The Receptor for Advanced Glycation End Products Promotes Pancreatic Carcinogenesis and Accumulation of Myeloid-Derived Suppressor Cells

Philip J. Vernon, Tara J. Loux, Nicole E. Schapiro, Rui Kang, Ravi Muthuswamy, Pawel Kalinski, Daolin Tang, Michael T. Lotze and Herbert J. Zeh III

*J Immunol* 2013; 190:1372-1379; Prepublished online 26 December 2012; doi: 10.4049/jimmunol.1201151

http://www.jimmunol.org/content/190/3/1372
Pancreatic ductal adenocarcinoma (PDA) is a largely lethal disease. The incidence and mortality rates in the United States are nearly identical, and patients who achieve a 5-y survival from time of diagnosis are rare (6%) (1, 2). Histopathologically, PDA progresses in a conserved fashion beginning with the emergence of malignant precursor lesions termed pancreatic intraepithelial neoplasia (PanIN) lesions, which progressively become more severely hyper- and dysplastic and finally culminate in frank invasive cancer (3, 4). Although immunotherapies such as cellular and peptide-based vaccines, monoclonal Abs against tumor signaling molecules, and recombinant cytokines have exhibited moderate successes in many tumor types, pancreatic cancer patients have yet to benefit substantially from these strategies (5). A significant obstacle to immunotherapeutic intervention is the immunosuppressive pancreatic tumor microenvironment (6). Immune suppression is facilitated by soluble factors such as TGF-β, IL-10, vascular endothelial growth factor, and Fas ligand derived from both the tumor and stromal compartments, the downregulation of NK cell-surface ligands such as MHC class I-related chain A/B in tumor tissue, and a marked increase in regulatory immune cell infiltrate consisting of regulatory T cells (Tregs), tumor-associated macrophages, and myeloid-derived suppressor cells (MDSCs) (6–8).

MDSCs are a recently identified heterogeneous population of hematopoietic cells of myeloid lineage (9, 10). Although normally present in relatively low quantities in the peripheral blood of healthy individuals, PDA patients exhibit high frequencies of these cells in the peripheral blood (11). Furthermore, MDSCs traffic to tumor tissue where they directly inhibit anti-tumor immune effector cells through a variety of mechanisms including depriving T cells of nutrients via arginase-I production and tryptophan and cysteine depletion, interfering with trafficking by inducing the downregulation of CD62L and α-selectin, and upregulating reactive nitrogen and oxygen species (NO synthase [NOS] and reactive oxygen species) (9, 10, 12, 13). MDSCs further support immune suppression in the tumor microenvironment by promoting reparative wound healing and angiogenesis and facilitating the recruitment of Tregs (14).

Identifying MDSCs based on cell surface marker expression in cancer patients has been difficult given the phenotypic heterogeneity. Several subsets have been characterized as CD11b+CD14+, CD11b+CD33+, and CD11b+Gr1−/80− that were not immunosuppressive in vitro. KCR mice also maintained a significantly less suppressive milieu evidenced by marked decreases in CCL22 in relation to CXCL10 and diminished serum levels of IL-6. The Journal of Immunology, 2013, 190: 1372–1379.

Department of Surgery, Hillman Cancer Center, University of Pittsburgh Cancer Institute, Pittsburgh, PA 15219

Received for publication April 2012. Accepted for publication November 19, 2012.

This work was supported by National Institutes of Health Grants PO1 CA 101944-04 (to M.T.L.), 5T32 CA 082084-13 (to Olivera J. Finn, University of Pittsburgh), and 3P30CA047904-22S1 (to Nancy Davidson, University of Pittsburgh Cancer Institute).

Address correspondence and reprint requests to Dr. Michael T. Lotze or Dr. Herbert J. Zeh, University of Pittsburgh, W1540 Biomedical Science Tower, G.27A Hillman Cancer Center, Pittsburgh, PA 15621 (M.T.L.) or Department of Surgery, University of Pittsburgh School of Medicine, Pittsburgh, PA 15219 (H.J.Z.). E-mail addresses: lotzemt@upmc.edu (M.T.L.) or zehh@upmc.edu (H.J.Z.).

The online version of this article contains supplemental material.

