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IgG4 Immunostaining and Its Implications in Orbital Inflammatory Disease

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Abstract

Objective: IgG4-related disease is an emerging clinical entity which frequently involves tissue within the orbit. In order to appreciate the implications of IgG4 immunostaining, we analyzed gene expression and the prevalence of IgG4-immunostaining among subjects with orbital inflammatory diseases.

Methods: We organized an international consortium to collect orbital biopsies from 108 subjects including 22 with no known orbital disease, 42 with nonspecific orbital inflammatory disease (NSOI), 26 with thyroid eye disease (TED), 12 with sarcoidosis, and 6 with granulomatosis with polyangiitis (GPA). Lacrimal gland and orbital adipose tissue biopsies were immunostained for IgG4 or IgG secreting plasma cells. RNA transcripts were quantified by Affymetrix arrays.

Results: None of the healthy controls or subjects with TED had substantial IgG4 staining. Among the 63 others, the prevalence of significant IgG4-immunostaining ranged from 11 to 39% depending on the definition for significant. IgG4 staining was detectable in the majority of tissues from subjects with GPA and less commonly in tissue from subjects with sarcoidosis or NSOI. The detection of IgG4+ cells correlated with inflammation in the lacrimal gland based on histology. IgG4 staining tissue expressed an increase in transcripts associated with inflammation, especially B cell-related genes. Functional annotation analysis confirmed this.

Conclusion: IgG4+ plasma cells are common in orbital tissue from patients with sarcoidosis, GPA, or NSOI. Even using the low threshold of 10 IgG4+ cells/high powered field, IgG4 staining correlates with increased inflammation in the lacrimal gland based on histology and gene expression.


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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant clinical and immunostaining data are within the paper and its Supporting Information files. The raw and normalized gene expression microarray data are available from the GEO database (accession numbers GSE58331 and GSM1407182 through GSM1407356).

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Competing Interests: Stephen R. Planck has read the journal’s policy and an author of this manuscript has the following possible competing interests: JTR previously consulted for Genentech and was a co-investigator on a studied funded by Genentech to evaluate the use of rituximab for orbital inflammatory diseases. Dr. Rosenbaum is a PLOS ONE Editorial Board member. This does not alter the authors’ adherence to PLOS ONE Editorial policies and criteria.

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Introduction

IgG4-related disease (IgG4-RD) was first described in patients with autoimmune pancreatitis who had elevated concentrations of IgG4 in serum [1]. Shortly thereafter, in 2003, extra-pancreatic lesions were described in patients with autoimmune pancreatitis, which led to the recognition of IgG4-RD as a systemic condition [2]. Since 2003, IgG4-RD has been described in a multitude of organ systems including the pancreas, biliary tree, salivary glands, kidneys, lungs, skin, prostate, and orbit [1,3–7]. Across the various organ systems, IgG4-RD is known to have a similar histopathological presentation which includes a dense lymphoplasmacytic infiltrate that is rich in IgG4+ plasma cells (IgG4+PC), storiform fibrosis, and obliterator phlebitis [8].

Ophthalmic disease is a common manifestation of IgG4-RD [9]. Patients with IgG4-immunostaining, may present with painless eyelid swelling, proptosis, or diplopia [10,11]. The lacrimal glands, the nasolacrimal duct, and the retrobulbar region may be affected [7,11–13]. A consensus report recommended the term, IgG4-related dacryoadenitis for disease in the lacrimal gland and IgG4-related orbital inflammation for disease that affects adipose tissue just posterior to the ocular globe [14].

Orbital inflammatory disease can affect orbital muscle, lacrimal gland, or adipose tissue. The most common systemic disease associated with orbital inflammation is hyperthyroidism attributable to Graves disease, also known as thyroid eye disease or TED. Sarcoidosis or granulomatosis with polyangiitis (GPA, previously known as Wegener’s granulomatosis) can also cause inflammation within the orbit. Many patients with orbital inflammatory disease are classified as having nonspecific orbital inflammation (NSOI, previously known as orbital pseudotumor). Little is known as to how each of these entities might be related to IgG4-RD.

