Smad7 induces plasticity in tumor-infiltrating Th17 cells and enables TNF-alpha-mediated killing of colorectal cancer cells

Angelamaria Rizzo, Vincenzo De Mare, Chiara Rocchi, Carmine Stolfi, Alfredo Colantoni, Markus F. Neurath, Thomas T. Macdonald, Francesco Pallone, Giovanni Monteleone and Massimo C. Fantini

Department of Systems Medicine, University of Rome “Tor Vergata”, Rome, Italy, 1First Medical Clinic, University of Erlangen, Erlangen, Germany and 2Barts and the London School of Medicine and Dentistry, London, UK

*To whom correspondence should be addressed. Tel: +39 (06) 7259 6150; Fax: +39 (06) 7259 6391; Email: m.fantini@med.uniroma2.it

Transplanting growth factor-beta (TGF-β) is deeply involved in colorectal cancer development and the disruption of the TGF-β signaling in dysplastic cells is required for tumor to grow. Nevertheless, tumor cells express TGF-β to escape the immune surveillance mediated by T cells. T-cell expression of Smad7, an intracellular inhibitor of the TGF-β signaling, protects against colitis-associated colorectal cancer. However, whether Smad7 in T cells might influence colorectal cancer growth independently of chronic inflammation and which T-cell subset is involved in this process is unknown. To address this issue, T-cell-specific Smad7 transgenic mice and wild-type (WT) littermates were subcutaneously transplanted with syngenic MC38 colon carcinoma cells. Smad7tg mice were resistant to tumor development compared with WT mice and protection was dependent on CD4+ T cells. Smad7 expression in T cells increased the number of tumor-infiltrating Thet/ROR-γt double-positive CD4 T cells characterized by the expression of tumor necrosis factor-alpha (TNF-α) and interferon-gamma but lower IL17A. The low expression of IL17A defines Th17 cells inducing Thet and IFN-γ expression while dampening IL17A. In turn, low intratumoral IL17A expression allows tumor necrosis factor-alpha (TNF-α)-mediated proapoptotic signaling in MC38 colorectal cancer cells.

Materials and methods

Cell lines

The C57Bl6/J MC38 murine colorectal cancer cell line was generously provided by Dr Ignacio Malero (Clinica Universidad de Navarra, Pamplona, Navarra, Spain). DLD-1 and HCT-116 human colorectal cancer cell lines were purchased from American Type Culture Collection (ATCC). All cell lines were maintained at 37°C in 5% CO2. The following cell culture media were used: Dulbecco’s modified Eagle’s medium for MC38, McCoy 5A medium for HCT-116 and RPMI 1640 medium for DLD-1. All media were supplemented with 2 mM l-glutamine, 100 U/ml streptomycin, 100 mg/ml penicillin and 10% heat-inactivated fetal bovine serum. All cell culture reagents were from Lonza (Ambrose, France).

Mice

All mice used were on C57Bl6/J specific pathogen-free conditions at the animal facility of the University of Rome Tor Vergata. All animal experiments were performed in accordance with the local institutional guidelines. Male mice (6–8 weeks old) were used for all the experiments.

Immunohistochemistry

Sections of snap-frozen tissues were used for indirect immunofluorescence using rat anti-mouse CD8 or CD4 (BD Pharmingen, Milan, Italy) or CD11c (eBioscience, San Diego, CA). Antigen detection was obtained with Tyramide (Cy3 and fluorescein isothiocyanate) according to the manufacturer’s instructions (PerkinElmer, Waltham, MA). The number of positive cells observed at high-power field was quantified by ImageJ software (NIH, Bethesda, MD). At least three high-power fields per section were counted. Statistical analysis was performed on four non-consecutive sections. Tumor sections were also stained with commercially available terminal deoxynucleotidyl transferase-mediated
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Results
Smad7 overexpression in T cells protects against MC38 tumors
To assess whether Smad7 overexpression in T cells could prevent the growth of colorectal cancer cells, we subcutaneously injected 2 × 10^5 syngenic MC38 colorectal cancer cells into both flanks of C57Bl6 WT and Smad7 transgenic (Smad7Tg) mice. Four weeks after tumor cell injection, macroscopic lesions were detectable in both the groups of mice. However, tumor incidence was significantly lower in Smad7Tg mice than that in the WT (WT 70% versus Smad7Tg 29%; Figure 1A). In addition, the tumors developing in the Smad7Tg mice were of a smaller size compared with those observed in the WT (Figure 1B).