Abbreviations used in this article: HMGB1, high-mobility group box 1; iNOS, inducible NO synthase; KC, Pdx1-Cre;KrasG12D/+; KCR, Pdx1-Cre;KrasG12D/+; RAGE, receptor for advanced glycation end products; MDSC, myeloid-derived suppressor cell; NOS, NO synthase; PanIN, pancreatic intraepithelial neoplasia; PDA, pancreatic ductal adenocarcinoma; RAGE, receptor for advanced glycation end products; Treg, regulatory T cell; WT, wild-type.

Copyright © 2013 by The American Association of Immunologists, Inc. 0002-2767/13/$16.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1201151

The Receptor for Advanced Glycation End Products Promotes Pancreatic Carcinogenesis and Accumulation of Myeloid-Derived Suppressor Cells

Philip J. Vernon, Tara J. Loux, Nicole E. Schapiro, Rui Kang, Ravi Muthuswamy, Pawel Kalinski, Daolin Tang, Michael T. Lotze, and Herbert J. Zeh, III
KrasG12D/+ transgenic mice were obtained from the Mouse Models of Human Cancers Consortium/National Cancer Institute Mouse Repository. To determine if RAGE plays a role in the accumulation of MDSCs during pancreatic carcinogenesis, we have backcrossed RAGE-null mice into the KC strain. The resultant Pdx1-Cre:KrasG12D/+ mice are termed KC. We demonstrate in this study that the targeted ablation of RAGE in the emerging KC mice is associated with a significant decrease in intratumoral and splenic MDSC frequency and a slower rate of tumor growth.

Materials and Methods

Mouse strains

Wild-type (WT) C57BL/6 mice were purchased from Taconic Farms (Hudson, NY). RAGE knockout (Rage-/-) mice (SVEV129 × C57BL/6) were obtained from Dr. Angelika Bierhaus (27) as a kind gift. Pdx-1-Cre and KrasG12D/+ transgenic mice were obtained from the Mouse Models of Human Cancers Consortium/National Cancer Institute Mouse Repository. The genotypes Pdx1-Cre:KrasG12D/+ (termed KC) and Rage-/- mice were crossed to generate KC mice. Genomic and recombination screens were done by PCR and analysis of GFP expression (data not shown).

Flow Cytometry

Flow cytometric analysis was performed on the C6 flow cytometer (Accuri Cytometers, Ann Arbor, MI) instrument provided by the University of Pittsburgh Cancer Institute Flow and Imaging Cytometry core facility and analyzed using FlowJo software (Tree Star, Ashland, OR). Murine spleens were homogenized through a 70-μm nylon filter (BD Biosciences, San Jose, CA) and washed with PBS. RBCs were lysed with RBC Lysing Buffer (Sigma-Aldrich, St. Louis, MO) according to the manufacturer’s instructions. Single cell suspensions were derived from the pancreas by mechanical separation and collagenase digestion (Sigma-Aldrich). The resulting single cell suspensions were then stained with the following fluorescently labeled Abs: CD11b, CD11c, F4/80, Gr1, IL-4Rα, and corresponding isotype controls (all from BD Biosciences). In cases, the cells were then permeabilized with 0.2% Triton-X and stained with Abs to intracellular inducible NOS (iNOS)/NOS type II and Arginase-I (BD Biosciences). In cases, the cells were then labeled with Alexa Fluor 488–conjugated anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL). After permeabilization and staining, the cells were washed and flow cytometric analysis was performed on the C6 flow cytometer (Accuri Cytometers, Ann Arbor, MI) instrument provided by the University of Pittsburgh Cancer Institute Flow and Imaging Cytometry core facility and analyzed using FlowJo software (Tree Star, Ashland, OR).

Histology

Harvested pancreatic tissue was formalin fixed (Sigma-Aldrich), stained with H&E, and mounted onto glass slides by the University of Pittsburgh Department of Pathology. Images were visualized and captured at a magnification of 10x by Nikon Eclipse E800 fluorescent microscope under bright field settings (Nikon, Melville, NY).

Coculture assays

A total of 4 × 10^5 bulk WT spleenocytes were obtained as previously mentioned from C57BL/6 mice. To activate effector splenocytes, cells were incubated with T-activator CD3/CD28 Dynabeads (Dynal AS, Oslo, Norway) at a ratio of 1:1 according to the manufacturer’s instructions. For coculture conditions, splenocytes from KC and KCR mice were incubated with oGrl or oCD11b microbeads and magnetically separated using MACS LS separation columns (all from Miltenyi Biotec, Leiden, The Netherlands) according to the manufacturer’s instructions and purity assayed by flow cytometry. A total of 4 × 10^5 (termed low) or 8 × 10^5 (termed high) Gr1+ or CD11b+ cells were incubated with bulk WT splenocytes for 96 h. The resulting supernatants were harvested for analysis.

