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Chronic Fibro-Inflammatory Responses in Autoimmune Pancreatitis Depend on IFN-α and IL-33 Produced by Plasmacytoid Dendritic Cells

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In previous studies, we found that human IgG4-related autoimmune pancreatitis (AIP) and murine AIP are driven by activation of plasmacytoid dendritic cells (pDCs) producing IFN-α. In the present studies we examined additional roles of pDC-related mechanisms in AIP pathogenesis, particularly those responsible for induction of fibrosis. We found that in murine AIP (MRL/Mp mice treated with polyinosinic-polycytidylic acid) not only the pancreatic infiltration of immune cells but also the development of fibrosis were markedly reduced by the depletion of pDCs or blockade of type I IFN signaling; moreover, such treatment was accompanied by a marked reduction of pancreatic expression of IL-33. Conversely, polyinosinic-polycytidylic acid–induced inflamed pancreatic tissue in murine AIP exhibited increased expression of type I IFNs and IL-33 (and downstream IL-33 cytokines such as IL-13 and TGF-β1). pDCs stimulated by type I IFN were the source of the IL-33 because purified populations of these cells isolated from the inflamed pancreas produced a large amount of IL-33 upon activation by TLR9 ligands, and such production was abrogated by the neutralization of type I IFN. The role of IL-33 in murine AIP pathogenesis was surprisingly important because blockade of IL-33 signaling by anti-ST2 Ab attenuated both pancreatic inflammation and accompanying fibrosis. Finally, whereas patients with both conventional pancreatitis and IgG4-related AIP exhibited increased numbers of acinar cells expressing IL-33, only the latter also exhibited pDCs producing this cytokine. These data thus suggest that pDCs producing IFN-α and IL-33 play a pivotal role in the chronic fibro-inflammatory responses underlying murine AIP and human IgG4-related AIP. The Journal of Immunology, 2017, 198: 000–000.

Clinicopathological analysis of autoimmune pancreatitis (AIP) as well as other inflammations often accompanying AIP such as sialadenitis or autoimmune cholangitis has established that these diseases are organ-specific manifestations of a systemic autoimmune fibroinflammatory disorder that can affect many organs, sometimes simultaneously. These inflammations are now called IgG4-related diseases (IgG4-RD) because the inflammation is in each case marked by the presence of elevated serum levels of IgG4 as well as a massive infiltration of affected organs with IgG4-expressing plasmacytes (1–4). Additionally, these inflammations are characterized by the presence of storiform fibrosis and, in some cases, obliterator phlebitis (1–4).

Despite the fact that elevated levels of serum IgG4 and infiltration of IgG4-expressing plasmacytes in affected organs are diagnostic of IgG4-RD, the pathophysiological role of this Ig subtype is poorly understood. One possibility is that the presence of IgG4 in IgG4-RD inflammations is an epiphenomenon of the unique fibro-inflammatory response characterizing this disease rather than its cause because this Ig subtype has only a limited ability to bind to complement and Fcγ receptors (5). This idea is favored by studies showing that IgG1, but not IgG4, purified from serum of patients with IgG4-RD causes pancreatic injury upon injection into neonatal mice (6). Contrary evidence, however, comes from studies showing that autoantigen-specific IgG4 Abs have been identified as a cause of small vessel vasculitis and membranous nephropathy (7, 8); it thus remains possible that IgG4 is playing a pathologic role in at least some of the manifestations of IgG4-RD.

One major line of investigation of IgG4-RD pathogenesis has been studies of T cell cytokine responses focusing quite logically on Th2 cytokines that might enhance B cell production of IgG4, such as IL-4, IL-10, and IL-13 (9–12). These studies disclosed an increased accumulation of Th2 cells or regulatory T cells producing IL-4, IL-10, and IL-13 in the peripheral blood and...
affected organs of patients with IgG4-RD (13, 14). Perhaps more importantly, they showed that there was a positive correlation between circulating numbers of T follicular helper 2 cells and serum levels of IgG4 in these patients (15). These findings thus suggested that various T cells generated during an abnormal adaptive immune response contribute to the pathogenesis of IgG4-RD; however, it remained possible that the development of these T cells is secondary to a more fundamental innate immune defect.

Initial evidence for the latter came from studies in which we showed that B cells can be induced to produce IgG4 by APCs activated by TLRs or nucleotide-binding oligomerization domain (NOD)–like receptor ligands (16, 17). Additionally, we showed that monocytes, basophils, and plasmacytoid dendritic cells (pDCs) isolated from patients with IgG4-RD enhance IgG4 production by B cells from healthy controls in a T cell–independent manner (16–18). These studies therefore showed that innate cell abnormalities could account for increased IgG4 production in IgG4-RD.

Further and more convincing evidence that innate cells participate in the pathogenesis of IgG4-RD came from studies of a murine model of AIP, that is, MRL/Mp mice treated with repeated injections of polyinosinic-polycytidylic acid [poly(I:C)] (19, 20). Such mice exhibit similar pathological findings to human IgG4-related AIP such as massive infiltration of immune cells, destruction of pancreas acinar architecture, and fibrosis. Utilizing this murine model of AIP, we found that the development of AIP is accompanied by pancreatic accumulation of pDCs producing IFN-α and that the development of AIP is markedly attenuated by the depletion of pDCs or the blockade of IFN-α signaling (18). In accordance with these murine studies, we also showed that pDCs isolated from patients with IgG4-RD enhance IgG4 production by B cells from healthy controls in an IFN-α–dependent manner. Thus, these studies provide strong evidence that both murine AIP and human IgG4-related AIP are characterized by activation of pDCs producing IFN-α.

