THE PENNSYLVANIA STATE UNIVERSITY
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TGFB1 OVEREXPRESSION IN BASAL KERATINOCYTES CAUSES UPREGULATION OF S100A8, S100A9 AND INFILTRATION OF MPO+ CELLS WITHIN THE SKIN

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Abstract

Transforming growth factor beta1 (TGFβ1) is a ubiquitous cytokine made by most cell types in the body. However, the effects of TGFβ1 are both diverse and largely context dependent. Overexpression of TGFβ1 has also been shown to cause hyperproliferative type disorders, with implications for cancer promotion through supporting chronic inflammation. Therefore, this study seeks to further understand the effect of TGFβ1 overexpression on skin condition and proliferation within both normal and inflamed skin. Utilizing a transgenic mouse model, TGFβ1 expression was induced at the keratin-5 promotor in the basal layer of the epidermis. Expression was induced for acute (five day) and chronic (two week) periods in both normal and inflamed, TPA-treated, skin. Through this experimentation, it was found that overexpression of TGFβ is able to induce increased levels of S100A8 and S100A9, MPO^+ cell recruitment to the epidermis, and epidermal thickness in both normal and chronically inflamed skin. These effects could be seen after only four days of TGFβ induction, and were amplified over time in normal skin. However, the exact mechanism of S100A8 and S100A9 upregulation remains unclear.

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Introduction

Transforming growth factor beta1 (TGFβ1) is a ubiquitous cytokine made by most cell types in the body. However, the effects of TGFβ1 are both diverse and largely context dependent. TGFβ is well known for its ability to suppress the immune response as an effector cytokine for the immunosuppressive, regulatory T-cells (Tregs). The regulatory functions of Tregs and TGFβ1 have been shown to be necessary to allow peripheral self-tolerance in prevention of autoimmunity, limit T-cell activation, and suppress inflammation[1,3]. The critical role of TGFβ1 in these processes has been well established as it has been shown that TGFβ1 knockout mice rapidly develop autoimmunity and inflammatory type diseases[1,4]. However, overexpression of TGFβ1 has also been shown to cause hyperproliferative type disorders, with implications for cancer promotion through supporting chronic inflammation. Therefore, this study in particular seeks to further understand the effect of TGFβ1 overexpression on skin condition and proliferation within both normal and inflamed skin.

TGFβ is produced in three different isoforms, each from different genes, TGFβ1, TGFβ2, and TGFβ3[3]. Due to the fact that TGFβ1 is the most highly expressed in vivo, any discussion of TGFβ will be referring to the properties of TGFβ1 in particular. When synthesized, TGFβ is held in a latent, inactive complex with both the latency-associated protein (LAP) and latent-TGFβ-binding protein (LBP). Importantly, the LBP aids in targeting TGFβ to the extracellular matrix[2]. Although it is known that TGFβ cannot signal through its receptor while associated with this latency complex, the mechanism by which it dissociates to an active form is not completely understood.

When active, TGFβ signals through binding to transmembrane serine/threonine kinase receptors, mostly via activin receptor-like kinase 5 (ALK5) and TGFβ receptor II (TGFβRII). In signaling, a TGFβ dimer will bind to an ALK5-TGFβRII complex at the membrane to a serine/threonine kinase receptor. This receptor then phosphorylates the intracellular proteins known as Smads, which are required to enter the nucleus in order to regulate transcription of the TGFβ target genes[1,3]. Currently, eight Smad proteins have been identified, which have then been divided into three groups: Receptor-associated (R-Smad1, 2, 3, 5, and 8), inhibitory Smads (I-Smad6 and 7), and a common Smad (Co-Smad4). In the TGFβ signaling pathway, phosphorylation of ALK5 leads to the formation of a complex of R-Smad2, R-Smad3, and Co-Smad4. This Smad complex is then able to enter the nucleus and recruit histone-acetyl transferase (HAT) coactivators or histone-deacetylase activity-containing (HDACs) corespressors to either initiate or repress gene transcription. It has also been established that the Smad complex alone binds rather weakly to DNA, and therefore must associate with a variety of other transcription factors such as FAST and AP-1 in order to effectively recruit HAT and HDACs. Interestingly, the inhibitory Smad I-Smad7 is not phosphorylated during TGFβ signaling, but instead competes with the R-Smads in binding to ALK5 and also recruits the E3 ubiquitinase Smurf1 to destroy ALK5, inhibiting the signaling pathway[1,3].

Upon signaling, the various effects of TGFβ can be observed. General effects reveal TGFβ as a regulatory cytokine, able to inhibit growth and differentiation for most cells, including epithelial cells. TGFβ also has distinct profibrotic properties both through induction of extracellular matrix proteins in fibroblasts such as collagen and fibronectin, as well as by inhibiting gene expression involved in extracellular matrix turnover, such as metalloprotease collagenases and stromelysin[2]. As mentioned above, the primary effect of TGFβ with regard

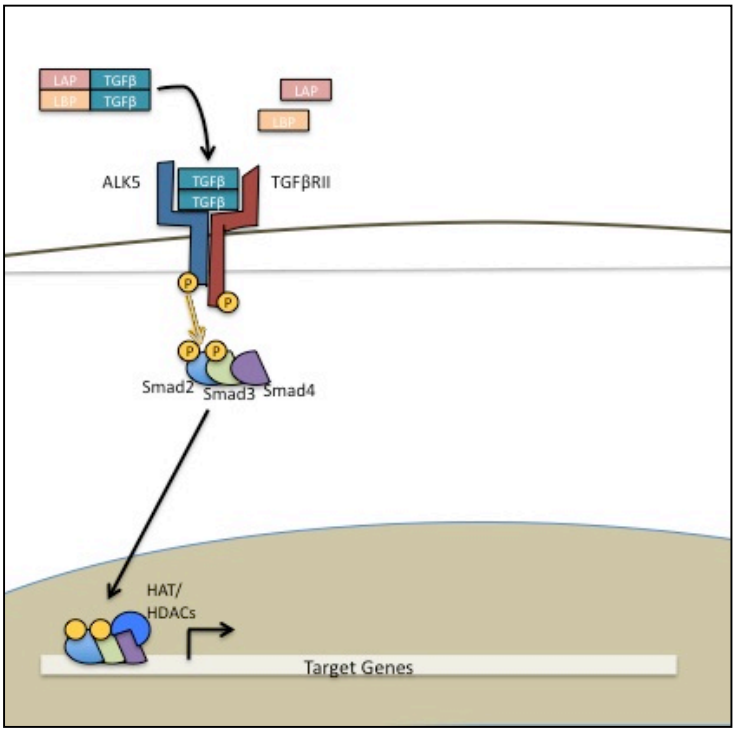

Figure 1: TGFβ Signaling Pathway Prior to binding a heterodimeric ALK5- TGFβRII receptor, TGFβ is bound inactive by latency-associated protein (LAP) and latent-TGFβ-binding protein (LBP). Upon binding to this receptor, TGFβ initiates phophorylation of an intracellular protein complex of Smad2, Smad3, and Smad4. This Smad complex is then able to enter the nucleus and recruit histone-acetyl transferase (HAT) coactivators or histone-deacetylase activity-containing (HDACs) corespressors to either initiate or repress gene transcription.

to the immune system is also inhibitory. T-cell proliferation, differentiation, and survival are all regulated by TGFβ. Interleukin-2 (IL-2) is one of the key cytokines signaling for growth and differentiation of T-cells during an immune response through the Ras/MAPK, JAK/STAT, and PI3/Akt pathways. TGFβ however, serves to block transcription of IL-2 by causing obstruction to the promoter region. This activity has been shown to operate in a Smad3 dependent manner, as Smad3 deficient mice are not susceptible to TGFβ-induced suppression of IL-2[1]. It has also been observed that in T-cells, TGFβ signaling leads to upregulation of cyclin-dependent kinase inhibitors (CDKIs), cell-cycle regulators, and downregulation of c-myc, a transcription factor promoting cell growth[1,3]. Hence, TGFβ is able to suppress T-cell proliferation via multiple mechanisms. It is important to note that while TGFβ holds anti-proliferative effects in naïve T-cells, mature T-cells are largely unaffected in terms of proliferation. This effect may be due in part to the decrease level of TGFβRII on the surface of mature T-cells as compared to naïve.