In vitro differentiation of MDSCs from murine bone marrow

MDSCs were generated in vitro using the following method. Fresh bone marrow flushed from the femurs and tibias of mice was resuspended in media supplemented with G-CSF (100 ng/ml) and GM-CSF (250 U/ml) and cultured for 3 d. On day 4, 80 ng/ml of IL-13 was added to the culture for an additional 24 h (28). All cytokines were from eBioscience (San Diego, CA). Harvested cells routinely were 50% CD11b+Gr1+ and were magnetically sorted as previously mentioned for functional analysis.

ELISA analysis

Supernatants from coculture experiments or serum from murine peripheral blood (obtained via direct cardiac puncture) was assayed for detectable levels of IL-2, IFN-γ, and IL-6 by ELISA Ready-SET-Go! Kits (eBioscience) according to the manufacturer’s instructions.

Statistical analysis

Data are expressed as means ± SEM of at least two independent experiments performed in triplicate. Statistical analysis was performed using a two-tailed Student t test. The p values <0.05 were assigned statistical significance.

Results

Targeted ablation of RAGE attenuates development of high-grade PanIN lesions in KC mice

To determine if RAGE contributes to pancreatic carcinogenesis, 20- and 35-wk-old WT, RAGE-null, KC, and KCR mice were sacrificed and their pancreata harvested and stained with H&E for visualization of PanIN lesion formation. At 20 wk, both the KC and KCR strains show the occurrence of atypical ducts. However, glandular and islet cells remain largely intact, and both strains exhibit a paucity of fibrotic and inflammatory tissue (Fig. 1A). At 35 wk of age, KC mice exhibit extensive atypical ductal morphologic within pancreatic tissue, the emergence of low- and high-grade PanIN lesions, and severe fibrogenesis indicative of emergent pancreatic carcinogenesis. In KC mice in which RAGE has been chromosomally ablated (KCR mice), the frequency of hyperplastic and dysplastic ductal epithelium is substantially decreased and the integrity of glands and β cells in the islets is largely retained (Fig. 1B). Furthermore, we have previously reported that these mice have a significantly enhanced survival rate (22). These findings suggest that RAGE directly or indirectly contributes to the pathogenesis of early pancreatic carcinogenesis.

RAGE promotes splenic MDSC accumulation during Kras-mediated pancreatic carcinogenesis

MDSCs accumulate within the spleens of KC mice as they progress toward ductal adenocarcinoma (17). To determine if the integrity of the RAGE signaling pathway is an important factor in this observation, WT, RAGE-null, KC, and KCR mice were sacrificed at 35 wk of age at which point animals expressing mutant Kras display evidence of pancreatic carcinogenesis manifested by the presence of low and high grade PanIN lesions within the pancreatic ducts (Fig. 1B). Spleens from these mice were processed into single-cell suspensions for flow cytometric analysis of im-
mune cell frequencies. Both WT and RAGE-null animals exhibit comparable basal levels of CD11b^Gr1^ MDSCs ranging from 3 to 4% (Fig. 2A) in the absence of apparent pathology. As has previously documented, Kras-driven pancreatic carcinogenesis resulted in a significant increase in splenic MDSC frequency to 20–35% (17). Interestingly, KCR mice fail to accumulate MDSCs with a relative paucity of CD11b^Gr1^ cells (Fig. 2A, 2B). This is significantly less than the KC strain (p = 0.0225) at comparable times. However other cells of myeloid-lineage such as myeloid dendritic cells (DCs) identified by the coexpression of CD11b and CD11c are elevated in both KC and KCR mice to similar extents, suggesting that the reduction in splenic MDSCs in the KCR strain is restricted to CD11b^Gr1^ cells.

