TNF-α-dependent Regulation of Acute Pancreatitis Severity by Ly-6C<sup>hi</sup> Monocytes in Mice<sup>§</sup>

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The roles of monocytes/macrophages and their mechanisms of action in the regulation of pancreatitis are poorly understood. To address these issues, we have employed genetically altered mouse strains that either express the human diphtheria toxin receptor (DTR) coupled to the CD11b promoter or have global deletion of TNF-α. Targeted, conditional depletion of monocytes/macrophages was achieved by administration of diphtheria toxin (DT) to CD11b-DTR mice. We show that in the absence of DT administration, pancreatitis is associated with an increase in pancreatic content of Ly-6C<sup>hi</sup> monocytes/macrophages but that this response is prevented by prior administration of DT to CD11b-DTR mice. DT administration also reduces pancreatic edema and acinar cell injury/necrosis in two dissimilar experimental models of acute pancreatitis (a secretagogue-induced model and a model elicited by retrograde pancreatic duct infusion of sodium taurocholate). In the secretagogue-elicited model, the DT-induced decrease in pancreatitis severity is reversed by adoptive transfer of purified Ly-6C<sup>hi</sup> monocytes harvested from non-DT-treated CD11b-DTR mice or by the transfer of purified Ly-6C<sup>hi</sup> monocytes harvested from TNF-α<sup>−/−</sup> donor mice, but it is not reversed by the transfer of Ly-6C<sup>hi</sup> monocytes harvested from TNF-α<sup>−/−</sup> donors. Our studies indicate that the Ly-6C<sup>hi</sup> monocyte subset regulates the severity of pancreatitis by promoting pancreatic edema and acinar cell injury/necrosis and that this phenomenon is dependent upon the expression of TNF-α by those cells. They suggest that therapies targeting Ly-6C<sup>hi</sup> monocytes and/or TNF-α expression by Ly-6C<sup>hi</sup> monocytes might prove beneficial in the prevention or treatment of acute pancreatitis.

The morbidity and mortality rates associated with acute pancreatitis are closely correlated with its morphological severity, but the processes that regulate pancreatitis severity are poorly understood. Previously reported studies have suggested that monocytes/macrophages might play an important role in regulating pancreatitis severity, but the methods employed in those studies to alter monocyte/macrophage number or function were relatively nonspecific and inefficient (1–8). As a result, definitive mechanistic studies exploring these important issues have not been possible, the monocyte subset responsible for regulating pancreatitis severity has not been identified, and the critical factors involved in that regulatory process are unknown.

In 2001, Saito et al. (9) showed that transgenic expression of the human diphtheria toxin receptor (DTR) in mice followed by administration of diphtheria toxin to those animals could be used to achieve targeted and conditional DT-induced cell injury. Human and mouse DTRs bind DT with widely differing affinities (the mouse with very low and the human with very high affinity) and, as a result, human cells are rapidly killed by exposure to even very low concentrations of the toxin, whereas mouse cells are highly resistant. In our studies, we have employed a transgenic mouse strain (CD11b-DTR mice) that expresses DTR coupled to the CD11b promoter. Coupling expression of DTR to the CD11b promoter leads to the selective expression of DTR by mouse cells belonging to the granulocyte-macrophage lineage. Theoretically, both granulocytes and monocytes/macrophages would be expected to be killed following exposure of these mice to diphtheria toxin but, perhaps because of their relatively low rate of protein synthesis, granulocytes from CD11b-DTR mice survive exposure to the toxin and only monocytes/macrophages are depleted when CD11b-DTR mice are given very small amounts of DT (25 ng/g, i.p.) (10, 11).

Increasing evidence from in vivo and in vitro studies points to key roles for monocytes/macrophages in regulating the injury response in diverse tissues. In injury studies of heart, kidney, and muscle in which there is organ repair, monocytes/macrophages have been shown to play roles in both augmenting the initial injury and subsequently promoting repair (10, 12, 13). To explain these diverse functions, it has been postulated that there are functional subsets of macrophages in the inflammatory tissue. Indeed, cultured macrophages or monocytes can be polarized by application of polarizing cytokines and known as M1 and M2 (14, 15). M1 macrophages evolve in response to interferon-γ and play a pro-inflammatory role, whereas M2 macrophages evolve in response to IL-4 or IL-13 and play a pro-reparative role. It was recently shown that in blood, there

