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Lesional CD4+ IFN-γ+ cytotoxic T lymphocytes in IgG4-related dacryoadenitis and sialoadenitis

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Abstract

Objectives—IgG4-related disease (IgG4-RD) is a chronic, systemic, inflammatory condition of unknown aetiology. We have recently described clonally expanded circulating $CD4^+$ cytotoxic T lymphocytes (CTLs) in IgG4-RD that infiltrate affected tissues where they secrete interleukin (IL)-1β and transforming growth factor -β1 (TGF-β1). In this study, we sought to examine the role of $CD4^+$ CTLs in the pathogenesis of IgG4-related dacryoadenitis and sialoadenitis (IgG4-DS) and to determine whether these cells secrete interferon-gamma (IFN-γ) at lesional sites.

Methods—Salivary glands of 25 patients with IgG4-DS, 22 patients with Sjögren's syndrome (SS), 12 patients with chronic sialoadenitis (CS) and 12 healthy controls were analysed in this study. Gene expression analysis was performed on submandibular glands (SMGs) from five patients with IgG4-DS, three with CS and three healthy controls. Infiltrating $CD4^+$ CTLs were examined by quantitative multicolour imaging in tissue samples from 20 patients with IgG4-DS, 22 patients with SS, 9 patients with CS and 9 healthy controls.

Results—In IgG4-DS tissues, nine genes associated with $CD4^+$ CTLs were overexpressed. The expression of granzyme A (GZMA) mRNA was significantly higher in samples from patients with IgG4-RD compared with corresponding tissues from SS and healthy controls. Quantitative imaging showed that infiltrating $CD4^+$ $GZMA^+$ CTLs were more abundant in patients with IgG4-

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DS than in the other groups. The ratio of $CD4^+GZMA^+$ CTLs in SMGs from patients with IgG4-DS correlated with serum IgG4 concentrations and the number of affected organs. A large fraction of $CD4^+GZMA^+$ CTLs in SMGs from patients with IgG4-DS secreted IFN-γ.

Conclusions—The pathogenesis of IgG4-DS is associated with tissue infiltration by $CD4^+GZMA^+$ CTLs that secrete IFN-γ.

INTRODUCTION

IgG4-related disease (IgG4-RD) is characterised by multiorgan inflammation, elevated serum IgG4 concentrations, tissue infiltration by $IgG4^+$ plasmacytes and storiform fibrosis in various organs, including the pancreas, salivary and lacrimal glands, lungs, thyroid, liver, kidneys, aorta, prostate, retroperitoneum and lymph nodes.[1–3] T cells are the most abundant cells in the lymphoplasmacytic infiltrate in IgG4-RD lesions and are thought to be the drivers of IgG4-RD pathogenesis.[3] 4 The finding of T helper 2 (T_{H2})-type cytokines within IgG4-RD tissue lesions led to suggestions that this disease may be caused by T_{H2} cells[56] and IgG4-RD has been frequently associated with allergic disorders.[7] Contrary to these results, some reports suggested that T_{H1} immune responses might play an important role in the pathogenesis of IgG4-RD.[8–10] However, these previous reports used single-colour staining of the tissues from these patients, which lacks the ability to directly identify T_{H1} or T_{H2} cells in disease tissues. Previous reports have all relied on indirect evidence to implicate $CD4^+$ T cell subsets in this disease and more direct analyses of T cells have only recently been undertaken.

In a previous study, we demonstrated that relative increases in circulating T_{H2} cells were only observed in a subset of patients with IgG4-RD who had a history of atopic disease and that non-atopic IgG4-RD subjects did not exhibit any expansions of circulating T_{H2} cells.[11] In a more recent study, we demonstrated clonal expansions of $CD4^+$ cytotoxic T lymphocytes (CTLs) in the blood of patients with IgG4-RD. We also used multicolour analyses of tissues to show that these $CD4^+$CTLs infiltrated tissue lesions and were the dominant $CD4^+$ T cells at disease sites, while $CD4^+GATA3^+$ T_{H2} cells were sparse.[12] Using T cell receptor beta chain repertoire analysis of $CD4^+$CTLs and T_{H2} cells obtained simultaneously from the peripheral blood of patients with IgG4-RD with a history of atopic disease, we also reported that $CD4^+$CTLs were clonally expanded, but T_{H2} cells were highly polyclonal.[12] These data, the first to directly analyse $CD4^+$ T cell subsets in IgG4-RD tissues using quantitative multicolour fluorescence, strongly suggest that T_{H2} cells do not contribute to the pathogenesis of IgG4-RD and that IgG4-RD represents the first chronic inflammatory disease that has been documented to be linked to tissue-infiltrating, clonally expanded $CD4^+$ CTLs.

IgG4-related dacryoadenitis and sialoadenitis (IgG4-DS), also known as Mikulicz's disease, is a condition in which the lacrimal and salivary glands are enlarged because of the infiltration of lymphocytes and other inflammatory cells. Mikulicz's disease is characterised by elevated serum IgG4 concentrations and infiltration into gland tissues of IgG4-positive plasma cells.[13] To clarify the pathogenesis of IgG4-DS, we compared gene expression in submandibular glands (SMGs) from patients with IgG4-DS, chronic sialoadenitis (CS) and

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healthy subjects using DNA microarray analysis. These results were subsequently validated by quantitative PCR and quantitative analyses using multicolour immunofluorescence staining. Our data indicate that IgG4-DS is associated with tissue infiltration by $CD4^{+}$GranzymeA $(GZMA)^{+}$ CTLs that secrete interferon-gamma (IFN-γ), but not by T_{H1} or T_{H2} cells. A correlation between infiltrating $CD4^{+}$ CTL numbers in disease tissues with the number of disease lesions and serum IgG4 levels was also observed.

PATIENTS AND METHODS

Study population

This study included 25 patients with IgG4-DS, 22 patients with active Sjögren's syndrome (SS), 12 patients with CS and 12 healthy controls. Three patients with oral squamous cell carcinoma (OSCC) were the source of control submandibular glands (SMGs) for microarray analyses. CS is a non-specific inflammatory disease of the salivary glands linked to sialolithiasis. All of these patients had been followed up between 2007 and 2015 at the Department of Oral and Maxillofacial Surgery of Kyushu University Hospital, a tertiary care centre. Open SMG biopsies were obtained from patients with IgG4-DS;[14] patients with CS underwent submandibulectomy, and control subjects with OSCC underwent neck dissection. The SMGs from patients with OSCC were histologically normal (see online supplementary figure E1), with no clinical evidence of metastasis. IgG4-DS was diagnosed according to the following criteria[1]: (1) persistent (longer than 3 months) symmetrical swelling of more than two lacrimal and major salivary glands; (2) high (>135 mg/dL) serum concentrations of IgG4 and (3) infiltration of IgG4-positive plasma cells into tissue ($IgG4^{+}$ cells/IgG^{+} cells >40%), as determined by immunostaining. All SMGs from patients with IgG4-RD had histopathological features of IgG4-RD (see online supplementary figure E1). None of the patients had any allergic disease.

