Tim-3 promotes tumor-promoting M2 macrophage polarization by binding to STAT1 and suppressing the STAT1-miR-155 signaling axis

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ABSTRACT

T cell Ig mucin-3 (Tim-3), an immune checkpoint inhibitor, shows therapeutic potential. However, the molecular mechanism by which Tim-3 regulates immune responses remains to be determined. In particular, very little is known about how Tim-3 works in innate immune cells. Here, we demonstrated that Tim-3 is involved in the development of tumor-promoting M2 macrophages in colon cancer. Manipulation of the Tim-3 pathway significantly affected the polarization status of intestinal macrophages and the progression of colon cancer. The Tim-3 signaling pathway in macrophages was explored using microarray, co-immunoprecipitation, gene mutation, and high-content analysis. For the first time, we demonstrated that Tim-3 polarizes macrophages by directly binding to STAT1 via residue Y256 and Y263 in its intracellular tail and inhibiting the STAT1-miR-155-SOCS1 signaling axis. We also identified a new signaling adaptor of Tim-3 in macrophages, and, by modulating the Tim-3 pathway, demonstrated the feasibility of altering macrophage polarization as a potential tool for treating this kind of disease.

Abbreviations: Arg-1, arginase 1; CAC, colitis-associated cancer; LPS, lipopolysaccharide; SOCS1, suppressor of cytokine signaling 1; STAT1, signal transducer and activator of transcription 1; Tim-3, T cell Ig mucin-3

Introduction

The gut is constantly exposed to commensal microbes, ingested antigens, and potential pathogens, and regulation of intestinal tolerance is therefore the main task of the immune system of the gut mucosa. Intestinal macrophages play a crucial role in monitoring the environment and in controlling the cellular and molecular networks of the immune system to maintain tissue homeostasis.\textsuperscript{1} Dysregulated macrophage function has emerged as an important aspect of the progression of intestine disorders, including inflammatory bowel diseases\textsuperscript{2,3} and colon cancer,\textsuperscript{4,5} raising the possibility of a therapeutic approach involving the targeting of intestinal macrophages. However, the mechanisms involved in regulating macrophage function in the physiopathology of intestinal diseases are not well understood. Macrophages are categorized as classically activated (M1, pro-inflammatory), which promote antitumor inflammation, or alternatively activated (M2, anti-inflammatory), which support tumor growth.\textsuperscript{6,7} Modulation of macrophage polarization is a feasible strategy to treat intestinal diseases, such as colon cancer.\textsuperscript{8,9}

The signals controlling intestinal macrophage polarization are not well defined and their identification will be of great value in understanding how these cells control the local microenvironment and affect intestinal homeostasis. The polarization state of macrophages is regulated, at least in part, by local concentrations of cytokines and chemokines, as well as by interactions of macrophages with normal and degraded components of the extracellular matrix.\textsuperscript{10} As for the intracellular mechanisms, some transcriptional factors have been shown to play a role in regulating macrophage differentiation and polarization. In particular, signal transducer and activator of transcription (STAT) family members, such as STAT1, and suppressor of cytokine signaling 1 (SOCS1) are reported to be important regulators of macrophage polarization.\textsuperscript{11,12} In addition, dynamically modulated expression of specific microRNAs (miRs), such as miR-155 and miR-146, regulates the physiological differentiation of macrophages.\textsuperscript{13-15} The targeting of molecular pathways regulating macrophage polarization therefore holds great promise for immunotherapy.

T cell Ig mucin-3 (Tim-3) is an immune regulator first identified on activated T effector cells, including Th1, Th17, and Tc1 cells. By inducing T cell tolerance or exhaustion, Tim-3 negatively regulates the responses of these T effector cells.\textsuperscript{16-18} Recent reports by ourselves\textsuperscript{19} and others have demonstrated that Tim-3 is also a negative regulator of innate immune cells, such as macrophages and dendritic cells. As Tim-3 is involved in the physiopathology of many immune disorders, including
tumors and chronic virus infections, it is now considered as an immunotherapeutic target. However, the molecular mechanism by which Tim-3 maintains immune homeostasis remains largely unclear, and, in particular, very little is known about the mechanism of Tim-3 signaling in innate immune cells.