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‡‡ The abbreviations used are: DTR, human diphtheria toxin receptor; DT, diphtheria toxin; BMC, bone marrow cell; PBMC, peripheral blood mononuclear cell.
are functionally distinct subsets of monocytes delineated by the marker Ly-6C (16). Ly-6C monocytes are released from bone marrow in response to distant organ injury and traffic directly to the injured site (16). These Ly-6C monocytes are believed to play important roles in initial responses to tissue injury, whereas Ly-6C monocytes may play a role in tissue repair. It has recently been suggested that the Ly-6C monocytes correspond to M1 and M2 macrophages, respectively (reviewed in Ref. 17), but that remains to be confirmed. In recent studies, we have demonstrated that the Ly-6C monocytes traffic to chronically injured kidney, where they differentiate into pro-inflammatory Ly-6C macrophages but also into Ly-6C pro-fibrotic macrophages (18). The role of Ly-6C or Ly-6C monocytes or macrophages in pancreatic injury remains unknown.

The studies described here have employed selective depletion of monocytes/macrophages achieved by administration of DT to CD11b-DTR mice and selective repletion of monocytes/macrophages in those mice achieved by adoptive transfer of monocytes/macrophages harvested from naïve donor mice to (a) identify the role played by monocytes/macrophages in regulating acute pancreatitis severity, (b) define the monocyte subset that is involved in this process, and (c) explore the possibility that monocytes/macrophages might regulate pancreatitis severity by a mechanism that involves generation of TNF-α. Our studies are the first to unequivocally show that monocytes belonging to the Ly-6C subset exert a profound pro-inflammatory effect in acute pancreatitis and the first to show that they do so by generating TNF-α.

EXPERIMENTAL PROCEDURES

Animals—FVB/N mice (20–25 g) were used in all experiments. Unless otherwise indicated, all of the animals used had been transgenically modified to express DTR coupled to the CD11b promoter as described previously (10). In selected experiments, FVB/N mice with global deletion of TNF-α expression (kindly provided by Dr. A. K. Verma, University of Wisconsin, Madison, WI) were also employed. All experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of Tufts Medical Center.

Induction of Pancreatitis—Secretagogue-induced pancreatitis was elicited by administering 12 hourly intraperitoneal injections of the cholecystokinin analog caerulein (50 μg/kg injection) (19). Bile acid-induced pancreatitis was elicited by retrogradely infusing sodium taurocholate (50 μl, 37 mmol) into the pancreatic duct of anesthetized mice as described previously by our group (20).

Quantitation of Pancreatitis Severity—The severity of pancreatitis in both models is maximal 12 h after the start of induction and persists at this level for at least an additional 12 h (21, 22). Accordingly, unless otherwise stated, mice were sacrificed to evaluate pancreatitis severity 24 h after the start of induction. At that time, blood was harvested for measurement of serum amylase activity, and pancreas samples were prepared for evaluation of pancreatic edema (defined as pancreatic water content above that observed in untreated control animals), pancreatic inflammation (defined as an increase in pancreatic myeloperoxidase content), and acinar cell injury/necrosis (defined as morphologic changes in hematoxylin/eosin-stained samples noted by an observer unaware of sample identity as described previously (23). Myeloperoxidase content was quantitated by ELISA (Hycult Biotechnology Uden, Netherlands). Acinar cell injury/necrosis was expressed as a percentage of total acinar cell mass. Preliminary experiments (24) showed that (a) DT administration to wild-type FVB/N mice does not alter the severity or course of pancreatitis; (b) pancreatitis is slightly more severe and consistent in female, as opposed to male, mice; and (c) the effects of DT administration are the same in both sexes. For these reasons, only female mice were used to quantitate pancreatitis severity, whereas mice of either sex were used as donors in adoptive transfer experiments.

Conditional, Targeted Depletion of Monocytes by Administration of DT—CD11b-DTR mice were given DT (25 ng/g i.p.) 16 h before the start of pancreatitis induction. Previously published studies (10, 11) have shown that this protocol leads to the transient but marked depletion of monocytes/macrophages but little or no change in the numbers of circulating granulocytes within 12 h of DT administration and that, in the absence of further DT administration, monocyte/macrophage repletion occurs over the subsequent 4–7 days.

