Alternatively activated macrophages promote pancreatic fibrosis in chronic pancreatitis

Jing Xue,

Stanford University School of Medicine, Department of Medicine, Division of Gastroenterology and Hepatology, Stanford, California, USA

Vishal Sharma,

Stanford University School of Medicine, Department of Medicine, Division of Gastroenterology and Hepatology, Stanford, California, USA

Michael H. Hsieh,

Department of Urology, Stanford University School of Medicine, Stanford University, Stanford, California, USA (now at Children's National Health System, Washington, DC, USA and the Biomedical Research Institute, Rockville, MD, USA)

Ajay Chawla,

Cardiovascular Research Institute and Departments of Physiology and Medicine, University of California, San Francisco, California, USA

Ramachandran Murali,

Departments of Medicine and Biomedical Sciences Cedars-Sinai Medical Center, Los Angeles, California, USA

Stephen J. Pandol, and

Departments of Medicine and Biomedical Sciences Cedars-Sinai Medical Center, Los Angeles, California, USA

Aida Habtezion

Stanford University School of Medicine, Department of Medicine, Division of Gastroenterology and Hepatology, Stanford, California, USA

Abstract

Chronic pancreatitis (CP) is a progressive and irreversible inflammatory and fibrotic disease with no cure. Unlike acute pancreatitis, we find that alternatively activated macrophages (AAMs) are dominant in mouse and human CP. AAMs are dependent on IL-4 and IL-13 signaling and we

show that mice lacking IL-4Rα, myeloid specific IL-4Rα, and IL-4/IL-13 were less susceptible to pancreatic fibrosis. Furthermore, we demonstrate that mouse and human pancreatic stellate cells (PSCs) are a source of IL-4/IL-13. Notably, we show that pharmacologic inhibition of IL-4/IL-13 in human ex-vivo studies as well as in established mouse CP decreases pancreatic AAMs and fibrosis. We identify a critical role for macrophages in pancreatic fibrosis and in turn PSCs as important inducers of macrophage alternative activation. Our study challenges and identifies pathways involved in cross talk between macrophages and PSCs that can be targeted to reverse or halt pancreatic fibrosis progression.

Introduction

Chronic pancreatitis (CP) is characterized by progressive and what is thought to be irreversible damage to the pancreas with end result of endocrine and exocrine insufficiency[1]. CP histologic features include chronic inflammation, fibrosis, acinar cell atrophy and distorted and/or blocked ducts[2,3]. The management of CP is challenging with focus on management of complications, and most patients remain symptomatic despite limited supportive therapy. Currently, there are no effective methods to limit progression or reverse this syndrome[4]. Recurrent acute pancreatitis or pancreatic insults lead to necroinflammation and are linked to the development of pancreatic fibrosis (the necrosis-fibrosis concept)[4]. Recent *in vitro* and *in vivo* studies demonstrate the central role of activated pancreatic stellate cells (PSCs) in CP associated fibrogenesis by regulating the synthesis and degradation of extracellular matrix (ECM) proteins[5,6]. PSCs are activated by many factors such as toxic factors associated with pancreatitis (e.g. ethanol) and/or by cytokines released from injured acinar cells and/or pancreas infiltrating leukocytes (such as macrophages and neutrophils)[7].

Macrophages are innate immune cells, for simplicity divided into two spectra of major types based on Siamon Gordon's scheme: 1) classically activated macrophages (M1), induced by IFNγ and/or LPS, characterized by the production of reactive oxygen and nitrogen species and thought to play a critical role in host defense and anti-tumor immunity; and 2) alternatively activated macrophages (M2), upon exposure to IL-4/IL-13, are characterized by cell surface expression of scavenger receptors CD206. Alternatively activated macrophages play key roles in dampening inflammation, promote wound healing, fibrosis, and tumorigenesis[8]. Recent studies highlighted the function of macrophages as master regulators of fibrosis[9]. Distinct macrophage populations contribute important activities towards the initiation, maintenance, and resolution phase of fibrosis[9,10]. Macrophages have been observed in close proximity to PSCs in human pancreatic fibrosis and their presence observed in rat model of chronic pancreatitis, although not well defined their potential role in chronic pancreatitis has been suggested[11,12]. Thus, the mechanism(s) by which cross-talk between activated stellate cells and macrophages trigger and sustain the fibrotic process during CP is not known. Delineating immune responses involved in the fibrotic processes will improve our understanding of disease pathogenesis and allow for designing novel therapeutics that can either treat and/or reverse the disease. Our study investigates and identifies macrophage characteristics and function in CP.

In this study, we demonstrate that progression to CP is associated with alternative activation of macrophages and show an important role for the IL-4/IL-13 pathway in a cross talk between macrophages and PSCs using in vivo and in vitro animal studies as well as ex-vivo human primary cells. Notably, blocking IL-4/IL-13 using a peptide antagonist we show a therapeutic effect in established experimental CP and proof-of-concept therapeutic *ex vivo* effect using human samples. These studies are likely to offer potential benefit in a disease for which currently no active therapeutic agent exists and as such the disease is deemed progressive and irreversible.

Results

Macrophages are increased in mouse and human CP

Studies on pathogenic mechanism of fibrosis in human chronic pancreatitis are restricted by limited availability of tissues obtained from surgery. Therefore, animal models, despite their limitation in recapitulating all aspects of human disease, have been useful to investigate the initiation and progression of CP[13,14]. In mice, hyper-stimulation of the pancreas with cholecystokinin analog caerulein leads to acute pancreatitis, and continuous acute injury to the pancreas drives chronic inflammation of the pancreas[4,14]. To generate experimental CP, we induced acute pancreatitis in a repetitive manner over 4 weeks (3 times per week). Mice undergoing repetitive treatment with caerulein revealed morphologic signs of CP with leukocyte infiltration, pancreatic fibrosis and acinar cell loss corresponding to small size of the pancreas relative to body weight (Supplementary Fig. 1a–c).

We next sought to investigate the immune responses in experimental CP. Using Luminex assay, we compared multiple cytokine and chemokine expression profiles in the pancreas from control and CP mice. As expected, the pro-fibrotic cytokine, TGFβ was increased in the pancreas of CP mice. However, pro-inflammatory cytokines (IL-1β, IL-6), which are known to be increased during acute inflammation, were down-regulated in CP. Chronic repeated caerulein administration and pancreas harvest three days after the last injection is consistent with the development of a chronic and not acute pancreatitis. Furthermore, macrophage-associated cytokines and chemokines (GMCSF, GCSF, CCL2/MCP-1, CCL7/MCP-3, CCL3/MIP1A) were up regulated, suggesting that monocytes/macrophages play an important role during CP. In contrast, no significant increase in CXCL1, a neutrophil chemoattractant with role in acute pancreatitis[15], was observed (Fig. 1a).

To understand the relevance of these observations to human CP, we compared them to normal pancreas tissue sections. Immunofluorescence analysis showed that CD68+ macrophages were increased in human CP (Fig. 1c). A similar trend was observed in mice; macrophage marker F4/80 analysis revealed that macrophages are abundant in CP as compared to the normal pancreas of mice (Fig. 1b). Furthermore, the number of pancreatic macrophages (CD11b+F4/80+) analyzed by flow cytometry was markedly increased in CP mice (Fig. 1d). Macrophage tissue infiltration occurs in two distinct ways: recruitment of monocyte precursors and proliferation of resident cells[16]. We used Ki-67 expression and BrdU incorporation to determine the proportion of proliferating macrophages. We found that BrdU+ and Ki-67+ macrophages were both increased in CP mice accounting for about 12 – 25% of the pancreatic macrophages (Fig. 1d), indicating proliferation of either resident

macrophages or recruited monocytes that differentiated to macrophage contributed to some of the macrophage accumulation in CP.

During acute pancreatitis monocytes are attracted to the injured pancreas prior to their differentiation into macrophages in a CCR2-dependent manner[17,18]. CCR2 ligands such as CCL2 (MCP1) and CCL7 (MCP3) are also elevated in CP (Fig. 1a). Considering the model of CP is based on repetitive acute injury, we hypothesized that in addition to proliferation, a significant proportion of macrophages in CP arise from monocyte recruitment. To confirm this hypothesis, we set up a competitive bone marrow chimeras by lethally irradiating recipient $CCR2^{WT}CD45.1^+CD45.2^+$ C57BL/6 mice and reconstituting them with a 1:1 mixture of bone marrow derived from CCR2 wild type ($CCR2^{WT}CD45.1^+$) and CCR2 knockout ($CCR2^{KO}CD45.2^+$) mice so that macrophages derived from $CCR2^{KO}$ and $CCR2^{WT}$ mice could be distinguished from one another as well as from those of the recipient mice based on the allotypic CD45 markers. Following 8 weeks of engraftment and induction of CP, we found that the number of $CCR2^{WT}$ macrophages greatly outnumbered $CCR2^{KO}$ macrophages. However, there was no difference in proliferation (BrdU incorporation) observed between $CCR2^{WT}$ and $CCR2^{KO}$ cells, supporting CCR2's role in monocyte recruitment as a significant contributor to macrophage accumulation in CP (Fig. 1e).

