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Transforming growth factor- β 1 regulated phosphorylated AKT and interferon gamma expressions are associated with epithelial cell survival in rhesus macaque colon explants

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Abstract

Transforming growth factor- β 1 (TGF- β 1) is an important immunoregulatory cytokine that plays an obligate role in regulating T-cell functions. Here, we demonstrated the role of TGF- β 1 in regulating the survival of intestinal epithelial cells (ECs) in rhesus colon explant cultures using either anti-TGF- β 1 antibody or recombinant TGF- β 1 proteins. Neutralization of endogenous TGF- β 1 using anti-TGF- β 1 antibodies induced apoptosis of both intestinal ECs and lamina propria (LP) cells. Additionally, endogenous TGF- β 1 blocking significantly increased expression of IFN γ , TNF α , CD107a and Perforin in LP cells compared to media and isotype controls. A significant decrease in pAKT expression was detected in anti-TGF- β 1 MAbs treated explants compared to isotype and rTGF- β 1 protein treated explants. Our results demonstrated TGF- β 1 regulated pAKT and IFN γ expressions were associated with epithelial cell survival in rhesus macaque colon explants and suggest a potential role of mucosal TGF- β 1 in regulating intestinal homeostasis and EC integrity.

Keywords

Apoptosis; Colon; Cytokine; Epithelial Cell; pAKT; Rhesus Macaque; TGF- β 1

1. INTRODUCTION

Transforming growth factor- β 1 (TGF- β 1), which is an important immunoregulatory cytokine with pleiotropic effects on cell migration, differentiation, proliferation and survival is also

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involved in regulating immune responses [1]. The active form of TGF- β 1 mediates its function after binding with activin like kinase (ALK)-5 and TGF- β receptor II (TGF- β RII). TGF- β 1 dimers bind to TGF- β RII, which recruits and phosphorylates TGF- β RI, and finally recruits and phosphorylates SMAD proteins, a family of conserved transcription factors [2]. SMAD2 and SMAD3 are upregulated and bind with SMAD4 forming a heterodimer that enters the nucleus where it can interact with various transcription factors, co-activators or co-repressors to modulate multiple gene expression. SMAD7 inhibits TGF- β 1 signaling by inhibiting the formation of SMAD2/SMAD3/SMAD4 complexes [3]. TGF- β 1 signaling via SMAD-independent signaling pathways can be mediated by various mitogen activated protein kinase (MAPK) pathways including Rho-like GTPase and phosphatidylinositol-3-kinase (PI3K)/AKT [4].

In human mucosal tissue, TGF- β 1 was shown to be produced spontaneously by ECs, mast cells, regulatory T-lymphocytes, and stromal cells that were responsible for maintaining mucosal homeostasis and tolerance [5]. Several studies demonstrated TGF- β 1 associated immune defects contribute to intestinal inflammation in inflammatory bowel diseases [6-8]. Inhibition of TGF- β 1 signaling by disruption of TGF- β RII, SMAD3, and SMAD4 expression or by overexpression of inhibitory SMAD7 induces mucosal inflammation [9-12]. Despite all these studies, the main source of TGF- β 1 in colon has not been well defined. The detailed role of TGF- β 1 in regulating intestinal homeostasis of normal healthy rhesus macaques (RMs) is also poorly documented, where the RM model is well recognized for understanding HIV/SIV pathogenesis, drug development and vaccine design [13]. The colon explant culture in RM model allows to study the pathophysiology at the whole tissue level and has an experimental advantage to assess the novel anti-inflammatory intervention modalities that can be applied in human trials.

Here, we have identified the major TGF- β 1 producing cells in RM colon. The role of TGF- β 1 in regulating intestinal ECs survival has been examined using either anti-TGF- β 1 monoclonal antibodies (MAbs) or recombinant TGF- β 1 (rTGF- β 1) protein in colon explant culture, and the population of mucosal cytokine(s) and degranulation molecule producing cells were correlated with total apoptotic ECs. Expression of SMAD3, SMAD7 and IL-12A transcription factors and pSMAD2/3, SMAD7 and pAKT proteins were measured in treated explant cultures to determine their role in TGF- β 1 signaling and ECs survival. We present evidence that mucosal TGF- β 1 plays an important role in maintaining mucosal integrity by regulating the expression of IFN γ and pAKT in colon tissues.