Because the Smad7 transgene is expressed by CD4, CD8 and NKT cells, we investigated whether resistance to tumor development observed in Smad7Tg mice was associated with a different distribution of these cell subsets in the tumor tissue. However, no difference in the frequency of CD4, CD8, NK and NKT (defined as CD3−CD49b+ and CD3+CD49b− cells, respectively) was observed in the tumors of both the groups of mice as shown by flow cytometry (Figure 1C and Supplementary Figure 1A, available at Carcinogenesis Online). CD4+ and CD8+ T cells were also present at the same levels in sections of tumors obtained from both groups of mice (Figure 1D and E). Interestingly, in the Smad7Tg, but not in WT mice, CD4+ cells appeared to cluster in close proximity with D11c+ dendritic cells (Figure 1D), suggesting a higher activation in transgenic CD4+ T cells. However, analysis of the activation markers expressed by CD4+ T cell isolated from tumors and spleens of tumor-bearing WT and Smad7Tg mice did not show any difference (Supplementary Figure 1B, available at Carcinogenesis Online). Indeed, the tumor-infiltrating CD4+ T cells in both groups of mice were highly activated as shown by the dominant CD69−CD26−CD49d− phenotype both in the WT and transgenic mice. Despite the similar T-cell infiltrate and activation status, analysis of cytokine mRNA expressed in the tumors showed higher levels of IFN-γ and TNF-α and lower levels of IL17A in the Smad7Tg mice as compared with the WT (Figure 2A). Indeed IFN-γ and TNF-α expressions were 10- and 3-fold, respectively, higher in the tumors in Smad7Tg mice compared with tumors in WT mice, whereas IL17A was reduced by 3-fold (Figure 2B). Intracellular cytokine staining of TILs showed that CD4+ but not CD8+ T cells contributed to the differential expression of IFN-γ and IL17A observed in the whole tumor tissue (Figure 2C). Indeed tumor-infiltrating CD8+ T cells did not express IL17A and the expression of IFN-γ was similar between WT and Smad7Tg cells. In contrast, IFN-γ expression was higher and IL17A expression was lower in intratumoral Smad7Tg CD4+ T cells as compared with WT mice. Similarly, more tumor-infiltrating Smad7Tg CD4+ T cells expressed TNF-α than WT (Figure 2D). Moreover, TNF-α cytokine expression was higher in the Smad7 transgenic mice compared with WT at the single-cell level (TNF-α-positive CD4+ cells mean fluorescence intensity (MFI) WT: 1028.67 versus Smad7Tg 1397.33; Figure 2D, right lower panel).

These data suggest that Smad7Tg mice preferentially generate intratumoral Th1 immune responses while limiting the accumulation of Th17 cells. However, intracellular expression of the Th1- and Th17-related transcription factors, Tbet and RORγt, showed no differences in the number of RORγt+Tbet+ Th1 cells (Figure 2E). In contrast, the number of RORγt-Tbet- Th17 cells was decreased 3-fold in WT mice and this was associated with a significant increase of RORγt-Tbet double-positive cells in the Smad7Tg CD4+ T cells. Functionally, the Smad7Tg RORγt-Tbet+ subset of CD4+ T cells were characterized by lower IL17A expression and a 6-fold increase in IFN-γ compared with the WT mice (Figure 2F, upper panels). This difference was even more pronounced in the RORγt-Tbet double-positive subset where ~40% of Smad7Tg versus 10% of the WT cells expressed IFN-γ and 15% of the Smad7Tg versus 40% of the WT cells expressed IL17A (Figure 2F, middle panels). In contrast, WT and Smad7Tg RORγt-Tbet+ Th1 cells equally expressed IFN-γ but not IL17A (Figure 2F, lower panels). These data indicate that tumors developing in the Smad7Tg mice are characterized by the accumulation of Th17 cells characterized by low expression of IL17A, which also express high levels of IFN-γ.
**CD4** but not **CD8** T cells mediate resistance against MC38 tumor development