The etiology of IgG4-RD remains unclear. Although the infiltration of IgG4+PC is a defining characteristic of the disease, there is no evidence that IgG4 is directly involved in the pathogenesis. In fact, some have hypothesized that IgG4, which does not fix complement, is expressed to dampen inflammation [15]. Intriguingly, an immune response to IgG4 reportedly exacerbates rheumatoid arthritis [16].

Three studies that sought to determine the prevalence of IgG4-immunostaining among patients with orbital inflammation found very discrepant results with prevalence ranging from 4 to 52% [7,17,18]. In part, this relates to the definition of a positive case. Some studies have used a threshold of 10 IgG4+PC/high powered field (hpf) [9,18]. Other studies have used thresholds of up to 30 IgG4+cells/hpf or a minimum ratio of IgG4+IgG+PC of 0.40 or a combination thereof [11,17]. Some have suggested that IgG4-immunostaining has immense clinical implications that frequently indicate a multisystem disease which is highly likely to respond to rituximab therapy [19,20]. Accordingly an understanding of the prevalence of IgG4 immunostaining among patients with orbital inflammation has potential clinical and therapeutic implications. We sought to clarify the implication of IgG4 immunostaining in the orbit by studying tissue from patients with a variety of orbital inflammatory diseases. We correlated the detection of IgG4+ plasma cells in tissue with the specific diagnosis as well as with inflammation, fibrosis, and gene expression.

Materials and Methods

Human subjects and tissues

This study was approved by the Institutional Review Boards (IRB) at Oregon Health & Science University, Columbia University, University of California San Diego, Wake Forest University, Medical College of Wisconsin, and Mount Carmel (Ohio) and by the University of British Columbia Clinical Research Ethics Board, the Royal Adelaide Hospital Research Ethics Committee, and the King Khaled Eye Specialist Hospital Human Ethics Committee/Institutional Review Board. This study was in compliance with the Helsinki Declaration. Formalin-fixed paraffin-embedded (FFPE) samples were obtained from 109 contributing centers. Data were analyzed anonymously and written informed consent was obtained when required by the local IRB or ethics committee.

The diagnoses of nonspecific orbital inflammation, sarcoidosis, granulomatosis with polyangiitis, thyroid eye disease, and normal pulmonary disease in addition to the orbital disease. One other subject with GPA had renal disease, but the others had a limited form of GPA. Among the 12 subjects diagnosed with sarcoidosis, all had non-caseating granulomata present in the lacrimal or orbital adipose tissue biopsy. Adenopathy was present on chest CT scan in six. In 5 subjects, results of CT scanning and/or biopsy outside of the orbit were not known or not performed. The control tissue was obtained during surgery on eyes with non-inflamed orbits, such as blepharoplasties and enucleations. Multiple specimens were evaluated for 7 patients, three of whom had both orbit and lacrimal gland biopsies. In total there were 119 tissue biopsies (74 orbital tissue, 45 lacrimal gland) used for histopathological and immunohistochemical analysis.

All samples were reviewed and scored without reference to the indications for biopsy or other clinical information. Two slides from each specimen were stained with hematoxylin and eosin for histopathological evaluation. These features were independently re-evaluated by two ocular pathologists (D.J.W. and H.E.G.). The features included degree of fibrosis, degree of inflammation, presence of obliterator phlebitis, and presence of storiform fibrosis. The degrees of fibrosis and degree of inflammation were quantified as absent (0), mild (1), moderate (2), or severe (3). For most samples, the presence or absence of lacrimal gland tissue was confirmed by the presence or absence of lacrimal gland mRNA.

Immunohistochemistry

Two serial sections from each specimen were used for immunohistochemical evaluation. One slide was stained for IgG4+ plasma cells (antibody from The Binding Site, San Diego, Ca, 1:15000 dilution) and the other for IgG+ plasma cells (antibody from Dako, 1:10,000 dilution). Immunostaining used Ventana automated instruments with Ultraview detection (Ventana, Tucson, AZ). Two surgical pathologists (M.L.T. and D.C.H.) independently counted the numbers of IgG4+ cells in three high power fields (hpf, 400X, about 0.3 mm²) and the IgG+ cells in the corresponding fields of the paired slides. These data are expressed as the mean number of positive cells per hpf. For the subjects for whom more than one specimen of the same tissue type was stained, the overall mean was used for the statistical analysis.