To further confirm our findings, we induced CP in non-irradiated wild type ($CCR2^{WT}$) and CCR2 knockout ($CCR2^{KO}$) mice. As shown in Supplementary Fig. 2, CCR2-deficiency limits pancreatic macrophage accumulation in CP, and no proliferation difference is observed between presence and absence of CCR2, which is consistent with the above bone marrow chimera findings. However, in the absence of CCR2 there is still monocyte/macrophage recruitment to the pancreas during CP, suggesting that CCR2-independent mechanism(s) also exist. Thus, both monocyte recruitment (via CCR2 dependent and independent mechanisms) and macrophage proliferation account for macrophage accumulation during CP progression.

AAMs are dominant in mouse and human CP

In order to determine the polarization state of macrophages in CP, SSC-A^{low}CD11b$^+$ monocytes/macrophages were sorted from both control and CP mice for gene expression analysis. Sorting strategy and purity of monocytes/macrophages are shown in Supplementary Fig. 3. Gene expression profile revealed an increase in M2-associated genes, such as YM1, CD206, CD301, IL-10, TGFβ, and PDGFβ in pancreatic monocytes/macrophages of CP as compared to controls (Fig. 2a). Moreover, the presence of alternatively activated macrophages in CP was further verified using flow cytometry as shown by increased M2-associated markers (CD206, IL-10 and IL-4Rα), and decreased or unchanged expression of M1-associated markers (MHCII and TNFα) (Fig. 2b). In contrast, by assessing dynamic gene expression of pancreatic macrophages during acute pancreatitis induction, classical activation profile with increased TNFα and decreased CD206, CD301 was found in acute pancreatitis mice (Supplementary Fig. 4a,b). Unlike TNFα, IL-10 expression can be seen as late response in M1s[19], which is consistent with our acute pancreatitis data (Supplementary Fig. 4a). Nevertheless, IL-10 in acute pancreatitis is

expressed at much lower level as compared to CP in pancreatic macrophages (Fig. 2), although not to the same extent, slightly higher level of YM1 expression is seen in CP as compared to acute pancreatitis. Arg1 has recently been shown to be expressed by both classic and alternatively activated macrophages[20]. Flow cytometric data was consistent with histologic analysis of pancreas from CP mice, based on immunofluorescence staining where a majority of F4/80$^+$ macrophages were positive for CD206 but not TNFα. Similar findings were observed in human CP tissues, where CD68$^+$ cells expressed CD206, but not TNFα (Fig. 2c,d).

PSCs promote alternative activation of macrophages

Macrophages are highly heterogeneous cells that can rapidly change their activation status and function in response to local microenvironment signals[21,22]. In light of the crucial role played by PSCs in CP, we asked whether PSCs might contribute to macrophage polarization and function. We isolated PSCs from CP mice and assessed their cytokine production by Luminex assay. Overall, the expression of several pro-inflammatory cytokines such as IFNγ, TNFα, and IL-1β was very low. In contrast, PSCs secreted higher levels of IL-4, IL-5, IL-13, IL-10 and TGFβ, indicating a Th2 and pro-fibrogenic cytokine bias (Fig. 3a).

To explore whether factors released by PSCs have the ability to change the activation and polarization status of pancreatic macrophages, we co-cultured bone marrow derived macrophages (BMDMs) with the PSCs *in vitro*. BMDMs after co-culture with activated PSCs exhibited alternative activation (M2) profile with increased CD206, CD301, IL-10, TGFβ and PDGFβ mRNA expression but had decreased expression of iNOS (inducible nitric oxide synthase, M1 marker) (Fig. 3b). Moreover, conditioned medium from the PSCs resulted in up-regulation of CD206, IL-10 and IL-4Rα, and down-regulation of MHCII and TNFα expression (Fig. 3c), suggesting that factors released by PSCs promoted macrophage polarization towards M2.

Th2 cytokines such as IL-4/IL-13 mediate alternate activation of macrophages via IL-4 receptor. Thus we tested whether the PSCs supernatant is mediating M2 polarization via IL-4Rα using BMDMs from WT and *IL-4Rα$^{-/-}$* mice. Compared with WT BMDMs, *IL-4Rα$^{-/-}$* BMDMs were unable to be alternatively activated by conditioned medium from the PSCs (Fig. 3d). Moreover, PSC conditioned medium was as good as exogenously added IL-4/IL-13, standard M2 polarizing conditions. The macrophages produced TGFβ and PDGFβ (Fig. 3b), previously shown to be potent activators of PSCs[6]. To investigate the effect of these factors on PSC mediated macrophage polarization, we treated PSCs with TGFβ and PDGFβ and examined the expression of IL-4/IL-13. Interestingly our study shows that PDGFβ and in particular TGFβ were capable not only at inducing αSMA but also IL-4 and IL-13 genes in the PSCs (Fig. 3e). Taken together, these results suggest that PSCs promote macrophage alternative activation in an IL-4Rα signaling dependent manner and the macrophages in turn have the ability to induce PSC activation and production of IL-4R ligands.

To further validate our finding in the human, we isolated PSCs from six patients (hPSCs) that underwent surgical resection (3 from normal pancreas, 3 from pancreatic cancer) of the pancreas. We confirmed that the cultured cells had a PSC phenotype by

immunofluorescence staining of GFAP and α-SMA (Supplementary Fig. 7a). Consistent with mouse PSCs, hPSCs secreted relatively higher levels of Th2 as compared to Th1 cytokines (Fig. 3f). Macrophages derived from circulating monocytes of healthy blood donors were co-cultured with supernatant of the hPSCs in an effort to translate the mouse studies. Conditioned medium from all six hPSCs increased CD206, but decreased TNFα expression in the human macrophages, indicating that factors released from hPSCs also promote macrophage polarization towards M2 (Fig. 3g).

IL-4Rα signal deficiency limits AAMs and protects against CP

To investigate the importance of macrophage alternative activation in CP, we induced CP in mice lacking IL-4 and IL-13. *IL-4/IL-13*$^{-/-}$ mice have been shown to lack alternatively activated macrophages in other disease models[23]. Unlike WT counterparts, *IL-4/IL-13*$^{-/-}$ mice were less susceptible to CP, as shown by the larger relative pancreas size (WT: from 5.95±0.11 to 1.71±0.05; *IL-4/IL-13*$^{-/-}$: from 5.98±0.20 to 2.89±0.07; Fig. 4a), lower fibrosis-associated gene expression in the pancreas such as *αSMA* (*α*-SMA) and *Col1α1* (Collagen1A1) by real-time PCR, and immunofluorescence analysis (Fig. 4b,c). Moreover, compared to WT mice macrophages isolated from the *IL-4/IL-13*$^{-/-}$ mice pancreas had lower expression of CD206, suggesting a decrease in alternative activation of pancreatic macrophages (Fig. 4d).

To determine whether the observed decrease in pancreatic fibrosis was a direct consequence of IL-4Rα signaling, we used mice with global deletion of IL-4Rα and *LysM*^{cre}*IL-4Rα*$^{flox/flox}$ mice, where IL-4Rα is deleted specifically in myeloid cells (i.e. macrophages and neutrophils)[23,24] to induce CP. As expected, pancreatic macrophages from *IL-4Rα*$^{-/-}$ and *LysM*^{cre}*IL-4Rα*$^{flox/flox}$ mice displayed impaired alternative activation under chronic inflammation (Fig. 4d). *IL-4Rα*$^{-/-}$ and *LysM*^{cre}*IL-4Rα*$^{flox/flox}$ mice pancreas were less fibrotic and had lower expression of fibrosis-associated markers (*α*-SMA and Collengen1A1) as compared to their WT counterparts (Fig. 4a–c). Notably, *IL-4Rα*$^{-/-}$ and *LysM*^{cre}*IL-4Rα*$^{flox/flox}$ mice showed no obvious difference in pancreatic fibrosis and alternative macrophage activation suggesting that the protective effect of IL-4Rα inhibition is mediated via absence of this receptor or signaling on myeloid cells or macrophages. Thus in this model of CP, alternatively activated macrophages are important contributors to disease pathogenesis.