To dissect the role of CD4 and CD8 T cells, Rag1 knockout (Rag1<sup>−/−</sup>) alymphocitic mice were adoptively transferred with either CD8<sup>+</sup> or CD4<sup>+</sup> T cells sorted from WT and Smad7Tg splenocytes a week before the injection of MC38 cells. When RAG1<sup>−/−</sup> mice received CD8<sup>+</sup> T cells (Supplementary Figure 2A, available at Carcinogenesis Online), no differences in tumor incidence and size were observed among the groups (Supplementary Figure 2B and C, available at Carcinogenesis Online). Moreover, CD8<sup>+</sup> T cells from Smad7Tg mice accumulated significantly less than WT cells in the tumor tissue (Supplementary Figure 2D, available at Carcinogenesis Online). In contrast, Rag1<sup>−/−</sup> mice adoptively cotransferred with CD4<sup>+</sup> and CD8<sup>+</sup> T from Smad7 Tg mice developed smaller tumors with a lower incidence as compared with mice receiving WT cells (Supplementary Figure 3A–C, available at Carcinogenesis Online), thus suggesting that tumor incidence is dependent on CD4<sup>+</sup>/CD8<sup>+</sup> cooperation in this model.

In order to assess whether the reduced tumor growth was caused by increased apoptosis, TUNEL staining was performed on sections of tumors isolated from the different groups. Interestingly, tumors developing in mice reconstituted with transgenic CD4<sup>+</sup> T cells showed multiple focal areas of apoptotic MC38 cells, whereas in mice reconstituted with WT cells, only rare isolated apoptotic cells were detected (Figure 3D). Both WT and Smad7Tg CD4<sup>+</sup> T cells equally accumulated in the tumor tissue, indicating that Smad7 overexpression in CD4<sup>+</sup> T cells is necessary to slow tumor growth but is not fully sufficient to recapitulate the phenotype observed in the Smad7Tg animals. Consistently, RAG1<sup>−/−</sup> mice adoptively cotransferred with CD4<sup>+</sup> and CD8<sup>+</sup> T from Smad7 Tg mice developed smaller tumors with a lower incidence as compared with mice receiving WT cells (Supplementary Figure 3A–C, available at Carcinogenesis Online), thus suggesting that tumor incidence is dependent on CD4<sup>+</sup>/CD8<sup>+</sup> cooperation in this model. In order to assess whether the reduced tumor growth was caused by increased apoptosis, TUNEL staining was performed on sections of tumors isolated from the different groups. Interestingly, tumors developing in mice reconstituted with transgenic CD4<sup>+</sup> T cells showed multiple focal areas of apoptotic MC38 cells, whereas in mice reconstituted with WT cells, only rare isolated apoptotic cells were detected (Figure 3D). Both WT and Smad7Tg CD4<sup>+</sup> T cells equally accumulated in the tumor tissue,
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A