Isolation of Cells for Flow Cytometry and Adoptive Transfer—To isolate cells from the pancreas, the gland was cut into fragments (<0.5 mm3) that were suspended in FACS buffer (Dulbecco’s phosphate-buffered saline with 10% calf serum and 5 mM EDTA). The fragments were triturated using a 1-ml pipette with an opening diameter of 0.5 mm, and the remaining large fragments were allowed to sediment for 15 s. The supernatant was harvested and reserved, whereas the sedimented large fragments, resuspended in FACS buffer, were again triturated. The resulting supernatant, combined with the reserved initial supernatant, was filtered through a Nitex filter (40 μm, Sefar America, Kansas City, MO) and centrifuged (250 × g, 4 °C, 25 min). The resulting pellet was then resuspended in FACS buffer for immediate use. In preliminary experiments, we found that this method of cell isolation yields single-cell suspensions that are of more consistent composition but the same numbers of leukocytes when compared with a method that involves collagenase digestion of the gland. For the isolation of bone marrow cells (BMC), bone marrow was harvested from femurs and tibias under sterile conditions and suspended in FACS buffer. The samples were triturated and resuspended in 5 ml of FACS buffer to obtain single cell suspensions. For isolation of peripheral blood mononuclear cells (PBMC), whole blood (0.5–0.8 ml) was used, and it was diluted with an equal volume of FACS buffer. Both the BMC and the PBMC samples were layered over a 2-ml cushion of Histopaque (1.083 g/ml, Sigma-Aldrich) and sedimented by centrifugation (30 min, 250 × g). The cells were harvested from the buffer-Histopaque interphase, washed in FACS buffer, resedimented, and used immediately.

Flow Cytometry—Isolated cells were preincubated with rat anti-mouse CD16/CD32 antibodies (BD Biosciences) for 10 min and then, at 4 °C, with specific antibodies conjugated with

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fluorescein (FITC), R-phycoerythrin, peridinin chlorophyll protein, and/or allophycocyanin. To determine cut-off values and true positive staining, cells were incubated with isotypic control antibodies conjugated with the same fluorophores (BD Biosciences). Immunostained cells were subjected to flow cytometry using a FACSCalibur (BD Biosciences).

Adoptive Transfer—Adoptive transfer was performed using either PBMC or BMC preparations. Unless otherwise stated, adoptive transfer studies involved infusing 300 μl of FACS buffer containing 10⁶ PBMCs or BMCs, obtained from single donor mice, into the lateral tail vein of each recipient mouse. In preliminary studies characterizing the CD45⁺ cells in those preparations, we found that they were predominantly composed of CD11b⁺ cells but that they also contained CD90.2⁺ T-cells (0.6 ± 0.3% of PBMCs, 13.5 ± 0.8% of BMCs), CD45R⁺ B-cells (14.5 ± 1.6% of PBMCs, 32.7 ± 3.0% of BMCs), and NK1.1⁺ natural killer cells (9.1 ± 1.4% of PBMCs, 15.3 ± 0.9% of BMCs). For this reason, in selected experiments, recipient FVB/N CD11b-DTR mice were adoptively transferred with monocytes that had been either depleted or enriched with monocytes of the Ly-6C⁺ subset and/or depletion of Ly-6G⁺ cells (i.e. granulocytes). Depletion and enrichment were achieved by either negative or positive selection cell sorting using anti-Ly-6C and/or anti-Ly-6G antibodies. Negative selec-

**FIGURE 1.** Effects of pancreatitis and administration of diphtheria toxin on Ly-6C⁺ monocytes/macrophages in the pancreas, bone marrow, and circulating blood of CD11b-DTR mice. CD11b-DTR mice were pretreated with either vehicle (black bars) or DT (white bars) and, 16 h later, they began receiving 12 hourly injections of either saline or caerulein (caer, 50 μg/kg). They were sacrificed 12 h after the start of pancreatitis induction. Monocytes/macrophages in the pancreas (A), bone marrow (B), and circulating blood (C) were isolated and subjected to flow cytometry as described under “Results.” In each panel, the four scattergrams report cytometry results obtained after gating to select only CD45⁺, CD11b⁺, and Ly6G⁺ cells. Circumscribed areas of interest include Ly-6Chi and 7/4⁺ cells, and the bar graph in each panel reports the quantitation of those cells. D, quantitation of Ly-6Chi monocytes in bone marrow and blood at varying times after administration of DT to CD11b-DTR mice in the absence of pancreatitis. Results shown reflect mean ± S.D. values from four mice in each group, and asterisks indicate p < 0.05 when DT- and non-DT-treated animals in each group were compared.
tion was achieved using biotinylated anti-Ly-6C (clone AL-21) antibodies and streptavidin-coated magnetic particles (IMag, BD Biosciences). Flow cytometric analysis of the resulting sample indicated that this method achieved more than 95% reduction of Ly-6C<sup>hi</sup> cells (from 27.5 to 1.3%) and more than 99% reduction of the CD11b<sup>hi</sup>/Ly-6C<sup>hi</sup> cells (from 11.06 to only 0.04%). Positive selection was achieved by FACS, and the resulting sample was, by definition, composed entirely of Ly-6C<sup>hi</sup> or Ly-6Chi monocytes.