To further confirm an IL-4Rα requirement for macrophage alternative activation in CP, we set up a mixed bone marrow chimera with a 1:1 bone marrow reconstitution from *IL-4Rα*^{WT}*CD45.1*$^{+}$ and *IL-4Rα*$^{-/-}$*CD45.2*$^{+}$ mice in order to compare IL-4Rα-sufficient and deficient macrophages in the same environment of CP (Supplementary Fig. 5a). In such a competitive environment, CD206 expression in *IL-4Rα*^{WT} macrophages (CD11b^{+}F4/80^{+}) was up regulated following CP induction, whereas, no significant difference in CD206 expression was observed in *IL-4Rα*$^{-/-}$ macrophages (Supplementary Fig. 5b). No difference in proliferation between *IL-4Rα*^{WT} and *IL-4Rα*$^{-/-}$ pancreatic macrophages was observed (Supplementary Fig. 5c). In sum, these findings indicate that IL-4R*α* signaling in macrophages is in part required for pancreatic macrophage alternative activation and fibrosis during CP development.

IL-4/IL-13 blockade ameliorates established CP

In light of the above findings and the importance of IL-4Rα signaling in experimental CP associated fibrogenesis, we wanted to test whether IL-4Rα signaling blockade can be used as a therapy in established disease. We obtained IL-4/IL-13 blocking peptide (a potent cyclic peptide ~1.5kD) from Dr. Murali's laboratory. We first tested the inhibitor in an *in vitro* titration assay, and at 1μM the inhibitor significantly decreased mouse IL-4/IL-13-induced M2 polarization and CD206 expression (Supplementary Fig. 6). We then tested the effect of the inhibitor in mice that were already subjected to repeated caerulein injection for 2 weeks, where significant decrease in pancreas size and α-SMA expression was present (Supplementary Fig. 1d–e). Compared with control treatment group, the inhibitor treatment limited pancreas fibrosis (Fig. 5a–e). Furthermore, blockade of alternative activation of pancreatic macrophages by the inhibitor was confirmed by flow cytometry (Fig. 5f). These observations, as a proof-of-concept, demonstrate potential for treating CP. We then tested the inhibitor's ability in blocking M2 polarization of human macrophage by the hPSCs. The peptide was indeed capable of inhibiting hPSCs-mediated M2 polarization of human macrophages (Fig. 5g).

Discussion

Generally CP is accepted as an irreversible and fibrotic disease, and current management is supportive at best with focus in controlling pain and complications associated with the exocrine and endocrine loss of functions[4]. Following the discovery and the central role of PSCs in pancreatic fibrosis, potential treatment approaches in CP have proposed inhibition or inactivation of PSCs[25]. However, the immune responses and immune cell contribution to PSC activation during CP progression remain poorly understood. In the current study, we explore the characteristics and role of macrophages, as well as aim to identify mechanisms for macrophage interaction with PSCs in CP. Our results show that alternatively activated macrophages play an important role in CP fibrogenesis and identify key pathway that can be potentially targeted.

Macrophages have been proposed as master regulators of inflammation and fibrosis in diseases such as liver fibrosis and systemic sclerosis[9]. Our study reveals that macrophages are increased in both mouse and human CP. CP is also associated with an increase in macrophage-related cytokines and chemokines, supporting for a critical role of macrophages in disease progression. Since macrophages differentiate as well as polarize in tissues and do not recirculate, our data using competitive bone marrow chimeras and proliferation studies suggest that both local (*in situ*) macrophage proliferation and monocyte recruitment contribute to the macrophage accumulation in CP.

In sharp contrast to acute pancreatitis where M1 predominate, CP favored alternative activation of macrophages. Macrophages represent a spectrum of activated phenotypes rather than a discrete stable subpopulation[26] and it is possible that macrophages in different activation states or mixed phenotypes coexist as have been shown in different physiological and pathological conditions[27]. Indeed, these phenomena may account for the varied expression level of some of the M2 markers (e.g. *YM1*). Macrophages in our CP model and those from PSC co-cultures had higher mRNA expression of TGFβ and PDGFβ, suggesting

possible role in directly promoting proliferation and activation of PSCs[6,28,29]. These macrophages express higher levels of tissue inhibitor metalloproteinase 2 (*TIMP2*) and matrix metalloproteinase 9 (*MMP9*) and thus may also regulate ECM turnover (Fig. 2a). Moreover, their expression of IL-10 and down-regulation of MHCII suggests immunosuppressive properties.

Several studies have documented macrophage plasticity with these cells switching from one functional phenotype to another in response to variable local microenvironmental signals[27,30–32]. In light of the central role of PSCs in CP progression, we took into account that the PSCs may be providing signals to maintain and facilitate the increased alternative activation observed in CP. In fact, relative to pro-inflammatory cytokines, PSCs (from both mouse and human pancreas) expressed higher levels of Th2 cytokines, IL-4 and IL-13, which are required for macrophage alternative activation[8,33]. Furthermore, using IL-4Rα sufficient and deficient mixed BM chimeras, we confirmed that IL-4Rα signaling is required for alternative activation of macrophages in CP.

Both *IL-R4α*^{−/−} and *LysM*[cre]*IL-4Rα*[flox/flox] mice had similar decreases in pancreatic fibrosis and PSC activation (α-SMA), suggesting that the contribution of IL-R4α signaling in fibrosis during CP is myeloid- or macrophage-dependent. *IL-4/IL-13*^{−/−} mice were even less susceptible to caerulein-induced CP. Similar to IL-4, IL-13 signals through the IL-4 receptor (a heterodimeric receptor composed of I IL-4Rα and IL-13Rα1), however IL-13 can also bind to IL-13Rα2 (which does not bind IL-4) to trigger downstream signals. IL-13 has been shown to be a major inducer of fibrosis in many chronic infectious and autoimmune diseases in part via the IL-13Rα2[34]. Inhibition of both IL-4 receptor and IL-13Rα2 signaling in the double knock out mice (*IL-4/IL-13*^{−/−}) as compared to inhibition of IL-4 receptor only in the *IL-4Rα*^{−/−} and *LysM*[cre]*IL-R4α*[flox/flox] mice may account for the increased protection against CP development observed in the *IL-4/IL-13*^{−/−} mice.

Functional macrophage polarization is observed *in vivo* under physiological and pathological conditions[21]. The phenotype of polarized M1 or M2 macrophages can, to some extent, be reversed *in vitro* and *in vivo*[35,36]. Therefore, reorienting and reshaping macrophage polarization has been considered as a therapeutic strategy for several diseases. In our study, we attempted to reshape macrophage polarization through genetically deficient mice or via blocking IL-4/IL-13 by pharmacological means. Consistent with results from IL-4/IL-13-deficient mice, treatment with the IL-4/13 blocking peptide following induction of CP decreased macrophage alternative activation and ameliorated pancreatic fibrosis. This immune-based therapy in experimental CP challenges the thinking that CP cannot be actively treated, reversed or halted from progressing. Future studies determining the contribution of IL-4 versus IL-13 will be of interest to further define and design specific target(s).

It's worth pointing out that some limitations still exist in correlating to human studies[22]: 1) limited availability of human CP tissues from surgical resections; 2) challenge of identification of equivalent macrophage subsets in humans due to lack of panels of markers. Despite these, our *in vitro* human PSCs and macrophage co-culture experiments are consistent with the results obtained in mouse studies. We show that activated mouse and

human PSCs secrete Th2 cytokines and enhance M2 polarization, leading to a potentially perpetual feed forward process (Figure 6). These findings are confirmed using IL-4Rα deficient macrophages, and IL-4/IL-13 blocking peptide in experimental mouse and human systems. Interfering with IL-4Rα signaling and/or PSC activation is likely to turn off this feed forward process in chronic pancreatitis. Our *in vitro* human studies, together with results observed from mouse studies, provide a proof-of-concept for potential targetable pathway and first step towards bench to bedside translation.

Methods

Mice

BALB/c, C57BL/6, SJL, B6.SJL, *IL-4Rα*^{−/−}, CCR2[KO] mouse strains were purchased from Jackson laboratory and bred in house. *LysM*[cre]*IL-4Rα*[flox/flox] [24] and *IL-4/IL-13*^{−/−} [37] mice on BALB/c background were generated as described. All experimental mice were age (6–8 weeks) and sex matched, and animal experiments were approved by Stanford University institutional animal care and use committees.

Mixed bone-marrow chimeras

Competitive mixed BM chimeric mice were generated by lethally irradiating *CCR2*[WT]*CD45.1*[+]*CD45.2*[+] C57BL/6 mice with 9.5 Gy γ radiation in two doses with ~3h apart, followed by i.v. Injection of 5×10^6 BM cells comprised of 1:1 mixture of cells from *CCR2*[WT]*CD45.1*[+] and *CCR2*[KO]*CD45.2*[+] mice. Chimeric mice were left to engraft for at least 8 weeks before further experimental manipulation.