2. MATERIALS AND METHODS

2.1. Animals and ethical statement

Eleven healthy, uninfected, normal male and female Indian RMs (*Macaca mulatta*) between 4.6-8.3 years of age and negative for HIV-2, SIV, type D retrovirus and STL-1 infection were used for this study. Animals were housed at the Tulane National Primate Research Center (TNPRC) and under the full care of TNPRC veterinarians in accordance with the standards incorporated in the Guide for the Care and Use of Laboratory Animals and with the approval of the Tulane Institutional Animal Care and Use Committee. Animal

procedures were performed only on sedated animals. Colon specimens were collected at the time of necropsy.

2.2. Colon explant cultures

Colon specimens, 8 cm in length were collected in ice-cold HBSS and were processed as described earlier [14-16]. Two to four mm² fragments of colon explants were cultured for 6h in 2ml RPMI-1640 containing BSA (0.01%), fungizone (1%), HEPES (25mM), and antibiotics (200 µg/ml streptomycin and 200 U/ml penicillin) in the presence of 5% CO₂ at 37°C [15, 16]. Mucosal explants were treated with either anti-TGF-β1 MAb (15µg/ml, R&D Systems, USA), rTGF-β1 protein (50ng/ml, R&D Systems) or isotype MAb (isotype control, 15µg/ml, Biolegend, USA). Explants without any treatment or isotype MAb were defined as media-only controls. Brefeldin A (Sigma, USA) was added 1h after treatment in explant cultures for *in situ* detection of cytokines and degranulation molecules. After incubation, explant cultures were either cryopreserved in Optimal Cutting Temperature (OCT) compound (Sakura Fintek, Inc, USA) or embedded in paraffin after proper fixation as described earlier [15-17]. The use of explant culture provides an alternative approach to understand the pathophysiologic condition at the whole tissue level. A successful explant culture requires condition that will maintain cell viability and preserve histological features as expressed in the intact tissue. Initially we cultured colon explant tissues collected from sacrificed normal Indian RMs for 0 h, 6 h, 12 h, and 24 h without any antibody/protein treatment and there were no significant changes in cell death or morphology between 6 h cultures compared to 0 h cultures. However, increased death and changes in morphology were evident in colon explants kept beyond 6 h [15].

2.3. Immunodetection of colon tissue

Tissue sections were processed for either immunofluorescent or immunohistochemistry staining with one or a combination of primary antibodies (Table S1) as previously described [15-17]. For immunofluorescent staining, tissue sections were stained sequentially for 2-3 colors by incubating first with the primary antibody for 1h, washed and stained further with Alexa Fluor 488-conjugated secondary antibodies (1:1000 dilution, Life Technologies, USA) for 30 min. Similarly, the slides were further stained with another primary antibody followed by Alexa Fluor 568-conjugated secondary antibodies (1:1000 dilution, Life Technologies). Nuclear staining was performed with anti-nuclear ToPro3 antibodies (1µM, Life Technologies). Stained tissue sections were mounted using Prolong® Gold antifade medium (Life Technologies) and scanned for imaging using a TCS SP2 confocal laser scanning microscope (Leica, Germany) equipped with three lasers. Negative control slides were incorporated in each experiment either by omitting the primary antibody or using isotype IgG1 and IgG (H+L) controls [15-17]. ImageJ (version 1.49d, NIH, USA) and Adobe Photoshop CS5 Extended (USA) were used to assign colors to the channels collected. For quantification of intestinal apoptotic (active caspase-3 (AC-3+), marker for apoptotic cells) ECs, a minimum of 10 fields were imaged using Nuance FX multispectral imaging system at 500-720nm spectral range and assigned color using Nuance Version 2.10 software (CRI, USA). AC-3+ enterocytes were expressed as percentages of the total enterocytes (ToPro3+Cytokeratin+). For quantifying cytokines and degranulation molecules, an average of five fields (400X magnification) were manually counted in each stained mucosal explant

tissue. We used quantitative fluorescence densitometry using ImageJ software to quantify pSMAD2/3 and SMAD7 protein expression in isotype, anti-TGF- β 1 MAbs and rTGF- β 1 protein treated colon tissues. A minimum 5 high power fields chosen at random (6 optical slices, 40X magnification, n=4) were quantified. The intensity of pSMAD2/3 and SMAD7 was represented as fluorescence pixel values.