RNA extraction and microarray

For each FFPE specimen, multiple 10 μm sections were collected and total RNA was extracted with miRNeasy FFPE kits.
FFPE Amplification and 3’ IVT Labeling kit (Affymetrix, Santa Clara, CA). 50 ng of RNA was used for the majority of samples with a minimum input of 20 ng RNA for samples in which RNA yields were limited. Biotin-labeled cDNA targets were hybridized with a GeneChip Human Genome U133 Plus 2.0 array (Affymetrix, Santa Clara, CA) according to standard Affymetrix protocol. This array contains over 54,000 probe sets for 47,000 transcripts and variants. Following hybridization, arrays were stained and scanned using the GeneChip Scanner 3000 7G system (Affymetrix). Image processing and initial quality control analysis were performed using Affymetrix GeneChip Command Console (AGCC) v. 3.1.1 and Affymetrix Expression Console v. 1.1 software, respectively.

Statistical analysis

Simple statistical tests were done with chi-square tests, Mann-Whitney U-test, or Student’s t-test as appropriate [21]. Affymetrix CEL files were preprocessed by the Robust Multiarray Analysis. After normalization, linear models were fitted to test potential differences in gene expression profiles due to elevated concentrations of IgG4 while controlling for diseases and batch effects. We used ‘affy’ and ‘limma’ packages of Bioconductor [http://www.bioconductor.org] in the R project for statistical computing [http://www.r-project.org].

Results

Altogether, 119 orbital adipose tissue and lacrimal gland tissue samples from 108 subjects were evaluated (Table 1). Our control group consisted of 22 subjects without orbital disease. The control disease includes 26 with TED, 6 with GPA, 42 with NSOI, and 12 with sarcoidosis. Of the 108 subjects, about one third was male. The collective median reported age at the time of biopsy was 54.0 years (range, 12.0–96.1 years). The ages shown in Table 1 reflect the mean age at biopsy based on IgG4+ status and gender or the mean age at biopsy based on IgG4+ immunostaining defined as at least 10 positive cells per hpf (Table 3). Examining only the NSOI samples from orbital adipose tissue, there was a trend for IgG4+ samples to come from older subjects, by Mann-Whitney U test (p = 0.078, excluding one 96.1 year old outlier). There was no real significant difference in age and IgG4 status for NSOI affecting the lacrimal gland.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Number of subjects</th>
<th>Age Median (Q1–Q3)</th>
<th>Male:Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (normal)</td>
<td>22</td>
<td>66.5 (58.9–71.0)</td>
<td>7:15</td>
</tr>
<tr>
<td>TED</td>
<td>26</td>
<td>55.0 (44.6–61.0)</td>
<td>7:20</td>
</tr>
<tr>
<td>GPA</td>
<td>6</td>
<td>47.1 (27.1–49.0)*</td>
<td>2:04</td>
</tr>
<tr>
<td>NSOI</td>
<td>42</td>
<td>48.1 (37.6–64.3)</td>
<td>15:27</td>
</tr>
<tr>
<td>Sarcoidosis</td>
<td>12</td>
<td>39.8 (31.7–49.4)*</td>
<td>4:08</td>
</tr>
</tbody>
</table>

*Ages are not available for 4 subjects.

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No storiform fibrosis or obliterator phlebitis was noted in any of the tissue samples. A comparison of the fibrosis and inflammation scores between IgG4+ and IgG4− cases showed a significant increase in fibrosis and inflammation in the lacrimal gland tissue among IgG4+ NSOI patients (Mann-Whitney U test, p = 0.014 and p = 0.005, respectively) (Figure 1). Although in the orbit the IgG4+ group included samples with IgG4 counts ≤0.5, these fibrosis and inflammation scores were not statistically different. For these comparisons, the correlation relied on the least stringent criterion to classify IgG4 status.

Correlation of IgG4 staining with inflammation or fibrosis

No storiform fibrosis or obliterator phlebitis was noted in any of the tissue samples. A comparison of the fibrosis and inflammation scores between IgG4+ and IgG4− cases showed a significant increase in fibrosis and inflammation in the lacrimal gland tissue among IgG4+ NSOI patients (Mann-Whitney U test, p = 0.014 and p = 0.005, respectively) (Figure 1). Although in the orbit the IgG4+ group included samples with IgG4 counts ≤0.5, these fibrosis and inflammation scores were not statistically different. For these comparisons, the correlation relied on the least stringent criterion to classify IgG4 status.