SS was diagnosed as described.[15] Each patient exhibited objective evidence of salivary gland involvement based on the presence of subjective xerostomia and a decreased salivary flow rate, abnormal findings on parotid sialography and focal lymphocytic infiltrates in labial salivary glands (LSGs) (see online supplementary figure E1). None of the patients with IgG4-DS, SS and CS had a history of treatment with steroids or other immunosuppressants, infection with HIV, hepatitis B virus or hepatitis C virus; none had sarcoidosis or evidence of lymphoma at the time of the study. All patients with IgG4-DS, SS and CS had strong lymphocytic infiltration in these tissues.

All subjects studied provided written informed consent.

Analysis of gene expression

Analysis of gene expression by microarray and its validation are described in the online supplementary methods.[16–21]

Multicolour immunofluorescence staining

Tissue samples were obtained from whole SMGs[14] of 16 patients with IgG4-DS (see online supplementary E4) and 6 with CS (see online supplementary table E5) and from LSGs of 15

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patients with active-SS (see online supplementary table E6) and 5 healthy controls (see online supplementary table E5).

These tissue samples were fixed in formalin, embedded in paraffin and sectioned. These specimens were incubated with antibodies to transforming growth factor -β1 (TGF-β1) (clone MAB246; R&D Systems), IFN-γ (clone sc-74108; Santa Cruz Biotechnology), GZMA (clone LS-C312742; LSBio), GATA3 (clone CM405A; Biocare), CD8 (clone ab85792; abcam) and CD4 (clone CM153A; Biocare) followed by incubation with secondary antibody using a SuperPicTure Polymer Detection Kit (Invitrogen) and an Opal 3-Plex Kit (Fluorescein, Cyanine3 and Cyanine5) (Perkin Elmer). The samples were mounted with ProLong antifade-containing 4′, 6-diamidino-2-phenylindole (DAPI) (Invitrogen). Images were acquired with the TissueFAXS platform (TissueGnostics).[12] TissueFAXS is an analytical instrument that combines the advantages of multichannel microscopy and automated high-resolution imaging with the scientific accuracy of flow cytometry (http://www.bga.su/info/TissueFAXS). Stained cells were automatically counted in the salivary gland specimens.

Quantitative image analysis

Staining of salivary gland specimens was quantified using TissueQuest software (TissueGnostics), with cut-off values determined relative to the positive controls. Tissue samples from IgG4-DS patient G19 were not quantified, because of the lack of infiltration of lymphocytes. Tissue samples from all healthy controls were not quantified, because of the lack of infiltration of $CD4^{+}GZMA^{+}$ CTLs.

Statistical analyses

Differences between groups were determined by the Mann– Whitney U test and Spearman rank correlations. All statistical analyses were performed using JMP Pro software, V.11 (SAS Institute, Cary, North Carolina, USA) for Mac. A p value <0.05 was considered statistically significant.

RESULTS

Gene expression profiles of SMGs in patients with IgG4-DS, CS and controls

We initially wished to determine whether transcriptome analysis from salivary gland biopsies from patients with IgG4-DS could provide useful information regarding the inflammatory exudate in this disease. This analysis was undertaken using total RNA. The clinical and pathological features of participating patients and healthy controls are shown in online supplementary tables E1 and E2. Hierarchical clustering by the pvclust method (A) and principal component analysis (B) using the quantile algorithm FARMS-normalised data are also shown (see online supplementary figure E2). Gene expression patterns were distinct in each of these three groups (see online supplementary figure E2).

Because gene expression patterns in tissues from patients with IgG4-DS and CS differed from those in healthy controls by clustering analysis, we therefore compared gene expression and identified genes differentially expressed between IgG4-DS and CS in

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pairwise comparisons. In IgG4-RD, 935 upregulated genes and 516 downregulated genes were identified as differentially expressed genes (DEGs) (p value <0.05) compared with CS. Gene ontology term analysis indicated that differentially expressed genes upregulated in patients with IgG4-DS encoded proteins that function in T/B cell activation, T cell differentiation, defence responses, inflammatory responses, responses to wounding, activation of immune responses, immune system development, regulation of innate immune responses, positive regulation of innate immune responses, lymphocyte-mediated immunity, B cell-mediated immunity, leucocyte-mediated immunity, CTL-mediated immune response against target cells, glycoprotein, immunoglobulin subtype, cytokine activity, chemotaxis and Th1/Th2 differentiation (see online supplementary figure E3).

$CD4^+$CTL-related gene expression in SMGs from patients with IgG4-DS

We recently reported the clonal expansion of $CD4^+$CTLs in the peripheral blood of patients with IgG4-RD.[12] In vitro studies have indirectly suggested that innate immune cells may also contribute to IgG4 production in this disease.[22] We therefore examined gene expression in SMGs focusing on genes expressed in $CD4^+$ T cells and innate immune cells. We noted the higher expression of a selected panel of genes expressed by $CD4^+$ T cells and macrophages in patient biopsies (figure 1A). We found that genes associated with $CD4^+$ CTLs, T follicular helper cells, type II innate lymphoid cells and macrophages were expressed to a higher extent in IgG4-DS samples than in samples from patients with CS and healthy controls (see figure 1A and see online supplementary table E7 and E8). As shown in online supplementary table E7, among the genes upregulated in IgG4-DS were the $CD4^+$CTL-related genes encoding T-bet (p=0.034 and p<0.001 compared with patients with CS and controls), SLAMF7 (p=0.029 and p<0.001, respectively), perforin (p=0.001 and p<0.001, respectively), GZMA (p=0.004 and p<0.001, respectively) and GZMB (p=0.005 and p<0.001, respectively) (see online supplementary table E7). In addition, the expression of genes encoding IFN-γ (p<0.001 each), tumour necrosis factor (p=0.019 and p<0.001; respectively), CCL4 (p=0.035), CCL5 (p<0.001) and TGF-β1 (p<0.001) differed significantly in patients with IgG4-DS compared with control. Interestingly, the expression of interleukin (IL)-4 mRNA was significantly higher in IgG4-DS than in control and CS samples (p<0.001 each). However, the expression of GATA3 mRNA was significantly lower in IgG4-DS than in controls (p<0.001), but not in CS (p=0.065). These data were consistent with our recent studies in the blood indicating that in IgG4-RD, $CD4^+$CTLs predominate in tissue sites and that $CD4^+GATA3^+$ T_{H2} cells are sparse in disease lesions.[12] The possibility that the increased IL-4 expression in disease tissues was from a non-T_{H2} cell remains to be explored.

The expression of $CD4^+$CTL-related genes was subsequently validated by quantitative PCR. Quantitative real-time PCR (RT-PCR) (figure 1B and online supplementary table E3) showed that the levels of GZMA mRNA was significantly higher in the salivary glands of patients with IgG4-DS compared with patients with SS (p=0.046) and healthy controls (p=0.011).