Pancreatitis Model and Treatment

Acute pancreatitis (AP) was induced with caerulein injection in mice as previously described[38,39]. Chronic pancreatitis (CP) was induced by repetitive caerulein injections[14,40]. In brief, mice were given 6 hourly intra-peritoneal (i.p.) injections of 50μg/kg body weight caerulein (Sigma-Aldrich) 3 days per week, for a total of 4 weeks. Mice were then sacrificed and analyzed 3 days after the last caerulein injection. For blocking peptide study all mice were given caerulein injection 3 days per week for a total of 4 weeks as above, and 2 weeks following start of the caerulein injection mice were either given vehicle control (PBS) or IL-4/IL-13 blocking peptide (50μg/mouse, 100μl daily for 5 days per week × 2 weeks) until sacrificed 4 weeks and 3 days later as above.

Human Samples

Human pancreatic tissues from patients with CP, pancreatic ductal adenocarcinoma (PDAC), and normal pancreas margins from patients with PDAC, and one from a patient with normal pancreas (patient had adjacent non-pancreatic tumor that required partial resection of the pancreas) were obtained from the Stanford tissue bank with Local Ethics Committee approval and patient consents.

Histology and Immunofluorescence

Mice were euthanized by CO_2 inhalation, and then pancreata were rapidly removed. Pancreas pieces were immediately fixed in 10% formalin or frozen in Tissue-Tek OCT compound. Fixed tissues were sectioned and used for H&E and Trichrome staining (performed by Histo-Tec Laboratory). Frozen tissues were also sectioned for immunofluorescence staining with indicated antibodies and analyzed with confocal microscopy.

Luminex Assay

The assay was performed in the Human Immune Monitoring Center at Stanford University[39]. Human 63-plex or Mouse 26 plex kits were purchased from Affymetrix and used according to the manufacturer's recommendations with modifications as described below. Briefly, samples were mixed with antibody-linked polystyrene beads on 96-well filter-bottom plates and incubated at room temperature for 2 h followed by overnight incubation at 4°C. Room temperature incubation steps were performed on an orbital shaker at 500–600 rpm. Plates were vacuum filtered and washed twice, then incubated with biotinylated detection antibody for 2 h at room temperature. Samples were then filtered and washed twice as above and resuspended in streptavidin-PE. After incubation for 40 minutes at room temperature, two additional vacuum washes were performed, and the samples resuspended in Reading Buffer. Each sample was measured in duplicate. Plates were read using a Luminex 200 instrument.

Antibodies and flow cytometry

All antibodies used for flow cytometry were purchased from Biolegend unless indicated. For surface staining, murine cells were stained with the following antibodies: APC-CD45.2 (109814, 1:200), PE/Cy7-CD4 (100528, 1:300), Percp/Cy5.5-CD11b (101228, 1:200), BV421-F4/80 (123137, 1:200), APC/Cy7-CD11C (117324, 1:200), PE-IL-4Rα (144803 1:100), AF488-CD206 (141710, 1:100), AF700-MHCII (107622, 1:300), PB-Ly6C (128014, 1:200), PE/Cy7-Ly-6G (127617, 1:300) PE-FcεRIα (134307, 1:200), AF488-ckit (105815, 1:200), APC-eFluor780-CD45.1 (47-0453-82, eBioscience, 1:200) and PE-Siglec-F (562068, BD Biosciences, 1:200). Human cells were stained with PE/Cy7-CD14 (301814,1:100), AF488-CD206 (321114, 1:50) and APC-CD68 (333810, 1:100). For intracellular cytokine staining, cells were activated with phorbol myristate acetate (PMA, 50ng/ml) and ionomycin (1μg/ml), in the presence of brefeldin A (10μg/ml, eBioscience) for 4 hours at 37°C before staining. The cells were then fixed and permeabilized using eBioscience kit following and manufacturer's guidelines. APC-IL10 (554468, 1:100) and isotype control (556924, 1:100) from BD Biosciences were used for intracellular staining. For intracellular TNFα staining, cells were incubated with or without LPS (100ng/ml, InvivoGen) in the presence of brefeldin A before surface staining. PE-TNFα (554419, 1:200) and isotype control (554685, 1:200) from BD Biosciences were used.

For detection of intracellular Ki-67 and BrdU, cells were stained for surface makers then fixed and permeabilized using Foxp3 staining buffer set (eBioscience). For BrdU staining, cells were first incubated with DNase for 1h at 37°C. The Cells were then stained with PE-BrdU (339812, 1:100) or AF488-Ki67 (558616, BD Biosciences, 1:50). Dead cells were

excluded from analysis using violet viability stain (Invitrogen). Flow cytometry data collection was performed on Fortessa LSRII (BD Biosciences) and analyzed using FlowJo software (Tree Star, Inc.).

Cell preparation and *in vitro* cultures

Pancreatic leukocytes were isolated using collagenase digestion method described previously for flow cytometry analysis[38]. Pancreatic stellate cells (PSCs) from CP mice were isolated by outgrowth method as described[41]. Primary human PSCs from normal margins and PDAC were isolated by gradient centrifugation and outgrowth method respectively[41,42]. Murine PSCs were cultured in DMEM/F12 (1:1) medium containing 10% FBS. Where indicated mouse PSCs were cultured with TGFβ (5ng/ml), PDGFβ (10ng/ml), or media control for 6h before collected for mRNA and qPCR analysis. Human PSCs were cultured in IMDM medium containing 20% FBS. Isolated PSCs were ready for use after the second passage. Conditioned medium (CM) from the PSC was collected after 2 days of culture and when cells reached 70–80% confluence. The CM was centrifuged to remove cellular debris prior to use. Bone Marrow Derived Macrophages (BMDMs) were prepared as previously described[39]. For human monocyte derived macrophage preparation, human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coat by Ficoll-Hypaque density gradient centrifugation, and then monocytes were further enriched by CD14+ magnetic beads (Miltenyi Biotec). Enriched monocytes were cultured with complete RPMI medium containing 50ng/ml human M-CSF. At day 6, human macrophages are ready for use[43]. PSCs and BMDMs co-culture experiments were performed in Transwell system (Corning) in DMEM/F12 medium with 10%FBS. After seeding 5×10^4 PSCs in the bottom well, 5×10^5 BMDMs were seeded on the upper mesh (pore size: 0.4μm). Macrophages were collected for analysis after 48 hours of the co-culture.

Quantitative RT-PCR

Pancreas or cells were lysed with Trizol reagent (Invitrogen) for total RNA preparation according to manufacturer's instructions. Briefly, cDNA was generated using GoScript reverse transcription system (Promega). Quantitative PCR was performed with an ABI-7900 Sequence Detection System (Applied Biosystems) using designed specific TaqMan probes and primers as follows: YM1 (Forward, 5′-TGGTGAAGGAAATGCGTAAA-3′; Reverse, 5′-GTCAATGATTCCTGCTCCTG-3′; Probe, 5′-AGCAGCCTTGGAATGTCTTTCTCCA-3′); FIZZ1 (Forward, 5′-AGGAACTTCTTGCCAATCCA -3′; Reverse, 5′-ACAAGCACACCCAGTAGCAG-3′; Probe, 5′-CCTCCTGCCCTGCTGGGATG-3′); Arg1 (Forward, 5′-AGACCACAGTCTGGCAGTTG-3′; Reverse, 5′-CCACCCAAATGACACATAGG-3′; Probe, 5′-AAGCATCTCTGGCCACGCCA-3′); CD206 (Forward, 5′-TGATTACGAGCAGTGGAAGC-3′; Reverse, 5′-GTTCACCGTAAGCCCAATTT-3′; Probe, 5′-CACCTGGAGTGATGGTTCTCCCG-3′); CD301 (Forward, 5′-ACTGAGTTCCTGCCTCTGGT-3′; Reverse, 5′-ATCTGGGACCAAGGAGAGTG-3′; Probe, 5′-CACTGCTGCACAGGGAAGCCA-3′); IL-10 (Forward, 5′-CCCAGAAATCAAGGAGCATT-3′; Reverse, 5′-TCACTCTTCACCTGCTCCAC-3′; Probe, 5′-TCGATGACAGCGCCTCAGCC-3′); TGFβ (Forward, 5′-CCCTATATTTGGAGCCTGGA-3′; Reverse, 5′-CTTGCGACCCACGTAGTAGA-3′;