While TGFβ holds distinct roles in T-cell proliferation, it also regulates the differentiation process of naïve T-lymphocytes into effector T-cells. As in proliferation, this regulation again is largely inhibitory. Differentiation of naïve T-cells into $CD4^+$ T-helper 1 (Th1) and T-helper 2 (Th2) cells are key to the cellular immune response. Th1 cells are marked chiefly by secretion of the cytokine interferon-γ (IFN-γ) in order to mount an immune response to intracellular pathogens, while Th2 cells, known to secrete the cytokines IL-4, IL-13, and IL-5, are essential in the destruction of extracellular pathogens[1]. TGFβ inhibits differentiation of these effector T-cells by blocking expression of transcription factors critical to their development. More specifically, the transcription factors T-bet and STAT1 are required for Th1 development, while GATA-3 and STAT6 are required for Th2 development. In Th1 differentiation, the presence of IL-12 secreted by the antigen presenting cells macrophages and dendritic cells is crucial to induce IFN-γ production by the T-cell, leading to a positive feedback loop between antigen presenting cell production of IL-12 and T-cell production of IFN-γ. IFN-γ is then required to induce STAT1 promotion of T-bet which then drives Th1 differentiation[3]. TGFβ however blocks Th1 development possibly through several mechanisms that ultimately lead to decreased expression of T-bet. One such mechanism is through decreasing levels IL-12 receptor on the T-cell to prevent IFN-γ production and the positive feedback loop. This would be accomplished by TGFβ inhibition of the transcription factor STAT4, which is needed to induce IFN-γ production.

Similarly, TGFβ prevents the development of Th2 cells by decreasing expression of GATA-3. While the mechanisms for this action are less clear, it has been suggested that TGFβ may prevent NFAT translocation into the nucleus to induce GATA-3 production. However, as in proliferation, mature, differentiated Th2 cells are resistant to TGFβ-mediated inhibition of their

cytokine production. Importantly, TGFβ does retain the ability to inhibit IFN-γ by mature Th1 cells, as IFN-γ conversely induces Smad7 production to inhibit TGFβ signaling[1,3].

Given that TGFβ exerts inhibitory effects on proliferation and differentiation of naïve T-cells, it follows that $CD8^+$ effector T-cells would also be inhibited by TGFβ. $CD8^+$ T-cells are a chief mediator of the adaptive immune response to intracellular pathogens. Known as cytotoxic T-lymphocytes, these cells produce inflammatory cytokines such as IFN-γ and possess distinct cytolytic activity through a perforin-granzyme pathway. However, TGFβ has been shown to prevent $CD8^+$ T-cells from expressing both perforin and IFN-γ, limiting their ability to destroy invading pathogens[1].

Another interesting and influential role of TGFβ in regulation of the cell-mediated adaptive immune response lies in the balance between immunosuppressive regulatory T-cells (Tregs) and the inflammatory T-helper 17 cells (Th17). Tregs are a $CD4^+$ $CD25^+$ subset of T-cells that are characterized by their ability to suppress the immune system response, as well as permit tolerance of immune cells in the body. These cells act primarily through the secretion IL-10 and TGFβ. Differentiation of Tregs occurs through induction of the transcription factor Foxp3. Foxp3 then, not only enhances expression of IL-10 and TGFβ, but also inhibits secretion of inflammatory cytokines such as IL-2, IL-4, IL-17, and IFN-γ[1]. TGFβ in particular can induce Foxp3 via a STAT5-dependent mechanism. Knockout studies have further shown that the Smad2/3 complex is important during this TGFβ signaling pathway. However, TGFβ induction of Foxp3 is dependent on the presence of IL-2, a cytokine more known for its T-cell proliferative properties and traditionally blocked by TGFβ as mentioned above. Conversely the proinflammatory cytokine IL-6 is able to inhibit Foxp3 expression through interaction with STAT3, though the exact mechanisms of this action are currently unknown[1,3].

On the other side of the spectrum, TGFβ causes differentiation of Th17 cells under the context of IL-6. Hence, whether a T-cell enters a Treg or Th17 cell lineage depends on the presence of either IL-2 or IL-6. Induction of Tregs antagonizes induction of Th17 cells due to the fact that Foxp3 antagonizes RORγt, the Th17-promoting transcription factor. Th17 cells are a unique subset of $CD4^+$ T-cells known for their proinflammatory roles via secretion of proinflammatory cytokines IL-17, IL-22, and IL-21. Notably, IL-21 and IL-23 are also important in maturation and maintenance of Th17 cells. This is because IL-6, IL-21, and IL-23 all have the ability to activate STAT3, which then induces RORγt to lead to Th17 polarization[1,3]. Further supporting the importance of IL-23 in ensuring Th17 maintenance is the upregulation of the IL-23 surface receptor in mature Th17 cells. The antagonization between immunosuppressive Tregs and response promoting Th17 cells allows the immune system to maintain an appropriate balance in its response.

The effect of TGFβ on the immune response is not limited to T-lymphocytes, but also influence innate immune cells such as dendritic cells, macrophages, mast cells, and granulocytes. Dendritic cells (DCs) are antigen-presenting cells (APCs) that span both innate and adaptive immunity by presenting antigen to T-cells in order to evoke a cellular adaptive immune response. TGFβ holds seemingly contradictory roles on dendritic cell development. It is well established that TGFβ is required for development of dendritic cells resident to epithelial cells of the epidermis, known as Langerhan's cells[1]. TGFβ knockout mice in fact lack Langerhan's cells. However, it also suppresses the activity of differentiated DCs as well as DC-T-cell responses. DCs differentiated by inflammatory cytokines or bacterial products in the presence of TGFβ express low levels of MHC class II and costimulatory molecules, indicative of an immature phenotype. These DCs also fail to produce IL-12, a cytokine typically released by mature

stimulated DCs that induces T-cell activation and differentiation. Lastly to note of DCs with respect to TGFβ is that although the current function is unknown, immature DCs express high levels of TGFβ, while mature DCs express low levels[1]. It has been suggested that DC TGFβ may act in an autocrine or paracrine fashion to maintain an immature state or T-cell tolerance.

A second important APC influenced by TGFβ are the phagocytic cells, macrophages. Macrophages are known for their ability to phagocytose apoptotic cells and microbes during infection, as well as produce inflammatory cytokines in response to such an infection. Macrophages in an immature state, known as monocytes, are typically five to ten times smaller, lack phagocytic abilities and hydrolytic enzymes, and are less complex. Interestingly, TGFβ influences macrophage developing by stimulating monocytes, yet inhibiting the actions of activated macrophages[1]. TGFβ is able to serve as a monocyte chemoattractant to the site of inflammation, increases levels of adhesion molecules on monocytes surfaces, and induces the destruction of vascular membranes to allow for efficient transmigration of monocytes. Further aiding monocyte stimulation and inflammation in the immune response, TGFβ induces expression of IL-1 and IL-6 in monocytes.