We previously found that Tim-3 is involved in regulating macrophage polarization, but the precise molecular mechanism and the therapeutic potential remain to be determined. In the present study, we found that Tim-3 may promote tumorigenesis of colon cancer by promoting M2 macrophage polarization. We also identified STAT1 as a new signaling adaptor of Tim-3, through which Tim-3 determines the phenotype and function of macrophages. A better understanding of the molecular mechanisms by which Tim-3 controls intestinal macrophage homeostasis will help in establishing new therapeutic strategies to treat colon cancer.

**Results**

**Dysregulated upregulation of Tim-3 is associated with biased M2 macrophage polarization in colon cancer**

To test the possible roles of Tim-3 in the physiopathology of colon cancer, Tim-3 mRNA expression was examined in colon tissues from colon cancer patients and from C57BL/6 mice with a mouse model of colitis-associated cancer (CAC). The results showed that, compared to controls, levels were significantly higher in both the patients (Fig. 1A) and the CAC mice (Fig. 1B). We then examined Tim-3 protein expression and function on intestinal macrophages from CAC mice. As shown in Fig. 1C, immunohistochemical analysis showed colocalization of Tim-3 with F4/80, a macrophage marker, in intestinal tissues from CAC mice. Interestingly, we found that upregulation of Tim-3 expression was associated with biased M2 macrophage polarization in CAC, as shown by increased expression of dectin-1 (M2) and decreased expression of CD16/32 (M1) on macrophages (Fig. 1D) and increased levels of Arg-1 and IL-10 mRNAs (M2) and decreased levels of NOS2 and IL-12 mRNAs (M1) in macrophages (Fig. 1E).

**Tim-3 overexpression polarizes macrophages toward the M2 type and promotes tumor growth in mice with CAC**

To further test the role of Tim-3 in the physiopathology of colon cancer and in intestinal macrophage polarization, we generated Tim-3 transgenic (TG) mice by overexpressing Tim-3 under the control of the CMV promoter; incorporation of Tim-3 DNA in the recipient was confirmed by PCR and Tim-3 expression on macrophages and T cells was confirmed by flow cytometry (Fig. S1), then established our CAC model in both wild-type (WT) and the Tim-3 TG mice. We found that Tim-3-TG mice with CAC showed a significantly decreased survival rate (Fig. 2A) and increased tumor growth (Fig. 2B) and tumor burden and tumor numbers (Fig. 2C). When the effects of Tim-3 overexpression on macrophage polarization in CAC mice were examined, intestinal macrophages isolated from Tim-3-TG mice showed significantly increased Tim-3 protein expression compared to those from WT mice and enhanced M2 polarization, as demonstrated by increased dectin-1 and decreased CD16/32 expression on macrophages (Fig. 2D) and increased Arg-1 and IL-10 mRNA levels and decreased NOS2 and IL-12 mRNA levels in macrophages (Fig. 2E). These...
data suggest that Tim-3 may promote the growth of colon cancer by polarizing macrophages toward the M2 type.

**Blockade of the Tim-3 pathway alters macrophage polarization and inhibits colon cancer growth**

We then examined whether blockade of the Tim-3 pathway altered progression of colon cancer by altering macrophage polarization. To test this, a different colon cancer model was established by transplanting CT-26 cells into Balb/C mice with or without blockade of the Tim-3 pathway by injection of a soluble form of Tim-3 (sTim-3-Ig). Interestingly, as shown in Figs. 3A and B, sTim-3-Ig inhibited the growth of the transplanted tumor in a dose-dependent manner and led to an enhanced pro-inflammatory response, as shown by increased levels of mRNAs for IFNγ, TNF-α, IL-6, and IL-1β in the total tumor-infiltrating cells (Fig. 3C). When tumor-infiltrating macrophages were isolated and analyzed, they showed significantly increased surface expression of Tim-3 compared to intestinal macrophages from naive mice (Fig. 3D). Blockade of the Tim-3 pathway in mice with CAC by injection of stTim-3 resulted in inhibition of the M2 macrophage response, as shown by decreased dectin-1 expression and increased CD16/32 expression on macrophages (Fig. 3E) and decreased levels of Arg-1 and IL-10 mRNAs and increased levels of IL-12 and NOS2 mRNAs in macrophages (Fig. 3F).}