To observe how the kinetics of MDSC accumulation are affected by the presence or absence of RAGE, mice from all strains were sacrificed at 20, 24, 26, and 35 wk of age and their spleenocytes assayed for MDSC frequency. As precursor lesions develop in the pancreas, the KC mice begin to accumulate high levels of MDSCs at 26 wk of age. This is significantly elevated when compared with KCR mice (p = 0.049) at the same time point. This becomes further accentuated by 35 wk of age (p = 0.023) (Fig. 2C). The absence of RAGE is associated with a significant lack of MDSC accumulation over time in these animals. These findings suggest that RAGE is critical for in vivo MDSC accumulation during pancreatic carcinogenesis. Of note, a study by Connolly et al. using the Kras-driven pancreatic neoplasia model with the additional p48 mutation found that splenic CD11b^Gr1^ expansion was not evident until 9 mo of age and that MDSCs isolated from non-tumor tissue did not exhibit a suppressive phenotype (29).

**RAGE is dispensable for the differentiation and suppressor activity of MDSC**

The failure of MDSCs to accumulate within the spleens of KCR mice could be due to a requirement for RAGE to develop MDSCs within the bone marrow compartment. To determine if RAGE is critical for either the differentiation of MDSCs from hematopoietic progenitor cells or their immune inhibitory activity, both KC and KCR mice were sacrificed at 20 wk of age and their spleenocytes harvested for MDSC phenotyping and functional analysis. At 20 wk of age, both KC and KCR mice exhibit small but measurable CD11b^Gr1^ MDSC populations as assessed by flow cytometry. These cells are present at levels comparable to WT and RAGE^−/−^ animals (2 to 3%) (Fig. 3A). These cells were stained for cell surface expression of the MDSC marker, IL-4Ra and the mature macrophage marker, F4/80 which is absent or expressed at low levels on MDSCs, which typically display an immature myeloid phenotype (18, 25). These cells were also stained for intracellular expression of iNOS/NOS type II and arginase-I, which facilitate the suppressive function of MDSCs on effector and Th cells (30, 31). CD11b^Gr1^ cells from both the KC and KCR strains expressed IL-4Ra, iNOS/NOS type II, and arginase-I (Fig. 3B). Interestingly, MDSCs derived from the spleens of KCR mice exhibited higher expression of both IL-4Ra and arginase-I than those derived from KC mice (Fig. 3A, 3B). As expected, MDSCs from neither strain expressed F4/80 (Fig. 3B).

To confirm that these cells that phenotypically resembled MDSCs were capable of suppressing T cell activity and to determine if RAGE was required for suppressor function, they were isolated by MACS based on their positivity of Gr1 and evaluated in an in vitro coculture assay. Bulk WT splenocytes were stimulated with anti-CD3/anti-CD28 activator beads alone or in a coculture with the isolated MDSCs from both the KC and KCR strains at a ratio of 1:1 (MDSC [low]) or 1:2 (MDSC [high]). After 96 h of coculture, the resultant supernatants were harvested and analyzed by ELISA for levels of the proliferative T cell survival cytokine, IL-2 and the Th1-polarizing cytokine IFN-γ as indicators of relative T cell proliferation and activity. As expected, stimulation with anti-CD3/anti-CD28 activator beads induced significant secretion of both IL-2 (p < 0.01) and IFN-γ (p < 0.05) from WT splenocytes (Fig. 3C). When cocultured with MDSCs at either ratio isolated from KC or KCR mice, the secretion of these cytokines was inhibited and remained at non-stimulated levels. Importantly, there was no significant difference between MDSCs derived from either strain in terms of their suppressive capacity (Fig. 3C). To further validate the lack of a requirement for RAGE in MDSC hematopoiesis, MDSCs were generated in vitro from both non-tumor bearing WT and RAGE^−/−^ mice. CD11b^Gr1^ cells were generated to equal extents (50%) in marrow from both strains (Supplemental Fig. 1A). Furthermore, these cells were sorted and evaluated for suppressor function and previously described. Cells from both strains were equally suppressive (Supplemental Fig. 1B). These findings suggest that RAGE is not critical for the development of MDSCs in vivo or their ability to exert suppressor function in vitro. Moreover, the failure of KCR mice to accumulate splenic MDSCs during pancreatic carcinogenesis is not due to an impaired ability to generate functioning MDSCs.