Although, however stimulatory the effects of TGFβ are in monocytes, it is equally inhibitory in mature macrophages, both in terms of phagocytosis and antigen presentation. In terms of phagocytosis, macrophages are able to uptake and destroy microbes much more efficiently when that microbe has been IgG opsonized. However, expression of two IgG receptors on macrophages, FcγRI and FcγRIII, is suppressed by TGFβ[1]. In vitro, TGFβ also inhibits macrophage development of reactive nitrogen and oxygen species, two important defenses against invading pathogens. In terms of antigen presentation, IFNγ-induced expression of MHC class II by macrophages is inhibited by TGFβ, along with the costimulatory molecule

CD40. Therefore, TGFβ greatly limits the ability of macrophages to drive an active immune response. Macrophages themselves though do secrete TGFβ under certain conditions. For example, during phagocytosis of apoptotic cells which functions to prevent an inflammatory response to programmed cell death.

Finally, TGFβ has important effects on neutrophils in the innate immune response. Neutrophils are granulocytic cells that act as a first line of defense against infection through the use of reactive oxygen species. They also contribute largely to inflammation via release of lipid mediators, inflammatory cytokines and chemokines, and oxidative burst. Most current research supports TGFβ as a chemoattractant for neutrophils to the site of infection[1]. However, similarly to its behavior in DCs and macrophages, TGFβ seems to only initially stimulate the immune response by recruiting neutrophils, while later in the inflammatory response, TGFβ down-regulates transendothelialcytosis by altering surface expression of E-selectin and VCAM-1.

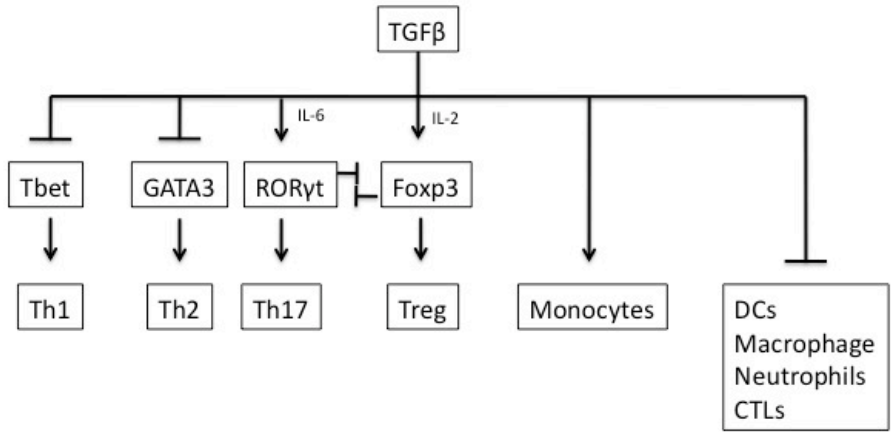

Figure 2: TGFβ Regulation of Immune Cells
Although mostly inhibitory, TGFβ has diverse effects on immune cell development. TGFβ inhibits production of Th1 cells, Th2 cells, DCs, Macrophages, Neutrophils, and CTLs. However it has been shown to both promote development and indirectly inhibit Th17 and Treg cells, regulating the Th17-Treg axis of the immune response. While these represent general properties, the influence of TGFβ in immune cell activity is often context-dependent.

In regulating the activity of immune cells that contribute to an inflammatory response, it is no surprise that TGFβ then in effect plays a key role in development of inflammation and inflammatory disease. The importance of TGFβ in the inflammatory response becomes especially apparent under conditions when TGFβ is either over or under expressed. Interestingly in the epidermis, both situations, over and under expression of TGFβ, result in severe hyperplasia, increased vascularization, and increased risk for neoplastic diseases such as cancer[4,5,6]. One model that allows for the controlled over or lack of expression of TGFβ is the tetO system (see Materials and Methods). Use of this model in the epidermis revealed that rapid, acute expression of TGFβ in the skin, for 24-72 hours, strongly inhibited cell proliferation in adult mice as expected. However, while TGFβ expression had anti-proliferative during acute induction, chronic expression led to hair loss and hyperplasia of the skin. This was accompanied by increased neutrophil infiltration, increased extracellular matrix presence, and thickening of the dermis[5]. Other studies using similar models have also demonstrated that TGFβ over expression leads to inflammatory skin lesions characterized by epidermal hyperproliferation and high infiltration of neutrophils, $CD4^+$ T-cells and macrophages in the epidermis of adult mice. This skin further presented with increased levels inflammatory cytokines IL-1, IL-6, IFNγ, TNFα, and IL-2 as well as macrophage and monocyte chemotactic factors, MIP1α and β, and MCP-1, respectively[6]. Recent data has suggested that in inflamed and premalignant skin, overexpressed TGFβ induces IL-17 expression from the infiltrated $CD4^+$ and γδ T-cells at the site of inflammation. Coupled with increased levels of IL-6, IL-23, and IFNγ, it was found that TGFβ is polarizing these $CD4^+$ T-cells to a Th17 phenotype, again promoting an inflammatory response[7].

Similarly, other studies have found that TGFβ expression is reduced or absent in mouse hyperproliferative skin, papillomas, and carcinomas, with less TGFβ correlating to higher malignancy rates of papillomas and carcinomas[4]. The dual nature of TGFβ in promoting and regulating the immune and inflammatory response makes it of great interest in inflammatory and neoplastic hyperproliferative diseases such as cancer. Cancerous cells do not respond to the inhibitory effects of TGFβ. Instead, the TGFβ signaling pathway now evokes a response promoting proliferation[8]. This altered response to TGFβ may be the result of disruptions in CDKIs, which typically regulate growth downstream of TGFβ. However, due to the fact that TGFβ plays controversial and not well-classified roles in inflammation, leading to hyperproliferative diseases in the skin, it is important to further investigate the effects and mechanisms of TGFβ in inflammatory models.

Materials and Methods

K5rTA and K14rTA tetoTGFβ Mouse Model

In order to study the effects of TGFβ overexpression in the skin, a transgenic mouse model was used that allowed for conditional expression of TGFβ. In this model, keratin 5 (K5) promoter driven expression of a tetracycline transactivator causes expression of the transactivator in the basal layer of the epidermis. These K5 mice were crossed with mice transgenic for a tetO regulated expression TGFβ. In mice containing both transgenes, referred to as double transgenic (DT) mice, the rTA binds the tetO sequence and induces expression of TGFβ in the basal layer of the epidermis, but only in the presence of doxycylcine, a tetracycline analogue. Mice not transgenic for tetO regulated TGFβ expression are referred to as single transgenic (ST) mice. Doxycycline will therefore not induce expression of TGFβ in ST mice.

TPA Treatments of Mice

In order to induce inflammation in the skin of mice, 12-O-Tetradecanoylphorbol-13-acetate (TPA, 5μg in 200μL) was applied to the back skin of mice biweekly for either 14 or 28 days, with three to four days between treatments. Mice used were eight to ten weeks old during experimentation, and mice of both genders were used in approximately equal proportions. Backs were shaved one day prior to treatment to allow treatment onto bare skin. Control mice in these experiments were treated with 200μL of acetone.