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$CD4^+GZMA^+$CTLs in IgG4-DS tissues

We scored salivary gland samples from a larger number of patients with IgG4-DS for the presence of $CD4^+$CTLs. We also sought to functionally characterise these $CD4^+$CTLs by analysing the cytokines secreted by these cells in vivo. Sections from 16 patients with IgG4-DS, 15 with SS, 6 with CS and 5 healthy controls were stained with antibodies to CD4 (red), GZMA (green) and GATA3 (magenta) and with DAPI (blue). A large number of $CD4^+$ cells (red) were colocalised with GZMA in IgG4-DS, but not in SS, CS and controls (figure 2A and see online supplementary figure E4). Although very few $CD4^+GZMA^+$ CTLs were detected in patients with SS, their localisation also differed between SS and IgG4-DS samples, being present only around ductal epithelial cells in SS samples. Furthermore, most of the $CD4^-GZMA^+$ cells (likely $CD8^+$CTLs or natural killer (NK) cells) in patients with SS were also detected around ductal epithelial cells (see figure 2A yellow arrow and see online supplementary figure E5). Although many $CD4^-GZMA^+$ cells (green) were present in CS samples, these cells did not colocalise with $CD4^+$ cells (red) (figure 2A). No $CD4^+GZMA^+$ CTLs were present in samples from healthy controls. Quantitative analysis of the distributions of $CD4^+GZMA^+$ CTLs in 15 patients with IgG4-DS, 15 with SS and 6 patients with CS showed that the percentage of $CD4^+GZMA^+$ CTLs was significantly higher in IgG4-DS than in SS ($p<0.001$) and CS ($p<0.001$) samples (figure 2B), whereas the percentage of $CD4^-GZMA^+$ cells was significantly higher in CS than in IgG4-DS ($p=0.001$) and SS ($p=0.001$) (figure 2B). Of the patients with IgG4-DS, patients G13 and G14 had some $CD4^+GATA3^+$ T_{H2} cell accumulations in these tissues (figure 2C), and patients G14 and G15 also had a history of allergy for radiopaque dye and anticholinergic drug. $CD4^+GATA3^+$ T_{H2} cells from an IgG4-DS subject (G14) are shown in online supplementary figure E4. Although $CD4^+GATA3^+$ T_{H2} cells were detected in all patients with IgG4-DS, the proportion of these cells was much smaller than that of $CD4^+GZMA^+$ CTLs.

The percentage of $CD4^+GZMA^+$ CTLs in IgG4-DS tissue positively correlated with serum IgG4 concentrations of the patients ($r=0.614$; $p=0.0154$) and also with the number of organs affected by IgG4-RD ($r=0.796$; $p=0.002$) (see figure 3A and see online supplementary figure E6). In contrast, the percentage of tissue $CD4^+GATA3^+$ T_{H2} cells was not correlated with serum IgG4 concentrations or the number of affected organs (see figure 3A and see online supplementary figure E6). As shown in figure 3B, although serum IgG4 concentrations did not show a strong correlation with the number of affected organs in IgG4-DS tissue, the patients with IgG4-DS with higher percentage of $CD4^+GZMA^+$ CTLs in these tissues exhibited higher serum IgG4 concentrations and larger number of affected organs. These are the first data to establish an association between the abundance of infiltrating $CD4^+$ CTLs and the extent of disease.

$CD4^+GZMA^+$ CTLs secrete IFN-γ and TGF-β1 in IgG4-DS tissues

Although $CD4^+$ CTLs express T-bet and IFN-γ RNA at relatively high levels, we had not previously analysed the ability of these cells to secrete IFN-γ at the disease sites.[12] Specimens from previously mentioned tissues from IgG4-DS (n=15), SS (n=6), CS (n=5) and healthy controls (n=3) were stained for CD4 (red), GZMA (green) and IFN-γ (magenta) and with DAPI (blue). We observed a large number of $CD4^+GZMA^+IFN\text{-}\gamma^+$ CTLs in the inflamed IgG4-DS, but not in inflamed SS and CS lesions (figure 4A and see online

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supplementary figures E7 and E8-A). Conversely, large numbers of $CD4^{+}GZMA^{-}IFN\text{-}\gamma^{+}$ T cells were detected in the inflamed SS and CS lesions (likely T_{H1} cells), but not in the inflamed IgG4-DS lesions. Distributions of $CD4^{+}GZMA^{+}IFN\text{-}\gamma^{+}$ CTLs and $CD4^{+}IFN\text{-}\gamma^{+}$ T cells in samples from 15 patients with IgG4-DS, 15 with SS and 6 with CS were analysed quantitatively. This analysis showed that the percentage of $CD4^{+}GZMA^{+}IFN\text{-}\gamma^{+}$ CTLs was significantly higher in IgG4-DS than in SS ($p<0.001$) and CS ($p<0.001$) samples (figure 4B, C) and that the percentage of $CD4^{+}GZMA^{-}IFN\text{-}\gamma^{+}$ T cells, consistent with a T_{H1} phenotype, was higher in SS and CS than in IgG4-DS samples (see online supplementary figure E8-B). A large number of $CD4^{+}GZMA^{+}IFN\text{-}\gamma^{+}$ CTLs were frequently detected in the lymphocytic infiltrate in inflamed IgG4-DS tissues (figure 4D and see online supplementary figure E9).

Based on the gene expression analyses, TGF-β1 was shown to be elevated in IgG4-DS tissues. To investigate the ability of $CD4^{+}$CTLs to secrete TGF-β1, specimens from previously mentioned tissues from IgG4-DS (n=15), SS (n=6), CS (n=5) and healthy controls (n=3) were stained for CD4 (red), GZMA (green) and TGF-β1 (magenta) and with DAPI (blue). Large numbers of $CD4^{+}GZMA^{+}TGF\text{-}\beta1^{+}$ CTLs were observed in inflamed lesions in biopsies from patients with IgG4-DS but not in inflamed lesions from patients with SS and CS (figure 5A and see online supplementary figure E10). A large number of $CD4^{+}GZMA^{-}TGF\text{-}\beta1^{+}$ T cells were also present in CS lesions (figure 5A). Quantitative analysis of $CD4^{+}GZMA^{+}TGF\text{-}\beta1^{+}$ CTLs in IgG4-DS tissues is shown (see online supplementary figure E11A). Quantitative analysis of the distributions of $CD4^{+}GZMA^{+}TGF\text{-}\beta1^{+}$ CTLs and $CD4^{+}TGF\text{-}\beta1^{+}$ T cells in samples from 15 patients with IgG4-DS, 15 with SS and 6 with CS showed that the percentage of $CD4^{+}GZMA^{+}TGF\text{-}\beta1^{+}$ CTLs was significantly higher in IgG4-DS than in patients with SS ($p<0.001$) and patients with CS ($p<0.001$) (figure 5B and see online supplementary figure E11B). However, $TGF\text{-}\beta1^{+}$ non-$CD4^{+}$CTL cells were also detected in IgG4-DS in large numbers (figure 5C). The percentage of tissue $CD4^{+}GZMA^{+}TGF\text{-}\beta1^{+}$ CTLs did not correlate with the number of affected organs (see online supplementary figure E11C). All subjects studied lacked a history of allergic disease and very few eosinophils were observed per high power field in IgG4-RD tissue samples (see inline supplementary figure E12).