Correlation of treatment with IgG4 staining

We employed the prescription of corticosteroid or other immunosuppressive as a surrogate index for severity, reasoning that more severe disease was more likely to prompt potentially toxic therapy. Using chi square analysis, patients with NSOI, GPA, or sarcoidosis had at least 10 IgG4+cells/hpf were not more likely to be treated with corticosteroid (p = 0.3) and not more likely to receive additional immunosuppression after the biopsy (p = 0.7). A higher percentage of the IgG4+ patients started corticosteroid treatment prior to their biopsy, whereas the opposite was true for the IgG4+ group (Figure 2). We further compared the
maximum dose of prednisone given prior to biopsy to those who were IgG4+ with those who were IgG4 negative. The values (52.5±18.9 mg/day and 55.8±27.1 mg/day) respectively did not differ between the two groups. Six patients received an injection of triamcinolone as part of their therapy, and these were equally divided between the IgG4+ and IgG4- groups.

Because no subject with TED had substantial staining for IgG4, we reviewed the therapy for these subjects in case the absence of staining was a result of treatment. Of the 26 subjects with TED in this study, two were receiving prednisone at the time of biopsy, 5 had previously been treated with prednisone between 3.5 months and 3.5 years before the biopsy, 13 had not received any medications, and in 6 subjects, the prior treatment was unknown. Thus treatment is an unlikely explanation for the absence of IgG4 staining among subjects with TED.

IgG4 staining and multisystem disease

IgG4 staining has been proposed as a marker of multisystem disease [2]. GPA by definition is a multisystem disease and sarcoidosis tends to involve multiple tissue sites. Fifteen subjects in our study had NSOI and IgG4 immunostaining. Of these 15, we were able to review the medical history of 12 in detail. None of these 12 had evidence for a multisystem disease, including the two subjects who had greater than 30 IgG4+ cells per hpf and an IgG4:IgG cell ratio of >0.4.

IgG4 immunostaining and gene expression

We compared RNA transcripts in tissues that stained for IgG4 plasma cells with tissues from subjects with NSOI, GPA, or sarcoidosis and little or no IgG4 staining. We used a positive staining threshold of at least 10 IgG4+ cells/hpf and a threshold for gene expression change of at least 1.5-fold difference with a false discovery rate adjusted p-value of <0.05. In lacrimal gland samples, we detected 98 probes sets with increased signals and 4 that had decreased signals. For tissue from the orbital adipose tissue, 100 probe sets had increased signals and 22 were decreased. The complete lists of probe sets indicating significant expression differences are in Tables S1 and S2 in File S1.

Examples of probe sets with elevated signals in IgG4+ orbital adipose tissue (Table 4) include several immune response related genes. Of the 100 probe sets with higher signals in IgG4+ orbital adipose tissue, 16 are for light or heavy chain immunoglobulin

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**Table 2.** A minority of subjects with inflamed orbits have markedly high IgG4+PC counts.

<table>
<thead>
<tr>
<th></th>
<th>IgG4+ PC/hpf</th>
<th>IgG4+ PC/hpf &gt;30 and IgG4+PC/IgG-PC ≥0.4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;10</td>
<td>10–29</td>
</tr>
<tr>
<td><strong>Lacrimal Gland</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7</td>
<td>-</td>
</tr>
<tr>
<td>TED</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>NSOI</td>
<td>15</td>
<td>4</td>
</tr>
<tr>
<td>Sarcoidosis</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td><strong>Orbital fat</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>15</td>
<td>-</td>
</tr>
<tr>
<td>TED</td>
<td>25</td>
<td>-</td>
</tr>
<tr>
<td>GPA</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>NSOI</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>Sarcoidosis</td>
<td>5</td>
<td>1</td>
</tr>
</tbody>
</table>

The number of subjects in each category is shown.

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**Table 3.** IgG4 status is independent of age and gender.