**Splenic myeloid cells derived from KCR mice exhibit a mature phenotype and do not exhibit suppressor function**

A lack of MDSC accumulation during pancreatic carcinogenesis in KCR mice could be due to a failure in MDSC recruitment. Alternatively, the absence of RAGE could result in the induction of the differentiation of MDSCs from an immature (Gr1^+^) to a mature (Gr1^−^) phenotype or a failure to prevent such a differentiation.
from occurring. To investigate the latter hypothesis, 35-wk-old WT, RAGE-null, KC, and KCR mice were sacked and their splenocytes analyzed for expression of CD11b and Gr1 and myeloid DC markers CD11b and CD11c from 35-wk-old WT (n = 2), RAGE-null (n = 2), KC (n = 8), and KCR (n = 5) mice. Note the boxes denoted MDSCs are distinct from the boxes denoting DC populations. (B) Scatter plots depicting the frequencies of splenic MDSCs identified by expression of CD11b, Gr1, and IL-4Ra and frequencies of splenic DCs of myeloid origin (CD11b^CD11c^) in mice from (A). (C) WT, RAGE-null, KC, and KCR mice were sacked at 20 (n = 2/strain), 24 (n = 2/strain), 26 (n = 2/strain), and 35 wk (n = 2, WT and RAGE-null; n = 8, KC; n = 5, KCR). The kinetics of splenic MDSC accumulation with the progression of pancreatic neoplasia (KC and KCR mice) is depicted.

FIGURE 2. RAGE ablation is associated with limited splenic MDSC accumulation during Ras-mediated pancreatic carcinogenesis. (A) Representative flow cytometry diagrams of splenocytes stained for coexpression of MDSC markers CD11b and Gr1 and myeloid DC markers CD11b and CD11c from 35-wk-old WT (n = 2), RAGE-null (n = 2), KC (n = 8), and KCR (n = 5) mice. Note the boxes denoted MDSCs are distinct from the boxes denoting DC populations. (B) Scatter plots depicting the frequencies of splenic MDSCs identified by expression of CD11b, Gr1, and IL-4Ra and frequencies of splenic DCs of myeloid origin (CD11b^CD11c^) in mice from (A). (C) WT, RAGE-null, KC, and KCR mice were sacked at 20 (n = 2/strain), 24 (n = 2/strain), 26 (n = 2/strain), and 35 wk (n = 2, WT and RAGE-null; n = 8, KC; n = 5, KCR). The kinetics of splenic MDSC accumulation with the progression of pancreatic neoplasia (KC and KCR mice) is depicted.
that lack suppressor activity and could contribute to both the paucity of classical MDSCs and the inhibited occurrence of malignant pancreatic lesions.

RAGE promotes a regulatory milieu within the emerging pancreatic tumor microenvironment

The presence or absence of host regulatory immune cell infiltrate in the pancreatic tumor microenvironment is in part regulated by factors present both within tumor tissue and circulating in the peripheral blood (33, 34). To investigate whether there were differences in the tumor microenvironments of both KC and KCR mice that might contribute to both the failure to accumulate MDSCs in the absence of RAGE and the influx of mature, nonsuppressive myeloid cells in KCR mice (Fig. 4), pancreatic tissue was harvested from 35-wk-old WT, RAGE-null, KC, and KCR mice and analyzed for mRNA levels of chemokines known to attract either immunoregulatory (CCL22) or immunostimulatory (CXCL10) immune cells by quantitative PCR (35, 36). To determine what the

FIGURE 3. RAGE is dispensable for the differentiation and suppressor activity of MDSC. (A) Representative flow cytometry diagrams of splenocytes from 20-wk-old KC (n = 2) and KCR (n = 2) mice stained for cosurface expression of MDSC markers CD11b and Gr1. (A and B) Flow diagrams of splenocytes gated for CD11b and Gr1 positivity and stained for surface expression of IL-4Rα and F4/80, and intracellular expression of iNOS/NOS type II and arginase-I from KC and KCR mice. (A) Graphical representation of the mean fluorescence intensity (MFI) of each marker (± SD). (B) MFI values illustrated on diagrams. Demarcations based on isotype controls (data not shown). (C) MDSCs from mice in (A) and (B) were magnetically separated and cocultured with bulk WT splenocytes stimulated with anti-CD3/anti-CD28 activator beads at ratios of 1:1 (MDSC [low]) and 2:1 (MDSC [hi]). Graphs depict IL-2 and IFN-γ levels detected in the resultant supernatants by ELISA (± SEM; *p < 0.05, **p < 0.01).