To quantify pAKT+ cells by immunohistochemistry staining, slides were stained with pAKT antibodies using the Mach 3 Rabbit AP-Polymer Detection Kit (Biocare Medical, USA). The negative control slide consisted of rabbit Ig fractions (Dako, USA) used at the same concentration as pAKT antibodies to determine the background staining. Labeling was developed using BCIP/NBT (Dako) chromogen system followed by nuclear Fast Red counterstain. The slides were then mounted with Vecta Mount AQ (Vector Laboratories, USA). An average of 13-20 fields (40X magnification) were used in each of the slides to quantify pAKT+ cells manually using SPOT3 live imaging software. The sites for all tissue evaluations were selected randomly from each tissue and counted by two different individuals blinded to the samples to avoid bias.

2.4. Morphometric analysis

Tissue sections of 5 μ m thick were processed from paraffin blocks, stained with Hematoxylin and Eosin (H&E), and measured for crypt length and breadth using Image-Pro Plus, v4.5 software as outlined previously [15-18].

2.5. Quantification of SMAD3, SMAD7 and IL-12A gene expression in colon explants

Total RNA was extracted from freshly cut OCT colon sections using Purelink FFPE RNA Isolation Kit (Life Technologies) as per manufacturer's instruction. RNA was purified using the RNA Clean & Concentrator-25 Kit (Zymo Research, USA) with on-column DNaseI treatment (Life Technologies) and finally quantified. Total RNA was converted to cDNA using SuperscriptIII first strand synthesis system (Life Technologies) using random hexamers. Quantification of SMAD3, SMAD7 and IL-12A transcripts was performed using Taqman Gene Expression Assays Hs00706299_s1, Hs00178696_m1, and Rh02621733_m1 respectively (Life Technologies). Gene expression was normalized against 18S rRNA expression for each sample using Taqman 18S rRNA Endogenous Control Assay (Life Technologies) and validation experiments were run to determine target dynamic range and ensure equal amplification efficiencies between all assays. cDNA was amplified as per manufacturer's protocols on an ABI 7900HT Fast PCR System (Life Technologies). Relative gene expression was determined across treatments using the comparative threshold cycle (C_T) method. Samples were calibrated to the mean of the isotype controls. Fold changes in the expression were calculated using 2^{-C_T} [16, 19].

2.6. Statistical analysis

Graphical presentation and statistical analysis of the data were performed using GraphPad Prism (Version 5.0f, GraphPad software, USA). Results between experimental groups were compared using nonparametric Kruskal-Wallis test. Dunn's multiple comparison test was used for post hoc analysis. The correlation between the frequency of cytokine expressing

cells and percentages of apoptotic enterocytes from all treatments was calculated using nonparametric Spearman's rank correlation.

3. RESULTS AND DISCUSSION

3.1. TGF- β 1 is produced by multiple cells and can be depleted by anti-TGF- β 1 antibody treatment in colon explant cultures

We assessed the source of TGF- β 1 in RM colon by multilabel confocal microscopy. T-cells (CD3+), B-cells/plasma cells (CD79a+), monocytes/macrophages (CD68+), monocytes/histiocytes (Mac387+), dendritic cells (CD11c+) and ECs (Cytokeratin+) were expressing TGF- β 1 in colon tissues (Figures 1A-F). Rhesus colon maintained a TGF- β rich environment and TGF- β 1+ cells were distributed in lamina propria (LP) and epithelial cells (Figures 1A-G), in agreement with previous reports [20, 21]. To verify that TGF- β 1 depletion was achieved with neutralizing anti-TGF- β 1 MAbs, we performed immunofluorescent staining using polyclonal TGF- β 1 antibody to detect TGF- β 1+ cells in tissues. As expected, anti-TGF- β 1 MAbs treatment in colon explants lead to the depletion of mucosal TGF- β 1 compared to isotype control treated explants (Figures 1G-H). The result confirmed the use of neutralizing anti-TGF- β 1 MAbs as an effective means of depleting intramucosal TGF- β 1 rather than inhibiting a self propagating TGF- β 1 induction cascade. TGF- β RII, a major TGF- β 1 signaling molecule was distributed in colon LP (Figure 1I) and expressed in the cellular membrane (Figure 1J) of leukocytes (CD45+, Figure 1K) and monocytes/macrophages (CD68+, Figure 1L).