<table>
<thead>
<tr>
<th></th>
<th>IgG4+</th>
<th>IgG4-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median(Q1–Q3)</td>
<td>Median(Q1–Q3)</td>
</tr>
<tr>
<td><strong>Lacrimal Gland</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSOI</td>
<td>44.2(43.3–50.5)</td>
<td>45.9(37.8–61.4)</td>
</tr>
<tr>
<td>Sarcoidosis</td>
<td>30.7(21.3–40.0)</td>
<td>35.1(32.1–37.5)*</td>
</tr>
<tr>
<td><strong>Orbital adipose tissue</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPA</td>
<td>48.1(41.3–50.37)*</td>
<td>27.1</td>
</tr>
<tr>
<td>NSOI</td>
<td>40.0(35.6–53.5)</td>
<td>69.3(44.2–72.8)</td>
</tr>
<tr>
<td>Sarcoidosis</td>
<td>—*</td>
<td>53.9(45.0–58.9)</td>
</tr>
</tbody>
</table>

*Ages are not available for 4 subjects.

doi:10.1371/journal.pone.0109847.t003
transcripts (Table S1 in File S1). These results are consistent with the inflammation scores shown in Figure 1 in that higher scores were more frequent in the IgG4+ tissues. Table 4 also lists examples of probe sets with lower signals in the IgG4+ orbit tissues.

Examples of lacrimal gland transcripts with differential expression in IgG4+ tissues are listed in Table 5. Unlike orbital adipose tissue, no increased expression of immunoglobulin genes was detected. This is likely due to high levels of immunoglobulin transcripts in normal lacrimal gland that might obscure detection of additional antibody transcripts. Nonetheless, the profile of genes with increased transcript levels in the IgG4+ lacrimal gland tissues is consistent with more lymphocytic activity than in the IgG4-tissues.

Discussion

To our knowledge, our study is the first study to compare the prevalence of IgG4 staining among various inflammatory conditions within the orbit. A recent report on IgG4 immunostaining in skin disease similarly concluded that IgG4 staining is detectable in a variety of clinical entities [22]. Wallace and colleagues [23] described patients with IgG4 associated pachymeningitis. Their series included patients who had either meningeval sarcoidosis or GPA, some of whom stained positively for IgG4 [23]. Strehl and colleagues found positive IgG4 staining in a variety of conditions including rheumatoid synovitis and the inflammatory response around a carcinoma. [24] We believe that ours is the first study to detect IgG4 immunostaining in tissue from patients with sarcoidosis affecting the orbit. A report from Japan showed that nearly 25% of patients with lymphoproliferative disease within the orbit have IgG4+ cells in orbital tissue [25]. Another study found that 50% of subjects with xanthogranuloma in the orbit show IgG4 immunostaining [25]. Chang et al. noted the presence of IgG4+ plasma cells in GPA patient biopsies taken from various sites including the majority of periorbital biopsies [26]. Similarly we reported that renal biopsies from patients with GPA can stain positively for IgG4 [27].

Considering only the non-TED orbital inflammation cases, we found the prevalence of IgG4+ immunostaining to be 36% based on a cutoff of \( \geq 10 \) IgG4+ PC/hpf. However, this cutoff is controversial in the literature. In 2008, Sato et al. published one of the largest IgG4-immunostaining studies [7]. Applying a cutoff of \( > 10 \) IgG4+cells/hpf, they found a prevalence of 19% among patients with ocular adnexal lymphoproliferative disorders. This paper was criticized for setting too low of a threshold for determining IgG4 status [28]. Most papers set a minimum threshold of 30 IgG4+PC/hpf [29]. Using this standard, the prevalence of IgG4-immunostaining would decrease to 15 of 64 (23%). Alternatively, some studies have used the more stringent criteria of \( \geq 10 \) IgG4+PC/hpf and an IgG4+/IgG+ ratio of \( \geq 0.4 \) [7,30]. Using this stringent criterion, the prevalence of IgG4-immunostaining in our study would be 7 of 64 (11%).

Our study is the first to analyze RNA transcript expression in tissue that contains IgG4 plasma cells. We based this analysis on the least stringent definition of IgG4 staining for a practical reason; other definitions of IgG4 positivity would have provided too few samples for accurate statistical analysis. We believe that this analysis lends support for defining significant IgG4 staining within the orbit using the criterion of at least ten cells staining per hpf.