Although pancreatic fibrosis, especially storiform fibrosis, is one of the typical pathological findings of human IgG4-related AIP (1–4), the molecular mechanisms leading to the development of such fibrosis have not been fully understood. One possibility is that it involves the secretion of IL-33, a cytokine produced by nonhematopoietic cells that has been shown to induce hepatic and intestinal fibrosis through its ability to promote the production of profibrogenic mediators such as IL-13 and TGF-β1 (21–23). In line with these findings, we recently reported that IL-33 secretion by pancreatic acinar cells under the influence of type I IFN plays an important role in the development of pancreatic fibrosis occurring in a model of conventional pancreatitis (24, 25). These findings led us in the present studies to explore the possibility that IL-33 production plays a key role in the pathogenesis of IgG4-related AIP, particularly that relating to fibrosis. In this study, we provide the evidence that chronic fibro-inflammatory responses in human IgG4-related AIP and murine AIP are mediated by pDCs producing both IFN-α and IL-33.

Materials and Methods

Induction of AIP in MRL/Mp mice

Female MRL/Mp mice were purchased from Japan SLC (Shizuoka, Japan) and reared at the Kyoto University animal facility under specific pathogen-free conditions. The ethical permission of this study was obtained by the Review Boards of Kyoto University Graduate School of Medicine and Kindai University Faculty of Medicine. Female MRL/Mp mice at 6 wk old were treated with an i.p. injection of poly(I:C) (100 μg; InvivoGen, San Diego, CA) twice a week for a total of 14 or 16 times to induce experimental AIP as previously described (18). To deplete pDCs and to neutralize type I IFN–mediated signaling pathways, mice were treated with Ab against bone marrow stromal cell Ag 2 (100 μg; 120G8; Dendritics, Lyon, France) and Ab against type I IFN receptor (IFNAR; 100 μg; BD Biosciences, San Jose, CA) prior to each poly(I:C) injection (18). Additionally, mice were treated with Ab against IL-33 receptor, ST2 (100 μg; R&D Systems, Minneapolis, MN), to neutralize IL-33–mediated signaling pathways prior to each poly(I:C) injection (24). Mice treated with rat IgG (100 μg; Sigma-Aldrich, St. Louis, MO) or mouse IgG (100 μg; Sigma-Aldrich) were used as control mice. Pancreatic levels of hydroxyproline were determined by the hydroxyproline assay kit (QuickZyme Biosciences, Leiden, the Netherlands) (24).

Histology, immunohistochemical, and immunofluorescence analysis

Pancreatic samples obtained from MRL/Mp mice treated with poly(I:C) were subjected to fixation with 10% formalin followed by H&E staining. Pathological assessment was performed by using a scoring system for AIP as previously described (18–20). Pancreatitis inflammation was scored as follows: 0, pancreas without mononuclear cell infiltration; 1, mononuclear cell aggregation and/or infiltration within the interstitium with no parenchymal destruction; 2, focal parenchymal destruction with mononuclear cell infiltration; 3, diffuse parenchymal destruction but some intact parenchymal areas retained; 4, almost all pancreatic tissue, except the pancreatic islets, destroyed or replaced with fibrosis or adipose tissue (18–20). Immunofluorescence staining was performed using anti–pDC Ag (PDCA)-1 Ab (eBioscience, San Diego, CA) and anti–IL-33 Ab (Abcam, Cambridge, MA) followed by incubation with Alexa Fluor 488– or 546–conjugated anti-rat or anti-mouse IgG (Life Technologies, Carlsbad, CA). For visualization of pancreatic fibrosis, anti–IL-33 Ab (Abcam), anti–fibronectin Ab (Abcam), and anti–smooth muscle actin (SMA) Ab (Abcam) were used as primary Abs as previously described (24). Sirius red staining was performed using a picrosirius red stain kit (Polysciences, Warrington, PA). At least two immunofluorescence and immunohistochemical images were taken by microscopy (Biozero, BZ-8100; Keyence, Osaka, Japan) from each slide. Positive areas were calculated by using hybrid cell count software (Keyence) according to the manufacturer’s protocol.

ELISA

Protein concentrations of cytokines were determined by ELISA. Concentrations of IFN-α and IFN-β were determined by ELISA kits from R&D Systems. Concentrations of IL-13 and IL-33 were determined by ELISA kits from eBioscience. Concentration of TGF-β1 was determined by an ELISA kit from Promega (Madison, WI).