FIGURE 4. Splenic myeloid cells derived from KCR mice exhibit a mature phenotype and do not exhibit suppressor function. (A) Representative flow cytometry diagrams of splenocytes from 35-wk-old KC (n = 6) and KCR (n = 5) mice stained for the cell-surface expression of the myeloid-lineage marker CD11b and the mature macrophage marker F4/80. (B) Scatter plot depicting the frequencies of splenic myeloid cells exhibiting immature phenotypes identified by the expression of CD11b and the lack of F4/80 expression in mice from (A). (C) CD11b+ splenocytes from mice in (A) and (B) were magnetically separated and cocultured with bulk WT splenocytes stimulated with anti-CD3/anti-CD28 activator beads at ratios of 1:1 (CD11b+ [low]) and 2:1 (CD11b+ [hi]). Graphs depict IL-2 and IFN-γ levels detected in the resultant supernatants by ELISA (± SEM, *p < 0.05).
relative levels of mRNA of these chemokines are in relation to each other, the ratio of CCL22 to CXCL10 was obtained. As expected, KC mice demonstrate a significant increase in CCL22: CXCL10, indicating a pronounced regulatory chemokine milieu in the pancreata of these mice. In the absence of RAGE, this ratio is significantly diminished (p = 0.037), suggested that the emerging tumor microenvironments of these animals is substantially less likely to recruit regulatory infiltrate (Fig. 5A).

We have recently reported that ablation of RAGE in the emerging tumor microenvironment attenuates STAT3-IL-6 pathway activation in the KC model (22). IL-6, a cytokine often associated with epithelial-derived cancers and general tissue damage, has also been implicated in MDSC recruitment and persistence through a STAT3-dependent signaling pathway (37–39). To compare serum IL-6 levels between the KC and KCR strains, peripheral blood was obtained via direct cardiac puncture of 35 wk old mice and assayed for IL-6 concentration by ELISA. Although serum IL-6 levels were relatively low in WT and RAGE-null mice, KC mice exhibited a significant increase in the concentration of the cytokine in their peripheral blood. This was not the case for the KCR strain, which exhibited significantly diminished levels of IL-6 (p = 0.038), which were comparable with both WT and RAGE-null mice (Fig. 5B, left panel). Further illustrating the role of IL-6 in the recruitment and survival of MDSCs, mice were divided into two groups, regardless of strain: those with <100 pg/ml serum IL-6 and those with >100 pg/ml IL-6. The MDSC frequencies in the spleens from mice from these two groups were then assayed by flow cytometry. Mice with >100 pg/ml of serum IL-6 were significantly more likely to have greater splenic MDSC accumulation (p = 0.022) (Fig. 5B, right panel). Single-cell suspensions derived from the pancreata of 35 wk old mice from both strains were examined for MDSC frequencies as well as expression of F4/80 (Fig. 5C). A failure to accumulate MDSCs in response to endogenous carcinogenesis was observed in the KCR strain. This was in contrast with the KC strain, which showed a demonstrable expansion of these cells as has been previously reported (17). Interestingly, nearly uniform F4/80 expression was observed in CD11b+ cells in the KCR strain, but not in KC mice, where the vast majority of CD11b+ cells remained negative for F4/80. Taken together, these findings suggest that RAGE has a critical role in the promotion of an immunoregulatory milieu within the emergent tumor microenvironment during pancreatic carcinogenesis. The lack of the regulatory chemokine CCL22 in relation to the immunostimulatory chemokine CXCL10, coupled with the normal serum IL-6 levels in response to the diminished incidence and timing of Kras-mediated pancreatic neoplasia could contribute to inhibited MDSC accumulation in the absence of RAGE expression.