Unlike the histopathological presentation in IgG4-RD in other organ systems, we did not find storiform fibrosis or obliterative phlebitis in any of our samples, regardless of the tissue type. A
Table 4. Examples of gene expression differences comparing IgG4+ to IgG4- orbital tissue from subjects with NSOI, GPA, or sarcoidosis.

<table>
<thead>
<tr>
<th>Probe Set</th>
<th>Gene Symbol</th>
<th>Fold Change</th>
<th>FDR P-value</th>
<th>Gene Title</th>
<th>A Gene Ontology Biological Process</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Probe sets with increased levels</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>211639_x_at</td>
<td>IGH; IGHA1; IGHA2; IGHD; IGHG1; IGHG3; IGHG4; IGHM; IGHV4-31</td>
<td>3.33</td>
<td>0.029</td>
<td>Immunoglobulin heavy locus</td>
<td>Immune response</td>
</tr>
<tr>
<td>216829_at</td>
<td>IGK; IGKC</td>
<td>3.06</td>
<td>0.013</td>
<td>Immunoglobulin kappa locus; immunoglobulin kappa constant</td>
<td>Immune response</td>
</tr>
<tr>
<td>205242_at</td>
<td>CXCL13</td>
<td>2.99</td>
<td>0.045</td>
<td>Chemokine (C-X-C motif) ligand 13</td>
<td>T and B cell chemotaxis</td>
</tr>
<tr>
<td>242020_s_at</td>
<td>ZBP1</td>
<td>2.80</td>
<td>0.048</td>
<td>Z-DNA binding protein 1</td>
<td>Positive regulation of type I interferon-mediated signaling pathway</td>
</tr>
<tr>
<td>234477_at</td>
<td>IGH1A; IGHV4-31</td>
<td>2.52</td>
<td>0.022</td>
<td>Immunoglobulin heavy constant alpha 1; immunoglobulin heavy variable 4-31</td>
<td>Immune response</td>
</tr>
<tr>
<td>205884_at</td>
<td>ITGA4</td>
<td>2.40</td>
<td>0.049</td>
<td>Integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)</td>
<td>Cell adhesion</td>
</tr>
<tr>
<td>217227_x_at</td>
<td>IGLV1-44</td>
<td>2.40</td>
<td>0.039</td>
<td>Immunoglobulin lambda variable 1-44</td>
<td>Immune response</td>
</tr>
<tr>
<td>216541_x_at</td>
<td>IGHG1; IGHM</td>
<td>2.18</td>
<td>0.045</td>
<td>Immunoglobulin heavy constant gamma; immunoglobulin heavy constant mu</td>
<td>Immune response</td>
</tr>
<tr>
<td>211648_at</td>
<td>IGH1G1; IGHM</td>
<td>1.98</td>
<td>0.034</td>
<td>Immunoglobulin heavy constant gamma 1; immunoglobulin heavy constant mu</td>
<td>Immune response</td>
</tr>
<tr>
<td>223565_at</td>
<td>MZB1</td>
<td>2.13</td>
<td>0.011</td>
<td>Marginal zone B and B1 cell-specific protein</td>
<td>Positive regulation of immunoglobulin biosynthetic process</td>
</tr>
<tr>
<td>204562_at</td>
<td>IRF4</td>
<td>2.08</td>
<td>0.026</td>
<td>Interferon regulatory factor 4</td>
<td>interferon-gamma-mediated signaling pathway</td>
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<td>1558561_at</td>
<td>HM13</td>
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<td>Histocompatibility (minor) 13</td>
<td>Proteolysis</td>
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<td>ITGB6</td>
<td>1.96</td>
<td>0.043</td>
<td>Integrin, beta 6</td>
<td>Cell adhesion</td>
