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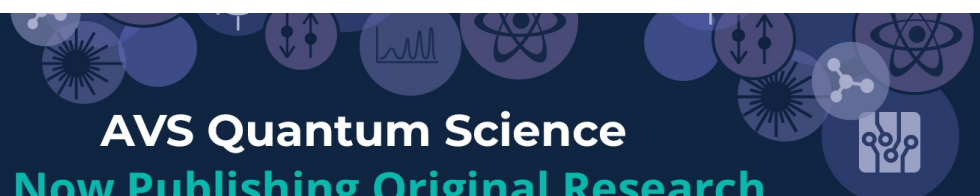
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ABSTRACT

Ferumoxytol nanoparticles are being used clinically for the treatment of anemia and molecular imaging in patients. It is well documented that while most patients tolerate ferumoxytol well, a small percentage of patients (i.e., 0.01%) develop severe allergic reactions. The purpose of our proof-of-concept study was to determine whether patients with or without hypersensitivity reactions have specific protein corona profiles around ferumoxytol nanoparticles. In a retrospective, institutional review board approved pilot study, we enrolled 13 pediatric patients (5 girls, 8 boys, mean age 16.9 ± 8.2 years) who received a ferumoxytol-enhanced magnetic resonance imaging and who did (group 1, $n = 5$) or did not (group 2, $n = 8$) develop an allergic reaction. Blood samples of these patients were incubated with ferumoxytol, and the formation of a hard protein corona around ferumoxytol nanoparticles was measured by dynamic light scattering, zeta potential, and liquid chromatography–mass spectrometry. We also performed *in vitro* immune response analyses to randomly selected coronas from each group. Our results provide preliminary evidence that *ex vivo* analysis of the biomolecular corona may provide useful and predictive information on the possibility of severe allergic reactions to ferumoxytol nanoparticles. In the future, patients with predisposition of an allergic reaction to ferumoxytol may be diagnosed based on the proteomic patterns of the corona around ferumoxytol in their blood sample.

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I. INTRODUCTION

Ferumoxytol (Feraheme®, AMAG Pharmaceuticals) is perhaps the most widely clinically applied nanoprobe to date, having been administered to more than 1×10^6 patients. Ferumoxytol is composed of iron oxide nanoparticles with a 6.4–7.2 nm iron oxide

core and a 28–32 nm carboxymethyl dextran coat.^{1–3} In addition to its Food and Drug Administration (FDA)-approved use as an iron supplement, ferumoxytol has been used “off label” as a contrast agent for magnetic resonance imaging (MRI) scans.^{4–8} Based on the published evidence to date, ferumoxytol does not cause direct or indirect renal toxicity^{1,3,9–11} and can, therefore, be applied in

patients with renal insufficiency. Therefore, ferumoxytol fills an important clinical need.^{1,3,9–11} However, rare severe adverse events (AEs) have been reported with an incidence of approximately 0.01%, including 18 cases of cardiac arrest.¹ As a result, the FDA issued a boxed warning about the risk of serious hypersensitivity reactions in response to ferumoxytol. A better understanding of the mechanisms that cause these AEs is urgently needed to develop preventive and curative interventions.

Ferumoxytol is coated by carboxymethyl dextran, which can trigger hypersensitivity reactions, including complement-like type II or III Coombs type reactions, type IV (i.e., T cell-mediated) reactions, or type I reactions with IgE formation after haptenization, especially after sensitization to dextran derivatives.¹² In addition, reports from phase III randomized controlled trials described a complement activation related pseudoallergy (CARPA) after rapid bolus injection of ferumoxytol,^{1,9,10,13} where intravenously administered iron exceeded the transferrin binding capacity, leading to complement activation by free iron and anaphylactic-like symptoms.¹⁴ However, it is not well understood why many patients tolerate ferumoxytol well and why a small percentage of patients develop life-threatening AEs.

It is now well accepted that the biological fate of nanoparticles, in terms of their safety and therapeutic efficacy, is determined by their biological identity (i.e., biomolecular/protein corona) in addition to their synthetic identity.^{15–18} After intravenous infusion of iron oxide nanoparticles, a layer composed of various biomolecules (e.g., proteins, lipids, nucleic acids, and metabolites) forms around the surface of the nanoparticles.¹⁹ This biomolecular corona (often called as “protein corona,” as the lion share of the layer is composed of proteins) serves as the first line of interaction with cells and biological mediators in the blood.^{20–22} Several investigators have described that the immune response against nanoparticles is triggered by specific compositions of the biomolecular corona at the surface of nanoparticles. For example, negatively charged poly(acrylic acid)-conjugated gold nanoparticles have a capacity to unfold fibrinogen and expose its active site to promote interaction with the integrin receptor, which, in turn, increases the NF- κ B signaling pathway and, thus, induce the release of inflammatory cytokines.²³

Although the role of the biomolecular corona on immune responses has been investigated,^{23,24} to the best of our knowledge, there is no report on whether specific proteins in the corona of ferumoxytol nanoparticles have a capacity to trigger hypersensitivity reactions. To close this gap, the purpose of our study was to determine whether patients with or without hypersensitivity reactions have specific protein corona profiles around ferumoxytol nanoparticles that could be linked to serious hypersensitivity reactions.