3.2. Immuno-neutralization of endogenous TGF- β 1 in colonic mucosal explants induces crypt morphological changes and apoptosis

The morphology of colonic mucosa was well preserved in the media control explant culture (Figure 2A). However, the LP of the anti-TGF- β 1 MAbs treated explant was mildly expanded with moderately increased numbers of lymphocytes and plasma cells compared to media only control explants (Figures 2A-B). The number of apoptotic cells in anti-TGF- β 1 MAbs treated explant increased both in LP and crypts and were characterized by pyknotic nuclei and eosinophilic cytoplasm compared to medial controls. Moderate to severe cytoplasmic vacuolar degeneration was observed in the majority of the goblet cells in crypts. No morphological changes in either isotype control or rTGF- β 1 protein treated explants were observed.

Crypt lengths of anti-TGF- β 1 MAbs treated explant were decreased ($262.4 \pm 7.2 \mu\text{m}$) compared to that of media ($272.9 \pm 10.8 \mu\text{m}$) and isotype controls ($283.1 \pm 12.3 \mu\text{m}$), although this change was not statistically significant. Similarly, no significant changes were observed in crypt breadth among the anti-TGF- β 1 MAbs treated ($57.8 \pm 1.4 \mu\text{m}$), isotype control ($64.6 \pm 2.4 \mu\text{m}$) and media control ($58.7 \pm 1.9 \mu\text{m}$) explant cultures from four independent experiments (Figures 2C-D). Our *in vitro* studies demonstrated the presence of endogenous TGF- β 1 is crucial for the maintenance of crypt morphology and the survival of epithelial cells.

3.3. Anti-TGF- β 1 antibody treatment induces increased proinflammatory, Th1 cytokines and degranulation molecules in LP lymphocytes and also induce enterocytes apoptosis

We quantified *in situ* proinflammatory (TNF α), Th1 (IFN γ) cytokines and degranulation molecules (CD107a and Perforin) in mucosal explant tissues treated with either anti-TGF- β 1 MAbs or rTGF- β 1 protein and compared with the cell counts observed in explants treated with either media or isotype controls (Figures 2E-H). Endogenous TGF- β 1 blocking with anti-TGF- β 1 MAbs in *ex vivo* experiments significantly increased expression of IFN γ , TNF α , CD107a and Perforin in LP cells per field compared to control explant cultures ($p < 0.0001$, Figures 2E-H) suggesting the increased localized expression of cytokines rather than the increased recruitment of cytokine producing cells from other tissues. Representative immunofluorescent images indicated up-regulation of IFN γ and Perforin expression at mucosal site (supplementary Figures 1A-B). No significant differences in cytokine expression were detected in explants when treated with rTGF- β 1 protein compared to controls (Figures 2E-H).