Isolation of pancreatic mononuclear cells and flow cytometric analysis

Pancreatic mononuclear cells (PMNCs) were isolated from the pancreas of MRL/Mp mice treated with poly(I:C) as previously described (18). PMNCs were stained with FITC, PE, or allophycocyanin-conjugated B220 Ab (eBioscience), PDCA-1 Ab (eBioscience), Gr-1 Ab (eBioscience), CD3 Ab (eBioscience), and CD11b Ab (BD Biosciences) and then subjected to flow cytometric analysis by using an Accuri C6 flow cytometer (BD Biosciences) and CFlow Plus software (BD Biosciences). In some experiments, pDCs were isolated from PMNCs using a mouse pDC isolation kit (Miltenyi Biotec, Auburn, CA). The purity of this method was >90% when pDCs were defined as B220+PDCA-1+ cells (18). Whole PMNCs, pancreatic pDCs, and pDC-depleted cell fractions (1 × 10^6/ml) were stimulated with poly(I:C) (25 μg/ml) and/or CpG (1 μM; InvivoGen) for 48 h for measurement of the production of IFN-α, IFN-β, and IL-33.

Immunofluorescence staining of human pancreas tissue

Pancreatic tissue samples resected from patients with IgG4-related AIP (n = 5), patients with chronic alcoholic pancreatitis (n = 5), and patients with pancreatic cancer (n = 5) were prepared as described previously (18). Noncancerous portions of pancreatic tissue from patients with pancreatic cancer were used as controls. Ethical permission for this study was obtained by the Review Boards of Kyoto University Graduate School of Medicine and Kindai University Faculty of Medicine. Immunofluorescence staining was performed by using anti–IL-33 Ab (Abcam), anti–amylose Ab (Abcam), and anti–blood DC Ag (BDCA)2 Ab (Miltenyi Biotec) followed by incubation with Alexa Fluor 488– or 546–conjugated anti-rabbit or anti-mouse IgG (Life Technologies).
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Statistical analysis

A Student t test was used to evaluate the significance of the differences. Statistical analysis was performed with Prism (GraphPad Software, La Jolla, CA). A p value <0.05 was regarded as statistically significant.

Results

MRL/Mp mice subjected to repeated administration of poly(I:C) develop AIP accompanied by fibrosis

To elucidate the molecular mechanisms accounting for the development of pancreatic inflammation and accompanying fibrosis in AIP, we used a well-established animal model of human IgG4-related AIP consisting of MRL/Mp mice treated with i.p. injections of poly(I:C) (see Materials and Methods). As shown in previous studies and mentioned in the Introduction, such mice develop a pancreatitis similar to that in human IgG4-related AIP (18, 26).

In initial studies we focused on the development of pancreatic fibrosis in MRL/Mp mice with AIP on the assumption that elucidation of this feature of AIP would disclose new innate mechanisms relating to AIP pathogenesis. Accordingly, we treated mice with i.p. injection of poly(I-C) and after the 16th injection harvested pancreatic tissue to measure collagen deposition and activation of pancreatic satellite cells (PSCs) by sirius red and α-SMA staining, respectively (24, 27). Upon pathological inspection as well as semiquantitative assessment of the extent of stained areas, the pancreatic tissues of poly(I:C)-treated MRL/Mp mice were quite positive for sirius red or α-SMA staining, whereas tissues of untreated MRL/Mp mice were more or less negative (Fig. 1A, 1B).

Additionally, repeated injection of poly(I:C) led to a marked increase in pancreatic tissue expression of collagen as assessed by the measurement of hydroxyproline as well as areas positive for fibronectin, a prototypical extracellular matrix protein associated with fibrosis (24) (Fig. 1A, 1B). These immunohistochemical and biochemical analyses show that the pancreatitis in MRL/Mp mice treated with repeated injections of poly(I:C) is accompanied by severe fibrosis.

Pancreatic fibrosis in MRL/Mp mice depends on the activation of pDCs

In further studies we sought evidence that fibrosis in AIP of poly(I:C)-treated MRL/Mp mice noted above is an integral part of the inflammatory program associated with AIP in this model and is therefore likely to be caused by the activation of pDCs and their production of type I IFNs shown previously to drive this experimental AIP (18). In this study, we took advantage of the fact that in previous studies we showed that administration of a pDC-depleting Ab (120G8 Ab) or neutralization of type I IFN signal transducing pathways by an IFNAR Ab to poly(I:C)-treated MRL/Mp mice efficiently prevented the development of the AIP-associated inflammatory changes in the pancreas of these mice (18). Thus, these previous findings allowed us to determine in the current study whether prevention of AIP-related inflammatory changes caused by accumulation of pDCs also affects the development of pancreatic fibrosis.

In initial studies along these lines we administered 120G8 Ab to poly(I:C)-treated MRL/Mp mice to prevent pDC accumulation into the pancreas. The specificity of this Ab for pDCs has been established in previous studies (28) as well as in current studies evaluating the effect of such administration on various cell types. As shown in Supplemental Fig. 1, flow cytometric studies of pancreatic cells from poly(I:C)-treated mice confirmed that administration of 120G8 Ab results in a greatly decreased percentage of B220lowPDCA-1+ cells (pDCs), but no significant change in the percentage of CD3+ T cells, B220+ B cells, Gr-1+ granulocytes, or CD11b+ myeloid cells as compared with administration of control Ab. The distant possibility that such treatment can also target conventional CD11c+ DCs producing type I IFN does not detract from the fact that its main target is pDCs producing this cytokine. We found that administration of 120G8 Ab prevented the development of AIP-associated pancreatic inflammation as well as the development of pancreatic fibrosis in poly(I:C)-treated MRL/Mp mice, with the latter assessed by tissue staining with sirius red, α-SMA, and fibronectin (Fig. 2A).