Probe, 5′-CCGCAGGCTTTGGAGCCACT-3′); iNOS (Forward, 5′-ACCTTGTTCAGCTACGCCTT -3′; Reverse, 5′-TCTTCAGAGTCTGCCCATTG-3′; Probe, 5′-TGCTCCTCTTCCAAGGTGCTTGC-3′); TNFα (Forward, 5′-CCAAAGGGATGAGAAGTTCC-3′; Reverse, 5′-CTCCACTTGGTGGTTTGCTA-3′; Probe, 5′-TGGCCCAGACCCTCACACTCA-3′); αSMA (Forward, 5′-CTCCCTGGAGAAGAGCTACG-3′; Reverse,5′-TGACTCCATCCCAATGAAAG-3′; Probe, 5′-AAACGAACGCTTCCGCTGCC-3′); Collegen1A1 (Forward, 5′-AGAAGGCCAGTCTGGAGAAA-3′; Reverse,5′-GAGCCCTTGAGACCTCTGAC-3′; Probe, 5′-TGCCCTGGGTCCTCCTGGTC-3′); Fibronectin (Forward, 5′-TGGTGGCCACTAAATACGAA-3′; Reverse, 5′-GGAGGGCTAACATTCTCCAG-3′; Probe, 5′-CAAGCAGACCAGCCCAGGGA-3′); TIMP1 (Forward, 5′-CCAGAAATCAACGAGACCAC -3′; Reverse, 5′-GGCATATCCACAGAGGCTTT -3′; Probe, 5′-TCTGCGGCATTTCCCACAGC -3′); MMP9 (Forward, 5′-TCCTTGCAATGTGGATGTTT -3′; Reverse, 5′-CTTCCAGTACCAACCGTCCT-3′; Probe, 5′-TGCAGAGCGCCCTGGATCTC -3′); GAPDH (Forward, 5′-TGTGTCCGTCGTGGATCTGA-3′; Reverse, 5′-CCTGCTTCACCACCTTCTTGA-3′; Probe, 5′-CCGCCTGGAGAAACCTGCCAAGTATG-3′). Samples were normalized to GAPDH and displayed as fold induction over untreated controls unless otherwise stated.

Statistical analysis

Unpaired Student's *t*-test was used to determine statistical significance unless otherwise indicated and *P* value of less than 0.05 was considered significant. Where indicated One-way ANOVA and Tukey's Post-hoc Test was used as described in figure legend. Values are expressed as mean ± s.e.m. (Prism 5; GraphPad Software). Unless indicated, results are from at least 3 independent experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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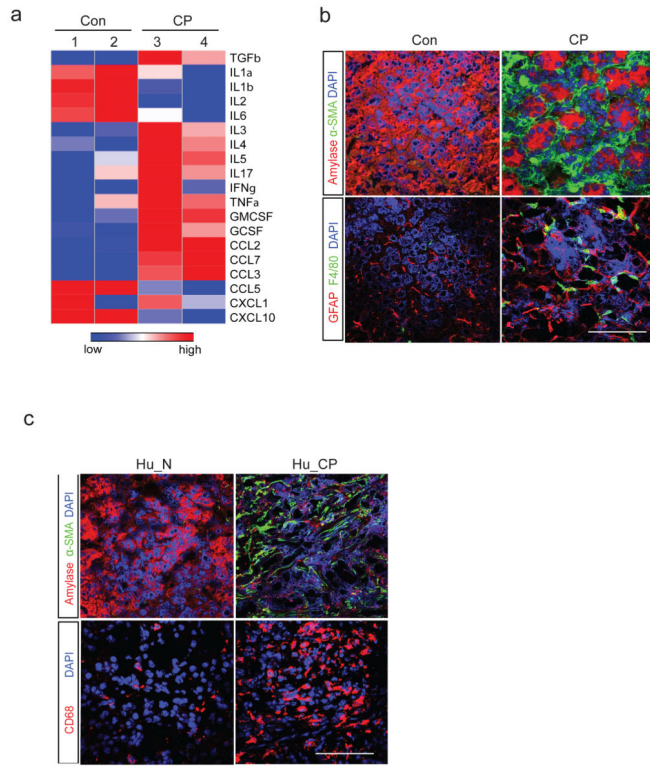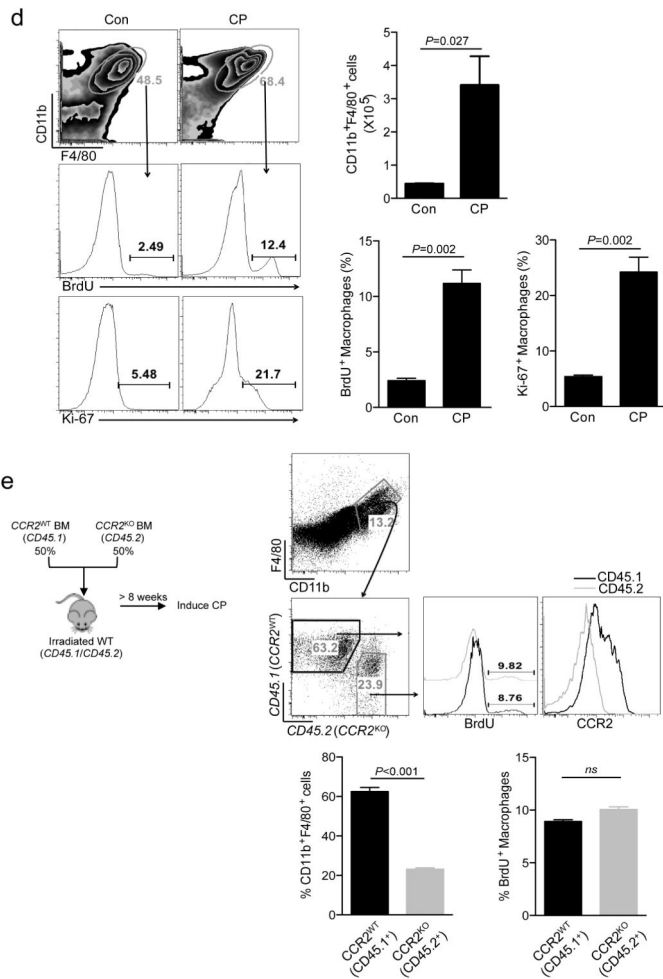
Figure 1. Macrophages are increased in mouse and human chronic pancreatitis