**Tim-3 signaling controls macrophage polarization in vitro**

The effects of Tim-3 signaling on macrophage polarization were then examined in vitro, using control RAW264.7 cells or RAW264.7 macrophages stably overexpressing Tim-3 or with Tim-3 knockdown or peritoneal macrophages from WT or Tim-3-TG mice; Fig. 4A shows Tim-3 mRNA levels in these different cell populations. Tim-3-overexpressing RAW264.7 macrophages (Fig. 4B) and peritoneal macrophages from Tim-3-TG mice (Fig. 4C) both showed an M2 macrophage phenotype, as evidenced by decreased levels of NOS2 mRNA and increased levels of Arg-1 mRNA compared to controls (left panels), and showed increased IL-4-induced IL-10 secretion (center panels) and a decrease in the LPS-induced increase in IL-12 mRNA levels (right panels), while RAW264.7 macrophages silenced for Tim-3 expression showed M1 polarization, as demonstrated by increased NOS2 mRNA levels, decreased Arg-1 mRNA levels, decreased IL-4-induced IL-10 secretion, and an increase in the LPS-induced increase in IL-12 mRNA levels (Fig. 4D). In addition, in vitro blockade of Tim-3 signaling in control RAW264.7 macrophages using stTim-3-Ig resulted in a biased M1 phenotype, as shown by increased NOS2 mRNA levels and decreased Arg-1 mRNA levels (Fig. 4F). Finally, peritoneal macrophages from untreated WT C57BL/6 mice incubated with IL-4 expressed higher levels of Tim-3 and Dectin-1 than those incubated with LPS and IFNγ (Fig. 4F). These in vitro data provide additional evidence that Tim-3 polarizes macrophages toward the M2 type.

**Tim-3 inhibits miR-155 expression in vitro and in CAC mice in vivo**

To explore the molecular mechanisms by which Tim-3 polarizes macrophages, a miRs array assay was used to compare miRNA profiles in control and Tim-3 knockdown RAW264.7 cells, and more than 100 differentially expressed miRNAs, including miR-155, miR-146, and miR-27a, were identified (Fig. 5A) (series entry, GSE76839, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE76839). We then
selected miR-155 for further studies, as it has been reported to be involved in regulation of macrophage polarization. Real-time PCR data confirmed upregulation of miR-155 expression in RAW264.7 cells either stably knocked-down for Tim-3 or treated with sTim-3-Ig (Fig. 5B) and significant downregulation in RAW264.7 cells stably transfected with Tim-3 or in peritoneal macrophages isolated from Tim-3-TG mice (Fig. 5C). These data show that Tim-3 signaling inhibits miR-155 expression both in vitro and in vivo. We then examined whether blockade of miR-155 activity affected Tim-3-induced polarization of RAW264.7 cells by transfecting the cells with a miR-155 inhibitor or control inhibitor, then treating them with Ig or sTim-3-Ig. As shown in Fig. 5D, incubation with sTim-3-Ig resulted in a significant increase in NOS2 mRNA levels and a significant decrease in Arg-1 mRNA levels in control inhibitor-treated cells, whereas the effect on NOS2 mRNA levels, but not Arg-1 mRNA levels, was significantly lower in miR-155 inhibitor-treated cells. These data show that miR-155 plays critical roles in Tim-3 signaling-mediated macrophage polarization.

We then examined miR-155 expression in the intestinal tissues of colon cancer patients by real-time PCR and found that it was lower in tumor tissues than in normal biopsy tissue controls (Fig. S2A). Decreased miR-155 expression was also seen in intestinal macrophages from Tim-3-TG mice with CAC compared to those from WT mice with CAC (Fig. S2B). These data show causative relations between increased Tim-3 expression, decreased miR-155 levels, and progression of colon cancer.

**STAT1 acts as a key signaling adaptor linking Tim-3 signaling to miR-155 during macrophage polarization**

To find the structural basis of the link between Tim-3, miR155, and the downstream signaling cascade, we generated several Tim-3 mutants, Y218A, Y256A, Y263A, Y272A, and Y256A/Y263A, in which tyrosine mutant has previously been shown to prevent phosphorylation.23,24 As shown in Fig. S3, in response to Gal-9 stimulation, HET293T cells transfected with control Tim-3 or the Y218A or Y272A mutant showed significantly decreased miR-155 expression compared to cells transfected with vector, while cells transfected with Y256A, Y263A, or the double mutant Y256A/Y263A did not. These mutagenesis studies show that Y256 and Y263 are required for the inhibitory effects of Tim-3 on miR-155 expression.