In parallel studies we found that the administration of an IFNAR-blocking Ab also prevented the development of pancreatic fibrosis (Fig. 2B). Taken together, these data strongly suggest that the development of pancreatic fibrosis in this experimental model of AIP and accompanying fibrosis is part of the inflammatory program underlying pancreatitis in this model and, as such, depends on type I IFN signaling pathways initiated by pDC activation.

Pancreatitis in MRL/Mp mice with AIP is associated with increased levels of type I IFNs and IL-33

In previous studies we established a model of pancreatitis reflecting patients with non–autoimmune chronic pancreatitis (i.e., conventional pancreatitis) in which mice are subjected to repeated administration of cerulein (cholecystokinin receptor [CCKR] agonist) and NOD1 ligand, FK156, or FKS65 (24, 25, 29). Using this model we showed that type I IFN signaling pathways mediate both pancreatic inflammation and fibrosis through induction of IL-33, IL-13, and TGFB1. We therefore hypothesized that type I IFNs derived from pDCs in MRL/Mp mice with AIP also cause inflammation and fibrosis in this model via a related cytokine mechanism.

To investigate this possibility we first determined whether AIP (and fibrosis) in MRL/Mp mice is accompanied by increased levels of type I IFNs as predicted in the pDC depletion studies or IFNAR blockade studies noted above and in a previous study (18), and this, in turn, is accompanied by increased expression of IL-33, IL-13, and TGFB1. Indeed, pancreatic expression of both IFN-α and IFN-β as assessed by ELISA was markedly increased upon repeated injection of poly(I:C), and this increase was parallel to an increase in the expression of IL-33, IL-13, and TGFB1 (Fig. 1A, 1C). Moreover, depletion of pDCs as well as blockade of IFNAR in MRL/Mp mice were accompanied by a marked reduction in IL-33 expression that occurred along with a reduction in pancreatic fibrosis (Fig. 2). These data therefore show that pancreatic inflammation and fibrosis in MRL/Mp mice with AIP are in fact accompanied by enhanced expression of cytokines previously associated with pancreatic inflammation in a model of conventional chronic pancreatitis.

Type I IFN–stimulated pDCs in MRL/Mp mice with AIP produce IL-33

In our previous report noted above, in which conventional pancreatitis induced by repeated administration of CCKR agonist and NOD1 ligand was studied, we showed that IL-33 in this model is produced by pancreatic acinar cells (24, 25). To determine whether acinar cells expressing amylase were also the source of IL-33 in MRL/Mp mice with AIP, we stained the inflamed pancreatic tissue with Abs to detect IL-33 in cells expressing either amylase or α-SMA (the latter PSCs) and found no dual staining cells (data not shown). We thus concluded that neither acinar cells nor PSCs were cellular sources of IL-33 in MRL/Mp mice with AIP.

Based on these data, we hypothesized that a pancreatic hematopoietic cell was the source of IL-33 production. To investigate
this idea, PMNCs were isolated from poly(I:C)-treated or untreated MRL/Mp mice and then stimulated with poly(I:C) and/or CpG in vitro. PMNCs from MRL/Mp mice treated with poly(I:C) in vivo produced greatly increased amounts of type I IFNs upon in vitro stimulation with poly(I:C) and/or CpG as compared with mice without in vivo treatment (Fig. 3A). In contrast, whereas these PMNCs produced increased amounts of IL-33 upon stimulation with CpG, they did not produce increased amounts of IL-33 upon stimulation with poly(I:C) (Fig. 3A); this is presumably because pDCs do not express the receptor for poly(I:C), TLR3 (30, 31). In related studies we found that IL-33 production by PMNCs from treated mice was greatly diminished by the addition of anti-IFNAR Ab to the culture (Fig. 3B).

In further studies addressing the source of the IL-33, we obtained pDC-enriched and pDC-depleted cell populations from the PMNCs by MACS sorting (enriched cells containing >90% pure pDCs by flow cytometry, data not shown) and then stimulated these cell populations with poly(I:C) and/or CpG. As expected from the studies described above, the purified pDC populations exhibited both robust IFN-α and IL-33 production upon stimulation with CpG but not poly(I:C), whereas the PMNCs depleted of pDCs exhibited markedly diminished IFN-α and IL-33 production upon stimulation with both stimulants (Fig. 3C).

In a final series of studies on the cellular origin of IL-33 in AIP, we performed dual immunofluorescence analysis of inflamed pancreatic tissue and found that most pancreatic pDCs expressing PDCA-1, a specific pDC marker, were positive for IL-33 staining (Fig. 3D). Taken together, these data provided evidence that pDC cell populations in the inflamed pancreas of poly(I:C)-treated MRL/Mp mice are not only the source of the IL-33, but also that such IL-33 production is dependent on type I IFN production by the pDCs.