DISCUSSION

We recently described the clonal expansion and accumulation of $CD4^{+}SLAMF7^{+}$ CTLs in the blood of patients with IgG4-RD and their infiltration into lesional tissue sites, suggesting that these cells play a role in the pathogenesis of this disease.[12] We also showed that while $CD4^{+}$CTLs are the dominant population in affected tissues, direct examination of T_{H2} cells in tissue revealed that these latter cells were sparse in IgG4-RD and only accumulated in the blood if there was a concomitant history of atopy. The results presented here confirm that active IgG4-DS is prominently linked to tissue infiltration by $CD4^{+}GZMA^{+}$ CTLs and that infiltration into tissue by $CD4^{+}GZMA^{+}$ CTLs that secrete IFN-γ (in addition to IL-1β and TGF-β as described before)[12] may contribute to the pathogenesis of IgG4-DS. $CD4^{+}$CTLs were virtually absent from lesional tissue samples from patients with CS and SS.

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Glandular lymphocytic infiltration is a progressive feature in SS, with most infiltrating cells being T cells, predominantly $CD4^+$ T cells. Other cells have also been found to infiltrate these tissues, including $CD8^+$ T cells, B cells, plasma cells and macrophages. IgG4-DS was once categorised as a subtype of SS, based on the histopathological similarities between the two diseases. Therefore, the epidemiological features, symptoms, organ involvement and serum abnormalities of IgG4-DS and SS have been frequently compared.[61923]

T_{H2} cell expansion in the circulation can be occasionally detected in IgG4-RD subjects, but only if they have a concurrent history of atopic disease.[11] 12 24 Our group showed that $CD4^+GATA3^+$ T_{H2} cells are relatively rare in IgG4-RD tissues in comparison with $CD4^+GZMA^+$ CTLs. IL-4 expression previously detected in disease tissues is likely from non-T_{H2} cells such as T follicular helper cells or type 2 innate lymphoid (ILC2) cells.

The expression of ILC2 and T_{FH} cell-related genes[2526] in IgG4-DS tissue was also increased as revealed by microarray analysis and we are currently analysing IgG4-RD tissues for the presence of T_{FH} cells and ILC2 cells.

In separate studies, Okazaki *et al*[8] examined the T_{H1}/T_{H2} balance of peripheral blood mononuclear cells in patients with autoimmune pancreatitis (AIP). The number of blood $CD4^+$ Tcells that secreted high levels of IFN-γ following in vitro reactivation was increased in patients with AIP compared with controls, whereas the number of IL-4-secreting $CD4^+$ T cells was not increased in patients with AIP. Furthermore, some reports reported that the serum IFN-γ/IL-4 ratio was higher in patients with IgG4-DS than those with SS.[9] They thus concluded that the pathogenesis of IgG4-RD might be mediated by a T_{H1}-predominant immune reaction.[8–10] Previous reports involved single-colour staining in the tissues from these patients, which would have been unable to distinguish $CD4^+$CTLs from T_{H1} cells. Some of these previous suggestions of T_{H1} cells in IgG4-RD may therefore have resulted from the erroneous categorisation of IFN-γ-secreting $CD4^+$CTLs as T_{H1} cells. Interestingly, our multicolour studies show that $CD4^+GZMA^-IFN\text{-}\gamma^+$ Tcells, which are likely 'true' T_{H1} cells, are relatively rare in IgG4-RD tissues in comparison with IFN-γ-secreting $CD4^+GZMA^+$ CTLs. In addition, $CD4^+GZMA^-IFN\text{-}\gamma^+$ T cells with a T_{H1} phenotype likely play an important role in the pathogenesis of SS. These data are consistent with our previous studies,[1920] although, since those studies also used single-colour staining approaches, we were unable to provide direct evidence for the existence of T_{H1} cells in tissues. In the case of IFN-γ secretion—it should be noted that apart from T_{H1} cells, this cytokine may be secreted by NK cells, natural killer T (NKT) cells, $CD8^+$ T cells and γδ T cells. However, our data suggest that the increased IFN-γ expression in IgG4-DS tissues is likely from $CD4^+$CTLs and not from T_{H1} cells.

The pathogenesis of fibrosis in IgG4-RD remains unclear. Although we observed populations of $CD4^+GZMA^+TGF\text{-}\beta1^+$ CTLs in tissue samples from patients with IgG4-DS, these cells were not detected in samples from patients with CS and SS. These cells may induce the fibrotic pathology observed in IgG4-RD. We have recently documented that $CD4^+SLAMF7^+$ CTLs in IgG4-RD tissues express IL-1β and TGF-β1.[12] Transgenic overexpression of IL-1β in the murine pancreas results in fibrotic pancreatitis.[27] $CD4^-TGF\text{-}\beta1^+$ cells were abundantly present in IgG4-DS SMG specimens, colocalising with areas of

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fibrosis. These data, taken together, suggest that $CD4^+$ CTLs may induce the fibrotic pathology seen in IgG4-RD.

In a previous study, we have demonstrated marked expansions of oligoclonal $CD19^+CD20^-CD27^+CD38^+$ plasmablasts in IgG4-RD subjects with active disease.[28] Since these plasmablasts express high levels of major histocompatibility complex (MHC) class II molecules, are depleted by rituximab and are also present in disease lesions, it is possible that they play an important role in the reactivation of the $CD4^+$CTLs.