Next, we investigated the signaling adaptor linking Tim-3 to miR-155. We focused on STAT1 as (1) it is an upstream transcriptional factor involved in miR-155 regulation25; (2) it is involved in regulating macrophage polarization12, 26; and (3) it contains an SH2 domain, while the intracellular tail of Tim-3 contains a highly conserved tyrosine- containing src homology 2 (SH2)-binding motif, and tyrosine residues within this motif can be constitutively phosphorylated.23,24

We first examined phosphorylation of Y256 and Y263 in HEK293T cells transfected with Tim-3 or the Y256A/Y263A Tim-3 mutant or co-transfected with Gal-9 and found that co-transfection with Gal-9 led to enhanced tyrosine phosphorylation on Tim-3 compared to transfection of Tim-3 alone; while mutant of Y256A/Y263A resulted in decreased phosphorylation (Fig. S4).
Figure 4. Tim-3 signaling regulates macrophage polarization in vitro. (A) Expression of Tim-3 in Tim-3- or vector control transfected RAW264.7 cells (left panel), in peritoneal macrophages isolated from wild type (WT) or Tim-3-TG mice (central panel), and in Tim-3 knockdown RAW264.7 cells (right panel) examined by real-time PCR. (B–D) RAW264.7 macrophages stably overexpressing Tim-3 (B), peritoneal macrophages from Tim-3-TG mice (C), Tim-3 knockdown (KD) RAW264.7 macrophages (D), or controls were tested for levels of NOS2 and Arg-1 mRNAs by real-time PCR (left panels) or were incubated with or without IL-4 (50 ng/mL) or LPS (100 ng/mL) for 12 h, then IL-10 secretion (center panels) or IL-12 mRNA levels (right panels) were measured by ELISA or real-time PCR, respectively. (E) RAW264.7 cells were incubated with 10 μg/mL of sTim-3-Ig or Ig (Con) for 6 h, then NOS2 (left panel) or Arg-1 (right panel) mRNA levels were measured by real-time PCR. (F) Peritoneal macrophages from control C57BL/6 mice were incubated for 24 h with either LPS (100 ng/mL) plus IFN-γ (100 ng/mL) or with IL-4 (50 ng/mL), then expression of Tim-3 (left panel) or Dectin-1 (right panel) was measured by flow cytometry. The data shown are representative of those obtained in three independent experiments. *p < 0.05, **p < 0.01. Isotype represents the isotype control for the antibodies used.
We then tested the hypothesis that Tim-3 might interact with STAT1 and regulate a downstream signaling cascade by examining STAT1 expression and phosphorylation in control RAW264.7 cells and Tim-3 knockdown RAW264.7 cells in the presence or absence of LPS stimulation or in control RAW264.7 cells in the presence or absence of sTim-3-Ig and (2) in peritoneal macrophages from WT or Tim-3-TG C57BL/6 mice in the presence or absence of LPS stimulation. Our results showed that knockdown of Tim-3 (Fig. 6A) enhanced the LPS-induced phosphorylation of STAT1 (Fig. 6B) and blockade of Tim-3 signaling increased STAT1 phosphorylation (Fig. 6C), while TG expression of Tim-3 (Fig. 6D) significantly inhibited LPS-induced phosphorylation of STAT1 (Fig. 6E). To examine whether there was a direct interaction between Tim-3 and STAT1, and, if so, whether residues Y256 and Y263 of Tim-3 played a role, HEK293T cells were co-transfected with STAT1 and either Tim-3 or the Y256A/Y263A double mutant, then the cell lysate was immunoprecipitated (IP) with an anti-Tim-3 antibody that binds to extracellular Tim-3, and the precipitate immunoblotted (IB) with anti-STAT1 antibody and the results showed that STAT1 bound to Tim-3, but not Y256A/Y263A-Tim-3 (Fig. 6F, left panel). Furthermore, when HEK293T cells were transfected with either Tim-3 or Y256A/Y263A-Tim-3, but not Y256A/Y263A-Tim-3, bound to endogenous STAT1 (Fig. 6F, central panel). Finally, we examined whether galectin-9 (Gal-9), the Tim-3 ligand, played a role in the interaction between Tim-3 and STAT1 by transecting HEK293T cells with Tim-3, then incubating the cells in the presence or absence of recombinant Gal-9 and the results showed that ligation of Tim-3 by Gal-9 increased the binding between Tim-3- and STAT1 (Fig. 6F, right panel). Together, these data show that (1) Tim-3 inhibits STAT1 phosphorylation; (2) Tim-3 binds to STAT1 and this requires residues Y256 and Y263; and (3) Tim-3 engagement by Gal-9 enhances the binding between Tim-3 and STAT1.