II. MATERIALS AND METHODS

A. Patient population

We performed a secondary analysis of data obtained through a prospective, nonrandomized, HIPAA-compliant clinical trial, which was approved by our institutional review board and performed under an investigator-initiated investigational new drug (IND) (111 154). We invited pediatric and young adult patients to participate if they met the following inclusion criteria: (1) age younger than 30 years, (2) scheduled or recent ferumoxytol administration, and (3) willingness to participate in the study and undergo safety

evaluations. Patients were excluded if they (1) could not donate at least 10 ml blood, (2) had hemochromatosis/hemosiderosis, or (3) were pregnant. We recruited 13 patients with cancer ($n = 11$) or ventricle septum defect, or arteriovenous malformation of the brain ($n = 1$, respectively). The patients included five girls (mean age 21.7 ± 6.4 years; range: 13–27) and eight boys (mean age 14.5 ± 8.2 years; range: 1–30). We administered ferumoxytol as part of a clinical trial, which investigated the value of ferumoxytol as an imaging agent for whole body MRI of cancer patients. Other iron supplements do not provide a combined positive contrast on T1-weighted MRI and negative contrast on T2-weighted MRI. This is a unique feature of ferumoxytol and was the basis for our imaging evaluations.

According to our IND protocol, we infused ferumoxytol diluted (1:4) slowly intravenously over 15 min and collected principal safety data. The patients had fasted for 4 h before the ferumoxytol infusion. We observed the patients for any objective signs of an allergic reaction (rash, flush), acquired vital signs (blood pressure, pulse rate, and oxygen saturation), and asked the patients for any subjective adverse events like itching or dizziness from baseline up to 1 h. We also checked symptoms during the MRI scan, up to 48 h after the ferumoxytol infusion, and at the follow-up visit at the hospital, usually within 1 week after the ferumoxytol administration. These safety data were recorded in a case report form, prescribed under our IND. For patients with AEs, we recorded the nature of the event (e.g., skin reaction, breathing difficulties, cardiovascular problems, or nonspecific symptoms, such as metallic taste and lower lumbar pain), including the first onset, evolution, and disappearance of symptoms. If treatment was needed, we noted the type of therapy and response. We rated AEs in accordance with the Common Terminology Criteria for Adverse Events v4.03 from Grade 1 “mild” through Grade 5 “death.”

B. Blood sample collection and preparation

Patients were asked to donate a 10 ml blood sample before intravenous ferumoxytol injection. We performed experiments on triplicate blood samples of ferumoxytol-exposed patients with ($n = 6$) or without ($n = 8$) observed hypersensitivity reactions to ferumoxytol. In order to imitate the clinical situation of ferumoxytol applied in patients for IV administration, we added ferumoxytol at calculated plasma concentrations as follows: The typical patient dose of 5 mg Fe/kg is assumed to distribute in the plasma volume (0.05 l/kg) and would yield a plasma concentration of 34–50 μ g Fe/ml.

C. Formation of protein corona

We incubated 100 μ l of ferumoxytol (1 mg/ml) nanoparticles with 900 μ l of patient-specific serum at 37 °C for 1 h and then centrifuged them to form a pellet of the particle–protein complexes. After aspiration of the supernatant (which contains excess/unbound proteins), we resuspended the pellet in 500 μ l of phosphate-buffered saline (PBS; at 15 °C) and centrifuged again for 3 min at a 13 000 relative centrifugal field at 15 °C to form a pellet of the hard corona-coated nanoparticles. We designed these centrifuge steps to remove the loosely bonded proteins from the composition of the protein corona. We resuspended the pellet in 500 μ l of PBS and examined all samples using dynamic light scattering

(DLS), zeta potential measurements, and liquid chromatography mass spectroscopy (LC-MS/MS) to characterize the proteomic content of protein corona. For basophil activation, the nanoparticles were incubated with the fresh blood.

D. Protein corona characterization

Size and surface charge. The hydrodynamic diameter of the nanoparticles was measured by DLS using a Malvern PCS-4700 micrograph, and the zeta potential of all samples was measured with a Malvern Zetasizer 3000HSA.

LC-MS/MS. Protease Max (Promega, Madison, WI, USA), an acid labile surfactant, was added to the nanoparticles, followed by vortexing and sonication. Next, the samples were reduced, alkylated, and digested overnight. The digestion was quenched by the addition of formic acid, followed by peptide concentration and purification. The peptide pools were dried, reconstituted, and injected into an in-house packed C18 reversed phase analytical column. The mass spectrometer was an Orbitrap Fusion set to acquire data in a dependent fashion. Fragmentation was performed on the most intense multiply charged precursor ions. All LC/MS data were analyzed for peptide composition using PREVIEW AND BYONIC v2.0 software (ProteinMetrics, San Carlos, CA, USA). Data were validated using standard reverse-decoy techniques. Peptide spectral matches and other supporting data were transferred for further analysis using custom tools developed in MATLAB (MathWorks, Natick, MA, USA) to provide visualization and statistical characterization.

A semiquantitative assessment of the protein amount was conducted through the application of a Spectral Counting (SpC) method. The Normalized percentage of Spectral Counts (NpSpC) of each protein, identified in the LC-MS spectra, was calculated by applying the following equation:²⁵

$$\text{NpSpC}_k = \left(\frac{\left(\frac{\text{SpC}}{M_w} \right)_k}{\sum_{i=1}^n \left(\frac{\text{SpC}}{M_w} \right)_i} \right) \times 100,$$

where NpSpC is the normalized percentage of spectral count (i.e., raw counts of ions) for protein k , SpC is the spectral count identified, and M_w is the molecular weight (in kDa) of the protein k . NpSpC was used for further downstream analysis of biomolecular coronas between allergic and healthy subjects.