We quantified the percentages of AC-3+ ECs out of total ECs in all four experimental colon explants and noticed a significant increase of apoptotic ECs (AC-3+ Cytokeratin+ ToPro3+) in anti-TGF- β 1 MAbs treated explants compared to other explant tissues ($P < 0.0001$, Figures 3A-B). Percentages of AC-3+ ECs were correlated with cytokines/CD107a /perforin positive cells for both untreated and treated explant tissues. Nonparametric Spearman's rank correlation coefficient analysis of these independent measures demonstrated a highly significant positive correlation of the increased IFN γ + cells and increased EC apoptosis (Figure 3C). Despite an increased expression of TNF α , CD107a and perforin in anti-TGF- β 1 MAbs treated cultures compared to other groups, no significant positive correlation between TNF α /CD107a/perforin positive cells and apoptotic ECs was detected (r value ranged from 0.27 to 0.32, $P > 0.05$). A recent report has also shown increased IFN γ gene expression in anti-TGF- β RII antibody (Ab) treated colon explant cultures collected from patients with colon carcinoma [22]. Based on the target cells, IFN γ , TNF α , Perforin and CD107a can induce either antiviral activity or damage ECs. *Ex vivo* studies with colon explants showed that the presence of TGF- β 1 plays a crucial role in maintaining mucosal integrity and ECs survival, while blocking of endogenous mucosal TGF- β 1 lead to localized increased proinflammatory, Th1 cytokine, and degranulation molecules resulting in both intestinal LP cell and EC apoptosis, which may potentially compromise the intestinal epithelial barrier and integrity compared to other treatment groups. These findings were in agreement with others [22-24] where mucosal TGF- β 1 was shown to be responsible for maintenance of intestinal immune-homeostasis through its anti-inflammatory activity. Loss of ECs and increased inflammation in inflammatory bowel disease is also mediated by increased effector T-cell responses like IFN γ and TNF α [25]. Increased IFN γ produced by LP T-cells and antigen presenting cells was also thought to be responsible for early enteropathy in an experimental colitis model [26]. The cytokine responses are the key pathophysiologic signals that regulate the initiation, evolution and ultimately resolution of the inflammation in different forms of inflammatory bowel diseases [27]. TGF- β 1 plays an important role in regulating Th1 pathway by the production of IFN γ , IL17 and IL22 cytokines that are key players in Crohn's and ulcerative colitis as seen in human and rodent models [27-30]. Our recent studies with colon explants and SIV-infected RMs suggested that IL-10 also plays an

important role in maintaining mucosal homeostasis by regulating IFN γ and TNF α production [15, 16]. Our experimental design does not exclude the role of other cytokine(s)/chemokine(s) that might be playing an important role in maintaining intestinal homeostasis and regulating ECs apoptosis that need future studies.

3.4. Anti-TGF- β 1 antibody treatment induces decreased pAKT expression in colonic tissues

Distribution and localization of pSMAD2/3 and SMAD7 proteins were examined in colon explants from three independent experiments. pSMAD2/3 and SMAD7 positive cells were predominantly observed in LP mononuclear cells and enterocytes (Figure 3D-I). The pSMAD2/3 protein was localized predominantly in the nucleus compared to SMAD7, where the SMAD7 protein was detected both in the cytoplasm and the nucleus (Figures 3E & H). Both pSMAD2/3 and SMAD7 proteins were also localized in the nucleus of enterocytes (Figures 3F & I). We quantified SMAD3 and SMAD7 transcription factors in isotype, anti-TGF- β 1 MAbs and rTGF- β 1 protein treated explants. There were no significant changes in SMAD3 and SMAD7 gene expression in either anti-TGF- β 1 MAbs or rTGF- β 1 protein treated explants compared to the isotype controls (Figure 4A). IL-12, a well-known T-cell stimulating cytokine was shown to be responsible for the induction of IFN γ and TNF α cytokines [31]. TGF- β 1 depletion after 12hrs of anti-TGF β II antibodies treatment showed IL-12 dependent IFN γ responses in colon explants from patients with colon cancer [23]. A slight increase in IL-12A expression was detected in both anti-TGF- β 1 MAbs (1.035 ± 1.69) and rTGF- β 1 protein (1.322 ± 1.92) treated cultures compared to isotype control (1.0 ± 1.43), however the difference in IL-12A expression in anti-TGF- β 1 MAbs treated cultures was not statistically significant (Figure 4A). We have also analyzed pSMAD2/3 and SMAD7 protein expression in explant tissues. Mean fluorescence pixel values of pSMAD2/3 (3.22 ± 0.77) and SMAD7 (4.63 ± 1.77) proteins in anti-TGF- β 1 MAbs treated explants were slightly lower compared to any other treatment groups (Figures 4B-C). However, neither the reduced pSMAD2/3 nor SMAD7 protein expression was statistically different compared to other treatment groups. An earlier study with cultured murine podocytes treated with TGF- β 1 induced an increased expression of SMAD7 protein expression [32]. However, we did not observe any significant upregulation of SMAD7 expression in rTGF- β 1 protein treated explant cultures compared to isotype controls. Increased TNF α and IFN γ were shown to interfere TGF- β signaling by NF-kappaB and STAT1 induced SMAD7 upregulation respectively [33, 34]. Our *ex-vivo* colon explants demonstrated the importance of TGF- β 1 in maintaining epithelial integrity and homeostasis at mucosal site, however, we were unable to detect SMAD2/3 downregulation and SMAD7 upregulation in anti-TGF- β 1 MAbs and rTGF- β 1 protein treated explants respectively suggesting that SMAD-independent pathway might be equally playing an important role in TGF- β signaling. Therefore, we measured pAKT protein expression in all the three treatment groups. A significant decrease in pAKT expression was detected in anti-TGF- β 1 MAbs treated explants compared to isotype and rTGF- β 1 protein treated explants (Figures 4D-G). Isotype antibody as well as rTGF- β 1 protein treated colon explants showed increased pAKT staining in the colon epithelium, myofibroblast, intraepithelial and cells from LP compared to anti-TGF- β 1 MAbs treated explants (Figures 4E-G). Weak pAKT staining was detected in the colon ECs treated with anti-TGF- β 1 MAbs. Several *in vitro* studies