In conclusion, $CD4^+GZMA^+$ CTLs, but not T_{H1} and T_{H2} cells, were significantly more abundant in the lesions of patients with IgG4-DS than of patients with SS and CS and healthy controls. These cells secrete IFN-γ, IL-1β and TGF-β1 in disease lesions, indicating reactivation by antigen in tissue sites, and the numbers of these cells correlate well with clinical disease activity, especially with the number of lesions. A schematic model for the pathogenesis of IgG4-RD is shown in figure 6. We are currently examining whether $CD4^+$CTLs-secreting IFN-γ are clonally expanded and represent the dominant infiltrating $CD4^+$ T cells in chronic inflammatory diseases other than IgG4-RD. However, additional research is required to further elucidate the pathogenesis of storiform fibrosis in IgG4-RD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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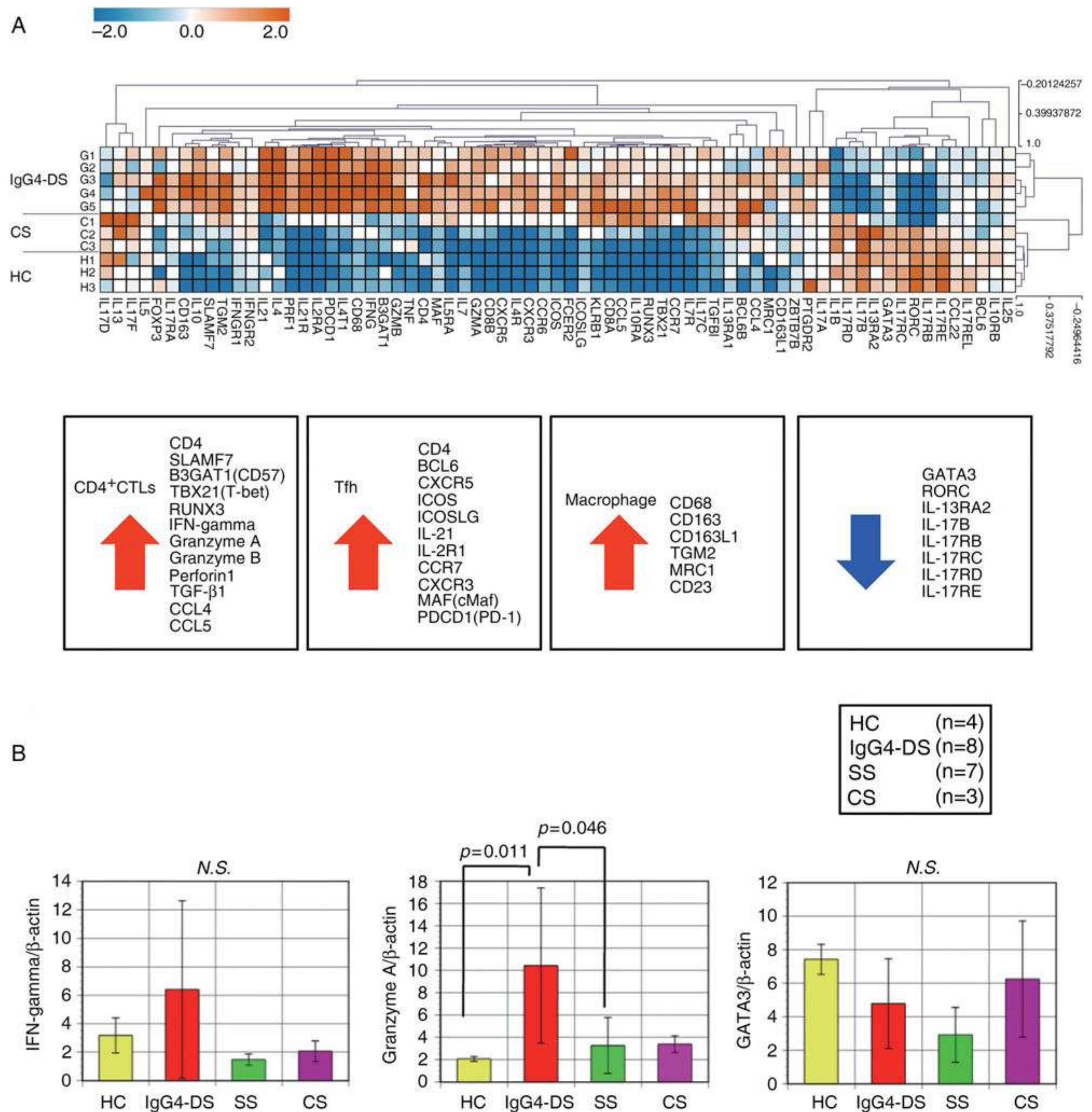

Figure 1.

$CD4^+$ cytotoxic T lymphocyte (CTL)-related gene expression in submandibular glands (SMGs) from patients with IgG4-related dacryoadenitis and sialoadenitis (IgG4-DS). (A) Heat map depicting differentially expressed immune-related genes in SMGs from five patients with IgG4-DS compared with SMGs from patients with chronic sialoadenitis (CS) and healthy controls (HCs). Only genes upregulated or downregulated by at least twofold are shown. p Values < 0.05 were considered significant. Upregulated genes representing $CD4^+$CTLs, $CD4^+$ T_{FH} cells and macrophages are highlighted. (B) Comparison of the mRNA expression levels of IFN-γ, granzyme A and GATA3 in the salivary glands from

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patients with IgG4-DS, patients with CS, patients with Sjögren's syndrome (SS) and controls using quantitative real-time PCR. Error bars represent SD. p Values are based on Mann–Whitney U test.

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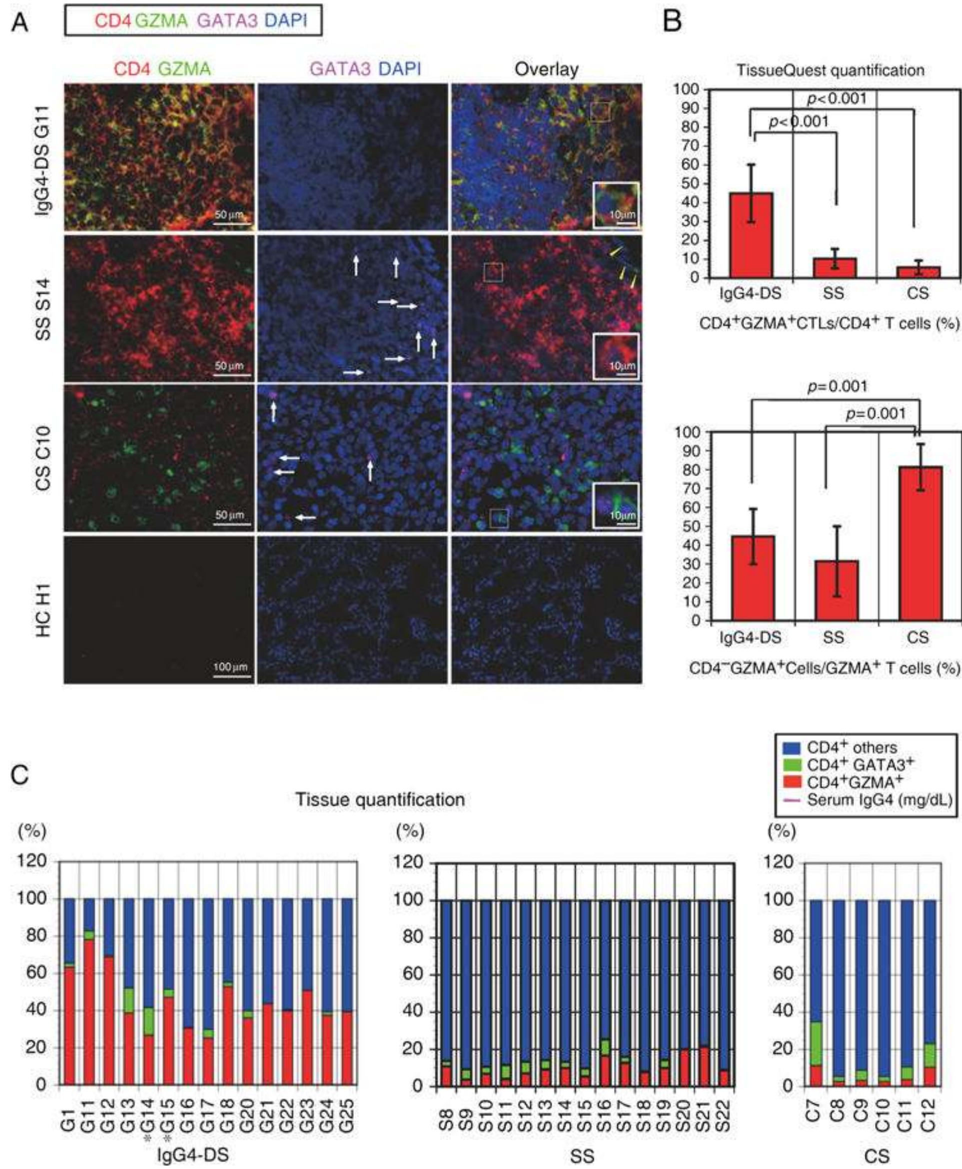

Figure 2.