B

C

D

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Fig. 2. (A) Cytokine mRNA relative expression was quantified by quantitative PCR in total RNA isolated from WT and Smad7Tg tumors at the end of the experiment. (B) Cytokine differential expression (fold change) in tumors developed in Smad7Tg mice relative to those developed in the WT. IL17A, IFN-γ (C) and TNF-α (D) expression was analyzed by flow cytometry in CD4+ and CD8+ T cells isolated from tumors developed in WT and Smad7Tg mice. Representative contour plots are shown and numbers in the quadrants represent frequencies of cell subpopulations (left panels). Bars indicate the average frequencies of subpopulations depicted in the contour plots and TNF-α MFI as indicated (±SD), obtained from three independent experiments (right panels). *Statistically significant differences between WT and Smad7Tg mice; P < 0.01. (E) Tbet and RORγ-t expression was analyzed in tumor-infiltrating CD4+ T cells by flow cytometry. Representative dot plots of WT and Smad7Tg cells are shown and frequencies of cell subpopulations are indicated in the quadrants (left panels). Bars indicate average frequencies of cell subpopulations as indicated (±SD) obtained from three independent experiments (right panels). (F) IL17A and IFN-γ expression was evaluated by flow cytometry in the cell subpopulations described in E. Representative contour plots of WT and Smad7Tg cells are shown and the frequencies of cells subpopulations are indicated in the quadrants (left panels). Bars indicate average frequencies of cell subpopulations as indicated (±SD) obtained from three independent experiments (right panels). *Statistically significant differences between WT and Smad7Tg cells, P < 0.01.
Fig. 3. (A) Immunodeficient Rag1−/− mice were adoptively transferred with either 5 × 10⁵ WT or Smad7Tg CD4+ T cells or left untransferred a week before the subcutaneous injection of 2 × 10⁵ MC38 cells. Mice were killed and tumor excised 4 weeks after tumor cell injection. Incidence (B) and size (C) of excised MC38 tumors was calculated at the end of each experiment. Three independent experiments were performed. *Statistically significant differences between the groups, P < 0.01. (D) Representative TUNEL staining of tumor sections obtained from mice adoptively transferred with either WT or Smad7Tg CD4+ T cells. Horizontal bars indicate 100 μm. (E) Representative CD4 staining of tumor sections obtained from the different groups of mice as indicated. Horizontal bars indicate 100 μm. Lower right panel: bars indicate the number of CD4+ T cells per high-power field (±SD) obtained from multiple tumor sections from the indicated groups of mice.
thus ruling out the possibility that the difference in tumor size and apoptosis depended on a different access of CD4+ T cells into the tumor (Figure 3E).

Smad7 overexpression in CD4+ T alters IFN-γ and IL17A expression in the tumor microenvironment

Similarly to what observed in WT and Smad7Tg mice, tumors developing in Rag1−/− mice reconstituted with transgenic CD4+ T cells showed higher levels of IFN-γ and reduced expression of IL17A compared with mice transferred with WT CD4+ T cells (Figure 4A). In these experiments, IFN-γ was 6-fold higher and IL17A was 10-fold lower in tumors developed in mice transferred with transgenic CD4+ T cells as compared with WT (Figure 4B). Intratumoral expression of TNF-α was also high in both CD4+ WT and Smad7Tg reconstituted mice. More than 10% of the WT CD4+ cells isolated from the tumors expressed IL17A (Figure 4C) and ~75% of the CD4+ IL17A+ cells coexpressed IFN-γ (8–9% of all gated CD4+ cells). About 70% of the CD4+ cells were IFN-γ single positive. In contrast, among tumor-infiltrating Smad7Tg CD4+ cells, the number of IL17A-expressing cells, independently of IFN-γ coexpression, was reduced by >10-fold. About 85–90% of the tumor-infiltrating WT and Smad7Tg CD4+ T cells expressed TNF-α (Figure 4D). However, among the TNF-α-expressing cells, the MFI was lower in the transgenic cells as compared with WT.

IL17A protects colorectal cancer cells from TNF-α-mediated apoptosis

In order to assess whether the intratumoral cytokine environment generated by the Smad7-overexpressing CD4+ T cells could induce apoptosis in tumor cells, MC38 cells were stimulated in vitro with supernatants of TILs isolated from tumors developed in mice adoptively transferred with either WT or Smad7Tg CD4+ T cells.

