included.

To identify potential proxy biomarkers, we displayed the strength of the interaction of the biomarker signal of the upper and lower airways in a chessboard pattern (2-sided Spearman rank correlation; Fig 1). Known pathomechanisms, such as cellular source of biomarkers and sputum cell counts, were used to augment these patterns and enrich for best biomarker selection.

Levels of many cytokines and chemokines in sputum correlated moderately to strongly ($r > 0.5$) with other mediators of sputum (intraorgan comparison; Fig 1, *A* and *B*), whereas the correlation of these mediators between sputum and nasal secretions showed a low to moderate relationship (interorgan comparison, $r < 0.5$). These correlations are mainly seen diagonally (Fig 1, *C*), which displays the correlation of one cytokine in the lower airways to the same cytokine in the upper airways. IL-24 seemed to be of special interest as a proxy because it showed moderate correlations between the upper and lower airways ($r = 0.53$, $P = .001$; Fig 1, *C*, and see Table E3 in this article's Online Repository at www.jacionline.org). Interestingly, the GINA score showed a strong negative correlation to nasal IL-24 levels ($r = -0.85$, $P = .001$). Furthermore, sputum IL-24 levels were increased in asthmatic patients in season (1084.0 ± 199.6 pg/mL) compared with out of season (641.2 ± 131.7 pg/mL; $P = .07$; Fig 2, *G*). Flow cytometric analysis of cellular components in sputum revealed a moderate negative correlation of $CD25^+/CD127^-$ regulatory T (Treg) cell counts to sputum IL-24 levels ($r = -0.50$, $P = .002$). We speculate that this decrease in numbers of Treg cells could be caused by T_H2-mediated suppression at the site of inflammation. This interpretation is supported by the finding that numbers of Treg cells in asthmatic patients were diminished independently of season (Fig 2, *C*). From a mechanistic point of view, this result supports our previous findings showing that type 2 immune responses inhibit induction of regulatory cell.[5]

As expected, correlation of levels of one cytokine with those of one of the other mediators was observed frequently in the intraorgan correlation grid (Fig 1, *A* and *B*); for example, levels of the type 2 cytokine IL-5 correlated with levels of the type 2 IL-4–inducible CCL-26 (eotaxin-3; nasal: $r = 0.726$, $P < .0001$; bronchial: $r = 0.771$, $P < .0001$). A moderate correlation was also observed for nasal CCL-26 and nasal IL-13 levels ($r = 0.57$, $P < .0001$). It is well known that IL-13 is able to seasonally promote IgE and IgG_4 production.[6] In fact, a weak correlation of sputum IL-13 levels and sputum $CD19^+$ B-cell counts was observed ($r = 0.41$, $P = .011$; Fig 2, *H*). In general, the proportion of total B cells in sputum within asthmatic patients showed a significant increase during the grass pollen season (20.03% ± 3.85%) compared with off season (9.80% ± 1.18%; $P = .036$; Fig 2, *A*).

Levels of the regulatory cytokine IL-10 moderately correlated with TNF-α expression levels in both the upper and lower airways ($r = 0.38$, $P = .024$; Fig 1, *C*) and even more so in the intraorgan comparison (lower airways: $r = 0.61$, $P < .0001$; upper airways: $r = 0.61$, $P < .0001$; Fig 1, *A* and *B*). Sputum IL-10 levels correlated weakly with the presence of $CD19^+$ B cells ($r = 0.36$, $P = .027$) in sputum (Fig 2, *I*). IL-10 in airway lining fluid can originate from previously described IL-10–producing regulatory B cells, which are capable of regulating type 2 inflammation or promoting the IgE to IgG_4 switch.[6] Furthermore, regulatory B cells are known to play a pivotal role in allergen-specific immunotherapy.[7] Surprisingly, we observed weak to moderate correlation for nasal IL-10 expression with the perceived stress burden of asthma patients assessed by PSQ score ($r = 0.42$, $P = .01$; see Fig E1 in this article's Online Repository at www.jacionline.org), which showed a strong correlation with GINA score ($r = 0.78$, $P = .003$) and moderate correlation with RQLQ score ($r = 0.58$, $P < .0001$). Nasal IL-10 levels also negatively correlated with total sputum leukocyte count ($r = -0.65$, $P < .0001$), which is considered a marker for inflammation in the lower airways (see Table E2). Although IL-10 levels in the upper airways can display clinically relevant marker functions, it shows only few or even negative (eg, periostin) correlations between the upper and lower airways.

Periostin has been proposed as a biomarker for comorbid chronic rhinosinusitis in patients with asthma, revealing its relevance in T_H2-driven diseases also in patients with local tissue inflammation.[8] Expression pattern differed from markers because it was mostly correlated negatively with other mediators, including itself, when compared with the upper airways. This negative interaction was not observed in intraorgan comparison with one exception, namely IL-1α. The important role of periostin in the lower airways is implied by the finding that periostin expression in sputum negatively correlated with $CD25^+CD127^-$ Treg cell ($r = -0.65$, $P < .0001$; Fig 2, *J*) and positively with sputum eosinophil ($r = 0.36$, $P < .034$) counts, whereas RQLQ scores seemed to be associated to levels of lower airway periostin ($r = 0.38$, $P = .021$; see Fig E1). Taken together, local periostin might prove useful to assess disease progression in the lower airways, but our data suggest periostin to be a less favorable proxy in the upper airways because of its negative relationship to the lower airways.