PCR Genotyping of Mice

All transgenic mice underwent genotyping by polymerase chain reaction of DNA extracted from 0.5cm tail samples taken from mice at 21 days of age. Tails were lysed using an Adult Tail Lysis Buffer composed of 100mM Tris-HCl, 5mM EDTA, 0.2% SDS, and 200mM NaCl and placement in a Thermomixer (Eppendorf) for 1 hour at 57˚C. Adult Tail Lysis Buffer was composed of 100mM Tris-HCl, 5mM EDTA, 0.2% SDS, and 200mM NaCl After lysis, 200µL of lysate was added to 200µL of isopropanol to pellet DNA, after which DNA was resuspended in 400 µL low TE Buffer for 1 hour at 70˚C in the Thermomixer. Upon completion of DNA isolation, PCR reactions were carried out to determine whether a mouse was single or double transgenic. Mice DNA that amplified primers for K5rTA, or K14rTA, and tetoTGFb1 were considered as double transgenic, while if only K5rTA or K14rTA amplified, mice were considered single transgenic.

Flow Cytometric Analysis

Leukocytes were extracted from skin tissue for analysis via enzymatic digestion of tumors using collagenase-I and IV (Worthington), hyaluronidase, and DNase-I in complete RPMI medium and incubated at 37˚C for 1 hour. Cells were stained using antibodies against CD45, CD11b, F4/80 (eBioscience), and Ly6G. (BD Pharmingen) in different combinations. CD45 was used to detect all leukocytes, F4/80 for macrophages, CD11b for granulocytes and macrophages, and Ly6G for neutrophils. For cytokine staining, LNs and tumor cells were stimulated with 50 ng/mL phorbol 12- myristate 13-acetate (PMA) and 1 mg/mL ionomycin (Sigma) in the presence of GolgiStop (BD Pharmingen) for 4.5 hours and stained with anti-mouse CD45, CD4, or γδ-TCR (eBioscience) and fixed overnight in 4% paraformaldehyde.

GolgiStop is necessary to inhibit release of cytokines from the cells. Therefore, as cells are stimulated to produce their respective cytokines, GolgiStop keeps these cytokines within the cells amplifying intracellular cytokine levels to allow to for easier detection of the cytokine profiles of the cell population.

After staining for IFNγ and IL-17 (BD Pharmingen) in 0.2% saponin buffer, cells were analyzed using a Cytomics FC500 (Beckman Coulter). Dead tumor cells were excluded from analysis using the Live/Dead fixable red dead cell stain kit (Invitrogen).

Inguinal lymph nodes were removed for mice upon necropsy and digested by gently teasing apart with forceps in 1mg/ml RPMI 1640 collagenase D. Lymph node cells were cultured in 1.5mL of complete RPMI media containing 5mM of β-mercaptoethanol. Cell staining was performed in 2mM EDTA in FCS buffer using combinations of fluorescent antibodies against CD45, CD11b, and FITC. All results were analyzed using the program FlowJo with single color samples of cells used for compensation values to limit background fluorescence.

RT-PCR Analysis of RNA Expression

RNA was extracted from skin tissue using 1mL Trizol reagent and placing in a shake homogenizer (Qiagen Tissue Lyser by Retsch) at 30 beats/min for 6-8 minutes. Collected RNA was resuspended in 70µL DEPC water. All RNA was DNase treated using DNA-free™ Kit (Applied Biosystems) before under going cDNA synthesis (Promega). cDNA was prepared using 1.0µg of DNase treated RNA in a 25µL reaction volume including 5.0µL 5x RT Buffer, 0.5µL 100mM dNTPs, 0.5µL 500µg/mL primers, 0.25 RT enzyme, and 18.2µL DEPC water. All cDNA was stored at -20˚C in between use. PCR reactions were completed using 18µL of Taq

Complete (GeneChoice), 1μL primer, and 1μL cDNA. All results of PCR were then detected by gel electrophoresis.

QPCR Analysis

RNA was collected, DNase treated, and used to make cDNA as described for PCR analysis. Each reaction was run in a generic 96-well PCR plate containing 10μL PerfeCTa SYBR Green SuperMix (Quanta Biosceiences), 4μL nanopure water, 1μL of the respective primer, and 25ng of cDNA. Reactions were run on a MyiQ iCycler (BioRad) under the conditions 95˚C for 2-3minutes for initial denaturation, then 95˚C for 10-15 seconds and 55-65˚C for 30 seconds for PCR cycling.

Protein Analysis

Protein was isolated from skin tissue samples using RIPA buffer and 1μL/mL of the protease inhibitors DTT, sodium ortho vanidate, sodium fluoride, PMSF, aprotinin, leupeptin, pepstatin, and β-glycerophosphate. RIPA buffer was composed of 25mM Tris-HCl, 150mM NaCl, 1% IPEGAL, 0.05% sodium deoxychloride, and 0.025% SDS in nanopure water. Samples were then placed in a shake homogenizer (Qiagen Tissue Lyser by Retsch) at 30 beats/min for 10-12 minutes and allowed to rotate for 1 hour at 4˚C. Lastly, samples were centrifuged and the supernatant was collected.

For western blot analysis, after boiling samples for 5 minutes at 100˚C, 20μg of each protein sample was run in a 10% acrylamide gel at 53V until the loading dye had reached the end of the gel. A 1M Tris-glycine and 0.001% SDS in distilled water running buffer was used. Bands

from the gel were transferred onto nitrocellulose paper in chilled transfer buffer composed of 1M Tris-glycine and 20% methanol in distilled water. Transfer was run at 100V for 1 hour.

The nitrocellulose membrane was next stained with Ponceau dye for 5 minutes at room temperature and unstained by washing several times with distilled water until most of the color had faded from the paper. The nitrocellulose was labeled with the date and percent gel before scanning a copy of the paper into a computer. After scanning was complete the bands were washed away using TBS-tween.

The nitrocellulose paper was then placed in a 5% blocking solution composed of 5% dry nonfat milk in TBS-tween for 1 hour on a Multi-Purpose Rotator (Barnstead). After 1 hour, the paper was rinsed briefly with TBS-tween and incubated with the primary antibody, which were pSmad2 and GAPDH, overnight on the rotator. Six 10-minute washes with TBS were completed before incubating with the secondary antibody for 1 hour on the rotator, after which six more 10-minute washes were completed as before.

To prepare the blot for development, 1mL of Stable Peroxide solution and 1mL of Dura Luminol/Enhancer were placed on the nitrocellulose paper for 2 minutes. Lastly, the blots were exposed onto Image-Tek H imaging film (AXS) for 3 minutes, 1 minute, 30 seconds, and 15 seconds in a dark room setting before development by a Konica SRX 101A.

MPO Staining

Skin tissue collected during necropsy was sent to Hershey Medical Center for sectioning and plating on glass microscope slides. These slides were returned with the tissues paraffinized. In order to begin myeloperoxidase (MPO) staining of these sections, present in myeloid derived cells such as dendritic cells, macrophages, and neutrophils, the tissue was first deparaffinized and

hydrated by placing the tissues through three washes in Histochoice 1x Clearing Agent (Histochoice), 95% ethanol, 90% ethanol, 75% ethanol, and three washes of 1x PBS each for 5-minute increments. Next, tissues were quenched with 3% H_2O_2 in methanol for 20 minutes at room temperature, after which the slides underwent another three 5-minute washes in PBS. Tissues were then incubated for 30 minutes at room temperature in 5% normal goat serum.