**Discussion**

In this study, we demonstrate that RAGE plays a critical role in promoting pancreatic neoplasia and the resultant immunosuppres-
sive tumor microenvironment. When RAGE is ablated in mice that spontaneously form PanIN lesions due to mutant Kras expression, the emergence and progression of these malignant lesions are substantially attenuated. Concurrently, the accumulation of splenic MDSCs that occurs over time in KC mice is limited in the absence of RAGE. Our findings suggest that the integrity of RAGE is critical for pancreatic carcinogenesis and responding MDSC accumulation. Importantly, RAGE is not required for the development of MDSCs from myelopoietic progenitor cells or their specific inhibitory activity, as MDSCs are found in both RAGE-null and KCR mouse strains and are phenotypically and functionally intact. Interestingly, during pancreatic neoplasia in the absence of RAGE, a substantial majority of myeloid cells (CD11b+) exhibit a more mature phenotype manifested by the expression of the mature macrophage marker F4/80 and a loss of Gr1 expression (18, 32). When isolated and evaluated in an in vitro suppression assay, these cells had notably lost their inhibitory activity. This suggested that RAGE is a factor in either myeloid cell plasticity or determining which myeloid subsets respond to emergent pancreatic carcinogenesis. However, alternative explanations for the maturation of myeloid cells toward a more mature F4/80+ phenotype exist. Inability to recruit more suppressive cells types or apoptosis of other myeloid subsets could explain this observation. Additionally, the chemokine milieu in the pancreatic tumor microenvironment was markedly less regulatory in KCR mice illustrated by the relative abundance of CXCL10 in relation to CCL22. The levels of IL-6, which have been implicated in the STAT3-dependent recruitment and retention of MDSCs (38), were also significantly decreased in the KCR strain, and these levels directly correlated with measured MDSC frequency within the emergent pancreatic tumor microenvironment.

The proinflammatory proteins S100A8/A9 induce MDSCs by interacting with RAGE and other glycoproteins on the surface of MDSCs and promote their migration via NF-kB–dependent signaling (18). In addition, RAGE overexpressed within the tumor and stromal compartments is concurrently ligated by S100A8/A9 synthesized by MDSCs, which induces a regulatory chemokine tumor gene profile and serves as a positive-feedback loop for the further recruitment of MDSCs (18, 25). Our findings suggest that RAGE ablation in the emerging tumor microenvironment leads to diminished signaling through the IL-6/STAT3 pathway. It is likely that the failure to accumulate MDSCs in KCR mice is due to a combined effect of RAGE deletion in both the tumor and bone marrow compartments in the global RAGE knockout strain. Future studies will examine targeted ablation in each exclusive compartment to further elucidate these mechanisms. Other cells of myeloid lineage (particularly dendritic cells and macrophages) have been shown by our group and others to secrete another RAGE ligand, HMGB1, in response to various maturational stimuli, therefore HMGB1 secretion by MDSCs represents an additional mechanism for MDSC recruitment to be explored (40, 41).

Our observations suggest an important mechanism driving an immunosuppressive environment during early pancreatic carcinogenesis. However, several questions remain concerning the relationship between cancer–related inflammation and the accrual of MDSCs. First, it is not clear if the pancreatic carcinogenesis that promotes the accumulation of MDSCs is an epiphenomenon or if the recruitment of MDSCs contributes to carcinogenesis by limiting effective immune editing. Secondly, the failure of various immunotherapies, particularly within the context of pancreatic cancer, is attributed to the presence of host regulatory immune cells like MDSCs and Tregs (6). It is not clear if targeting MDSC function in these settings may render these therapies more effective. Our results demonstrating a critical role for RAGE in MDSC accumulation in the context of pancreatic carcinogenesis identifies a novel pathway that can be explored to address these questions possibly by assessing established strategies such as IL-2 administration in this model (42). Lastly, given the role of RAGE in promoting autophagy within tumor cells and evidence of immune cell-mediated autophagy (particularly T and NK cells), it would be interesting to explore whether interactions with MDSCs modulate these observations in KCR mice (21).

Acknowledgments
We dedicate this manuscript to the memory of Angelika Bierhaus, Ph.D., our friend and benefactor. She was a pioneering RAGE biologist, generous and kind in her dealings with colleagues, who provided us the mice with which we completed this work and who sadly died on April 15, 2012 after a long and courageous battle with cancer. Angelika had a great love of life, and she was generous, kind, and warm hearted. She was always full of plans for scientific endeavors, even when her disease began to take its toll. She remained steadfast that she would not be defeated, but despite recently celebrating her 50th birthday, she was aware her remaining time was short. She dedicated herself to research, but despite her incredible strength, she was not able to overcome the disease that tragically took her life. We also thank the University of Pittsburgh Cancer Institute Flow and Imaging Cytometry core facility as well as the University of Pittsburgh Cancer Institute Animal Facility.

Disclosures
The authors have no financial conflicts of interest.

References