demonstrated that AKT pathway antagonizes SMAD3 activation where TGF- β 1 enhances ECs survival by increased activation of AKT [4, 35-37]. AKT plays an important role in controlling cell survival and apoptosis [4]. Our data suggest that TGF- β 1 induced AKT activation is necessary for the ECs survival. The absence of TGF- β 1 signaling in anti-TGF- β 1 MAb treated explants prevented the induction of AKT activation, which might have induced upregulation of TNF α , IFN γ , CD107a, Perforin and AC-3 proteins, and led to increased EC apoptosis.

An early increased intestinal EC apoptosis and damage to the intestinal epithelial barrier contributes to generalized HIV/SIV induced immune-cell activation during acute and chronic infection [16, 17, 38-42]. However, this was associated with increased production of TGF- β 1 in peripheral blood, lymph nodes and intestine during HIV infection that was thought to be a key contributor to the dysfunction of CD4+ and CD8+ T-cells and disease progression [43-46], which suggests that the TGF- β 1 failed to perform immunoregulatory function in HIV infection. HIV-tat induced TGF- β 1 upregulation was thought to be responsible for immunosuppression in HIV infected patients [47]. TGF- β 1 had also been demonstrated to enhance the susceptibility of macrophages to HIV-1 infection by increasing the expression of CXCR4 co-receptors in primary human monocyte-derived macrophage culture suggestive of a significant role of TGF- β 1 in promoting viral replication [48]. An appropriate balance between inflammatory and anti-inflammatory cytokine responses are crucial for maintenance of a successful immune responses in HIV infection [49]. In conclusion, our results demonstrate TGF- β 1 regulated pAKT and IFN γ expressions are associated with epithelial cell survival in RM colon explants and suggest a potential role of mucosal TGF- β 1 in regulating intestinal homeostasis and EC integrity. It is important to monitor and further explore the pathway (both SMAD-dependent and SMAD-independent) of mucosal TGF- β 1 in regulating HIV/SIV pathogenesis and HIV/SIV enteropathy, which may lead to the development of improved therapeutic strategies to prevent epithelial cell damage and systemic immune activation during acute and chronic HIV/SIV infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AC-3	active caspase-3
EC	epithelial cells
MAb	monoclonal antibody
pAKT	phosphorylated AKT

RM	rhesus macaque
TGF-β	transforming growth factor-beta
rTGF-β	recombinant TGF- β
Th1	T-helper 1

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Highlights

- TGF- β plays an important role in regulating T-cell functions.
- Anti-TGF- β antibody treatment induced increased epithelial cell apoptosis of colon.
- TGF- β maintains mucosal homeostasis by regulating cytokines/degranulation molecules.

Figure 1.

Expression of TGF- β 1 in normal colon tissue by multilabel confocal microscopy in multiple cell types including CD3+ T-cells (A), CD79a+ B-cells/plasma cells (B), CD68+ monocytes/macrophages (C), Mac387+ monocyte/histiocytes (D), CD11c+ dendritic cells (E) and epithelial cells (F). TGF- β 1 producing cells were detected in lamina propria from colon tissues treated with isotype controls only (G). However, the expression of TGF- β 1 diminished in anti-TGF- β 1 monoclonal antibodies (MAbs) treated colon explant cultures (H). The majority of the TGF- β RII expressing cells were detected in colon lamina propria (I) and the expression of TGF- β RII was detected in the cellular membrane (J). The majority of the TGF- β RII expressing cells were positive for leukocytes (CD45+, K) and some of the TGF- β RII+ cells were also positive for monocyte/macrophage markers (CD68+, L) as detected by multilabel confocal microscopy. The labels of each image were indicated at the Y-axis of each image. TGF- β 1 and TGF- β RII positive cells in lamina propria were indicated by yellow arrows.