STAT1 acts upstream of miR-155 and promotes miR-155 transcription and it as therefore reasonable to hypothesize that STAT1 acts as an adaptor mediating the Tim-3-induced inhibition of miR-155 expression. To test this, we examined the effect of sTim-3-Ig blockade of Tim-3 signaling in cultured RAW264.7 cells on miR-155 expression in the presence or absence of the STAT1 inhibitor fludarabine. As shown in Fig. 6G, the increase in miR-155 expression caused by blockade of the Tim-3 pathway was partially inhibited by fludarabine. These data show that STAT1 is involved in the Tim-3-induced decrease in miR-155 expression.

To examine whether binding of STAT1 to Tim-3 mediates the downstream signaling cascade of Tim-3, U2OS cells transfected with a STAT1-EGFP vector were used. As shown in Fig. S5, STAT1 was found throughout the cytoplasm in untreated cells (top row) and translocated into the nucleus in response to IFNγ stimulation (center row) and this translocation was inhibited by a JAK inhibitor (bottom row).

To examine whether Tim-3 affected the nuclear translocation of STAT1, control Tim-3 or Y256A/Y263A-Tim-3 fused to a red marker protein was transfected into STAT1-transfected U2OS cells and the results, shown in Fig. 6H, showed that, following IFNγ stimulation, STAT1 nuclear translocation in Tim-3-transfected cells was considerably lower than that in vector-transfected and Y256A/Y263A-Tim-3 transfected cells. These data show a suppressive effect of Tim-3 on the nuclear translocation of STAT1.

Finally, we examined STAT1 phosphorylation in intestinal macrophages from mice with CAC or controls, and found that it was lower in cells from CAC mice (Fig. S6A). In addition, STAT1 phosphorylation was lower in Tim-3-TG mice with...
CAC than that in WT mice with CAC (Fig. S6B). These data show that STAT1 is a critical transcriptional factor affecting the status of macrophages in colon cancer in vivo and that this process can be regulated by Tim-3.

SOCS1 is involved in a signaling cascade downstream of Tim-3-STAT1-miR-155 and mediates macrophage polarization

Finally, the signaling cascade downstream of the Tim-3-STAT1-miR-155 axis was investigated. We focused on SOCS1, as it is a critical mediator promoting the production of the immunoregulatory cytokine IL-10 and enhancing M2 macrophage polarization and is reported to be a target of miR-155.28 Silencing of Tim-3 in Tim-3 knockdown RAW264.7 cells and blockade of Tim-3 signaling in RAW264.7 cells both decreased SOCS1 protein expression (Figs. 7A and B) and decreased SOCS1 mRNA expression (Figs. 7C and D), while TG expression of Tim-3 in mice increased SOCS1 mRNA levels (Fig. 7E). Furthermore, in response to Gal-9 stimulation, SOCS1 mRNA levels were higher in HET293T cells transfected with Tim-3 than in cells transfected with Y256A/Y263A-Tim-3 (Fig. 7F), showing that residues 256 and 263 of Tim-3 are involved in Tim-3-enhanced SOCS1 expression.

To determine whether there was a direct interaction between miR-155 and SOCS1 in macrophages, RAW264.7 cells were transfected with miR-155 inhibitor or control inhibitor and the effects on SOCS1 mRNA levels examined by real-time PCR. As shown in Fig. 7G, transfection with miR-155 inhibitor increased SOCS1 mRNA levels, suggesting that Tim-3 increases SOCS1 expression by inhibiting miR-155 expression. In addition, Tim-3-overexpressing RAW264.7 cells incubated with the STAT1 inhibitor showed significantly increased SOCS1 mRNA levels (Fig. 7H), suggesting that STAT1 is involved in the Tim-3-mediated upregulation of SOCS1. We also noted a negative regulatory effect of SOCS1 on STAT1 following Tim-3 signaling, as the enhanced STAT1 phosphorylation seen in RAW264.7 cells after Tim-3 blockade (Fig. 6C) was inhibited by overexpression of SOCS1 (data not shown). Finally, in peritoneal macrophages from Tim-3-TG mice, which showed increased SOCS1 expression (Fig. 7E), transfection with SOCS1 siRNA resulted in a significant decrease in mRNA levels for IL-10 (Fig. 7I) and Arg-1 (Fig. 7J).