Pancreatic inflammation and fibrosis in murine AIP depends on secretion of IL-33

Having shown that IL-33 secretion and other profibrotic cytokines occur in MRL/Mp mice with AIP in a type I IFN–dependent manner, we next wanted to determine the role of IL-33 in the development of both inflammation and fibrosis in the AIP. To this end, MRL/Mp mice were administered Ab against the receptor for IL-33, ST2, to neutralize IL-33 function by blockade of its
signaling pathway, as previously described (24). We found that administration of anti-ST2 Ab (as compared with control Ab) significantly reduced the level of inflammation in MRL/Mp mice with AIP as assessed by H&E staining and pathology scores (Fig. 4A). Additionally, administration of anti-ST2 Ab (as compared with control Ab) markedly reduced the level of pancreatic fibrosis in MRL/Mp mice with AIP as assessed by quantitative hydroxyproline assay and tissue staining with α-SMA or fibronectin (Fig. 4B–D). This reduction in pancreatic fibrosis by administration of anti-ST2 Ab was accompanied by, and was thus at least partially due to, reduction in the secretion of IL-13 and TGF-β1, that is, factors previously shown to be induced by IL-33, either directly or indirectly (24). Finally, as shown in Fig. 4E, the cause of the reduced inflammation in MRL/Mp mice with AIP due to the blockade of IL-33–mediated signaling pathways was revealed in studies that showed that such blockade led to decreased pancreatic accumulation of pDCs defined as PDCA-1+ B220low cells (18). Such a reduction in pDC accumulation induced by the blockade of IL-33–mediated signaling pathways might be explained by an effect on the attenuation of pancreatic inflammation rather than a specific effect on the function of pDCs because administration of ST2 Ab in the model of chronic pancreatitis results in greatly reduced amounts of proinflammatory cytokine production, including reduction in IL-6, TNF-α, and MCP-1 as shown in previous studies (24).

Taken together, these data suggest that murine experimental AIP is characterized by pDC-mediated type I IFN production followed by the secretion of proinflammatory and/or profibrotic cytokines such as IL-33, IL-13, and TGF-β1 and that IL-33 is a potent inducer of both pancreatic inflammation and pancreatic fibrosis as in the case of experimental chronic pancreatitis induced by CCKR and NOD1 agonists (24).

**Pancreatic acinar cells expressing IL-33 are increased in human chronic pancreatitis and IgG4-related AIP**

Having identified the profibrogenic and proinflammatory properties of IL-33 in murine AIP, we assessed the expression of this cytokine in human pancreatic diseases. These studies were facilitated by access to surgical pancreatic specimens obtained from patients with chronic alcoholic pancreatitis (n = 3) and human IgG4-related AIP (n = 3) as well as from one control patient as previously described (18). In baseline studies of levels of fibrosis we found that tissue specimens from patients with both forms of pancreatitis exhibited considerable but equivalent levels of fibrosis as assessed by α-SMA staining (Fig. 5A). In contrast, expression of α-SMA was barely seen in tissue specimens from the noncancerous portions of the pancreas with pancreatic cancer (Supplemental Fig. 2A). Thus, human chronic pancreatitis and IgG4-related AIP generate comparable levels of fibrosis.

We next evaluated the expression of IL-33 in acinar cells in tissue specimens from chronic alcoholic pancreatitis (n = 4) and IgG4-related AIP patients (n = 4) (as well as in control tissues). We found that most amylase+ acinar cells in tissue specimens from both patients with chronic pancreatitis and IgG4-related AIP were positive for IL-33 staining (Fig. 5B), whereas pancreatic acinar cells in tissue samples from one control patient (noncancerous portions of the pancreatic tissue with pancreatic cancer) were negative for IL-33 staining (Supplemental Fig. 2B). Additionally,
semiquantitative enumeration of acinar cells expressing IL-33 in these pancreatic specimens performed by counting the numbers of amylase"IL-33+ cells, amylase"IL-33- cells, and amylase-IL-33+ cells in high-power fields (HPFs) revealed no significant difference in IL-33+ acinar cell numbers in tissues from patients with human chronic pancreatitis and IgG4-related AIP (Fig. 5B). These studies thus revealed that human chronic pancreatitis and IgG4-related AIP are similar to murine chronic pancreatitis induced by administration of CCKR agonist and NOD1 ligand mentioned above in which pancreatic acinar cells were also found to express IL-33; however, such acinar cells expressing IL-33 were not found in the murine model of AIP.