For the primary and secondary antibody incubations, a 1:500 solution of Polyclonal Rabbit anti-Human IgG in PBS and a 1:2000 dilution of Biotin-conjugated Goat anti-Rabbit IgG (Vector BA-1000) in PBS were used, respectively, each for 30 minutes at room temperature. Slides underwent three 5-minute washes in PBS after each incubation. Next, tissues were stained by placing in Vectastain ABC (Elite) for 30 minutes, followed by three 5-minute washes in PBS. DAB-Substrate (Vector Laboratories) was then applied to the tissues for 5 minutes while protected from direct light. Excess substrate was washed off in distilled water. Finally, nuclei were counterstained using Gill's hematoxylin for 2 minutes. The excess stain was again washed off using distilled water. MPO counts of the tissue sections were performed using a Nikon Eclipse E200 microscope at 100x magnification at ten random fields. The counts were averaged to obtain values for each sample and treatment group.

Epidermal Thickness Measurements

Skin tissue collected during necropsy was sent to Hershey Medical Center for sectioning and plating on glass microscope slides. H&E staining was also performed by Hershey Medical Center. These tissues were then viewed using an Olympus CKX41 microscope at 100x magnification. The length measurement application of Spot Advanced software was used to

measure epidermal thickness in H&E tissue sections of all tissue samples. Five measurements over 7 random fields per sample were taken.

Statistical Analysis

For all data, a two-tailed T-test performed to determine statistical significance of the data. The average values of the control and experimental groups for each test were used in this calculation. Differences in results were considered statistically significant only when possessing a p-value <0.05.

Results

Acute TGFβ Induction in Normal Skin Causes Increased MPO$^+$Cells, S100A8, and S100A9

Groups of ST and DT K5rTA mice were administered doxycycline feed for five days, inducing expression of TGFβ transgene basal layer in the skin of the DT mice. On the sixth day, mice were sacrificed and skin tissue from the backs of these mice was collected. Ethanol fixed sections were then stained for MPO$^+$ cells as described in Materials and Methods. Counts of MPO cells revealed significantly increased levels of MPO$^+$ cells present in the epidermis of DT mice as compared to the ST control group ($p<0.0001$). The count values were approximately 5 MPO$^+$ cells/field compared to 1 MPO$^+$ cell/field, respectively (Figure 3). Importantly, these MPO$^+$ cells are clustered around the hair follicle, near the basal keratinocytes where TGFβ is expressed.

RNA extracted from collected tissues was also analyzed for inflammatory cytokines in order to assess how acute TGFβ induction influences inflammation in the skin. Notably, QPCR analysis of demonstrated significantly increased levels S100A8 ($p=0.001$) and S100A9 ($p=0.03$) genes in the skin of DT mice as compared to the ST group (Figure 3). S100A8 and S100A9 are two pro-inflammatory, neutrophil-secreted cytokines. Previous research has shown that these two proteins are strongly upregulated in the epidermis during skin cancer, as well as other human epithelial tumors[9]. It is also important to note the known chemotactic properties of S100A8 and S100A9. S100A8 and S100A9 are both expressed by and attract neutrophils, activated monocytes, and macrophages, promoting further leukocyte recruitment via a positive feedback type mechanism[9]. Therefore, these two cytokines are of particular interest for this study due the fact that they hold proinflammatory and carcinogenic roles, as does TGFβ.

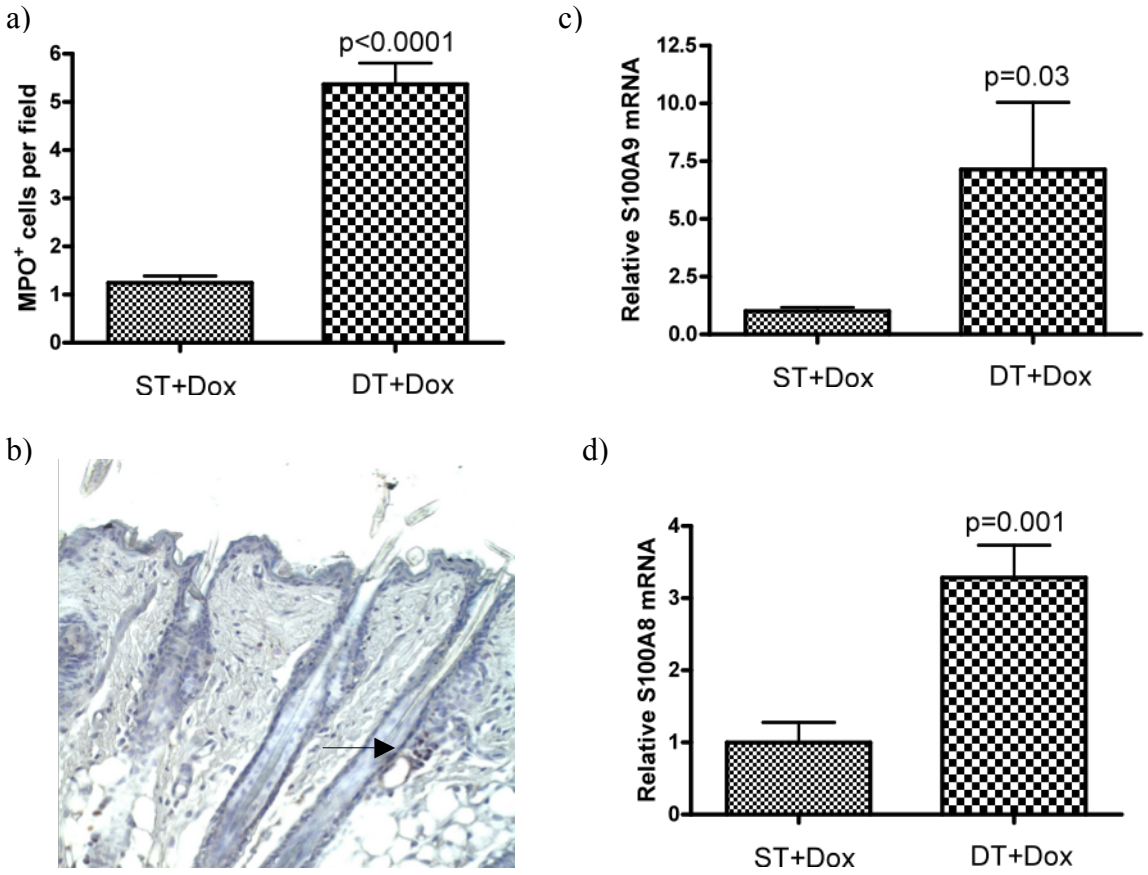

Figure 3: Acute TGFβ Induction in Normal Skin Causes Increased MPO$^+$Cells, S100A8, and S100A9

ST and DT mice were administered doxycycline for 5 days and skin tissue was collected on day 6. No skin treatments were performed. a) MPO cell staining was performed on ethanol fixed tissue sections from ST and DT mice. Values are averages of each group. For each tissue section, MPO$^+$ cells were counted for 10 random fields at 40x magnification. b) Picture of MPO stained tissue section from a DT mouse. c and d) S100A8 and S100A9 mRNA transcript was measured by QPCR for all mice. Values given are averages for each group.