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**Fig. 4.** (A) Cytokine mRNA relative expression was quantified by quantitative PCR in total RNA isolated from tumors isolated from RAG1−/− mice adoptively transferred with either WT or Smad7Tg CD4+ T cells or left untransferred at the end of the experiment. (B) Differential expression (fold change) of cytokine mRNA in tumors developed in mice transferred with Smad7Tg CD4+ T cells mice relative to those developed in the presence of WT cells. IL17A, IFN-γ (C) and TNF-α (D) expression was analyzed in tumor-infiltrating CD4+ T cells by flow cytometry. Representative contour plots of TILs isolated from tumors developed in Rag1−/− mice adoptively transferred with WT or transgenic CD4+ T cells are shown and numbers in the quadrants represent frequencies of cell subpopulations (left panels). Bars indicate the average frequencies of subpopulations depicted in the contour plots and TNF-α MFI as indicated (±SD) obtained from three independent experiments (right panels). *Statistically significant differences between the groups, P < 0.01, ns: not significant.
After 12 h of stimulation with supernatant of Smad7Tg TILs at 1 to 100 dilution, 65% of the MC38 cells underwent apoptosis as shown by AV and PI double-positive staining (Figure 5A). In contrast, only 25% of MC38 cells stimulated with medium or supernatant of WT TILs underwent apoptosis. After 48 h, the number of apoptotic cells reached 90% among MC38 cells stimulated with supernatant of Smad7Tg TILs, whereas only 35% of unstimulated and WT-TILs supernatant-stimulated MC38 cells underwent apoptosis (Figure 5B). Supernuant of ex vivo sorted Smad7 Tg but not WT CD4+ T cells isolated from the tumors induced apoptosis in MC38 cells in vitro, whereas no apoptosis was observed in the presence of CD4+ supernatants (Supplementary Figure 4A, available at Carcinogenesis Online). In order to assess which factors expressed in the supernatant of Smad7Tg TILs mediated apoptosis, the same experiments were performed in the presence of different cytokine-specific neutralizing antibodies. Neutralization of IFN-γ did not affect apoptosis induced by the supernatant of transgenic TILs (Figure 5C). In contrast, neutralization of TNF-α reduced apoptosis of MC38 cells stimulated with supernatant of transgenic TILs to levels of unstimulated and WT supernatant-stimulated cells. The combined neutralization of IFN-γ and TNF-α did not further increase the effect mediated by anti-TNF-α alone. Surprisingly, neutralization of IL17A increased apoptosis induced by the WT supernatant to levels observed in cells stimulated with transgenic TILs supernatant and this effect was reversed by the combined neutralization of IL17A and TNF-α. These data suggest that TNF-α expressed in the supernatants of both WT and Smad7Tg TILs is sufficient to induced apoptosis but this process is prevented in WT mice by the high expression of IL17A. To assess whether Smad7 in T cells can reduce IL17A expression in T cells, WT and Smad7Tg Tg Th17 cells were generated in vitro. Transgenic Th17 cells were characterized by higher frequency of RORγt−/−Tbet double-positive cells and 3-fold lower expression of IL17A than WT cells while high expression of TNF-α was observed in both WT and Smad7Tg cells (Supplementary Figure 4B and D, available at Carcinogenesis Online). Consistently, supernatants generated by transgenic Th17 cells induced more apoptosis in MC38 in a TNF-α-dependent manner as compared with WT supernatants (Supplementary Figure 4C, available at Carcinogenesis Online), thus indicating that Smad7-induced suppression of IL17A in Th17 cells is associated with the ability to induce apoptosis in MC38 cells.

TNF-α-induced apoptosis in MC38 cells was not affected by the addition of IFN-γ, whereas it was inhibited by the addition of IL17A (Figure 5D). Neither IL17A nor IFN-γ alone had any effect on apoptosis of MC38 cells and IFN-γ did not affect the IL17A-mediated block of TNF-α-induced apoptosis. TNF-α signaling is known to initiate a proteolytic cascade mediated by members of the caspase family of intracellular factors leading to apoptosis (18). Accordingly, TNF-α significantly induced activation of Caspase 8 in MC38 cells as shown by significantly higher number of cleaved Caspase 8-positive cells detected after stimulation with TNF-α by flow cytometry (Figure 5E). IFN-γ neither induced Caspase 8 activation alone nor increased the number of Caspase 8-positive cells induced by TNF-α. In contrast, IL17A prevented TNF-α-induced Caspase 8 activation. Caspases inhibition as obtained by using the pan-caspase inhibitor Q-VD-OPH prevented apoptosis in MC38 cells stimulated with TNF-α alone or in combination with IFN-γ (Figure 5F). These data suggest that apoptosis induced by the supernatant of TILs from mice reconstituted with transgenic CD4+ T cells depends on Caspase 8 activation. To address this issue, we analyzed Caspase 8 activation in MC38 cells stimulated with TIL supernatants. As expected, supernatants from transgenic TILs induced 3-fold more Caspase 8-positive cells than WT (Figure 5G). Moreover, caspase inhibitors prevented apoptosis in MC38 cells stimulated with transgenic TILs, thus indicating that apoptosis induced by transgenic CD4+ T cells requires the activation of Caspase 8 (Figure 5H). In order to assess whether IL17A could prevent TNF-α-mediated apoptosis in other cell systems, we tested the effect of IL17A in DLD-1 and HCT-116 human colorectal cancer cell lines. Similarly to MC38 cells, IL17A prevented, at least in part, the apoptosis induced by TNF-α in both DLD-1 and HCT-116 cell lines (Supplementary Figure 5, available at Carcinogenesis Online).