III. Immune responses

A. Basophil activation assay

Since mast cells are present in nearly all extracerebral tissues, basophils can be used as “surrogate” allergy effector cells. Importantly, the activation of these cells can be identified from whole blood through flow cytometry using validated and published methods.²⁶ To test whether ferumoxytol can directly activate basophils, we examined the effect of ferumoxytol on CD63 expression on gated basophils, an indicator of basophil reactivity. Specifically, we incubated 300 ng/ml free iron to evaluate the potential activation of CARPA, media alone, 15 mg/ml carboxymethyl dextran, different concentrations of ferumoxytol (100, 1.5, and 15 mg/ml),

100 mg/ml of patient’s plasma with ferumoxytol (i.e., protein corona), polyclonal IgE (as a positive control), and anti-IgE (1 mg/ml Omalizumab) with whole blood-derived basophils to search for evidence of an IgE-mediated reaction.

B. Complement activation related pseudo allergy (CARPA)

Especially with intravenous iron products, complement activation related pseudo allergy (CARPA) has been reported as another possible pathway mediating hypersensitivity reactions due to the release of free, labile iron. Therefore, we included free iron in all immunological assays to test whether free iron or ferumoxytol or ferumoxytol/protein corona is responsible for the main mechanisms associated with the immune reactions to ferumoxytol. Nanoparticle features such as surface charge, size, and composition directly activate the complement system leading to similar symptoms as a true type I hypersensitivity reaction.

C. T-cell activation assay

To test the possible role of a type IV response, we performed T-cell activation assay using validated and published methods.²⁷ We stained effector CD4+CD3+ T cells and cytotoxic CD8+CD3+ T cells with carboxyfluorescein succinimidyl ester (CFSE). We then subjected the cells to a proliferation assay over 3 days of incubation with anti-CD28/CD3 beads. We added 100 mg/ml of the patient’s plasma (i.e., ferumoxytol + protein corona) or 15 mg/ml ferumoxytol alone to the *in vitro* culture over 3 days. We stained for the following activation markers: CD25, CD69, CD137, CD154, and interferon gamma (IFN γ).

D. Statistical analyses

We tested differences in quantitative measurements between experimental groups with a Student’s t-test. Statistically significant differences were assumed for a p-value of less than 0.05. All statistical analyses were done using STATA RELEASE 14.2 (StataCorp LP, College Station, TX). Based on our previous experience, a sample size of five patients for the allergy and eight patients for the non-allergy group provides 90% power at 5% error to detect significant differences in protein and cellular assays between patients with and without allergic reactions.

IV. RESULTS

A. Biomolecular/protein corona formation

All patient blood serum samples exposed to ferumoxytol showed the formation of coronas around ferumoxytol nanoparticles as initially confirmed by DLS and zeta potential. Ferumoxytol nanoparticles in saline demonstrated a mean diameter of 25.3 ± 2.5 nm and a mean zeta potential of 27.7 ± 2.5 . The mean sizes of nanoparticles incubated with serum from patients were significantly increased to 53.5 ± 2.1 nm and 51.5 ± 2.0 nm for patients who demonstrated an allergic reaction or remained healthy (respectively). The formation of a biomolecular corona reduced the zeta potential of the nanoparticles to 17.5 ± 2.1 and 16.0 ± 3.1 for patients who demonstrated an allergic reaction or remained healthy