Figure 2.

Anti-TGF- β 1 monoclonal antibodies (MAbs) treatment induced damage to colonic crypts and increased inflammatory cytokine production in colon explants. Destruction of colonic epithelial cells was observed in explant cultures in the presence of anti-TGF- β 1 MAbs (B) compared to media alone explants (A) in H&E stained sections. Red arrows indicated disruption of the crypt epithelial cells. However, no significant differences in colonic crypt length (C) and crypt breadth (D) were observed between anti-TGF- β 1 MAbs treated explants and other treatment groups (media controls, isotype controls and recombinant TGF- β 1 (rTGF- β 1) protein treated cultures; $n=4$; 6-10 crypts were measured for length and breadth for each sample). Anti-TGF- β 1 MAbs treatment induces increased IFN γ (E), TNF α (F), CD107a (G) and perforin (H) production. The horizontal lines denoted the mean frequencies ($\pm$ standard errors) of each treatment group. Statistically significant differences between each treatment group were shown.

Figure 3.

Epithelial cell (EC) apoptosis correlates with markers of cytotoxicity. (A) Lamina propria cell and enterocyte apoptosis were detected by multi-label immunofluorescent confocal microscopy, where increased apoptosis of colonic ECs was detected in anti-TGF- β 1 monoclonal antibody (MAbs)-treated cultures compared to other treatment groups. The labels of each image are indicated at the top of the image. Apoptotic ECs and lamina propria cells are indicated by white and yellow arrows respectively. (B) Scattered plots (with mean $\pm$ standard errors) of apoptotic ECs in colonic explant cultures with different treatments are shown (n=3; A minimum 10 fields were measured for each sample). Statistically significant differences between each group of treatment were shown. (C) A positive correlation of apoptotic ECs with mucosal markers of cytotoxicity was shown (n=3). Spearman's rank correlation coefficient analysis of cytokine molecules/field and percentages of apoptotic enterocytes from all treatment groups of colon explant culture was performed. Phosphorylated SMAD2/3+ (pSMAD2/3+) cells were prevalent in cells from lamina propria and crypt epithelium (D & F), whereas the majority of the SMAD7+ cells were detected in lamina propria cells (G). Most of the pSMAD2/3+ cells (E) are localized within the cell cytoplasm whereas SMAD7+ cells (H) were localized both in cell cytoplasm and nucleus. ECs were also positive for pSMAD2/3 (F) and SMAD7 (I) localized mostly in the nucleus. Red arrows indicated either pSMAD2/3+ or SMAD7+ cells. Labels were shown in the Y-axis of each figure (D-I).

Figure 4.

Relative fold changes (mean $\pm$ standard errors) of SMAD3, SMAD7 and IL-12A gene expression were shown in colon explants in the presence of either anti-TGF-β1 monoclonal antibody (MAbs) or recombinant TGF-β1 (rTGF-β1) protein (n=3). All samples were normalized against 18S rRNA expression, and fold changes are relative to mean expression levels in isotype control samples. None of the changes in treated cultures were statistically significant (P>0.05). Immunofluorescence pixel values of pSMAD2/3 (B) and SMAD7 (C) expression (with mean $\pm$ standard errors) in different colon explant cultures were shown. (D) Scatter plots (with mean $\pm$ standard errors) of phosphorylated AKT (pAKT)+ cells in different colon explant cultures were shown. Statistically significant differences between each treatment group were shown. Detection of pAKT+ cells in colon explant cultures treated with isotype control (E), anti-TGF-β1 MAbs (F) and rTGF-β1 protein (G) were shown. pAKT+ cells were detected by immunohistochemistry staining using anti-pAKT antibodies with nuclear fast red counterstain. Red arrow denoted the presence of pAKT+ cells (dark brown color) distributed in lamina propria, intraepithelial, myofibroblast and epithelial cells.