pDCs expressing IL-33 accumulate in the pancreas of human IgG4-related AIP

Finally, we focused on the expression of IL-33 in pDCs in chronic fibro-inflammatory disorders of the pancreas, again utilizing surgical pancreatic tissue specimens obtained from patients with chronic alcoholic pancreatitis (n = 5), human IgG4-related AIP (n = 5), and control patients (nontumorous portions of the pancreatic tissue from patients with pancreatic cancer, n = 5). In this case we found accumulations of BDCA2+ pDCs in tissues from patients with IgG4-related AIP, but not in tissues from patients with chronic pancreatitis or controls (Fig. 6A). To verify these tissue staining results we then performed semiquantitative enumeration of pDCs expressing IL-33 in these pancreatic disorders by counting the numbers of BDCA2+IL-33+ cells, BDCA2+IL-33- cells, and BDCA2-IL-33+ cells in HPFs. We found that the numbers of pDCs expressing IL-33, which were defined as IL-33+BDCA2+ cells, were significantly higher in the pancreas of patients with IgG4-related AIP, as compared with those with chronic pancreatitis or controls (Fig. 6B). Because pancreatic accumulation of BDCA2+ pDCs expressing IFN-α was seen in patients with IgG4-related AIP but not in patients with chronic pancreatitis in our previous report (18), these data support the idea that human IgG4-related AIP is uniquely characterized by the pancreatic infiltration of pDCs producing both IFN-α and IL-33 and, as such, is similar to AIP in MRL/Mp mice.
Although massive infiltration of lymphocytes and plasmacytes accompanied by fibrosis is a well-established histological feature of human AIP, the molecular mechanisms accounting for the development of these chronic fibro-inflammatory effects are still poorly understood (1, 32). In this study, we established, to our knowledge for the first time, that IL-33 is a major proinflammatory factor in mice with experimental AIP [MRL/Mp mice administered poly(I:C)], particularly with respect to fibrosis formation. The various studies leading to this conclusion consisted of the following: first, the development of murine AIP was shown to be associated with pancreatic infiltration of pDCs and with enhanced expression of both type I IFNs and IL-33 (as well as profibrotic factors downstream of IL-33, IL-13, and TGF-β1). Second, the depletion of pDCs in these mice by a pDC-depleting Ab (120G8 Ab) or the blockade of type I IFN signaling by anti-IFNAR Ab led to marked reduction in the expression of IL-33. Third, PMNCs isolated from mice treated with poly(I:C) produced a large amount of type I IFN and IL-33 upon stimulation with poly(I:C) and/or CpG in vitro, and pDCs were identified as producers of these cytokines. Fourth, such IL-33 production was dependent on type I IFN signaling because it was blocked by the presence of anti-IFNAR Ab. Fifth, and finally, the blockade of IL-33–mediated signaling pathways by a neutralizing Ab against its receptor, ST2, led to a significant reduction in pancreatic inflammation and its associated fibrosis. These studies provide further evidence of the importance of pDCs in the pathogenesis of murine AIP in that they show that this cell is the origin of two major cytokines causing the fibro-inflammatory responses, type I IFN and IL-33. Note that in parallel with these findings, we found that pancreatitis in human IgG4-related AIP is also marked by infiltration of pancreatic tissue with pDCs producing IL-33 and IFN-α (but not tissue from patients with chronic alcoholic pancreatitis). This implies that pDC production of type I IFN and IL-33 is involved in the immunopathogenesis of human IgG4-related AIP.

IL-33, the cytokine shown in this study to be produced by pathogenic pDCs in AIP, is a member of the IL-1 family of cytokines that usually resides in the nucleus of a cell bound to chromatin and that is released into the extracellular milieu upon cell

**FIGURE 4.** IL-33–mediated signaling pathways are involved in the development of chronic fibro-inflammatory responses of the pancreas. MRL/Mp mice (n = 7) were treated with anti-ST2 Ab (100 μg, n = 7) or control Ab (100 μg, n = 8) prior to each poly(I:C) (100 μg) injection. Mice in each group received poly(I:C) injection twice a week for a total of 14 times. (A) Pancreas tissues were stained with H&E. Representative image of the pancreas (original magnification ×400) and pathological scores of AIP are shown. (B and C) Pancreas tissues were stained with sirius red, anti-α-SMA Ab, and anti-fibronectin Ab. Representative images of sirius red, α-SMA, and fibronectin staining are shown. Original magnification in (B) ×400. The areas positive for sirius red, α-SMA, and fibronectin are shown (C). (D) Concentrations of hydroxyproline, IL-13, and TGF-β1 in the pancreas lysates are shown. (E) Pancreatic accumulation of pDCs in mice treated with anti-ST2 Ab or control Ab. Representative dot plots of flow cytometric analysis. PMNCs were stained with B220 and PDCA-1. pDCs were defined as B220<sup>+</sup>PDCA-1<sup>+</sup> cells and the total numbers of pDCs in each mice were determined. Results are shown as mean ± SE. *p < 0.05, **p < 0.01, as compared with control Ab–treated mice.
death or, alternatively, by “unconventional” release mechanisms (33). Acting via the ST2 receptor on a variety of cells, including Th2 and group 2 innate lymphoid cells, it induces Th2 cytokine responses and other proinflammatory responses (23). Indeed, recent studies by Furukawa et al. (34) implicate the role of IL-33 as an inducer of Th2 responses in IgG4-RD. However, it also activates Th1 responses in cytotoxic antiviral T cells (35). IL-33 has an ambiguous relationship to gut inflammation: on the one hand, polymorphisms in the IL-33 and ST2 genes appear to be risk factors in both Crohn disease and ulcerative colitis (36), and there is increased IL-33 expression in ulcerative colitis tissues (37); on the other hand, IL-33-deficient mice are more susceptible to experimental gut inflammation possibly because IL-33 is necessary for optimal IgA responses that regulate the gut microbiome (38). The important role of IL-33 in the pathogenesis of experimental AIP shown in the present study, as well as in experimental mouse conventional pancreatitis (24, 39), is not yet completely understood. With respect to IL-33 involvement in the underlying AIP-associated inflammation, IL-33 secretion could be contributing to the production of Th2 cytokines in AIP mentioned in the Introduction and in this way to the generation of autoantibodies that are produced in this disease. As shown in our previous studies, such autoantibodies could form immune complexes that stimulate the formation of neutrophil extracellular traps (NETs) by neutrophils followed by NET induction of IFN-α by pDCs (18). IL-33 can also be more directly involved in development of the pancreatic inflammation by its induction of Th1 cytokines that, as mentioned above, stimulate the function of cytotoxic T cells. To these hypothetical functions of IL-33 in AIP, one can add a more definite function, the ability of IL-33 to induce various downstream cytokines previously demonstrated to have profibrotic functions, IL-13 and TGF-β1 (40, 41). This was shown by the dramatic decrease in fibrosis in murine AIP caused by the blockade of IL-33 signaling and the accompanying downregulation of pancreatic expression of IL-13 and TGF-β1. Thus, one of the most interesting findings in this study is the identification of IL-33 as the major mediator of fibrosis in experimental AIP and, by extension, to human IgG4-related AIP.