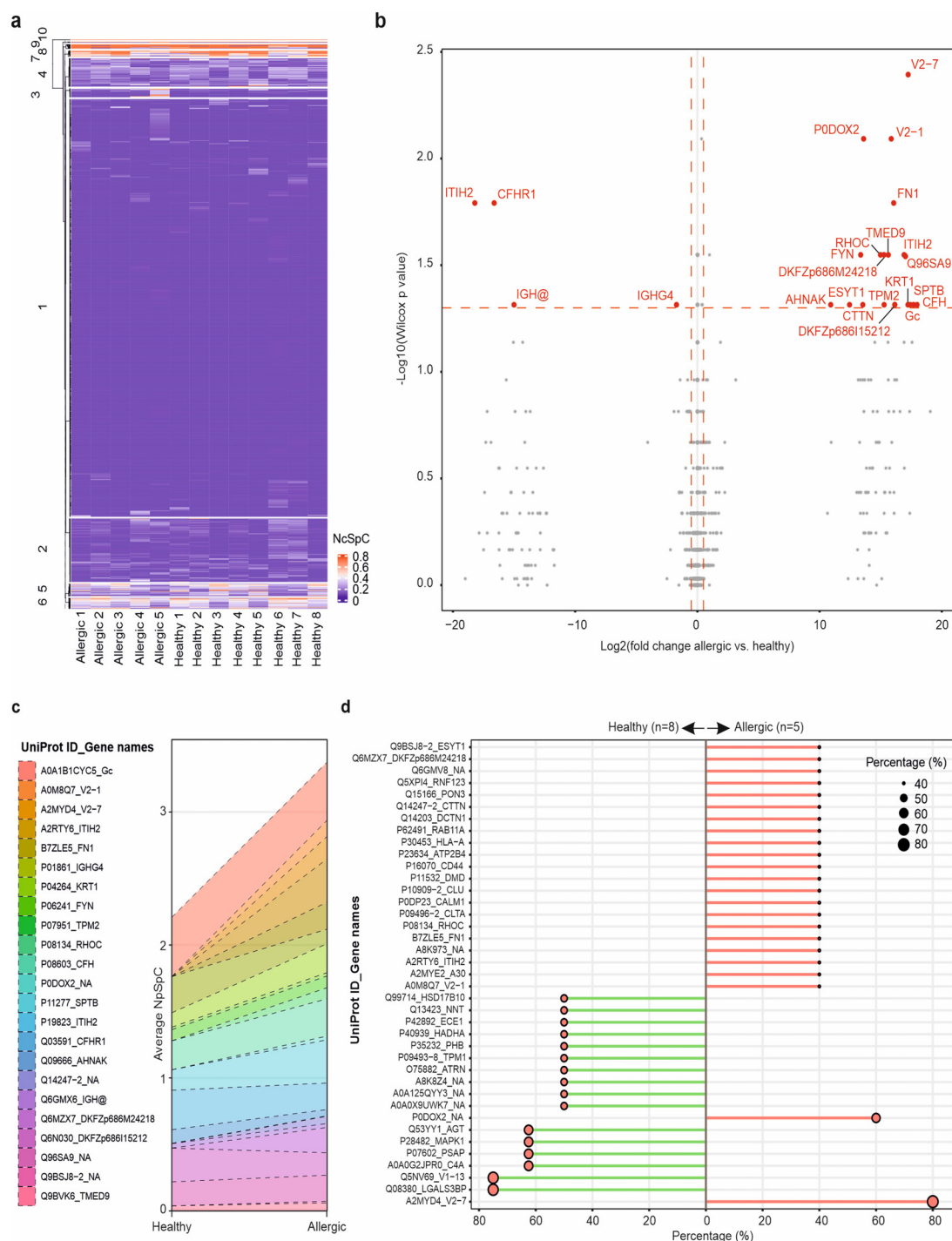


FIG. 1. (a) Plasma protein NpSpC profile shows the similarities and differences across the allergic and healthy individuals. (b) 23 proteins are over- or under-represented in allergic vs healthy subjects (excluding allergic sample 5; the nonquantified proteins were given a value of $1e-6$). (c) Stack plot shows the average NpSpC of the 23 proteins from volcano plot on (b), in protein coronas obtained from healthy vs allergic subjects. (d) Proteins found exclusively in the biomolecular/protein corona of allergic or healthy patients are shown in the pine plot. For instance, a percentage of 80 on the allergic side means that V2-7 is quantified in four out of five allergic samples and in none of the healthy samples.

(respectively). Our statistical analysis revealed that there were no significant differences ($p > 0.05$) between the two groups of patients.

B. Protein corona samples are personalized

To identify the type and quantity of the participating proteins in the composition of the biomolecular corona, we performed LC-MS/MS analysis. The LC-MS/MS results for all the subject coronas are available in Table 1 in the supplementary material.⁵³ These experiments led to the quantification of 1717 unique proteins in the 13 samples. Figure 1 in the supplementary material⁵³ shows the number of quantified proteins in individual samples. Only 5.5% of all proteins are found in the protein coronas of all individuals in the current study. As shown in the heatmap in Fig. 1(a), the remaining proteins are not quantified in every case (in the heatmap, the nonquantified values are adjusted as zeroes). The proteins enriched in each of the 10 clusters can be found in Table 2 in the supplementary material.⁵³ Figure 2(a) in the supplementary material⁵³ shows the correlation within and between allergic and healthy corona samples that are not significant, further supporting the personalized protein corona concept. This finding is more corroborated by the fact that allergic sample number 5 has more than 150 proteins that are not quantified in any other sample. However, since this subject showed an allergic reaction, and each allergy case might show a specific response, we did not exclude this sample from downstream analyses [except for Figs. 1(b) and 1(c)].

C. Proteins enriched in the protein corona of allergic versus healthy subjects

We compared proteins in coronas obtained from allergic versus healthy subjects using Wilcoxon nonparametric statistics. As shown in Fig. 1(b), 23 proteins pass the significance threshold (Wilcoxon p value < 0.05) and a twofold change in NpSpC. Out of these proteins, 19 were over-represented in coronas from allergic subjects, with V2_7, Immunoglobulin alpha-2 heavy chain (UniProt identifier: P0DOX2, no gene name assigned) and V2_1 as the most significant outliers. Figure 1(c) shows the relative difference in NpSpC for these 23 proteins in coronas from healthy with allergic subjects.

The proteins that show over- or under-representation in the protein coronas of allergic patients might give hints on the cause of allergic reactions. We first made an upset plot [Fig. 2(b) in the supplementary material⁵³] to visualize the protein quantification profiles across the samples. As shown, there are many proteins that are uniquely quantified in a subset of samples. Therefore, we analyzed the data to identify the proteins that are only detected in the protein corona of allergic patients and vice versa. As shown in the pine plot, we found 23 proteins that were specifically enriched in the corona samples of allergic subjects with a percentage $> 40\%$ (quantified in at least two out of five allergic subjects, and not in any healthy samples) [Fig. 1(d)]. The same procedure was performed for differentiating proteins quantified only in healthy subjects [Fig. 1(d)], please note the difference in the interpretation of percentages, as eight samples exist here compared to five in allergic]. The results can be found in Table 3 in the supplementary material.⁵³