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<tr>
<td>201688_s_at</td>
<td>TPD52</td>
<td>1.86</td>
<td>0.019</td>
<td>Tumor protein D52</td>
<td>B cell differentiation</td>
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<tr>
<td>225435_at</td>
<td>SSR1</td>
<td>1.86</td>
<td>0.020</td>
<td>Signal sequence receptor, alpha</td>
<td>Activation of signaling protein activity involved in unfolded protein response</td>
</tr>
<tr>
<td><strong>Probe sets with decreased levels</strong></td>
<td></td>
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<tr>
<td>217187_at</td>
<td>MUC5AC</td>
<td>−2.77</td>
<td>0.015</td>
<td>Mucin 5AC, oligomeric mucus/ gel-forming</td>
<td>Extracellular fibril organization</td>
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<tr>
<td>201044_x_at</td>
<td>DUSP1</td>
<td>−2.34 −1.75</td>
<td>0.038 0.014</td>
<td>Dual specificity phosphatase 1</td>
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<td>210226_at</td>
<td>NRA4A</td>
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<td>0.035</td>
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<td>Positive regulation of endothelial cell proliferation</td>
</tr>
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<td>207008_at</td>
<td>CCR2</td>
<td>−1.82</td>
<td>0.038</td>
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<td>Dendritic cell chemotaxis</td>
</tr>
<tr>
<td>1559960_x_at</td>
<td>SYCE1L</td>
<td>−1.77</td>
<td>0.030</td>
<td>Synaptonemal complex central element protein 1-like</td>
<td>Meiosis</td>
</tr>
<tr>
<td>226765_at</td>
<td>SPTBN1</td>
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<td>0.047</td>
<td>Spectrin, beta, non-erythrocytic 1</td>
<td>Mitotic cytokinesis</td>
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<td>227404_at</td>
<td>EGR1</td>
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<td>Early growth response 1</td>
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<td>206869_at</td>
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<td>Chondroadherin</td>
<td>Cartilage condensation</td>
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<tr>
<td>1553572_a_at</td>
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<td>Oxygen transport</td>
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<tr>
<td>208333_at</td>
<td>LHX5</td>
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<td>0.025</td>
<td>LIM homeobox 5</td>
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<td>216904_at</td>
<td>COL6A1</td>
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<td>0.029</td>
<td>Collagen, type VI, alpha 1</td>
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<tr>
<td>229207_x_at</td>
<td>RNF187</td>
<td>−1.62</td>
<td>0.017</td>
<td>Ring finger protein 187</td>
<td>Positive regulation of cell proliferation</td>
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Table 4. Cont.