**IL17A expression by CD4+ T cells is sufficient to prevent tumor apoptosis in vivo**

In order to confirm the role of IL17A in neutralizing tumor cell apoptosis *in vivo*, CD4+ T cells isolated from the spleens of either WT or IL17A-deficient (IL17A−/−) mice were adoptively transferred into Rag1−/− mice before injection of MC38 cells. At the end of the experiment, tumors developing in mice transferred with IL17A−/− CD4+ T cells were significantly smaller in comparison with those developed in mice transferred with WT cells (Figure 6A). Similarly, to mice transferred with Smad7Tg cells, intratumoral IL17A−/− CD4+ cells expressed IFN-γ and TNF-α but not IL17A (Figure 6B and C). Consistent with the antiapoptotic effect of IL17A observed in the previous experiments, supernatants of activated TILs isolated from mice transferred with IL17A−/− CD4+ cells induced more apoptosis in MC38 cells as compared with the WT in a TNF-α-dependent manner (Figure 6D and Supplementary Figure 5, available at Carcinogenesis Online).

**Discussion**

A defective TGF-β signaling is commonly observed in colorectal cancer cells, nevertheless these cells express high levels of TGF-β to escape immune system surveillance. Interestingly, allele variants of *smad7* associated with an increased risk to develop colorectal cancer are characterized by low Smad7 expression (14,15). A possible explanation for such an association is that low Smad7 expression might enhance the TGF-β signaling in T cells, thus allowing tumors to escape immnosurveillance. At support of this hypothesis, we have recently demonstrated that Smad7 is downregulated in the lamina propria CD4+ T cells of patients affected by inflammatory bowel disease complicated by colorectal cancer and that Smad7 overexpression in T cells is protective in a model of colitis-associated colorectal cancer. In this system, protection was dependent on cell-mediated cytotoxicity induced by high expression of IFN-γ.

Here, we show that Smad7 overexpression in T cells protects against metastatic colorectal cancer generated by the subcutaneous transplant of syngenic MC38 cancer cells in an IFN-γ-independent manner. Smad7Tg mice showed reduction of both tumor incidence and growth, thus indicating that Smad7 expression in T cells might exert a double effect by contrast the initial formation as well as the growth of metastatic foci. Interestingly, the adoptive transfer of transgenic CD4+ but not CD8+ T cells in immunodeficient Rag1−/− mice significantly reduced tumor growth but did not alter tumor incidence. In contrast, the cotransfer of Smad7Tg CD4+ and CD8+ T cells significantly reduced tumor incidence and growth, thus suggesting that the cooperation between CD4+ and CD8+ cells is required to inhibit the initial phase of tumor growth. IFN-γ has been shown to enhance the expression of MHC class I on tumor cells, an essential event for cytotoxic T lymphocytes activation and killing activity (19). One possibility is that the higher IFN-γ expressed by Smad7Tg CD4+ T cells might induce stronger activation of CD8+ T cells, thus enhancing the clearance of tumor foci. Moreover, CD4+ T cells are critical for primary response of CD8+ T cells in *vivo* and to sustain the expansion of the CD8+ memory T cells pool (20,21). We did not observe differences in tumor incidence and size between untransferred and WT CD4+ transferred mice. One possibility is that in untransferred mice, the expression of TNF-α, the cytokine responsible for MC38 cell apoptosis, is lower as compared with WT-transferred mice. On the other hand, the proapoptotic effect of the TNF-α expressed in WT CD4+–transferred mice could be neutralized by the concomitant expression of IL17A.