Discussion

In the present study, we demonstrated that increased Tim-3 expression was associated with biased M2 macrophage...
polarization in colon cancer and promoted tumor growth. We then focused on exploring the molecular mechanisms by which Tim-3 polarizes macrophages. As summarized in Fig. 7K, Tim-3 promotes M2 macrophage polarization by binding STAT1 via residues Y256 and Y263, then inhibits the STAT1-miR-155 signaling axis, resulting in increased SOCS1 activity and Arg-1 and IL-10 expression. This is the first report demonstrating that STAT1 acts as a signaling adaptor of Tim-3 in macrophages. More importantly, our results showed that blockade of the Tim-3 pathway inhibited both the polarization of tumor-supporting macrophages and colon cancer growth, demonstrating the feasibility of modulating the Tim-3 pathway and altering macrophage polarization to treat this kind of disease.

The effects of Tim-3 on macrophage polarization and tumor pathogenesis were first examined in vivo. Using CAC and CT-26 tumor models, we demonstrated that Tim-3 promoted the polarization of tumor-promoting M2 macrophages in colon cancer. Although Tim-3 is also expressed on T cells and we cannot exclude the possibility that Tim-3 induces tumor tolerance by suppressing the function of T cells\(^\text{39}\), our data did demonstrate that Tim-3-induced macrophage tolerance in tumor models in vivo. Our findings therefore shed new light on the mechanisms by which Tim-3 induces tumor tolerance in colon cancer. A previous report showed that PDL1 expression by tumor-associated macrophages is a key mechanism for disarming the T cell antitumor response in hepatocellular carcinoma\(^\text{40}\) and we argue that Tim-3-mediated M2 macrophage polarization in colon cancer may have a similar effect.

Biased M2 macrophage polarization also contributes to tumor progression and metastasis in colon cancer and other tissues\(^\text{31-33}\) and macrophage re-education has been considered as a microenvironment-targeted therapy\(^\text{34,35}\). For this purpose, factors determining the diversity of macrophages are under intensive investigation. For example, Wang et al.\(^\text{36}\) showed that Notch signaling determines the M1/M2 polarization of macrophages in the antitumor immune response, and Pyonteck et al.\(^\text{10}\) demonstrated that the CSF-1/CSF-1R pathway mediates macrophage polarization and that inhibition of this pathway reduces M2 macrophage levels and blocks glioma progression. Here, we demonstrated that Tim-3, an immune checkpoint inhibitor, is involved in macrophage polarization. Although a recent report by Yan et al.\(^\text{37}\) showed that Tim-3 is involved in the activation and function of tumor-infiltrating macrophages in hepatocellular carcinoma, a finding consistent with our previous finding that Tim-3 is involved in macrophage polarization,\(^\text{32}\) the molecular mechanism by which this is achieved remains largely unclear. Local factors leading to increased Tim-3 expression in colon cancer were not investigated in the present study. However, manipulation of the Tim-3 pathway altered colon cancer progression, suggesting that Tim-3 can be used as a new therapeutic target to re-program tumor-associated intestinal macrophages to an antitumor phenotype and restrict colon cancer growth.

The therapeutic potential of Tim-3 called for the definition of the precise molecular mechanism of Tim-3-mediated immune suppression. Recently, Kuchroo et al. showed that CEACAM1 is a heterophilic ligand of Tim-3 and is required for Tim-3 to mediate T cell inhibition\(^\text{38}\) and that Bat3 acts as a safety catch, which blocks Tim-3-mediated inhibitory signals.\(^\text{39}\) However, these studies focused on the mechanism by which Tim-3 inhibits T cell activity and very little is known about how Tim-3 signals in innate immune cells and no adaptor(s)
mediating the inhibitory effects of Tim-3 in innate immune cells have been previously identified. Here, for the first time, we have demonstrated that STAT1 functions as a signaling adaptor of Tim-3 in innate immune cells. We suggest a scenario in which Tim-3 binds to STAT1 through residues Y256 and Y263 and inhibits STAT1 phosphorylation and nuclear translocation, resulting in inhibition of the STAT1-miR-155 signaling axis in macrophages.

We also examined the relationship between Tim-3 dysregulation and miR-155 expression and STAT1 activity in colon cancer in vivo. Our data in Figs. S2 and S6 support causative relations between increased Tim-3 expression, inhibited miR-155 expression, and suppressed STAT1 activation in vivo. A previous report showing that tumor growth is enhanced in miR155−/− mice supports the tumor suppressing role of miR-155 in vivo. In addition, both STAT1 and miR-155 play critical roles in polarizing macrophages, and our present findings support a scenario in which Tim-3 signaling alters the progression of colon cancer by modulating macrophage polarization in vivo.