Chronic TGFβ Induction in Normal Skin Causes Increased MPO$^+$Cells

DT and ST groups were also administered doxycycline feed for four weeks, establishing chronic TGFβ induction in the DT mouse group. It is important to evaluate both acute and chronic TGFβ induction to assess context dependence of TGFβ in inflammation. This is evaluate how quickly the effects of TGFβ can be observed, as well as how cells respond to these effects over the long term. After four weeks of treatment, the mice were sacrificed and skin sections were stained for MPO$^+$ cells. MPO counts performed revealed increased levels of MPO$^+$ cells in the DT mice when compared to ST mice, approximately 15 MPO$^+$ cells/field compared to 3 MPO$^+$ cells/field, respectively (Figure 4). Due to low sample size of each group however, no statistics were performed for this data.

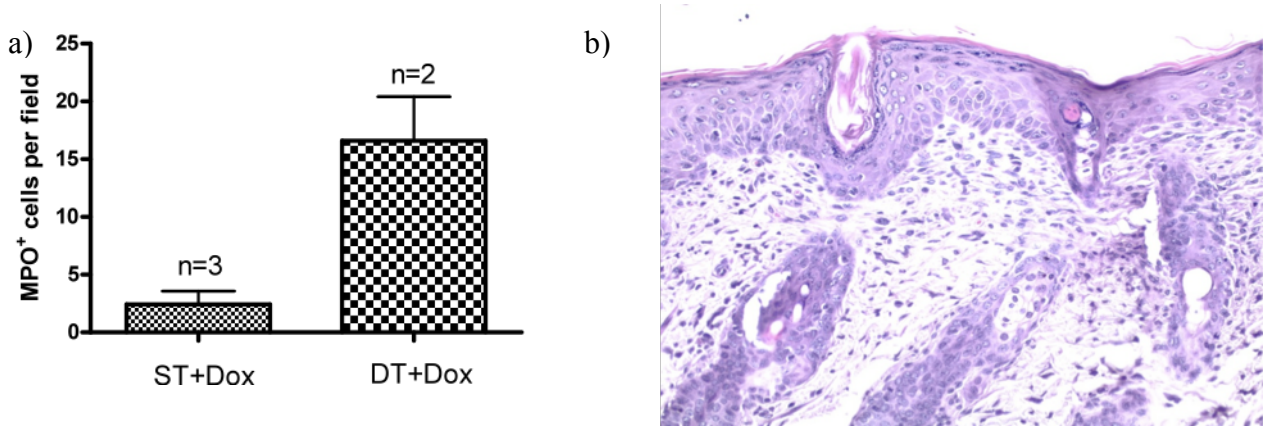

Figure 4: Chronic TGF β Induction in Normal Skin Causes Increased MPO+Cells
ST and DT mouse groups were administered doxycycline for 28 days and skin tissue was collected on day 29. No skin treatments were performed. a) MPO cell staining was performed on ethanol fixed tissue sections from ST and DT mice. Values are averages of each group. For each tissue section, MPO$^+$ cells were counted for 10 random fields at 40x magnification. b) H&E stained tissue section of a DT tissue sample showing increased thickness of the epidermis.

Acute TGFβ Induction Within Chronically Inflamed Epidermis Causes Increased MPO^+Cells, Epidermal Thickness, S100A8, and S100A9

TPA was administered to the shaved backs of ST and DT mice biweekly for 14 days to cause chronic inflammation, and then doxycycline feed was administered for the final three days of the TPA treatment period. MPO staining of collected tissue from these mice demonstrated a significant increase ($p=0.02$) in the number of MPO^+ cells in the DT group compared to the ST mouse group, 31 MPO^+ cells/field compared to 9 MPO^+ cells/field, respectively (Figure 5). Flow cytometric analysis of these tissue samples was also performed. Significantly higher percentages of $CD11b^+$cells ($p=0.03$) and $CD11b^+Ly6G^+$neutrophils ($p=0.009$) were observed (Figure 5). $CD11b^+$ cells indicate the granulocytes macrophages and neutrophils. Similarly, a significant increase ($p=0.0003$) in epidermal thickness of the DT mice was also observed, 75μM compared to 43μM, respectively (Figure 5). Lastly, significantly increased levels of the proinflammatory cytokines S100A8 ($p=0.03$) and S100A9 ($p=0.03$) were found in the DT mouse group as measured by QPCR (Figure 5e,f).

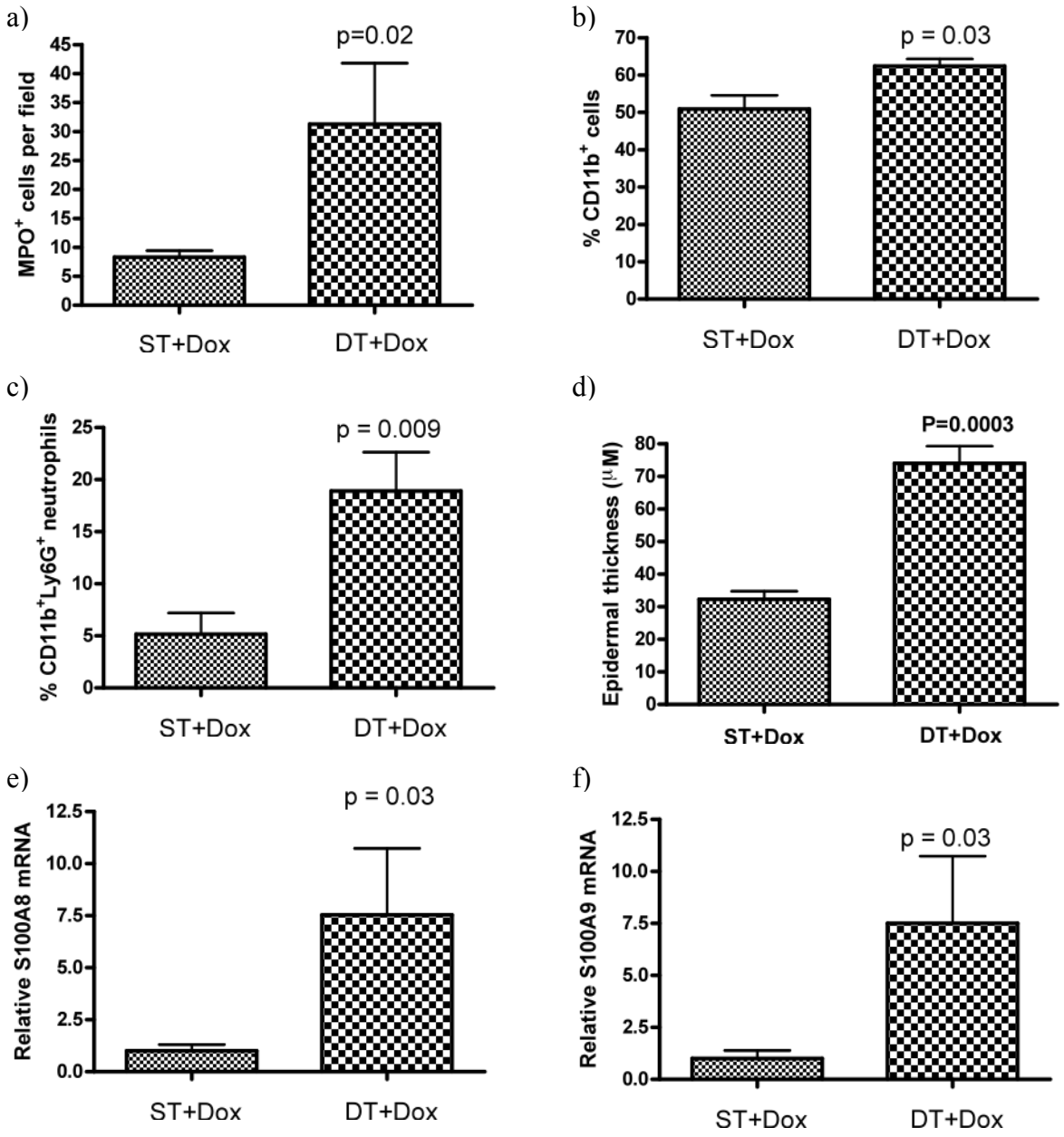

Figure 5: Chronic Inflammation with Acute TGFβ Induction Causes Increased MPO$^+$Cells, Epidermal Thickness, S100A8, and S100A9