In conclusion, the concept of united airways and consequently representative nasal proxy biomarker analytes holds particularly true for type 2 cytokines, specifically IL-24, which we have previously shown to be an epithelial type 2 cytokine.[1] Periostin and IL-10 levels correlate moderately with clinical symptoms and might be of limited use as proxy biomarkers because of the conflicting correlations between mediators of the upper and lower airways. These findings provide not only promising biomarker candidates but also correlation matrices, which might be reflective of underlying asthma endotypes. Larger clinical studies are needed to fully assess the validity and practability of cytokine measurements in nasal lining fluid to characterize asthmatic patients.

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Genetically engineered cell factories produce glycoengineered vaccines that target antigen-presenting cells and reduce antigen-specific T-cell reactivity

To the Editor:

Allergen-specific immunotherapy is a promising approach to reduce or remove allergic symptoms by inducing tolerance.[1] As an alternative to current allergy vaccines derived from extracts of natural allergens, recombinant vaccines offer the opportunity for *de novo* antigen design,[2] with enhanced targeting to antigen-presenting cells (APCs) and potentially increased ability to induce tolerance.[3,4]

Using the type 1 allergen Bet v 1 as our model antigen, we evaluated the effects of combining a nonglycosylated antigen to a glycomodule[5] to target the APC-expressed family of C-type lectin receptors (CLRs). CLRs are known to recognize glycan structures and promote rapid internalization of antigens on binding (see Fig E1, *A*, in this article's Online Repository at www.jacionline.org).[6,7] We used our recently developed glycoengineered cellular platform[8] to produce Bet v 1 with defined carbohydrates and evaluated APC uptake, efficacy using patient-derived T cells, and induction of tolerance in a murine model.

Currently, allergen-specific immunotherapy is primarily administered subcutaneously or sublingually. Focusing on human skin, we examined the CLR expression pattern in skin-localized APCs. We observed expression of the major CLRs, including mannose receptor (MR), dendritic cell–specific intercellular adhesion molecule 3–grabbing nonintegrin (DC-SIGN), and macrophage galactose-type lectin (MGL), in the dermal compartment of skin biopsy specimens (see Fig E1, *B*). Next, we examined expression of MR, DC-SIGN, and MGL in *in vitro*–differentiated human monocyte-derived dendritic cells (moDCs) and macrophages (M1 and M2) by means of transcriptomics and flow cytometry (see Fig E1, *C-E*). Expression of CLRs peaked after 3 days of differentiation, with simultaneous and significant expression of DC-SIGN, MR, and MGL in moDCs (see Fig E1, *D* and *E*). Cytokine measurements confirmed the nature of the APCs (see Fig E1, *F*) and their relevance as a cellular model.

To identify the optimal carbohydrate structure or structures for specific APC targeting, we evaluated uptake of chemically synthesized carbohydrate derivatives (N-acetylgalactosamine polyacrylamide [GalNAc-PAA], N-acetylglucosamine–PAA, Lewis X–PAA, and mannose-PAA) and a GalNAc glycosylated molecule with varying numbers of GalNAc residues (see Fig E2, *A* and *B*, in this article's Online Repository at www.jacionline.org). Both mannosylated and high-density GalNAc glycosylated structures were readily taken up by the APCs, and therefore GalNAc and mannose structures were selected for further studies.

We next designed Bet v 1 allergen constructs for the expression of full-length Bet v 1 fused to glycomodules, with consensus glycosylation motifs ensuring the incorporation of either O- or N-linked glycans. The fusion constructs were expressed in glycoengineered Chinese hamster ovary (CHO) cell lines and *Pichia pastoris* (Fig 1, *A*, and see Fig E2, *C-G*, and Table E1 in this article's Online Repository at www.jacionline.org). This strategy allowed us to generate fusion proteins decorated with high-mannose N-linked glycans from MGAT1 knockout CHO cells, linear α1-2 O-linked mannose residues from *P pastoris* (Pichia), or O-GalNAc residues from COSMC knockout CHO cells for efficient targeting of MR, DC-SIGN, and/or MGL on APCs (Fig 1, *A*, and see Fig E2, *C-G*).

Additionally, we expressed the O-glycan fusion construct in *Escherichia coli* and inserted GalNAc residues by means of *in vitro* glycosylation. Glycosylation profiles were verified by using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) of released glycans supplemented with lectin profiling (see Fig E2, *C-G*). As a control for glycan-mediated effects, we treated fractions of the glycoallergens with periodate, a treatment known to effectively destroy lectin recognition (see Fig E2, *E*).

Because moDCs were the only cell type to express high levels of DC-SIGN, as well as MR and MGL, we used moDCs to evaluate uptake of glycan-modified Bet v 1 fusion proteins. Internalization of all glycan-modified Bet v 1 fusion proteins was enhanced 1.5- to 6-fold compared with that of their