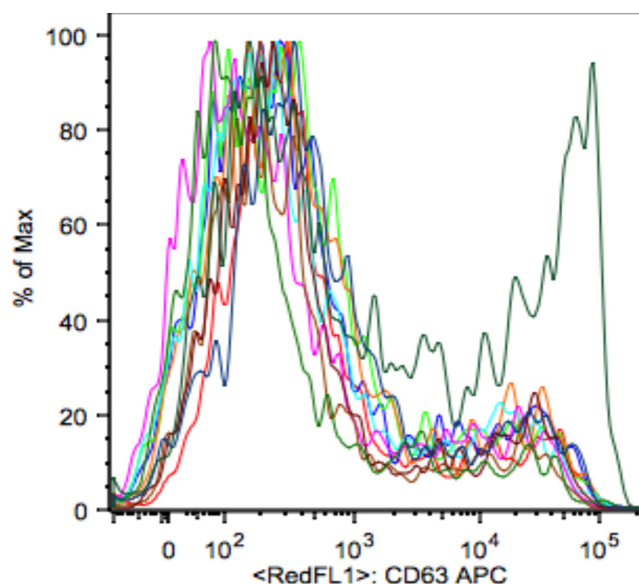


FIG. 2. Ferumoxytol does not activate healthy whole blood-derived basophils: Flow cytometry analysis of 300 ng/ml free iron (light blue), media alone (pink), 15 mg/ml carboxymethyl dextran (blue), or 300 ng/ml (green), 1.5 mg/ml (red), or 15 mg/ml ferumoxytol (black), incubated with healthy control whole blood-derived basophils. Median fluorescence intensity of the CD63 marker (an activator of basophils) is graphed on the x axis; less than 10^3 is negative.

Although proteins exclusively found in the protein corona of healthy subjects are also interesting, here we will only focus on proteins found exclusively in coronas of allergic individuals. The top target is V2-7 that was present in all four allergic patients but in none of the other samples (Table 3 in the supplementary material⁵³). We, therefore, hypothesize that this protein can be the potential candidate for causing an allergic reaction. Although the percentage contribution of the V2-7 protein is low (0.17%), the protein might have been exposed at the outer layer of protein corona. Several reports demonstrated that the exposure of the active site of proteins is more important to direct their functions rather than their concentration in the corona profiles.^{24,28} The second protein that was detected in 60% of coronas from allergic samples was “Immunoglobulin alpha-2 heavy chain” (UniProt identifier: P0DOX2), involved in humoral immunity. The other proteins are depicted in Fig. 1(d).

D. Basophil activation

Since mast cells are present in nearly all extracerebral tissues, basophils can be used as “surrogate” allergy effector cells.²⁹ Importantly, these cells can be identified from whole blood through flow cytometry using validated methods.²⁹ To test whether ferumoxytol can directly activate basophils, we examined the effect of ferumoxytol on CD63 expression on gated basophils, an indicator of basophil reactivity. Following incubation of healthy whole blood-derived basophils with free iron, media alone,

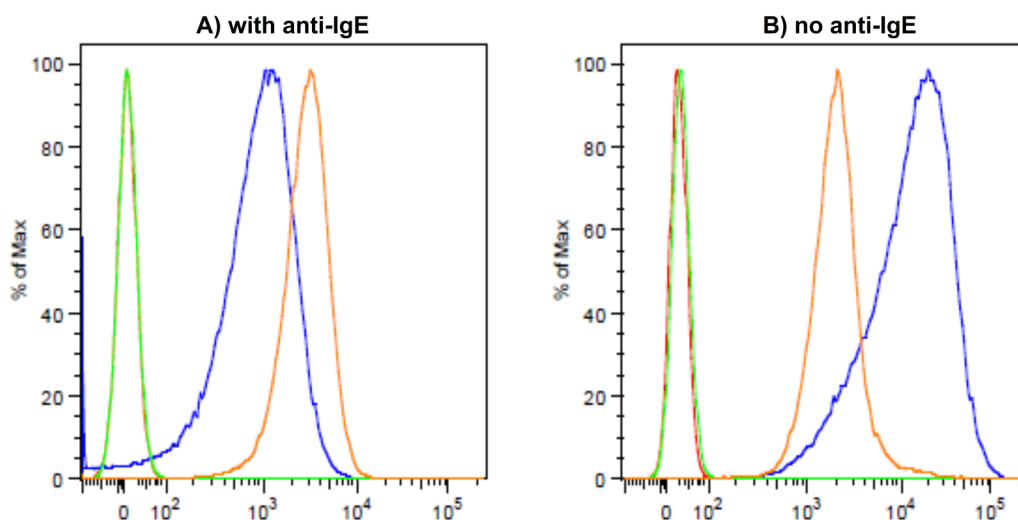


FIG. 3. Ferumoxytol does not cause an IgE-mediated immune response: Patient's plasma with ferumoxytol + protein corona (blue) was incubated with whole blood-derived basophils with (a) and without anti-IgE (b). Negative controls used were media alone (green), ferumoxytol alone (overlap with green), and plasma from healthy controls (overlap with green). Positive control used was polyclonal IgE (orange). Flow cytometry indicates that plasma proteins of patient's ferumoxytol corona can activate basophils; however, in the presence of anti-IgE, basophil response was significantly decreased as determined by CD63 log fold changes [CD63 expression via median fluorescence intensity (x axis) on gated basophils was detected by validated methods to indicate basophil reactivity].

carboxymethyl dextran, or ferumoxytol, we did not find any activation of gated basophils (Fig. 2). By comparison, incubation of healthy whole blood-derived basophils with polyclonal IgE as a positive control led to a significant activation of gated basophils. These data suggest that the ferumoxytol reactions are not due to the direct activation through nonimmune pathways.

We next examined blood samples from three patients who had an allergic reaction to ferumoxytol within 2 h of infusion to search for evidence of an IgE-mediated reaction (Fig. 3). Results showed that ferumoxytol alone does not activate basophils. However, the plasma proteins of the randomly selected patient's ferumoxytol corona did activate basophils. In the presence of anti-IgE, we could decrease the basophil response significantly as determined by CD63 logfold changes.