Shaved backs of ST and DT mice were treated with 200μg TPA biweekly for 14 days. All mice were administered doxycycline feed after day 11. Tissues were collected on day 15. a) MPO cell staining was performed on ethanol fixed tissue sections from ST and DT mice. Values are averages of each group. For each tissue section, MPO$^+$ cells were counted for 10 random fields at 40x magnification. b and c) Flow cytometry was used to detect amounts of CD11b$^+$ cells and CD11b$^+$Ly6G$^+$ neutrophils present in the leukocyte population. d) Spot Advanced software was used to measure epidermal thickness in H&E tissue sections of all tissue samples. Five measurements over 7 random fields per sample were taken. Value given in average thickness detected for each group. e and f) S100A8 and S100A9 mRNA transcript was measured by QPCR for all mice. Values given are averages for each group.

Chronic Inflammation and Overexpression of TGFβ Causes Increased Epidermal Thickness, S100A8, and S100A9

TPA was administered to the shaved backs of ST and DT mice biweekly for 14 days, while doxycycline feed was administered for the last 12 days of TPA treatment. RNA harvested from the treated skin tissue exhibited significantly increased levels S100A8 (p=0.02) and S100A9 (p=0.05) cytokines in the DT mouse group when compared to the ST group (Figure 6). Epidermal thickness measurements performed on H&E sections of skin revealed a significant increase (p=0.03) in epidermal thickness in DT mice compared to ST mice, as well, 43μM compared to 30μM, respectively (Figure 6).

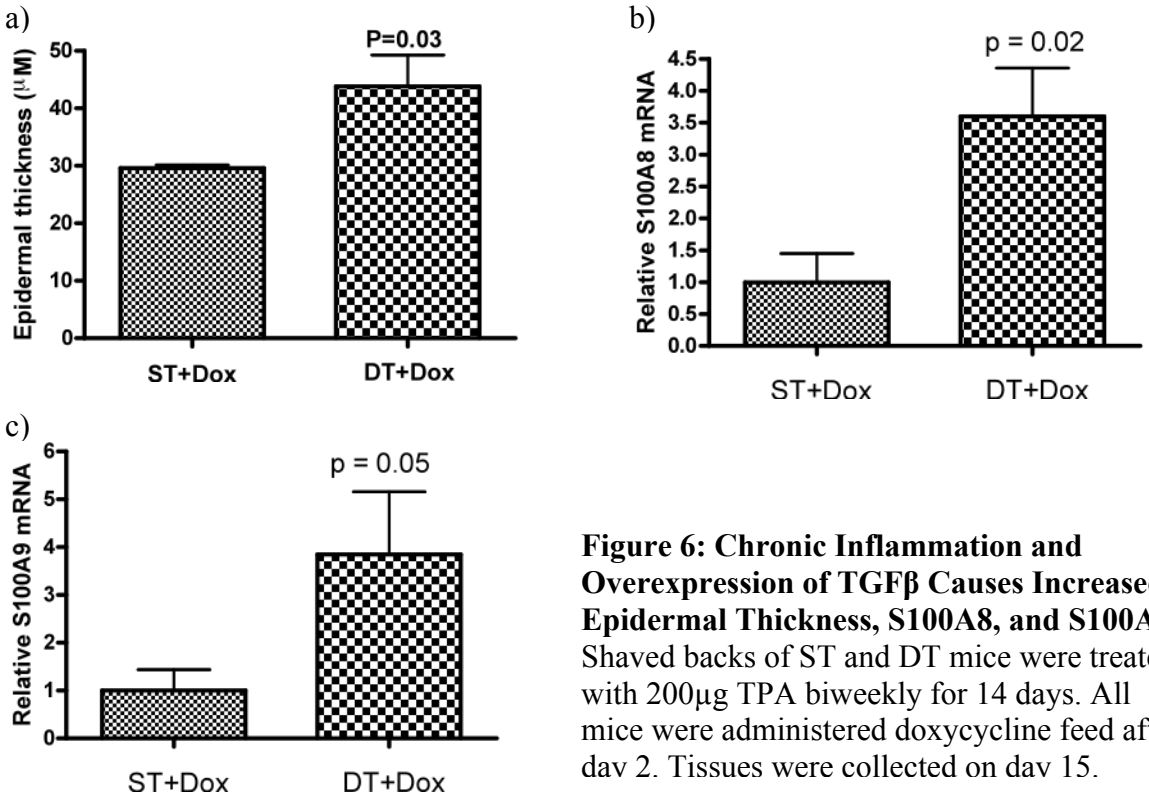

Figure 6: Chronic Inflammation and Overexpression of TGFβ Causes Increased Epidermal Thickness, S100A8, and S100A9 Shaved backs of ST and DT mice were treated with 200μg TPA biweekly for 14 days. All mice were administered doxycycline feed after dav 2. Tissues were collected on dav 15.

a) Spot Advanced software was used to measure epidermal thickness in H&E tissue sections of all tissue samples. Five measurements over 7 random fields per sample were taken. Value given in average thickness detected for each group. b and c) S100A8 and S100A9 mRNA transcript was measured by QPCR for all mice. Values given are averages for each group.

Chronic Inflammation and Overexpression of TGFβ Causes Increased MPO$^+$Cells

TPA was administered to the shaved backs of ST and DT mice biweekly for 28 days, while doxycycline feed was administered for the last seven days of TPA treatment. Additionally, a DT mouse control group was used that was not administered doxycycline. The number of MPO$^+$ cells in epidermis of the doxycycline treated DT mice, 13 MPO$^+$ cells/field, was significantly higher ($p<0.05$) than for either the doxycycline treated ST mice, 7 MPO$^+$ cells/field, or non-doxycycline treated DT mice, 7 MPO$^+$ cells/field (Figure 7).

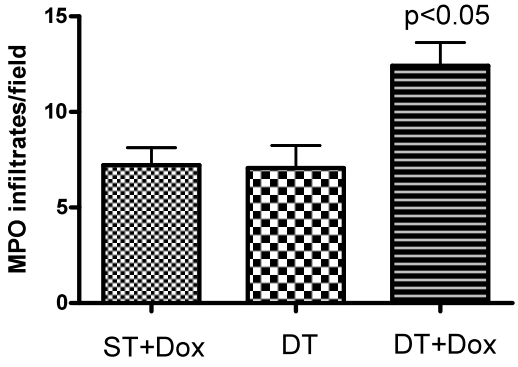

Figure 7: Chronic Inflammation and Overexpression of TGFβ Causes Increased MPO$^+$Cells
Shaved backs of ST and DT mice were treated with 200µg TPA biweekly for 28 days. Groups of ST and DT were administered doxycycline feed after day 21. Tissues were collected on day 29. A group of DT mice was also used what was not given doxycycline in their feed. MPO cell staining was performed on ethanol fixed tissue sections from ST and DT mice. Values are averages of each group. For each tissue section, MPO$^+$ cells were counted for 10 random fields at 40x magnification.