(a) Heat map of cytokine and chemokine expressions in the pancreas lysates from control (Con) and chronic pancreatitis (CP) mice quantitated via Luminex analysis. (b) Representative immunofluorescence images of pancreas from control and CP mice stained with amylase (acinar cells), α-SMA and GFAP (PSCs), F4/80 (macrophages) and DAPI. Scale bars, 50μm. (c) Representative immunofluorescence images of human pancreas from paired normal and CP tissue stained with amylase, α-SMA, CD68 (macrophages) and DAPI. Scale bars, 50μm. (d) Pancreatic leukocytes were isolated from control and CP mice and analyzed by flow cytometry for macrophages (gated on $CD45.2^+$ cells first as shown in Supplementary Fig. 3, then $CD11b^+F4/80^+$) numbers, BrdU incorporation, Ki-67 expression (n 3 per group). (e) $CCR2^{WT}CD45.1^+CD45.2^+$ C57BL/6 mice were lethally irradiated and reconstituted with a 1:1 mixture of BM cells from $CCR2^{WT}CD45.1^+$ and $CCR2^{KO}CD45.2^+$ mice over 8 weeks. Mice were injected with caerulein to induce CP as described in the methods, and macrophages (gated on $CD11b^+F4/80^+$ without prior CD45.2 gating) were analyzed by flow cytometry following pancreatic leukocyte isolation. Representative flow cytometry plots and bar graphs depicting the proportion of macrophages and BrdU incorporation originating from $CCR2^{WT}CD45.1^+$ (black line) versus from $CCR2^{KO}CD45.2^+$
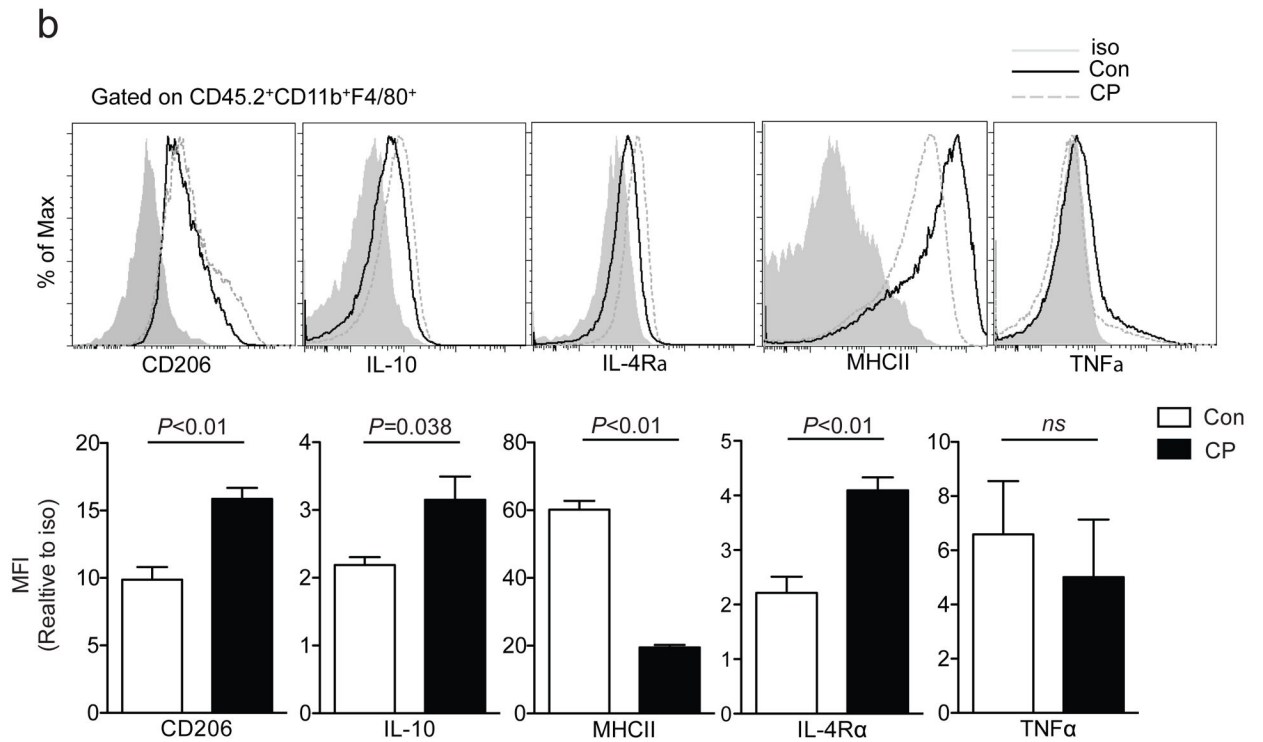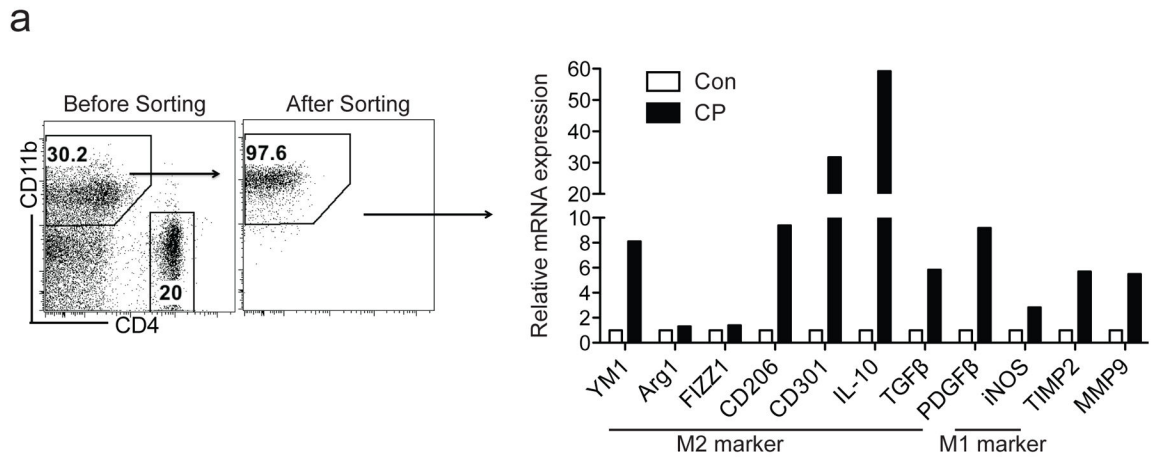
(grey line) and CCR2 expression are shown. $n=3$ for each group; mean± s.e.m., ns, not significant (unpaired two-tailed student's t-test).
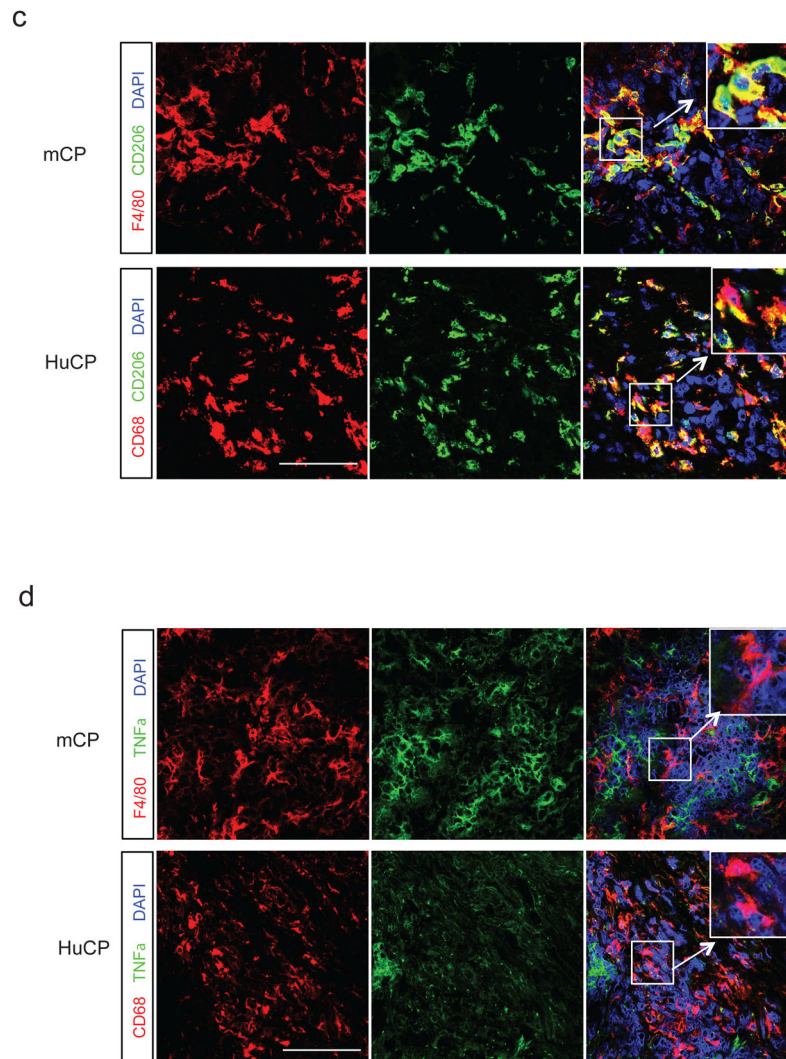
a

b

Gated on CD45.2+CD11b+F4/80+

c

d

Figure 2. Alternatively activated macrophages are dominant in mouse and human chronic pancreatitis

(a) Pancreatic leukocytes from control and CP mice were isolated and sorted for SSC-$A^{low}CD11b^+$ monocytes/macrophages (5 mice were pooled for each group). Markers of alternatively and classically activated macrophages were assessed by quantitative PCR. Expression of the genes was normalized to their relative expression in control mice. (b) Expression of alternative activation markers (CD206, IL-10, and IL-4Rα) and classical activation markers (MHCII and TNFα) in pancreatic macrophages of indicated mice were monitored by flow cytometry. MFI, mean fluorescence intensity; Data presented as mean± s.e.m. (unpaired two-tailed student's *t*-test). (c) Representative immunofluorescence images of mouse and human CP tissues co-stained with macrophage F4/80 (mouse) or CD68 (human), CD206 (M2 marker), and DAPI (nuclei). (d) Representative immunofluorescence images of mouse and human CP tissues, co-stained with F4/80 or CD68, TNFα (M1 marker) and DAPI. Scale bars, 50μm.
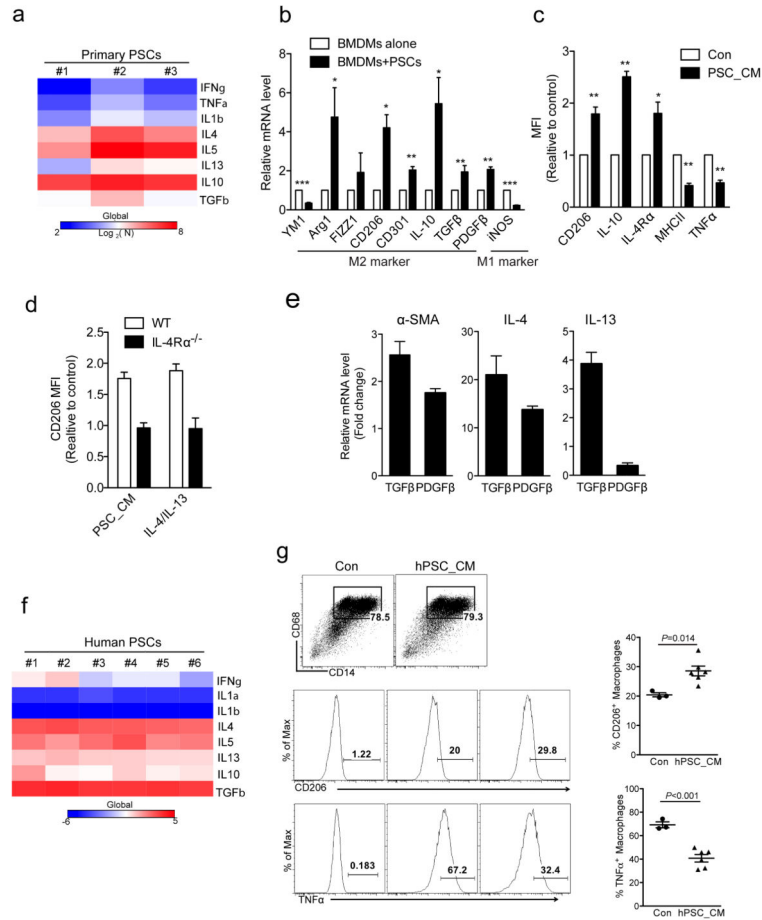
Figure 3. Mouse and human pancreatic stellate cells promote alternative macrophage polarization