Finally, our data demonstrated that SOCS1 is involved in the signaling cascade downstream of the Tim-3-STAT1-miR-155 axis in macrophages. SOCS1 is a transcriptional factor controlling the activity of M2 macrophages, in part, by inhibiting the JAK-STAT pathway. Our finding that transfection with SOCS1 inhibited Tim-3 blockade-induced STAT1 phosphorylation (data not shown) suggests negative feedback regulation by SOCS1 of the JAK-STAT pathway. As Tim-3-induced upregulation of Arg-1 and IL-10 was prevented by SOCS knockdown, our data explain how Tim-3 signaling controls macrophage polarization. In such a scenario, Tim-3 may regulate macrophage activity according to the following scheme: (1) in the constitutive state, Tim-3 competes with JAK for binding to STAT1 through the constitutively phosphorylated residues Y256 and Y263, then inhibits STAT1 phosphorylation and nuclear translocation and (2) this leads to decreased miR-155 expression, a lower inhibitory effect on SOCS1 expression, a subsequent increase in IL-10 and Arg-1 expression, and, finally, polarization of macrophages to the M2 phenotype.

In summary, this study identifies mechanisms by which Tim-3 determines intestinal homeostasis in colon cancer and how Tim-3 signaling operates in innate immune cells. We identified STAT1 as a signaling adaptor of Tim-3 in macrophages and demonstrated that Tim-3 controls macrophage polarization by inhibiting the STAT1-miR-155 signaling axis. These findings have potential clinical implications. We demonstrated the feasibility of re-educating macrophage polarization by modulating the Tim-3 pathway for treating this kind of disease.

Materials and methods

Mice

Male (6- to 8-week-old) C57BL/6 mice and Balb/C mice (both from Jackson Laboratory (Bar Harbor, ME, USA)) and Tim-3-TG mice, developed by Cyagen Biosciences Inc. China, were used under specific pathogen-free conditions.

Induction of colitis-associated cancer

CAC was induced as described previously. Briefly, WT or Tim-3-TG C57BL/6 mice were injected intraperitoneally (i.p.) with azoxymethane on day 0, then, starting on day 5, underwent four cycles of drinking water containing 2% DSS (MP Biochemicals) for 1 week and plain water for 2 weeks, ending on day 84, then normal drinking water was provided until day 100, when they were killed and macrophages analyzed by flow cytometry.

Immunohistochemistry

Paraffin-embedded slides were deparaffinized and immersed overnight in a 80° C water bath in 10 mM sodium citrate buffer containing 0.1% Tween 20 for antigen unmasking. Slides were incubated for 1 h at 37°C with primary antibody against Tim-3 (Abcam) or F4/80 (BD Biosciences) in PBS containing 1% BSA and 10% goat serum, then with biotinylated secondary antibody (Dako) at room temperature for 1 h. Streptavidin-HRP (BD Pharmingen) was then added, and, after 40 min the sections were stained with DAB substrate and counterstained with hematoxylin.

Cell culture and transfection

The mouse macrophage cell line RAW264.7, human embryonic kidney cell line HEK-293T, and human osteosarcoma cell line U2OS were obtained from ATCC (Manassas, VA). RAW264.7 cells stably overexpressing Tim-3 were generated in our laboratory, as described previously. The U2OS cell line stably expressing Stat1-EGFP was a kind gift from Prof. Lili Wang, State Key Laboratory of Toxicology and Medical Countermeasures, Beijing, China. Mouse colon tumor-infiltrating macrophages were elicited as described previously. All cells were maintained in complete DMEM medium in a humidified 5% CO2 atmosphere at 37°C. For cell transfection, Tim-3 cDNA was cloned into pcDNA3.1 to generate Tim-3-wt-Flag and Tim-3-wt-RFP. Gal-9 cDNA was cloned into pcDNA3.1. Overlap PCR was used to generate the point mutation constructs Y218A-Tim-3, Y256A-Tim-3, Y263A-Tim-3, Y272A-Tim-3, and Y256A/Y263A-Tim-3, in which the indicated tyrosine residues in the tail region of Tim-3 were replaced by alanine. To transiently express SOCS1, mouse SOCS1 cDNA was cloned into pcDNA3.1 and transfected into RAW264.7 cells. The recombinant fusion protein mouse Tim-3-Ig (sTim-3-Ig) and control Ig were prepared as described previously. Recombinant Gal-9 protein was purchased from R&D Corp (USA). All lines were grown according to the supplier’s recommendation.