Expression of CD4$^+$GranzymeA (GZMA)$^+$ cytotoxic T lymphocytes (CTLs) in IgG4-related dacryoadenitis and sialoadenitis (IgG4-DS) salivary glands. (A) Immunofluorescence staining of CD4 (red), GZMA (green), GATA3 (magenta) and DAPI (blue) in tissues from a patient with IgG4-DS (G11), a patient with Sjögren's syndrome (SS) (S14), a patient with chronic sialoadenitis (CS) (C10) and a healthy control (HC) (H1). (B) Quantification of CD4$^+$GZMA$^+$ cells, CD4$^+$ T cells, CD4$^-$GraA$^+$ cells and GZMA$^+$ cells in tissue biopsies from 15 patients with IgG4-DS, 15 patients with SS and 6 patients with CS. Error bars represent SD. p Values are based on Mann–Whitney U test. (C) Quantification of CD4$^+$GZMA$^+$, CD4$^+$GATA3$^+$ and CD4$^+$GZMA$^-$GATA3$^-$ cells in tissue biopsies from 15 patients with IgG4-DS (patients with a history of allergy are marked with asterisks), 15 patients with SS and 6 patients with CS.

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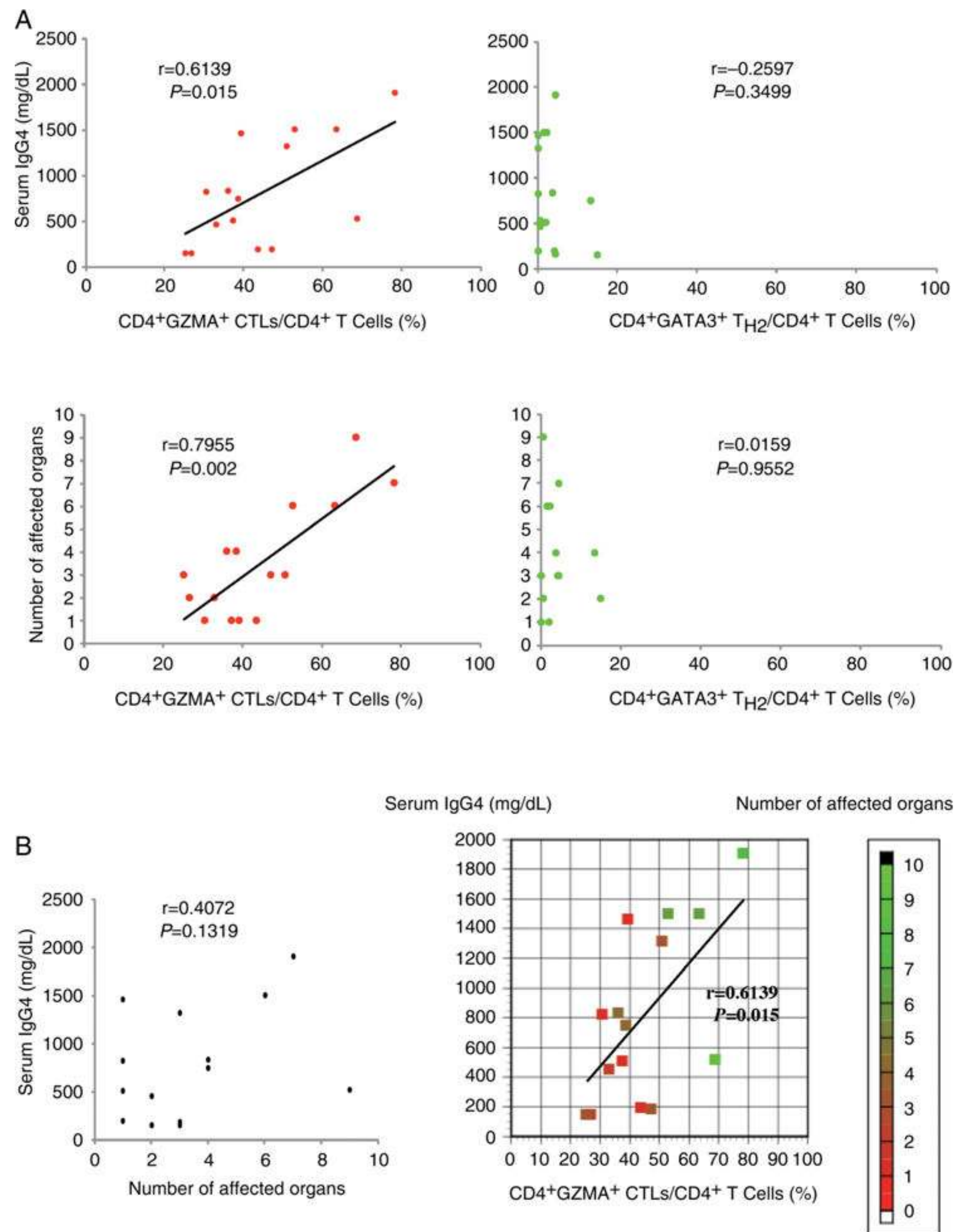

Figure 3.

The abundance of CD4$^+$GranzymeA (GZMA)$^+$ cytotoxic T lymphocytes (CTLs) in tissue biopsies from patients with IgG4-related dacryoadenitis and sialoadenitis (IgG4-DS) correlates with serum IgG4 concentrations and the number of affected organs. (A) Correlations of the proportions of CD4$^+$GZMA$^+$CTLs and CD4$^+$GATA3$^+$ T helper 2 (T_{H2}) cells in submandibular glands (SMGs) from patients with IgG4-DS with their serum IgG4 levels (n=15). Correlations of the proportions of CD4$^+$GZMA$^+$CTLs and CD4$^+$GATA3$^+$$T_{H2}$ cells in SMGs from patients with IgG4-DS (n=15) with the number of affected organs. The correlation coefficients and p values were determined using Spearman's rank correlations. (B) Correlations between serum IgG4 levels from patients with IgG4-DS (n=15) and the number of affected organs. The correlation coefficients and p values were determined using

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Spearman's rank correlations. The higher percentage of $CD4^{+}GZMA^{+}$ CTLs in SMGs from patients with IgG4-DS (n=15) correlated with higher serum IgG4 concentrations and larger numbers of affected organs.