(a) Culture supernatants from primary PSCs isolated from CP mice were collected and quantitated via Luminex analysis, cytokine data presented as a heat map. (b) Analysis of macrophage activation associated genes in BMDMs co-cultured in the presence or absence of PSCs for 2 days. Bar graphs represent BMDM gene expression in the presence of PSCs relative to absence of PSCs (BMDMs alone). (c) BMDMs were cultured with PSCs conditioned medium (PSC_CM) or control medium (Con) for 24h, and expression of CD206, IL-10, IL-4Rα, MHCII and TNFα determined by flow cytometry. (d) BMDMs from WT or $IL-4R\alpha^{-/-}$ mice were cultured with PSC_CM or IL-4/IL-13 (as a positive control for alternative macrophage polarization) for 24h, and the expression of CD206 was examined by flow cytometry. (e) Mouse PSCs were cultured with TGFβ (5ng/ml), PDGFβ (10ng/ml), or controls for 6h and indicated genes expression was determined by qPCR. Representative bar graphs show relative mRNA expression over control treatments. (f) Culture supernatants from primary PSCs derived from PDAC pieces (#2,4,6), normal margin of patients with PDAC (#3 and 5), and normal pancreas (#1; patient with non-pancreatic tumor requiring partial resection of the pancreas) were collected and quantitated via Luminex analysis. Data presented as a cytokine heat map expression. (f) Monocyte derived human macrophages were exposed to conditioned medium from human PSCs (hPSC_CM) for 24h. The
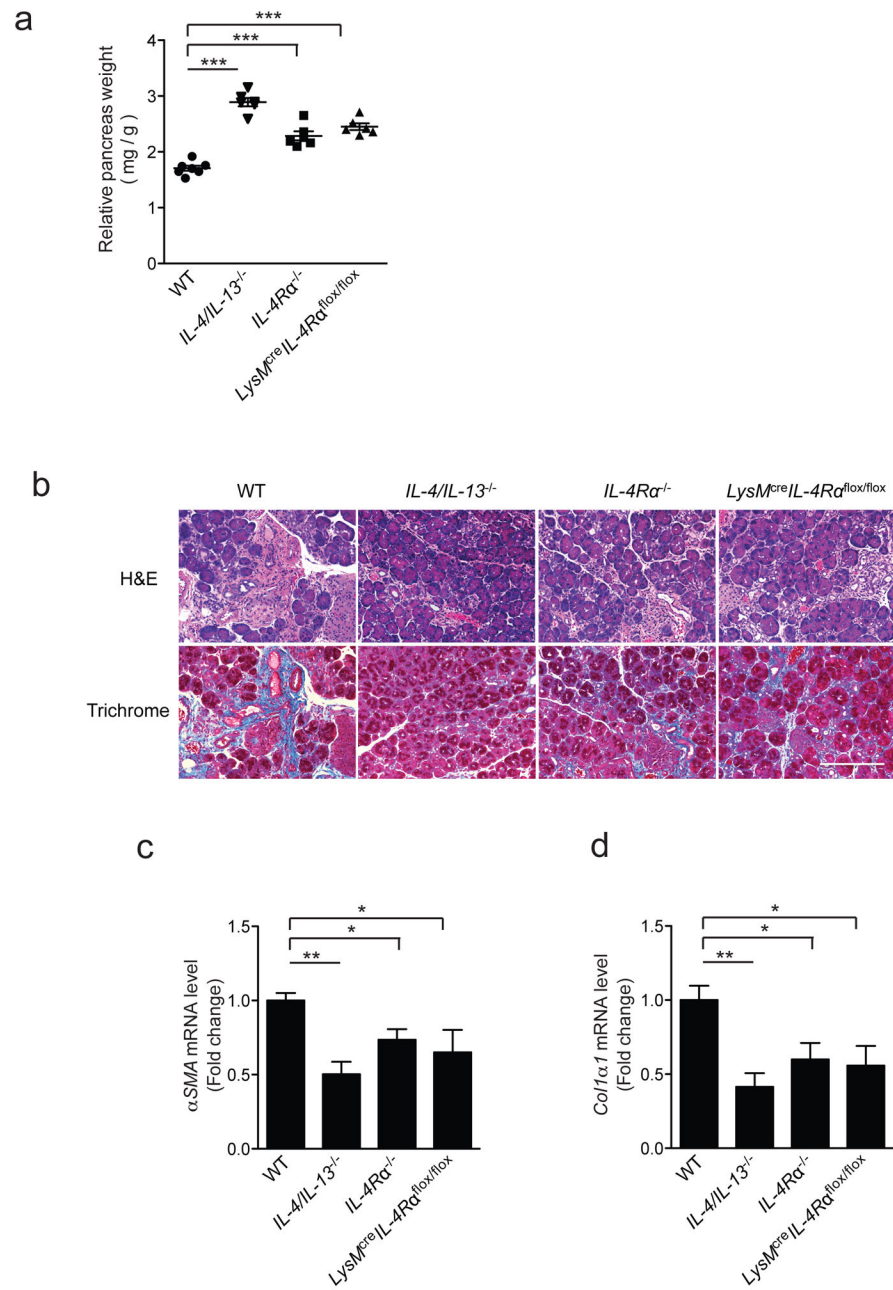
expression of CD206 and TNFα were examined by flow cytometry. *$P<0.05$, **$P<0.01$, ***$P<0.001$. Data presented as means ± s.e.m. (unpaired two-tailed student's *t*-test).
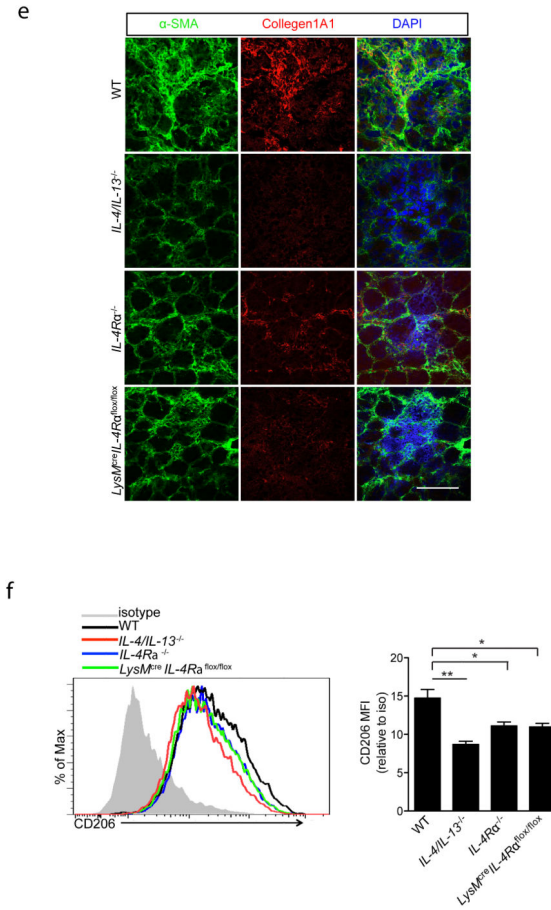
a