Analysis of Data—Data are expressed as mean ± S.D. values. They report results obtained from at least three, and usually more, independently evaluated animals in each group. The significance of differences was evaluated using a two-tailed Student’s t test for paired values and one-way analysis of variance when multiple groups were being compared. Significant differences were defined as those with p < 0.05. To allow for pooling of data from multiple animals, data from flow cytometric studies quantitating BMCs were expressed as “percentage of CD45<sup>+</sup> cells.” Those from studies quantitating cells extracted from the pancreas were expressed as “number of cells per total pancreas.”

RESULTS

Effects of Pancreatitis on Ly-6C<sup>hi</sup> Monocyte/Macrophage Content of Pancreas, Bone Marrow, and Blood—Preliminary studies were performed using immunohistochemistry to quantitate monocytes/macrophages (i.e. F4/80<sup>+</sup> cells) in the pancreas during pancreatitis (see supplemental Fig. 1). Those studies indicated that monocytes/macrophages are increased within the pancreas within 24 h of pancreatitis induction. To further characterize this process and permit identification of monocyte/macrophage subsets, we chose to extract intra-pancreatic leukocytes from the pancreas and evaluate those cells by flow cytometry. As shown in Fig. 1, very few Ly-6C<sup>hi</sup> monocytes/macrophages are found in the untreated mouse pancreas, but the number of Ly-6C<sup>hi</sup> monocytes/macrophages found in
the pancreas is markedly increased 24 h after the start of pancreatitis induction. At the same time, after the start of pancreatitis induction, the number of bone marrow Ly-6C^hi monocytes is decreased, and the number of blood Ly-6C^hi monocytes is increased (Fig. 1). This pattern of Ly-6C^hi monocyte/macrophage distribution is compatible with the conclusion that those cells are mobilized from the bone marrow and traffic, via the circulating blood, to the pancreas during induction of pancreatitis.

**Effects of DT Administration on Ly-6C^hi Monocyte/Macrophage Content in Pancreas, Bone Marrow, and Blood**—Our preliminary immunohistochemical studies indicated that DT administration to CD11b-DTR mice prevents the pancreatitis-associated increase in monocytes/macrophages (i.e. F4/80^+ cells) in the pancreas (see supplemental Fig. 1). To further characterize the effects of DT administration, all of our subsequent studies employed flow cytometry. Those studies indicate that in the absence of pancreatitis, DT administration to CD11b-DTR mice leads to a reduction in the number of bone marrow and blood Ly-6C^hi monocytes (Fig. 1, B and C). As shown in Fig. 1D, this reduction in bone marrow and blood content of Ly-6C^hi monocytes occurs rapidly (i.e. <6 h) after DT administration and persists for at least 24 h. Importantly, DT administration also prevents the pancreatitis-associated rise in pancreatic Ly-6C^hi monocyte/macrophage content (Fig. 1A) and significantly reduces the pancreatitis-associated rise in blood Ly-6C^hi monocyte content (Fig. 1C), but it does not further reduce the pancreatitis-associated reduction in bone marrow Ly-6C^hi monocyte content (Fig. 1B). These observations are compatible with the conclusion that DT administration to CD11b-DTR mice prevents trafficking of Ly-6C^hi monocytes to the pancreas via the blood during induction of pancreatitis by depleting Ly-6C^hi monocytes in the bone marrow.

**Effect of DT Administration to CD11b-DTR Mice on Severity of Pancreatitis**—Pancreatitis was induced in CD11b-DTR mice either by giving repeated (12×) hourly i.p. doses of the cholecystokinin analog caerulein (50 μg/kg/injection) (19) (Fig. 2A) or by retrogradely infusing the bile salt sodium taurocholate into the pancreatic duct (20) (Fig. 2B). Both models are characterized by pancreatic edema, hyperamylasemia, pancreatic inflammation, and acinar cell injury/necrosis, which are maximal 12 h after the start of pancreatitis induction and remain constant in severity over the ensuing 12 h (21–23). When administered, DT was given 16 h prior to induction of pancreatitis. As shown in Fig. 2, A and B, DT administration to CD11b-DTR mice significantly reduces the extent of pancreatic edema and acinar cell injury/necrosis in both models, but it does not alter the extent of pancreatic inflammation or hyperamylasemia in either model. Furthermore, DT administration does not have any effect in the caerulein-induced trypsinogen activation (supplemental Fig. 2). As shown in Fig. 2C, pancreatitis-associated edema and acinar cell injury/necrosis are reduced when pancreatitis is induced 1 day after DT administration but not when pancreatitis is induced 4 or 7 days after DT administration. These observations are compatible with the conclusion that DT administration to CD11b-DTR mice reduces the severity of pancreatic injury (i.e. edema and injury/necrosis), but not hyperamylasemia or pancreatic inflammation, in these two dissimilar models of acute experimental pancreatitis.