Tumor protection observed in the Smad7 transgenic mice was associated with a misbalance between IFN-γ and IL17A expression in Th17 cells. In the Smad7Tg mice, the number of CD4+ T cells coexpressing RORγt and Tbet resulted significantly higher in comparison with WT both in *vivo* and in *vivo*-generated Th17 cells. In *vivo*, IFN-γ expression in RORγt−/−Tbet double-positive cells was increased by 4-fold, whereas the number of Tbet single-positive Th1 cells and the...
Fig. 5. (A) MC38 cells were stimulated with medium or 1:100 diluted supernatants of ex vivo activated TILs isolated from tumors developed in adoptively transferred Rag1\(^{-/-}\) mice. After 12 h, MC38 cells were stained with AV and PI and analyzed by flow cytometry. Representative density plots are shown from three independent experiments. Numbers in the quadrants indicate the frequency of cell subpopulations. (B) AV/PI staining was evaluated in MC38 stimulated as in (A) at different time points as indicated. (C) AV/PI staining of MC38 cells after 24 h stimulation with supernatants of activated TILs isolated from the different groups of mice in the presence of IL17A, TNF-\(\alpha\) and IFN-\(\gamma\) neutralizing antibodies as indicated. (D) AV/PI staining of MC38 cells after 24 h stimulation with IL17A, TNF-\(\alpha\) and IFN-\(\gamma\) as indicated. (E) Caspase 8 staining of MC38 cells after 24 h stimulation with Caspase inhibitors as indicated. (F) AV/PI staining of MC38 cells stimulated with medium or supernatants obtained from activated TILs isolated from the different groups of adoptively transferred Rag1\(^{-/-}\) mice. Representative density plots are shown and numbers above the gates represent the frequency of Caspase 8-positive cells. (H) AV/PI staining of MC38 cells stimulated with medium or supernatants of 24 h-activated TILs from the different groups of adoptively transferred Rag1\(^{-/-}\) mice in the presence or absence of the pan-caspase (Q-VD-OPH) inhibitor as indicated. Representative density plots are shown from three independent experiments. Bars in B, C, D and F indicate the average of AV/PI-positive MC38 cells and E indicates the average of Caspase 8-positive MC38 cells (±SD) obtained from three independent experiments.
expression of IFN-γ in these cells did not differ between the groups. At least two subsets of Th17 cells have been identified based on their pathogenic potential in models of autoimmunity (22). A group of Th17 cells, characterized by the expression of RORγ-t, IL17A and IL10, showed low pathogenic potential in experimental autoimmune encephalomyelitis models and resulted protective against Staphylococcus aureus infection, on the contrary, a subset of Th17 cells characterized by the coexpression of RORγ-t and Tbet and by the expression of low IL17A and high IFN-γ showed high pathogenic potential in experimental autoimmune encephalomyelitis and mediated immunity against Candida albicans infection (23,24). In the Smad7Tg mice, tumor growth was contrasted by the accumulation of IFN-γ-expressing RORγ-t/Tbet double-positive cells, thus indicating that the Smad7-mediated inhibition of the TGF-β signaling might induce highly pathogenic Th17 cells. Accordingly, it has been shown that RORγ-t/Tbet double-positive Th17 cells are generated by IL6, IL1β and IL23 in the absence of TGF-β, whereas the differentiation of low pathogenic RORγ-t single-positive Th17 cells requires the TGF-β signaling. Therefore, it is tempting to speculate that while the generation of low pathogenic Th17 cells might sustain tumor growth, the shift toward a high pathogenic phenotype caused by the overexpression of Smad7 might be protective against tumor. Highly pathogenic Th17 cells also developed in Rag1−/− mice reconstituted with transgenic CD4+ T cells and the tumors developing in these mice were small and characterized by multiple clusters of apoptotic cells. In contrast, only few scattered apoptotic cells were detected in the tumors developing in mice transferred with WT cells. In line with in vivo data, the supernatant of Smad7Tg but not WT CD4+ TILs was able to induce apoptosis in MC38 cells in vitro and the same effect was obtained using the supernatant of in vitro-generated transgenic Th17 cells. Taken together, these data indicate that Smad7 overexpression in CD4+ T cells modifies the phenotype of Th17 that is responsible for tumor cell apoptosis.