b

c

d

Figure 4. Global or Myeloid-specific IL-4Rα deficiency is protective against chronic pancreatitis
Wild-type (WT), *IL-4/IL-13*$^{-/-}$, *IL-4Rα*$^{-/-}$ and *LysM*^{cre}*IL-4Rα*$^{flox/flox}$ mice were subjected to caerulein-induced CP. (a) Relative pancreas weight (pancreas weight/body weight) are shown. n=6–7 per genotype, means ± s.e.m., ***P<0.001, *ns*, not significant, *P*<0.05 considered significant (one-way ANOVA, Tukey's post-hoc test). (b) Histologic feature of pancreas from the different genotype of mice are shown using H&E and Trichrome staining. Scale bar: 200μm. (c, d) Quantitative RT-PCR analysis of fibrosis-associated genes *αSMA* (α-SMA) and *Col1α1* (Collagen1A1) in the pancreas of indicated mice is shown. Means ± s.e.m. *P<0.05, **P<0.01, *ns*, not significant (one-way ANOVA, Tukey's post-hoc test). (e) Representative immunofluorescence images of pancreas from each genotype of mice are shown (co-stained with α-SMA, Collagen1A1 and DAPI). (f) Flow cytometry analysis of pancreatic macrophages (CD11b$^+$F4/80$^+$) CD206 expression (MFI) from the different genotype of mice is shown. Means ± s.e.m., *P<0.05, **P<0.01, *ns*, not significant (one-way ANOVA, Tukey's post-hoc test).
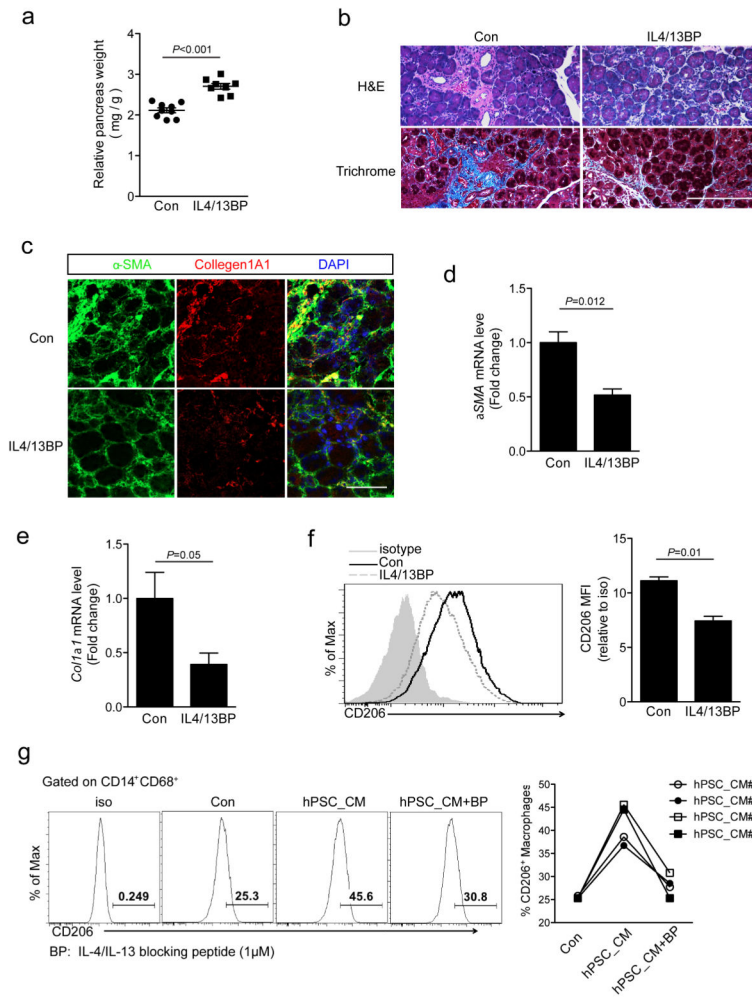
Figure 5. IL-4/IL-13 blocking peptide ameliorates established chronic pancreatitis

IL-4/IL-13 blocking peptide (IL4/13BP) was administrated to mice 2 weeks after starting CP induction (caerulein i.p., 50μg/mouse, 5 days per week) and mice euthanized as previous after 4 weeks of caerulein injections. (a) Relative pancreas weight from control (Con) and IL4/13BP treated mice are shown. n=8–9 per group, means ± s.e.m. (unpaired two-tailed student's *t*-test). (b) Representative of pancreas H&E and Trichrome staining. (c) Representative immunofluorescence images of pancreas from indicated mice (co-stained with α-SMA, Collagen1A1 and DAPI). (d, e) RT-PCR analysis of *α-SMA* and *Col1α1* (Collagen1A1) genes expression in the pancreas of indicated mice. Means ± s.e.m. (unpaired two-tailed student's *t*-test) (f) Flow cytometry analysis of CD206 expression (MFI) by pancreatic macrophages (CD11b^{+}F4/80^{+}) isolated from indicated mice. Means ± s.e.m. (unpaired two-tailed student's *t*-test). (g) Human macrophages as above were cultured with control medium (Con), conditioned medium from hPSCs (hPSC_CM) or hPSC_CM pre-treated with 1μM IL4/13BP(hPSC_CM+BP) for 24h. Expression of CD206 (% CD206^{+} macrophages) was analyzed by flow cytometry.
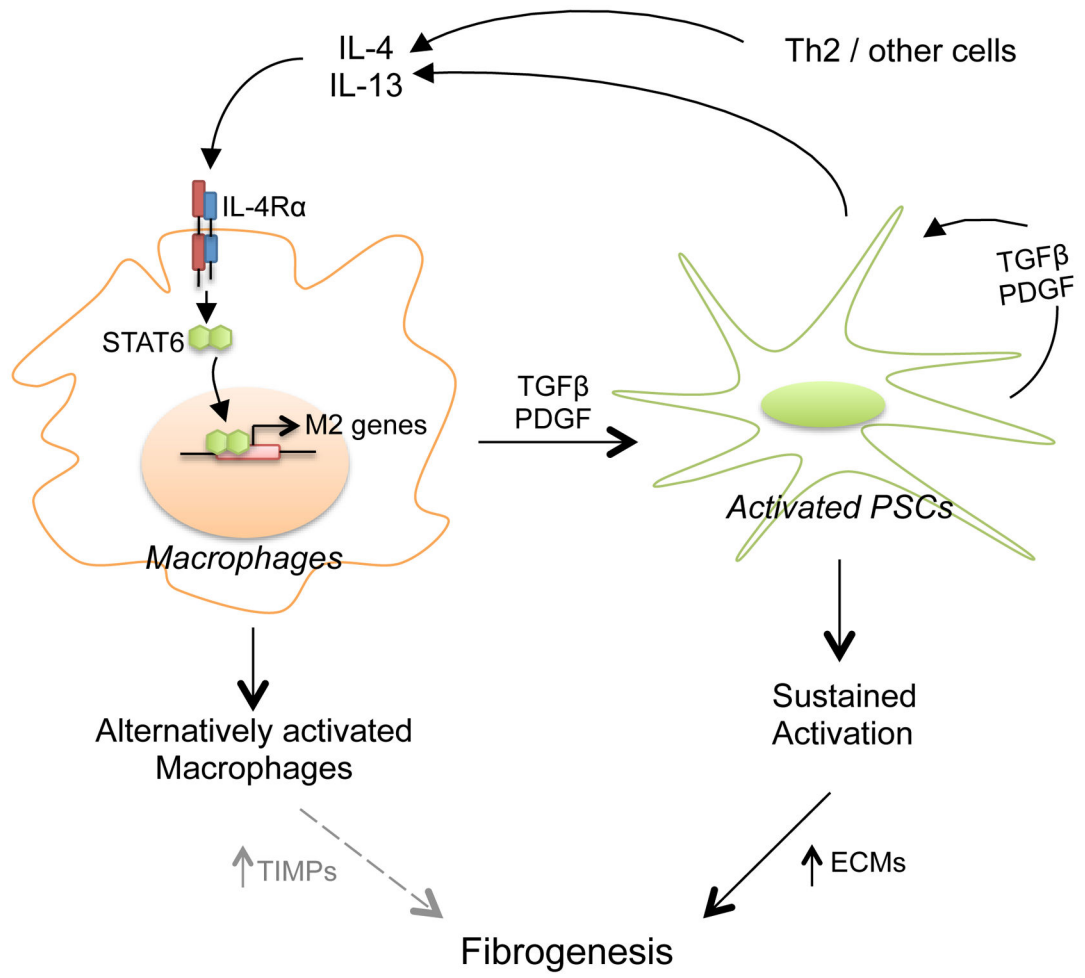
Figure 6.
Schematic representation of macrophage and pancreatic stellate cell interation in chronic pancreatitis