The blockade of IL-33–mediated signaling pathways by ST2 Ab led to decreased pancreatic accumulation of pDCs defined as PDCA-1+B220low cells (Fig. 4E). Whereas in this study the mechanism accounting for such reduction in pDC accumulation is not addressed, in our previous study (24), we showed that blockade of IL-33 signaling in the model of chronic pancreatitis results in greatly reduced amounts of proinflammatory cytokine production, including reduction in IL-6, TNF-α, and MCP-1. Thus, the reason why pDCs are reduced with anti-ST2 Ab treatment appears not to be due to a specific effect on pDCs but rather to a global effect on the attenuation of pancreatic inflammation.

One question arising from the present study is whether IL-33 produced by pDCs enhances IgG4 production by B cells. In this regard, Akiyama et al. (15) reported that there is no correlation between serum levels of IL-33 and IgG4 in patients with IgG4-RD. This study thus suggested that it is unlikely that IL-33

FIGURE 5. Pancreatic acinar cells express IL-33 in chronic alcoholic pancreatitis and IgG4-related AIP. (A) Surgical pancreas specimens obtained from patients with chronic alcoholic pancreatitis (n = 3) and IgG4-related AIP (n = 3) were stained with anti-α-SMA Ab. Representative images of α-SMA staining (top, original magnification ×400) and the areas positive for α-SMA staining (bottom) are shown. (B) Surgical pancreas specimens were obtained from patients with chronic alcoholic pancreatitis (n = 4) and IgG4-related AIP (n = 4). Immunofluorescence stainings show the presence of IL-33–expressing acinar cells (top, original magnification ×1200). Pancreas tissues were stained with anti–IL-33 Ab (green) and anti-amylase Ab (red). Nuclei were counterstained with DAPI. The numbers of cells positive for IL-33 and/or amylase were counted in HPFs. Results are shown as mean ± SE.
enhances IgG4 production by B cells; however, additional studies of IL-33 effects on B cells in the presence of type I IFN are necessary to be sure of this conclusion.

In our previous studies of an experimental model of chronic pancreatitis induced by repeated injection of CCKR agonist, cerulein, in combination with NOD1 ligand, FK156, or FK565 (24, 29), we elucidated important aspects of the molecular mechanisms leading to the chronic fibro-inflammatory responses likely causing non–autoimmune conventional acute and chronic pancreatitis in humans. A key feature of this model is that initial acinar cell injury caused by CCKR-induced intra-acinar cell activation of pancreatic enzymes leads to the translocation of commensal organisms bearing NOD1 ligand into the circulation and thereby NOD1-induced acinar cell production of type I IFN. The latter then played a critical role in the pathogenic process by promoting the influx of inflammatory macrophages into the pancreas and the induction of cytotoxic cytokines that cause further acinar cell injury and release of IL-33. As shown by the fact that the chronic pancreatitis and associated fibrosis induced in this model are markedly attenuated by administration of an Ab that blocks IL-33 signaling (anti-ST2 Ab), the IL-33 thus generated makes a major contribution to the underlying pathologic responses driving the pancreatitis. These characteristics of the murine model of conventional pancreatitis are similar to those of the murine model of AIP in poly(I:C)-treated MRL/Mp mice in that in both models a type I IFN/IL-33 axis plays a critical role in the development of the pancreatic inflammation; this conclusion is highlighted by the fact that AIP is also markedly attenuated by the blockade of type I IFN and IL-33 by anti-IFNAR and anti-ST2 Abs, respectively. Note, however, that as discussed below, the cellular source of the IL-33 in the two models and in the human diseases they represent is somewhat different.

Whereas the present study identified pancreatic pDCs as the key cellular element mediating murine experimental AIP, it did not address the nature of the activators of pDCs in either murine or human AIP. One possible activator already alluded to above is NETs, which we have previously identified in the pancreas of both murine and human AIP (18). These are web-like structures composed of extracellular DNA, chromatin, and molecules derived from neutrophil granules (42, 43) that can be induced to form in the pancreas by neutrophil exposure to Ab–Ag complexes, possibly those composed of Ags released by the neutrophils and Abs.