E. Effector memory t cells

To test the possible role of a type IV response, we performed T-cell assays using validated and published methods.^{30,31} Briefly, we stained effector CD4+CD3+ T cells from the same patient described in Sec. IV D and effector T cells from an age-matched healthy control (Fig. 4) with CFSE. We then subjected the cells to a proliferation assay over 3 days of incubation with anti-CD28/CD3 beads. We found that the patient's plasma was associated with CD4 + T-cell proliferation; ferumoxytol alone did not significantly increase proliferation compared to media alone (26.9% with patient plasma; 8.2% with ferumoxytol alone; 8.3% with media alone). Since many factors in the plasma could potentially activate T cells, this experiment does not rule out other mechanisms. However, we were intrigued that incubation of ferumoxytol alone versus plasma from healthy control subjects did not significantly change the

proliferation quantification (16.5% versus 14.9%) from media alone (15.3%). These data suggest that corona-coated ferumoxytol but not ferumoxytol alone could induce the proliferation of effector memory T cells, consistent with a type IV hypersensitivity reaction.

V. DISCUSSION

Patient-specific immune responses can vary depending on the type, size, and coating of the particle in question. For example, *in vitro* studies have shown that engineered nanoparticles such as quantum dots, silica, and superparamagnetic iron oxides activate Th2-type macrophages by enhancing interleukin (IL)-10 and inhibiting tumor necrosis factor alpha (TNF α) expression.^{32–35} Other reports indicate that superparamagnetic iron oxide nanoparticles induce production of TNF α and nitric oxide by macrophages *in vitro*.³⁶ These results are akin to *in vivo* alterations, in which macrophages alter their polarization to favor a pro-inflammatory Th1 phenotype.³⁷ These previous investigations focused on the nanoparticles themselves, but not the patient-specific biomolecular corona. To our knowledge, there is no prior report on the effects of the biomolecular corona around nanoparticles in patients with and without immune responses to these nanoparticles. Since nanoparticle-based drugs are increasingly translated to clinical practice,^{38–42} a better understanding of the effect of the biomolecular corona on potential adverse reactions could have broad clinical implications.

For immediate intravenous (IV) iron hypersensitivity, several pathogenic models have been implicated. Rather than IgE-mediated mechanisms,⁴³ high molecular weight iron dextran products have been repeatedly shown to cause immune complex anaphylaxis (Coombs and Gell type III) due to pre-existing dextran-reactive

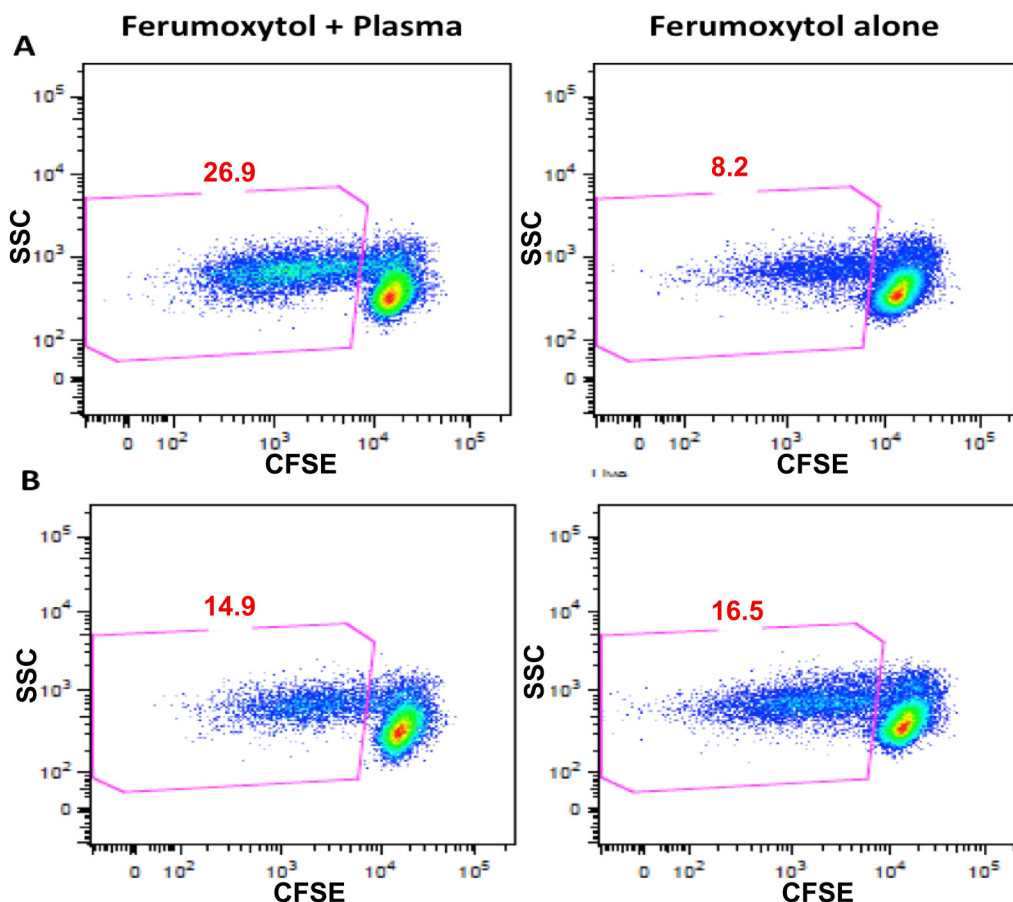


FIG. 4. Ferumoxyl + protein corona but not ferumoxyl alone induces proliferation of effector memory T cells, consistent with a type IV hypersensitivity reaction. CD4+CD3+ T cells from a patient with an allergic reaction against ferumoxyl (a) and an age-matched healthy control (b) were stained with CFSE and incubated for 3 days with ferumoxyl and patient's plasma (left panel), or ferumoxyl alone (right panel). Flow cytometry analysis showed that ferumoxyl with the protein corona (from the patient plasma) was associated with CD4+ T-cell proliferation, whereas ferumoxyl alone did not increase proliferation above background (background not shown). The y axis represents side scatter (SSC) indicating cell granularity (%) of proliferating populations. The x axis (CFSE) distinguishes proliferating vs nonproliferating populations.