**Effects of Adoptive Transfer on Pancreatitis Severity and Pancreatic Content of Ly-6C^hi Monocytes/Macrophages after DT Treatment**—As shown in Fig. 3, the reduction in both pancreatic edema and acinar cell injury/necrosis (i.e. measures of pancreatitis severity) that follows DT administration to CD11b-DTR mice is reversed when those mice are adoptively transferred with either PBMCs or BMCs isolated from naive donor mice. Dose-response studies indicate that complete restoration of pancreatitis severity is achieved by the adoptive transfer of 10^6 cells, whereas lesser numbers of transferred cells do not completely reverse the decrease in pancreatic edema and acinar cell injury/necrosis that follows quantitation as described under "Results." Results reflect mean ± S.D. values obtained from 3–9 mice per group. Asterisks denote p < 0.05 when compared with results obtained from mice not treated with DT. NS, not significant.
Ly-6Ch\textsuperscript{i} monocytes/macrophages were evaluated in experiments reported in Fig. 4. As already shown (Fig. 1A), pancreatitis induction in the absence of DT administration is associated with a marked rise in the pancreatic content of Ly-6Ch\textsuperscript{i} monocytes, and this rise is prevented by prior administration of DT. As shown in Fig. 4, adoptive transfer of either BMCs or PBMCs to DT-treated mice prevents this effect of DT administration, and the pancreatic content of Ly-6Ch\textsuperscript{i} monocytes in DT-treated mice after adoptive transfer is not different from that observed in non-DT-treated animals.

Taken together, the observations reported in Figs. 3 and 4 unequivocally indicate that the severity of pancreatitis is critically dependent upon the presence of the Ly-6Ch\textsuperscript{i} monocyte subset. Pancreatitis severity is reduced when those cells are depleted by DT treatment of CD11b-DTR mice, and the severity of pancreatitis in those DT-treated mice is restored by adoptive transfer of FACS-purified Ly-6Ch\textsuperscript{i}Ly6G\textsuperscript{−} monocytes harvested from TNF-\textalpha\textsuperscript{−/−} (wild-type) mice (Fig. 5A). The severity of pancreatitis is also reduced in FVB/N CD11b-DTR mice that have been pretreated with DT, and the severity of pancreatitis in those DT-treated mice is restored by adoptive transfer of FACS-purified Ly-6Ch\textsuperscript{i}Ly6G\textsuperscript{−} monocytes harvested from TNF-\textalpha\textsuperscript{−/−} mice (Fig. 5B). In agreement with these results, DT administration leads to reduced TNF-\textalpha\textsuperscript{levels during acute pancreatitis, but adoptive transfer restored the levels of TNF-\textalpha\textsuperscript{present in the pancreas (supplemental Fig. 4). In contrast, the severity of pancreatitis in the DT-pretreated mice is not restored when those mice are adoptively transferred with purified Ly-6Ch\textsuperscript{i}Ly6G\textsuperscript{−} monocytes harvested from TNF-\textalpha\textsuperscript{−/−} mice (Fig. 5B). These observations indicate that the ability of Ly-6Ch\textsuperscript{i} monocytes to regulate pancreatitis severity and, thus, to restore pancreatitis severity after DT treatment of CD11b-DTR mice is dependent upon the ability of those monocytes to express TNF-\textalpha. Furthermore, because FACS was used to positively select for Ly-6Ch\textsuperscript{i} cells and to eliminate Ly-6G\textsuperscript{+} cells in these studies, our findings exclude the possibility that contaminating Ly-6G\textsuperscript{+} granulocytes in the Ly-6Ch\textsuperscript{i} cell fraction could account for the observed restoration of pancreatitis severity.

DISCUSSION

At least two distinct monocyte subsets have been identified: a “resident monocyte” subset with the Ly-6Ch\textsuperscript{i}CX3CR1\textsuperscript{hi} phenotype and an “inflammatory monocyte” subset with the
Ly-6<sup>Ch</sup>iCX3CR1<sup>lo</sup> phenotype (24). Monocytes are believed to enter the circulation from the bone marrow as inflammatory monocytes and, in the absence of an inflammatory stimulus, these cells are thought to be transformed, within the circulating blood compartment, into resident monocytes. Auffray et al. (16) have suggested that resident monocytes patrol endothelial surfaces and react to inflammatory stimuli as local “first responders.” In the absence of inflammation, however, they enter and populate non-inflamed tissues as resident tissue macrophages. The onset of an inflammatory state, on the other hand, triggers the generation of factors that can accelerate mobilization of inflammatory monocytes from the bone marrow and promote their direct trafficking to inflamed tissues, where they function as inflammatory macrophages.