**FIGURE 6.** Pancreatic pDCs express IL-33 in IgG4-related AIP, but not in chronic alcoholic pancreatitis. Surgical pancreas specimens were obtained from patients with chronic alcoholic pancreatitis (n = 5), IgG4-related AIP (n = 5), and pancreatic cancer (n = 5). Noncancerous portions of the tissues from pancreas cancer patients were used as controls (n = 5). Pancreatic tissues were stained with anti–IL-33 Ab (green) and anti-BDCA2 Ab (red). Pancreas tissues were counterstained with DAPI. (A) Representative images of immunofluorescence staining. Original magnification ×1200. (B) The numbers of cells positive for IL-33 and/or BDCA2 were counted in HPFs. Results are shown as mean ± SE. **p < 0.01, as compared with chronic alcoholic pancreatitis; *p < 0.01, as compared with controls.
generated by the pathologic process (18). In support of this possibility, we have shown in vitro studies that NETs induce pDCs obtained from patients with human IgG4-related AIP to produce IFN-α and stimulate pDC induction of IgG4 by cocultured B cells (18). In addition, NETs, microbe-associated molecular patterns derived from intestinal microflora may also serve as activators of pDCs in IgG4-related AIP. In support of this notion, pDCs isolated from poly(I:C)-treated MRL/Mp mice produced a large amount of IFN-α and IL-33 upon stimulation with CpG, a substance mimicking bacterial dsDNA. The possibility that microbe-associated molecular patterns act as pDC activators in AIP is attractive because it suggests that this form of pancreatic inflammation is initiated and/or driven by gut bacterial components. Evidence that this is in fact the case comes from preliminary studies in which we have observed that bowel sterilization by a broad range of antibiotics prevented the development of experimental AIP in MRL/Mp mice (data not shown). However, further studies defining the gut microbiome in AIP as well as the demonstration that gut bacteria are translocated into the circulation and can thus contact pancreatic cells will be required to fully establish this concept.

Our purification and depletion studies utilizing PMNCs obtained from tissues of MRL/Mp mice with experimental AIP as well as immunofluorescence studies of these tissues revealed that pDCs are the main cellular sources of IL-33 in this model. Such production depended on type I IFN signaling because pancreatic expression of IL-33 was markedly reduced either by the depletion of pDCs or by the neutralization of type I IFN signaling. This fits well with the finding that transcription of IL-33 requires transactivation of IFN regulatory factor 7, a critical transcription factor highly expressed in pDCs (44). Interestingly, pancreatic pDCs from MRL/Mp mice with AIP do not respond to poly(I:C) likely due to the fact that pDCs do not express a receptor that recognizes dsRNA, TLR3 (30, 31). This, at first sight, appears to be at odds with the fact that stimulation of MRL/Mp mice with poly(I:C) is shown in these and previous studies to be the inducer of pancreatic pDCs that are responsible for the AIP developing in these mice. The resolution of this apparent contradiction lies in the fact that poly(I:C) is a type I IFN inducer via its stimulation of TLR3 on conventional DCs (45). Such type I IFN production by conventional DCs can lead to the induction of Flt3 ligand, a factor necessary for differentiation and expansion of pDCs (46); moreover, type I IFN enhances the inductive effect of Flt3 ligand on pDCs (47). Thus, poly(I:C) administration via its effect on type I IFN production can induce differentiation and expansion of pDCs when administered to MRL/Mp mice, even though it cannot directly stimulate pDCs because the latter lack TLR3 expression.

IL-33 is not expressed in pancreatic acinar cells in this model although it is expressed in acinar cells of humans with IgG4-related AIP. The latter finding indicates that in the human disease acinar cells also contribute to IL-33 production, although in this situation such production may be redundant. A somewhat different situation regarding IL-33 secretion exists in relationship to pancreatitis in both patients with chronic alcoholic pancreatitis and in the murine model of chronic pancreatitis described extensively above in which pancreatitis is induced by administration of CCK agonist and NOD1 ligand (24, 25). In this form of pancreatitis acinar cells are the main, if not sole, source of IL-33 secretion, and pDCs producing this cytokine are not observed. Because in the murine model of chronic pancreatitis, administration of anti-ST2 Ab and consequent blockade of IL-33 signaling resulted in reduced inflammation and prevented fibrosis, it is reasonable to conclude that IL-33 from this cellular source is functionally capable of mediating fibrosis and perhaps other proinflammatory functions in human chronic pancreatitis (24, 25).

In conclusion, our findings show that both human IgG4-related AIP and murine AIP are characterized by pancreatic accumulation of pDCs producing IFN-α and IL-33. In the case of murine AIP, we provide evidence that chronic fibro-inflammatory responses in AIP can be prevented by the depletion of pDCs, by the neutralization of type I IFN signaling pathways, and by the neutralization of IL-33 signaling pathways. Thus, patients with IgG4-related AIP can be treated with the blockade of type I IFN or IL-33–mediated signaling pathways. Confirmation of this idea awaits human clinical studies addressing the efficacy of anti-ST2 Ab or anti-IFNAR Ab in human IgG4-related AIP.

Disclosures
The authors have no financial conflicts of interest.

References