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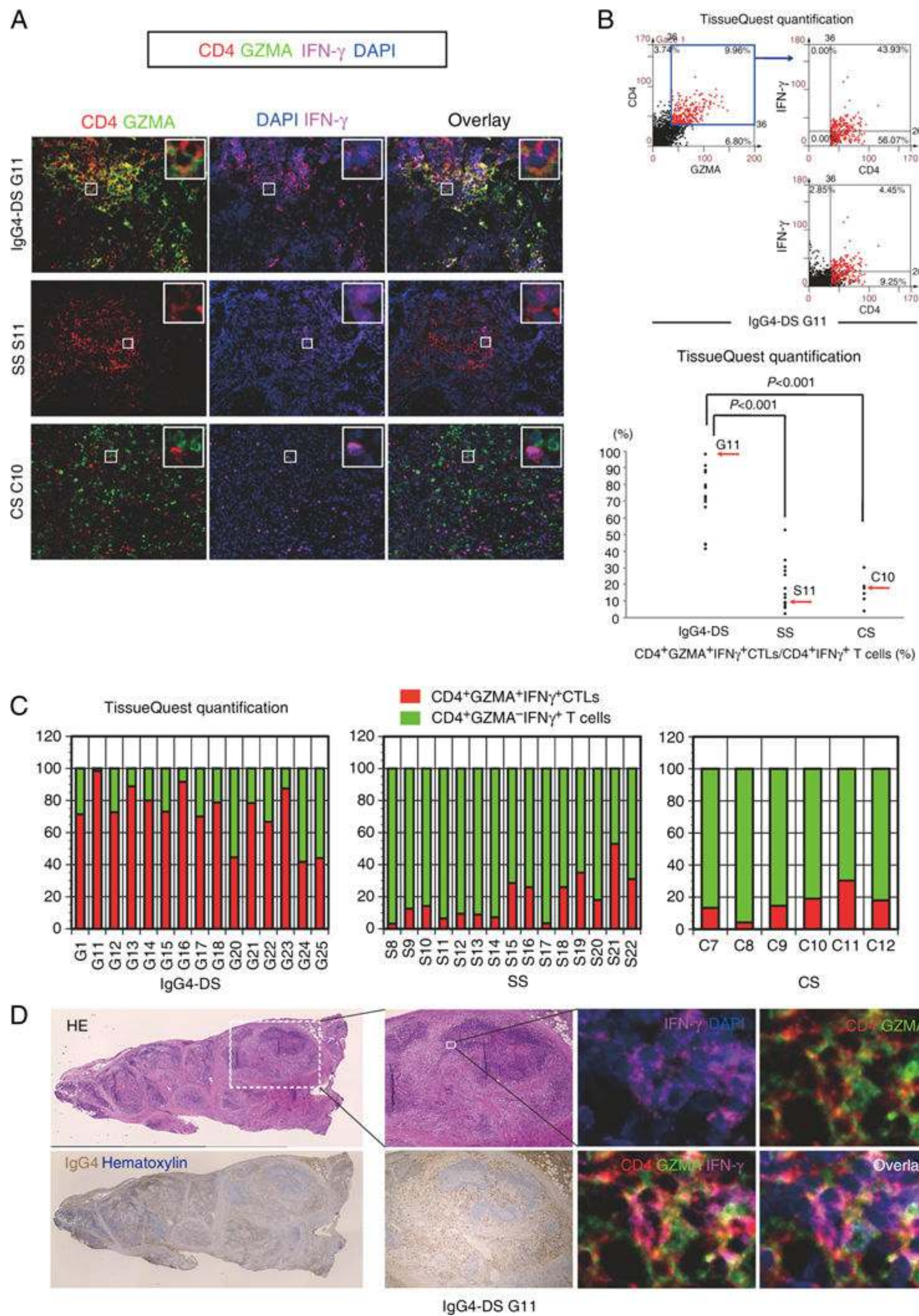

Figure 4.

Expression of CD4$^+$GranzymeA (GZMA)$^+$ cytotoxic T lymphocytes (CTLs) secreting IFN-γ in IgG4-related dacryoadenitis and sialoadenitis (IgG4-DS) salivary glands. (A) Immunofluorescence staining of IFN-γ-producing CD4$^+$GZMA$^+$ cells in the tissues of an IgG4-DS subject (G11), a Sjögren's syndrome (SS) subject (S11) and a chronic sialoadenitis (CS) subject (C10). CD4 (red), GZMA (green), DAPI (blue) and IFN-γ (magenta) staining are shown. (B) Scatter plots in the upper panel depicts the mean fluorescence intensity per cell quantified using TissueQuest software for each fluorescent immunostain (see IgG4-DS

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G11). Quantification of $CD4^+GZMA^+IFN\text{-}\gamma^+$ CTLs and $CD4^+IFN\text{-}\gamma^+$ T cells in tissue biopsies from 15 patients with IgG4-DS, 15 patients with SS and 6 patients with CS. Significant differences between groups were determined by the Mann–Whitney U test. (C) Quantification of $CD4^+GZMA^+IFN\text{-}\gamma^+$ CTLs and $CD4^+GZMA^-IFN\text{-}\gamma^+$ T cells in tissue biopsies from 15 patients with IgG4-DS, 15 patients with SS and 6 patients with CS. (D) Localisation of $CD4^+GZMA^+IFN\text{-}\gamma^+$ CTLs in infiltrating lymphocytes in inflamed tissue forms an IgG4-DS subject. Staining with H&E and IgG4 in a IgG4-DS subject (G11). Counterstaining was performed with Mayer's haematoxylin (blue).