The course and outcome of an acute pancreatitis attack are directly related to the severity of that attack, so much so that virtually all of the morbidity and mortality of pancreatitis is limited to patients with a severe attack. Despite the obvious clinical importance of severity in clinical pancreatitis, the factors that regulate pancreatitis severity are poorly understood. Although previous studies have suggested that monocytes/macrophages might play an important role in regulating pancreatitis severity (1, 4, 6–8), the ability of those studies to address issues related to specific monocyte subsets and to address mechanistic issues was quite limited. To overcome those limitations, we have employed a highly efficient method of achieving targeted and conditional depletion of monocytes combined with modern techniques of flow cytometry, FACS, and adoptive transfer to address these issues. Our studies have focused on the Ly-6<sup>Ch</sup>i monocyte subset because of studies by others that have indicated that those cells play important roles in regulating the severity of various other inflammatory states (18, 25–30). Our studies have also employed genetically modified mice to explore the role of TNF-α in the regulation of pancreatitis severity because of earlier studies that have suggested that TNF-α might play a pro-injurious role in acute pancreatitis (4, 7, 31, 32).

The studies reported here indicate that Ly-6<sup>Ch</sup>i monocytes are mobilized from the bone marrow to the pancreas during acute pancreatitis but that this phenomenon can be prevented if Ly-6<sup>Ch</sup>i monocytes are depleted by administration of DT to CD11b-DTR mice (Fig. 1). Depletion of Ly-6<sup>Ch</sup>i monocytes by DT administration prevents the pancreatitis-associated rise in pancreatic Ly-6<sup>Ch</sup>i monocyte content, and this phenomenon is associated with a reduction in pancreatic injury (i.e., edema and acinar cell injury/necrosis) that occurs in two dissimilar experimental models of pancreatitis (Fig. 2). Our studies also show that both the rise in pancreatic Ly-6<sup>Ch</sup>i monocyte content and the severity of pancreatic injury during pancreatitis can be restored by adoptive transfer of Ly-6<sup>Ch</sup>i monocytes to DT-treated CD11b-DTR mice (Figs. 3 and 4). Taken together, our studies unequivocally indicate that Ly-6<sup>Ch</sup>i monocytes play a critical pro-injurious role in regulating the severity of pancreatic injury during acute pancreatitis.

To our knowledge, ours are the first studies that have explored the role of a distinct and well characterized monocyte
subset, i.e. inflammatory monocytes, in the regulation of pancreatitis severity. It is interesting to note that although pancreatic edema and acinar cell injury/necrosis during pancreatitis are markedly reduced by depletion of Ly-6C^hi monocytes, pancreatic inflammation during pancreatitis were not altered when Ly-6C^hi monocytes were depleted or when their rise within the pancreas during pancreatitis was prevented. These observations suggest that trypsinogen activation, hyperamylasemia, and pancreatic inflammation during pancreatitis are regulated by mechanisms that differ from those that regulate pancreatic edema and acinar cell injury/necrosis, i.e. the latter by mechanisms involving Ly-6C^hi monocytes/macrophages but the former by mechanisms that are not dependent on those cells. Previous studies reported by our group have also suggested that the various pancreatic manifestations of acute pancreatitis may be differentially regulated (22).

Studies reported by several other groups have indicated that TNF-α plays an important role in promoting pancreatic injury during pancreatitis by showing that pancreatitis severity can be reduced by global genetic deletion of TNF-α receptor (7), by administration of anti-TNF-α antibodies (33), or by pharmacological interventions with agents known to abrogate the release of TNF-α (34). Our own studies also support this conclusion by showing that pancreatitis severity is reduced by global genetic deletion of TNF-α (Fig. 5A).