**Fig. 6.** (A) Size of MC38 tumors developed in Rag1−/− adoptively transferred with either WT or IL17A-deficient (IL17A−/−) CD4+ T cells was calculated at the end of the experiment. Three independent experiments were performed. IL17A/IFN-γ (B) and TNF-α/IFN-γ (C) coexpression was evaluated in tumor-infiltrating CD4+ T cells by flow cytometry. Representative contour plots of Rag1−/− mice adoptively transferred with either WT or IL17A−/− CD4+ T cells are shown (upper panels). Bars indicate the average frequencies of subpopulations depicted in the contour plots (±SD) obtained from three independent experiments (lower panels). (D) AV/PI staining of MC38 cells stimulated for 24h with medium or 1:100 diluted supernatant of 24h-activated TILs isolated from tumors developed in the adoptively transferred Rag1−/− mice in the presence or absence of neutralizing anti-TNF-α antibody as indicated. Representative density plots from three independent experiments are shown. Numbers in the quadrants indicate the frequencies of cell subpopulations.
Apoptosis was dependent on TNF-α and negatively controlled by IL17A. Indeed the block of IL17A was sufficient to restore the TNF-α-dependent killing capacity of WT TILs supernatant. The effect of IL17A was direct as shown by the reduced apoptosis observed in TNF-α-stimulated murine MC38 and human DLD-1 and HTC-116 colorectal cancer cell lines after the addition of IL17A to the culture medium. Finally, by adoptively transferring IL17A−/−CD4+ T cells in Rag1−/−, we confirmed the role of IL17A in preventing tumor growth in vivo. Indeed, tumors developed by mice transferred with IL17A−/−CD4+ T cells were significantly smaller than those developing in mice receiving WT cells. Moreover, supernatants of TILs containing IL17A-deficient CD4+ T cells killed MC38 cells more efficiently than WT cells in vitro. Although our data indicate that IL17A might act as an important survival factor for colorectal cancer cells, the role of IL17A in cancer development is controversial. Indeed, although high IL17A expression in patients affected by colorectal cancer is associated with a poor prognosis (11), the intratumoral accumulation of Th17 cells and high levels of IL17A are associated with a longer survival in patients affected by gastric cancer (25) and to a lower pathologic Gleason score in patients affected by prostate cancer (26), thus suggesting that the role of Th17 and IL17A expression might be context dependent. The reason of such variability is currently unknown but our data suggest that the effect of IL17A is strictly dependent on the cytokine milieu. Indeed, in our experimental setting, IL17A expressed by CD4+ T cells blocked apoptosis induced by TNF-α in TNF-α-sensitive cells. In contrast, the enhanced MC38 cell growth observed in conventional (i.e. non-conditional) IL17A knockout mice (27) and A.Rizzo and M.C.Fantini, unpublished results was associated with low TNF-α expression thus suggesting that sources of TNF-α other than T cells might be necessary to induce an effective antitumor immune response. TNF-α receptor belongs to a family of transmembrane receptors able to initiate a series of intracellular events leading to apoptosis. Activation of Caspase 8, a member of the caspase family of intracellular factors, represents one of the earliest events of TNF-α-induced apoptosis. In our system, IL17A prevented the activation of the Caspase 8 induced by the TNF-α. Although the intracellular mechanism leading to the inhibition of Caspase 8 activation is unclear, IL17A was shown to induce antiapoptotic molecules such as Bcl-2 and Bcl-xL in B16 melanoma and MB49 bladder carcinoma cell lines (28), thus indicating that IL17A might block apoptosis in MC38 cells at different levels. These data confirm and extend the relevance of Smad7 in the immune response against cancer and underscore the importance to define the survival mechanisms operating in cancer cells in order to tailor an effective therapeutic regimen. Indeed, it is tempting to speculate that in the presence of tumor-infiltrating 'low pathogenic' Th17 cells, the combined neutralization of IL17A and administration of TNF-α might determine a strong anticancer effect, thus representing an effective therapeutic approach.

Supplementary material

Supplementary Figures 1–6 can be found at http://carcin.oxfordjournals.org/

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