antibodies.⁴⁴ Hypersensitivity reactions to IV irons have similarities to the red man syndrome, which is related to IV vancomycin administration.⁴⁵ It is a nonimmunologic reaction and is thought to involve direct stimulation of mast cells or basophils. Also, some infusion reactions from biologic drugs show similarities [e.g., toxicity caused by high doses or acute cytokine release (cytokine storm) caused by cell activation or cell death].⁴⁴ Free iron toxicity can lead to transient facial flushing, chest oppression, headache, nausea, and back pain through a free-iron-like reaction.⁴⁶ Deep understanding of the underlying mechanism(s) associated with ferumoxyl reactions for each individual patient will be important for the development of successful preventive actions. One potential approach might be the development of diagnostic *in vitro* tests based on the biomolecular corona, such as a “protein corona sensor array.”^{47–49} Future studies have to show if the concept of a “personalized protein corona” can be confirmed in a larger number of patients and if the information can potentially be useful for creating

personalized management plans.⁵⁰ Future studies would also have to show if a much more complicated protein corona test could be done in a time efficient manner and would provide improved outcomes compared to generic premedication of patients with a history of allergies.

We recognize several limitations of our study: Results from patients with allergic reactions are based on blood samples from six patients. Severe allergic reactions to ferumoxyl are rare with an incidence of 0.01%–0.02% in adult patients.^{1,51} Our study was only a proof-of-concept and not designed to be a clinical efficacy study, which would require a greater number of subjects to obtain statistical significance for a clinical outcome measure (which typically have a wide range of variations). Instead, this proof-of-concept study focused on generating preliminary evidence for differences in the biomolecular corona in patients with and without allergic reactions to ferumoxyl. Future prospective studies will have to confirm this observation in a larger number of patients.

Our patients had mild allergic reactions to ferumoxytol and none of our patients have a severe allergic reaction. We do not know if the protein corona in patients with a severe allergic reaction would be different from patients with a mild reaction. If such a difference would exist, it would be clinically highly valuable.

We were not able to perform tryptase, histamine, or platelet-activating factor tests because the blood was drawn outside a 4-h window after the observed reaction. Nonetheless, the described comparison of biomolecular corona is an important first step in elucidating additional biological variables that might affect hypersensitivity mechanisms related to ferumoxytol.

A number of biological variables can affect corona formation, such as protein composition of the blood, body temperature, co-morbidities, and time period of exposure.^{16,52} To keep *in vitro* parameters consistent, we performed all assays for samples of different patients using identical ferumoxytol dose and concentration. Future investigations will compare biomolecular corona assay readouts from different time points of the same patient as well as assay results after *in vitro* and *in vivo* corona formations. Due to limited amounts of blood samples from pediatric patients, we also were not able to test the reproducibility of protein corona readouts of individual patients. Further studies should conduct multiple protein corona analyses from the same blood sample and evaluate the reproducibility of the results. Comparative experiments need to be conducted with plasma from male and female patients with various levels of allergic reactions and other co-morbidities to evaluate the effect of sex and gender in protein corona formations. In addition, disease stage, co-medications, and co-morbidities need to be considered to determine if patients with specific diseases have an increased risk of developing a protein corona that can trigger allergic reactions to ferumoxytol.

In summary, our preliminary *ex vivo* investigations provided some information on the biomolecular corona around ferumoxytol in patients with and without the allergic reaction to ferumoxytol. A major prospective investigation will be needed to identify specific protein patterns in the protein corona structure of ferumoxytol nanoparticles that are responsible, at least in part, for the activation of allergic pathways.