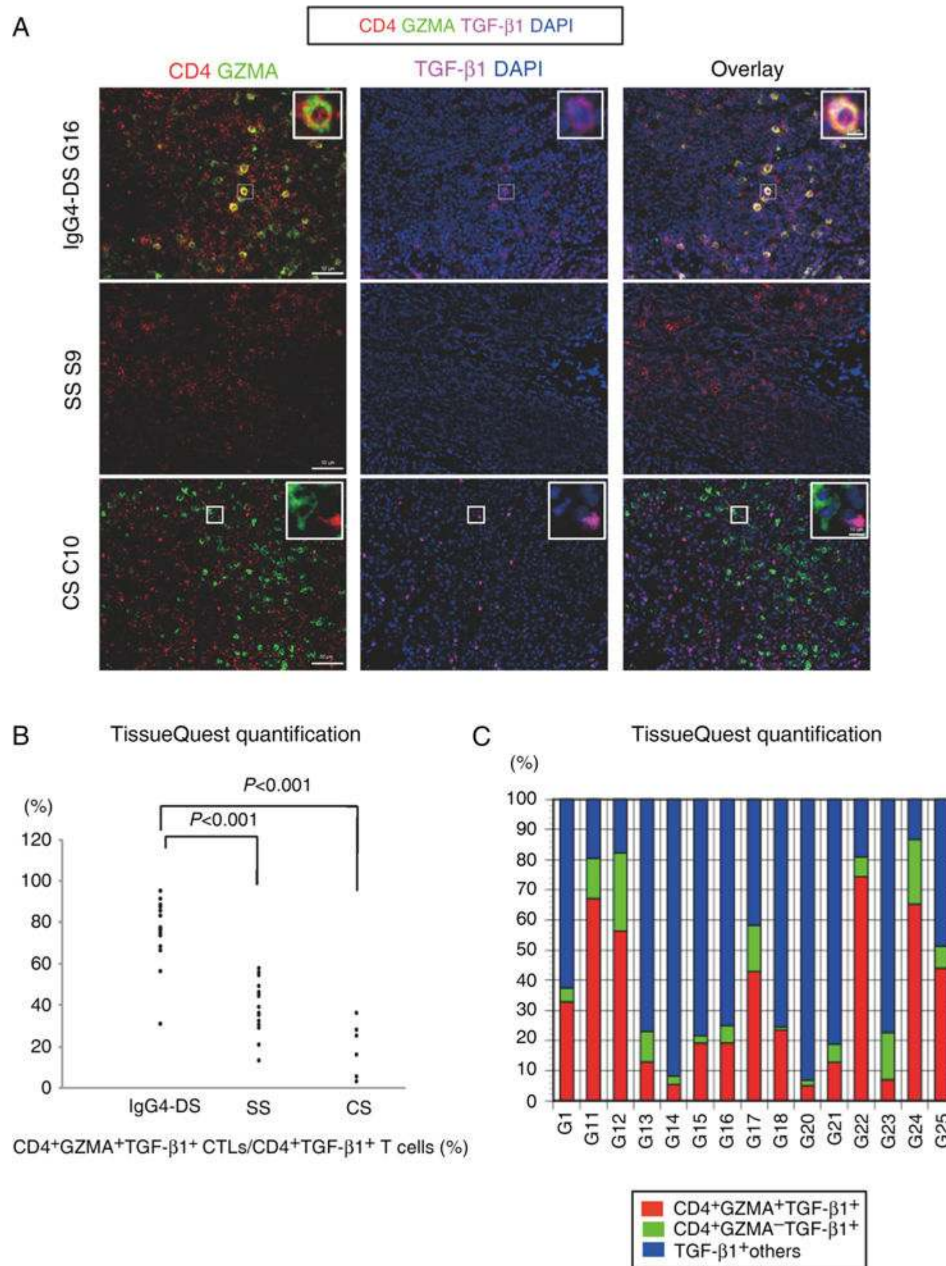

Figure 5.

Expression of CD4^{+}GranzymeA (GZMA)$^{+}$ cytotoxic T lymphocytes (CTLs) secreting TGF-β1 in IgG4-related dacryoadenitis and sialoadenitis (IgG4-DS) salivary glands. (A) Immunofluorescence staining of TGF-β1-producing CD4^{+}GZMA^{+} cells in the tissues of a IgG4-DS subject (G16), a Sjögren's syndrome (SS) subject (S9) and a chronic sialoadenitis (CS) subject (C10). CD4 (red), GZMA (green), DAPI (blue) and TGF-β1 (magenta) staining are shown. (B) Quantification of CD4^{+}GZMA^{+}TGF-β1 and CD4^{+}GZMA^{-}TGF-β1 cells in tissue biopsies from 15 patients with IgG4-DS, 15 patients with SS and 6 patients with CS. Significant differences between groups were determined by Mann–Whitney U test. (C)

Quantification of $CD4^+GZMA^+$ TGF-$\beta1^+$, $CD4^+GZMA^-$TGF-$\beta1^+$ and $CD4^-$TGF-$\beta1^+$ cells in tissue biopsies from 15 patients with IgG4-DS.

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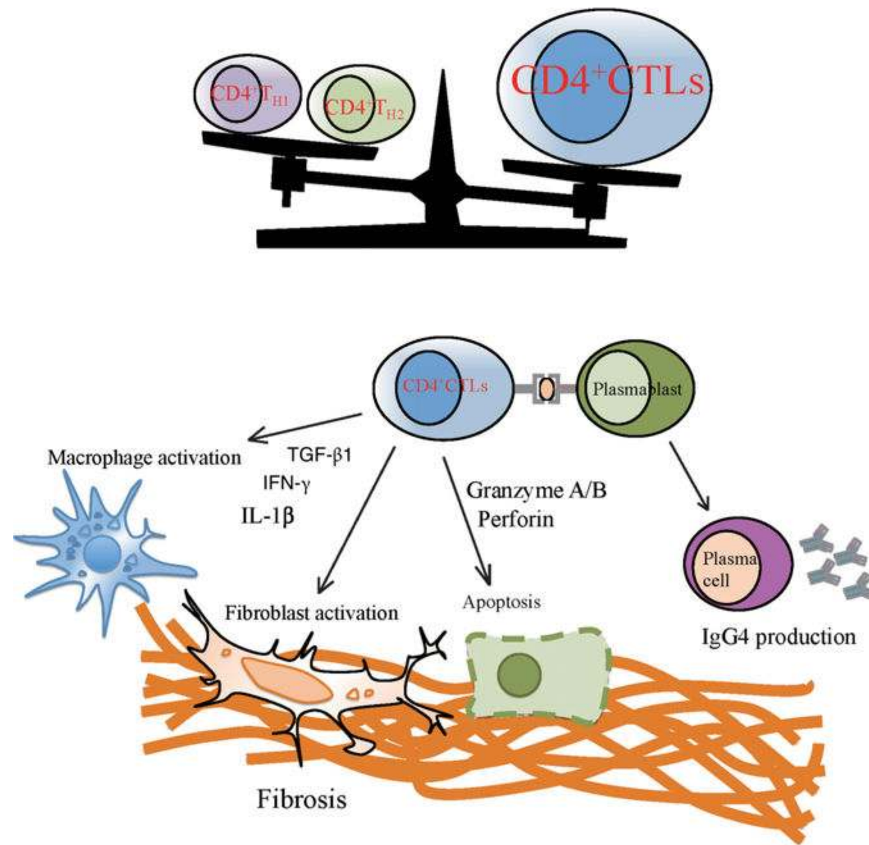

Figure 6.

Schematic model of CD4$^+$ cytotoxic T lymphocytes (CTLs) in IgG4-related disease (RD) pathogenesis. Clonal expansion of CD4$^+$ CTLs and their infiltration into tissue sites may be the cause of this disease state. Reactivation of these CD4$^+$ CTL cells may require antigen presentation by plasmablasts or other activated B cells in tissue sites. Activated CD4$^+$ CTLs presumably mediate fibrosis and inflammation as a result of cytokine secretion or possibly the induction of cell death. The mechanisms by which CD4$^+$ CTLs may cause disease remain speculative.