Tumor transplantation and Tim-3 blockade

The mouse colon carcinoma cell line CT26 was obtained from ATCC (Manassas, VA) and the cells injected (1 × 10⁶/mouse) subcutaneously into one flank of 6-week-old male nude mice (Jackson Laboratory), then, after 2 weeks, the mice were sacrificed and the tumor tissues collected and cut into several similar-sized pieces, which were transplanted subcutaneously into 6-week-old male Balb/C mice.
In some studies, starting on the day of tumor transplantation, sTim-3-Ig or Ig (0, 200, or 500 μg/mouse) was injected i.p. every other day till day 32, and tumor size was measured every 2 d.

**FACS analysis and macrophage sorting**

Tumor-infiltrating cells were stained with allophycocyanin (APC)-conjugated rat anti-mouse CD11b mAb (clone M1/70), FITC-conjugated rat anti-mouse F4/80 mAb (clone BM8), and/or phycoerythrin (PE)-conjugated rat anti-mouse Tim-3 mAb (clone GL3) (all from eBioScience) diluted in 2% FBS in PBS, then, after two washes with PBS/2% FBS, were analyzed by flow cytometry in a FACS Calibur (BD Biosciences). To isolate macrophages from tumor-infiltrating cells, antibodies against mouse F4/80 and CD11b (eBioScience) and FACS cell sorting were used.

**miRNA microarray analysis**

Differentially expressed miRNAs in Tim-3 siRNA- or control siRNA-transfected RAW264.7 cells were profiled using Affymetrix miRNA microarray chips following the manufacturer’s protocol. Briefly, collected samples was subjected to Gene Chip miRs array analysis using a GeneChip Scanner 3000 with GeneChip Operating Software (GCOS) and analyzed. Candidate miR(s) was further identified by quantitative real-time PCR analysis.

**Quantitative real-time RT-PCR**

For human studies, the study protocol was approved by the Ethics Committee of the General Hospital of the PLA, Beijing, China, and all patients gave their written informed consent. In both humans and mice, gene expression was analyzed by two-step QRT-PCR. The relative expression of a gene was determined using the 2−ΔΔCt method, with GAPDH as the internal control. The primers for miR-155 were provided by RiboBio (Guangzhou, China). Other primers used, including those for human and mouse Tim-3, are listed in Table S1.

**ELISA**

The concentration of IL-10 in cell-free supernatants was measured using a sandwich ELISA according to the manufacturer’s protocol (eBiosciences, San Diego, CA).

**Western blots**

Western blotting was performed as described previously19 to evaluate levels of phospho-STAT1, STAT1, SOCS1, and GAPDH using rabbit primary antibodies (CST, USA), horseradish peroxidase-conjugated anti-rabbit IgG antibodies (KPL, Gaithersburg, MD, USA), and enhanced chemiluminescence kits (Amersham Biosciences).

**Co-immunoprecipitation of STAT1 with Tim-3**

HEK-293T cells were cotransfected with the STAT1 construct and either the control Tim-3 or Y256A/Y263A-Tim-3 construct using Lipofectamine 2000 (Invitrogen), then Tim-3 immunoprecipitation with anti-Tim-3 antibodies was performed at 48 h post-transfection and the precipitated protein immunoblotted for STAT1 as described previously.35

**High-content analysis**

U2OS cells stably expressing STAT1-GFP were transfected with the Tim-3-RFP or Y256A/Y263A-Tim-3-RFP construct on 96-well assay plates (Corning) using Lipofectamine 2000 (Invitrogen) and incubated with or without IFNγ for 30 min at 48 h post-transfection, then were fixed for 20 min in 4% formaldehyde at room temperature, the nuclei stained with Hoechst 33342 at 4°Covernight, and the cells imaged and analyzed using a GE IN Cell Analyzer 2000 High-Content Cellular Analysis System (GE Healthcare Bio-Sciences Corp.). To measure STAT1 translocation, the ratio of the GFP signal intensity in the nucleus/cytosol was calculated.

**Statistical analysis**

SPSS software (version 20.0) was used for statistical procedures. Data are expressed as the mean ± standard deviation. Differences between groups were analyzed using the Kruskal–Wallis test and ANOVA. Survival rate and tumor growth were analyzed using a log-rank test. A p value less than 0.05 was considered significant.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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