ACKNOWLEDGMENTS

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DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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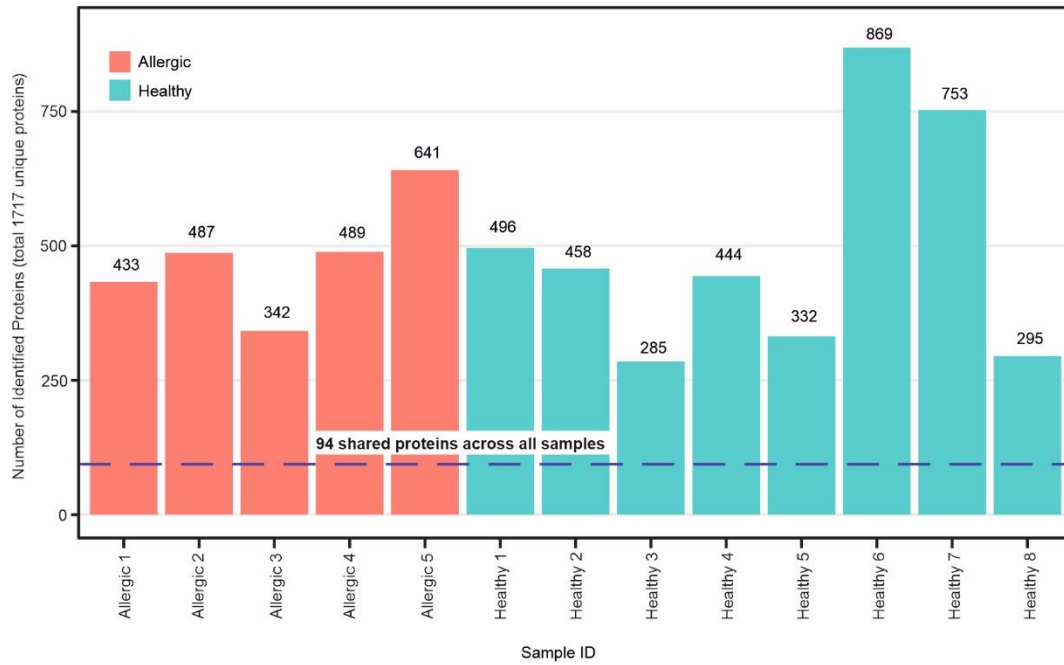
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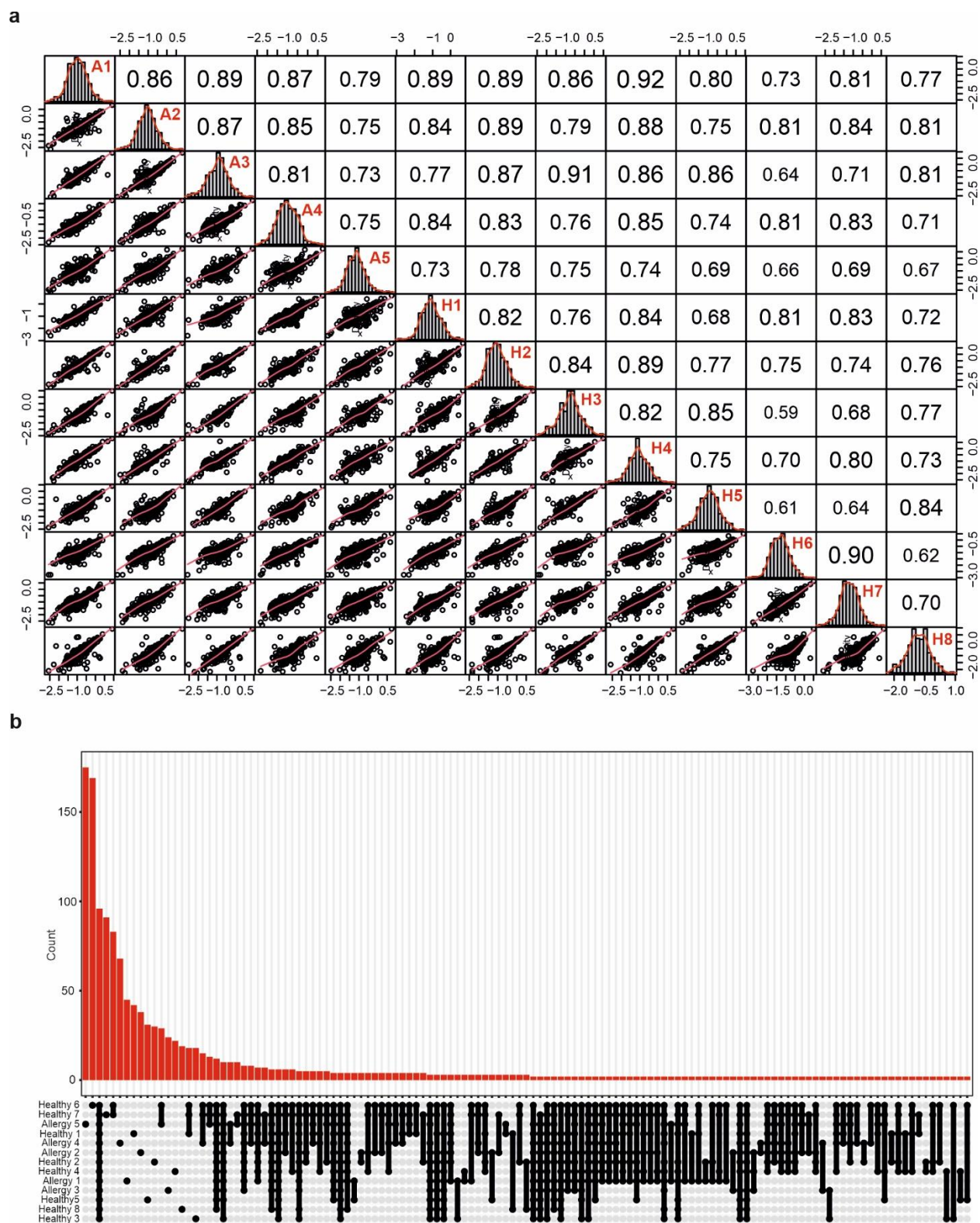
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Supporting information



Supp Fig. 1. The total number of quantified unique proteins and the number of proteins quantified in each sample. Note the higher number of proteins quantified in the corona of allergic subject number 5.



Supp Fig. 2. a, Correlation plot shows correlation of samples within and between different

individuals. The numbers within the boxes indicate correlations between 0 and 1 (highest correlations). All the correlations were significant (p value < 0.001). **b**, The upset plot shows the number of proteins that are quantified across all the samples or those that are uniquely quantified in a subset of samples. For instance, more than 150 proteins were quantified only in the protein corona of allergic subject number 5.

Supplementary Table 1. The LC-MS/MS results for all the coronas obtained from allergic and healthy subjects.

Supplementary Table 2. The proteins associated with each cluster in the heatmap in **Figure 1a**.

Supplementary Table 3. Proteins found exclusively in the biomolecular/protein corona of allergic patients.