The source of the TNF-α that plays this important role in regulating pancreatic severity during pancreatitis has remained uncertain despite previous studies that have explored this issue. Norman and co-workers (4, 8) showed that TNF-α expression in macrophages within the pancreas during pancreatitis is increased and that, under in vitro conditions, isolated macrophages generate TNF-α in response to exposure to activated pancreatic digestive enzymes. Those observations would suggest that the critical TNF-α that regulates pancreatitis severity is generated by macrophages. On the other hand, studies reported by Gukovskaya et al. (31) showed that pancreatic acinar cells can both generate and respond to TNF-α, suggesting that the critical TNF-α might be produced by acinar cells during evolution of acute pancreatitis. Our own findings, reported here, clearly favor the former of these two mechanisms. We found that pancreatic injury during pancreatitis is reduced by global genetic deletion of TNF-α and that the severity of pancreatitis in TNF-α−/− mice is restored when those animals are adoptively transferred with purified Ly-6C^hi monocytes harvested from TNF-α+/+ (but not TNF-α−/−) donors (Fig. 5A). In addition, we found that the reduction in pancreatic injury that follows DT treatment of CD11b-DTR mice with DT is reversed when those DT-treated animals are adoptively transferred with purified Ly-6C^hi monocytes harvested from TNF-α+/+ (but not TNF-α−/−) donors (Fig. 5B). Our studies do not challenge the possibility that pancreatic acinar cells (or other cells within the pancreas) may generate (and respond to) TNF-α during pancreatitis and that TNF-α contributes to pancreatic injury. They suggest that the TNF-α that regulates the extent of pancreatic injury (i.e. pancreatic edema and acinar cell injury/necrosis) during pancreatitis arises primarily from Ly-6C^hi inflammatory monocytes. It is tempting to speculate that those inflammatory monocytes generate TNF-α after first trafficking to the pancreas during pancreatitis, but our studies do not allow us to exclude the possibility that Ly-6C^hi monocytes might generate the critical TNF-α after trafficking to other, non-pancreatic, sites during pancreatitis.

We have shown that depletion of Ly-6C^hi monocytes and genetic deletion of TNF-α cause comparable reductions in the magnitude of pancreatic edema and acinar cell injury/necrosis during pancreatitis (edema by roughly 30–40%; injury/necrosis by roughly 50%) (Figs. 2 and 5). It is, perhaps, noteworthy that (a) the magnitude of these reductions in pancreatic injury brought about by either depletion of Ly-6C^hi monocytes or ablation of TNF-α is similar and (b) neither depletion of Ly-6C^hi monocytes nor ablation of TNF-α provides complete protection against injury during pancreatitis. Taken together, these observations lead us to speculate that in addition to TNF-α generated by Ly-6C^hi monocytes, there are additional mechanisms responsible for the regulation of pancreatic injury during pancreatitis. Identification of those mechanisms would represent fertile ground for future studies exploring the mechanisms responsible for regulating pancreatitis severity.

In summary, our studies indicate that pancreatic edema and acinar cell injury/necrosis, but not hyperamylasemia or pancreatic inflammation, during acute pancreatitis are regulated by the Ly-6C^hi monocyte subset and that the ability of those cells to promote pancreatic injury during pancreatitis is dependent upon their ability to express TNF-α. Our observations suggest that Ly-6C^hi monocytes and/or their expression of TNF-α may represent suitable targets for therapies designed to prevent or treat acute pancreatitis.