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<tr>
<th>Probe Set</th>
<th>Gene Symbol</th>
<th>Fold Change</th>
<th>FDR P-value</th>
<th>Gene Title</th>
<th>A Gene Ontology Biological Process</th>
</tr>
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<td>1555380_at</td>
<td>ADAMTS4</td>
<td>−1.62</td>
<td>0.013</td>
<td>ADAM metalloproteinase with thrombospondin type 1 motif, 4</td>
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<td>203792_x_at</td>
<td>PCGF2</td>
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<td>0.036</td>
<td>Polycomb group ring finger 2</td>
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<td>206128_at</td>
<td>ADRA2C</td>
<td>−1.60</td>
<td>0.043</td>
<td>Adrenoceptor alpha 2C</td>
<td>Activation of MAPK activity by adrenergic receptor signaling pathway</td>
</tr>
</tbody>
</table>

Probe sets were selected for expression increases or decreases and ample annotation. A relevant Gene Ontology Biological Process (http://geneontology.org/) is listed for each gene.

doi:10.1371/journal.pone.0109847.t004

Table 5. Examples of gene expression differences comparing IgG4+ to IgG- lacrimal gland tissue from subjects with NSOI, GPA, or sarcoidosis.

<table>
<thead>
<tr>
<th>Probe Set</th>
<th>Gene Symbol</th>
<th>FC</th>
<th>FDR p value</th>
<th>Gene Title</th>
<th>Gene Ontology Biological Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>228599_at</td>
<td>MS4A1</td>
<td>3.51</td>
<td>0.004</td>
<td>Membrane-spanning 4-domains, A1</td>
<td>B cell activation</td>
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<td>221969_at</td>
<td>PAX5</td>
<td>3.48</td>
<td>0.005</td>
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<tr>
<td>217422_x_at, 38521_at, 204581_at</td>
<td>CD22, CD22 molecule</td>
<td>2.87, 2.48</td>
<td>0.016, 0.028, 0.009</td>
<td>CD22 molecule</td>
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<tr>
<td>219014_at</td>
<td>PLAC8</td>
<td>2.83</td>
<td>0.030</td>
<td>Placenta-specific B</td>
<td>Regulation of cell proliferation</td>
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<tr>
<td>209995_s_at</td>
<td>TCL1A</td>
<td>2.74</td>
<td>0.031</td>
<td>T-cell leukemia/lymphoma 1A</td>
<td>Multicellular organismal development</td>
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<tr>
<td>1558662_s_at, 222915_s_at</td>
<td>BANK1, B-cell scaffold protein with ankyrin repeats 1</td>
<td>2.55, 2.44</td>
<td>0.012, 0.020</td>
<td>B cell activation</td>
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<tr>
<td>221601_s_at</td>
<td>FAIM3</td>
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<td>0.023</td>
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<td>235400_at</td>
<td>FCRLA</td>
<td>2.37</td>
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<td>Fc receptor-like A</td>
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<td>1564310_a_at</td>
<td>PARP15</td>
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<td>Poly (ADP-ribose) polymerase family, member 15</td>
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<td>0.032</td>
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<td>35974_at, 204674_at</td>
<td>LRMP, Lymphoid-restricted membrane protein</td>
<td>2.26, 2.19</td>
<td>0.009, 0.008</td>
<td>Vesicle targeting</td>
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<tr>
<td>211861_x_at</td>
<td>CD28</td>
<td>2.18</td>
<td>0.038</td>
<td>CD28 molecule</td>
<td>Inflammatory response to antigenic stimulus</td>
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</tbody>
</table>

Probe sets were selected for expression increases of more than 2.1 fold or decreases of more than 1.5 fold, p values <0.5, and sample annotation. A relevant Gene Ontology Biological Process (http://geneontology.org/) is listed for each gene.

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rituximab [31]. Thus therapeutic response also might not
be orbital inflammatory disease respond favorably to therapy with
the entity of IgG4-RD. We recently reported that 70% of patients with
IgG4-RD distinct from GPA, sarcoidosis, or NSOI. Since NSOI already
includes the histological implication of increased fibrosis and the
inflammation allowing us to compare the prevalence of IgG4
inflammation, including orbital myositis, and trigeminal nerve involvement.

In summary, we conducted a multi-centered, international study on the prevalence of IgG4-immunostaining in orbital
inflammation allowing us to compare the prevalence of IgG4+ PC infiltration in different entities affecting the orbit. Unfortunately,
due to the limitations of our database and incomplete clinical information, we were unable to examine the relationship between our cases and IgG4 serum concentrations. In addition, by
the nature of our multi-centered approach, we were reliant on
diagnoses from different institutions. In a minority of instances we
lacked complete information on the therapy or diagnostic evaluation. Although the clinical implications of IgG4 staining in
the orbit require further investigation, our data support the rationale to target B cells in therapy. Regardless of whether IgG4-
immunostaining is considered a distinct clinical entity or a subset
of other entities, subjects with an IgG4+ tissue infiltrate display an
identifiable gene expression profile consistent with a heightened
inflammatory response. Our study should help physicians better
interpret the implications of IgG4 staining in orbital tissue.

Supporting Information

File S1 Figure S1, Representative images illustrating the variability of IgG4 and IgG3 staining in orbit adipose tissue from
subjects with NSOI. Table S1, Comparison of gene expression in
IgG4+ and IgG4− orbit adipose tissues. Table S2, Comparison of
gene expression in IgG4+ and IgG4− lacrimal gland tissues.
(DOCX)

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work.

Author Contributions

Conceived and designed the experiments: AJW CAH JTR SRP. Performed the experiments: AJW DGH DJW HEG MLT PS. Analyzed the data: AJW CAH DC DJW JTR SRP. Contributed reagents/materials/
analysis tools: BSK CC DOK DPE DS EAS GJH HAH HAK JAF JDN
MK PJD PJP RAD RPY VAW. Wrote the paper: AJW BSK CAH CC DC
DCH DJW DOK DPE DS EAS GJH HAH HAK JAF JDN JTR MK MLT PJD PJP PS RAD RPY SRP VAW. Project leader: JTR.

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leads to rapid decline of serum IgG4 levels and prompt clinical improvement in

IgG4 in Orbital Inflammatory Disease