REFERENCES

1. Shifrin, A. L., Chirmule, N., Zhang, Y., and Raper, S. E. (2005) Surgery 137, 545–551
2. Fink, G. W., and Norman, J. G. (1996) J. Surg. Res. 63, 369–373
3. Gloor, B., Blinman, T. A., Rigberg, D. A., Todd, K. E., Lane, J. S., Hines, O. J., and Reber, H. A. (2000) Pancreas 21, 414–420
4. Norman, J. G., Fink, G. W., and Franz, M. G. (1995) Arch. Surg. 130, 966–970
5. Norman, J., Yang, J., Fink, G., Carter, G., Ku, G., Denham, W., and Livingston, D. (1997) J. Interferon Cytokine Res. 17, 113–118
6. Yang, J., Denham, W., Tracey, K. I., Wang, H., Kramer, A. A., Salhab, K. F., and Norman, J. (1998) Shock 10, 169–175
7. Denham, W., Yang, J., Fink, G., Denham, D., Carter, G., Ward, K., and Norman, J. (1997) Gastroenterology 113, 1741–1746
8. Iffayr, C., Mendez, C., Denham, W., Carter, G., and Norman, J. (2000) J. Gastrointest. Surg. 4, 370–378
9. Saito, M., Iwawaki, T., Taya, C., Yonekawa, H., Noda, M., Inui, Y., Meekada, E., Kimata, Y., Tsuru, A., and Kohno, K. (2001) Nat. Biotechnol. 19, 746–750
10. Duffield, J. S., Forbes, S. J., Constandinou, C. M., Clay, S., Partolima, M., Vuthooorri, S., Wu, S., Lang, R., and Iredale, J. P. (2005) J. Clin. Invest. 115, 56–65
11. Cailhier, J. F., Partolima, M., Vuthooorri, S., Wu, S., Ko, K., Watson, S., Savill, J., Hughes, I., and Lang, R. A. (2005) J. Immunol. 174, 2336–2342
12. Pulichino, A. M., Wang, I. M., Caron, A., Mortimer, J., Auger, A., Boie, Y., Elias, J. A., Kartono, A., Xu, L., Menetski, J., and Sayegh, C. E. (2008) Am. J. Respir. Cell Mol. Biol. 39, 324–336
13. Frangogiannis, N. G., Dewald, O., Xia, Y., Ren, G., Haudek, S., Leuecker, T., Kraemer, D., Taffet, G., Rollins, B. J., and Entman, M. L. (2007) Circulation 115, 584–592
14. Mosser, D. M., and Edwards, J. P. (2008) Nat. Rev. Immunol. 8, 958–969
15. Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A., and Locati, M.
16. Auffray, C., Fogg, D., Garfa, M., Elain, G., Join-Lambert, O., Kayal, S., Sarnacki, S., Cumano, A., Lauvau, G., and Geissmann, F. (2007) *Science* 317, 666–670
17. Geissmann, F., Manz, M. G., Jung, S., Sieweke, M. H., Merad, M., and Ley, K. (2010) *Science* 327, 656–661
18. Lin, S. L., Castaño, A. P., Nowlin, B. T., Lupher, M. L., Jr., and Duffield, J. S. (2009) *J. Immunol.* 183, 6733–6743
19. Lampel, M., and Kern, H. F. (1977) *Virchows Arch. A. Pathol. Anat. Histol.* 373, 97–117
20. Laukkarinen, J. M., Van Acker, G. J., Weiss, E. R., Steer, M. L., and Perides, G. (2007) *Gut* 56, 1590–1598
21. Frossard, J. L., Hadengue, A., Spahr, L., Morel, P., and Pastor, C. M. (2002) *Crit. Care Med.* 30, 1541–1546
22. Van Acker, G. J., Perides, G., Weiss, E. R., Das, S., Tsichlis, P. N., and Steer, M. L. (2007) *J. Biol. Chem.* 282, 22140–22149
23. Laukkarinen, J. M., Weiss, E. R., van Acker, G. J., Steer, M. L., and Perides, G. (2008) *J. Biol. Chem.* 283, 20703–20712
24. Geissmann, F., Jung, S., and Littman, D. R. (2003) *Immunity* 19, 71–82
25. Swirski, F. K., Weissleder, R., and Pittet, M. J. (2009) *Arterioscler. Thromb. Vasc. Biol.* 29, 1424–1432
26. King, I. L., Dickendesher, T. L., and Segal, B. M. (2009) *Blood* 113, 3190–3197
27. An, G., Wang, H., Tang, R., Yago, T., McDaniel, J. M., McGee, S., Huo, Y., and Xia, L. (2008) *Circulation* 117, 3227–3237
28. Arnold, L., Henry, A., Poron, F., Baba-Amer, Y., van Rooijen, N., Plonquet, A., Gherardi, R. K., and Chazaud, B. (2007) *J. Exp. Med.* 204, 1057–1069
29. Swirski, F. K., Libby, P., Aikawa, E., Alcaide, P., Luscinskas, F. W., Weissleder, R., and Pittet, M. J. (2007) *J. Clin. Invest.* 117, 195–205
30. Tacke, F., Alvarez, D., Kaplan, T. J., Jakubzick, C., Spanbroek, R., Lodra, J., Garin, A., Liu, J., Mack, M., van Rooijen, N., Lira, S. A., Habenicht, A. J., and Randolph, G. J. (2007) *J. Clin. Invest.* 117, 185–194
31. Gukovskaya, A. S., Gukovsky, I., Zaninovic, V., Song, M., Sandoval, D., Gukovsky, S., and Pandol, S. J. (1997) *J. Clin. Invest.* 100, 1853–1862
32. Bhatia, M., Brady, M., Shokuhi, S., Christmas, S., Neoptolemos, J. P., and Slavin, J. (2000) *J. Pathol.* 190, 117–125
33. Grewal, H. P., Mohey el Din, A., Gaber, L., Kotb, M., and Gaber, A. O. (1994) *Ann. Surg.* 167, 214–219
34. Hughes, C. B., el-Din, A. B., Kotb, M., Gaber, L. W., and Gaber, A. O. (1996) *Pancreas* 13, 22–28