Discussion

TGFβ is a unique cytokine, with diverse and often opposing functions dependent on context. It has been given increased attention recently regarding its role in the immune system, as disregulation of TGFβ has been associated with both inflammatory disease and hyperplastic disorders. Chronic inflammation is also positively correlated with promotion of cancer development. In this regard, TGFβ is an important cytokine in the field of cancer research due to its known roles in regulating both proliferation and inflammation. Specifically in this study, TGFβ has been viewed for its influence on inflammation and immune cell behavior in the epidermis of mice using a doxycycline controlled keratin-5 promoter transgene system. To develop a sense of the context dependence of TGFβ function in inflammation, TGFβ was overexpressed acutely and chronically in both normal and inflamed skin.

Recent cancer research has focused on two proinflammatory, neutrophil-secreted cytokines, S100A8 and S100A9. This interest was established after discovery that these two proteins are strongly upregulated in the epidermis during skin cancer, as well as other human epithelial tumors. Previous research has demonstrated that S100A8 and S100A9 are also significantly upregulated within keratinocytes upon TPA induction of inflammation in the skin. S100A8 and S100A9 are potent chemokines for neutrophils, activated monocytes, and macrophages. Since S100A8 and S100A9 are also expressed by these cell types, this causes further leukocyte recruitment via a positive feedback type mechanism. Therefore, these two cytokines are of particular interest for this study due the fact that they hold proinflammatory and carcinogenic roles, as does TGFβ.

An initial interesting feature of TGFβ overexpression in normal skin is that at both acute and chronic time points, overexpression of TGFβ caused significantly increased levels of MPO^+ cells in the epidermis. Although the numbers of MPO^+ cells for both time points were considerably lower when compared to the values observed in the epidermis for the TPA-treated inflamed skin, a distinct increase in MPO infiltration is observed in the normal skin between chronic and acute TGFβ expressing skin. This indicates that though the chemotactic properties of TGFβ expression in the epidermis are amplified over time, the effect is still observable four days after expression. Strengthening the link between TGFβ directly influencing recruitment of MPO^+ cells, it can be seen that these cells tend to be localized at the basal epithelium, exactly where to K5 promoter is causing expression of TGFβ.

Therefore, we measured expression of cytokines associated with MPO^+ cells and TGFβ that may give rise to an inflammatory response since this increase in MPO^+ cells could be indirect through an increase in proinflammatory cytokines. Among those examined by QPCR, S100A8 and S100A9 were the primary cytokines found to be significantly upregulated in this skin. This data and previous studies support the idea that S100A8 and S100A9 are both expressed by and chemotactic for MPO^+ cells. These cytokines can then give rise to an inflammatory response, which over time could promote development of neoplasia in the skin. Increased expression on S100A8 and S100A9 also helps to explain why the number of MPO^+ cells increases in the normal skin from acute to chronic induction of TGFβ. As noted above, the expression by and chemotactic properties of S100A8 and S100A9 on MPO^+ cells result in a positive feedback mechanism of leukocyte recruitment. Therefore, as more S100A8 and S100A9 are expressed, more leukocytes will follow, further increasing the amount of these

proinflammatory cytokines and subsequent chemotaxis of MPO^+ cells. This positive feedback mechanism in ways echoes the disregulation of uncontrolled growth associated with neoplasia.

We then tested the effect of acute and chronic TGFβ induction within the context of chronically inflamed skin in order to assign contextual roles to TGFβ in inflammation. Again, significantly increased levels of MPO^+ cells were observed in chronically inflamed skin overexpressing TGFβ both acutely and chronically. This is compared to the ST mice, which exhibited chronically inflamed skin without induction of TGFβ, indicating that TGFβ further exacerbates the inflammatory response evoked by treatment with TPA.

For the acute TGFβ induction, the MPO cells present were further classified through flow cytometry. Both $CD11b^+$ and $CD11b^+Ly6G^+$neutrophils were significantly increased by induction of TGFβ. Furthermore, QPCR analysis of RNA from these tissues showed that TGFβ induction significantly increased levels of S100A8 and S100A9, as well. These pieces of data operate cooperatively given that S100A8 and S100A9 are known to recruit and be secreted by macrophages and neutrophils. Therefore it is not surprising to find these cells and cytokines simultaneously upregulated, as was seen in the normal skin.

To supplement the upregulation of inflammatory cytokines and increased infiltration of inflammatory cells to the epidermis, epidermal thickness measurements were taken for the chronically inflamed skin. Overexpression of TGFβ caused a significant increase in epidermal thickness when compared to mice not expressing the tetoTGFβ transgene. This was true for inflamed skin after both acute and chronic TGFβ induction. This data again supports that TGFβ contributes to an inflammatory response, amplifying the effects of TPA treatment alone.

An interesting result regarding TGFβ induction in inflamed skin is that although TGFβ overexpression intensified the effects of inflammation during both acute and chronic TGFβ

induction, the extent of this exacerbation was noticeably less for chronic inflammation compared to the acute time point. The number of MPO^+ cells and epidermal thickness were distinctly lower for the DT mouse group during chronic TGFβ induction than in acute induction. This incites the question of what may occur should the for longer TGFβ induction time in inflamed skin. Perhaps this declining effect may be indicative of the diverse nature of opposite TGFβ functions, both promoting and suppressing inflammation depending on the context. Nevertheless, the data from this study still supports that TGFβ increases the inflammatory response in both normal and inflamed skin.

Although this study reveals a clear relationship between TGFβ leading to an increased inflammatory response, the mechanism of this action has not been completely elucidated. Several points can be made however, placing key pieces of the puzzle together. Though, the order of these pieces will require further investigation. It is apparent that overexpression of TGFβ results in increased levels of S100A8, S100A9, MPO^+ cells in the epidermis, and epidermal thickness. It is likely fair to attribute the increase in epidermal thickness to the well-established proinflammatory properties of S100A8, S100A9, and MPO^+ cells in general.

An area of ambiguity exists though regarding induction of S100A8 and S100A9 and the recruitment of MPO^+ cells. Both TGFβ alone and S100A8 and S100A9 are chemotactic for neutrophils and macrophages. Neutrophils and macrophages are also known to secrete S100A8 and S100A9 as part of the previously mentioned positive feedback mechanism of leukocyte recruitment. However, previous research has also shown that inflamed keratinocytes have upregulated expression of S100A8 and S100A9. Therefore, it is important to question whether the presence of S100A8 and S100A9 is primarily immune cell or keratinocyte derived.

To investigate this, in-vitro keratinocyte cultures of ST and DT cells were set up during which doxycycline was added for three days. After three days, the levels of S100A8 and S100A9 were measured between ST and DT groups (data not shown). The data from this analysis was overall inconclusive and will be repeated in the future to establish whether the keratinocytes are producing significant levels of S100A8 and S100A9 alone upon induction of TGFβ.

One possibility is that both the immune cells and inflamed keratinocytes are contributing substantially to the levels of S100A8 and S100A9. However, the relative increases of S100A8 and S100A9 mRNA levels detected by QPCR in the TGFβ overexpressing mice are not significantly different between normal and inflamed skin. It would be logical to believe that if the keratinocytes were major contributors to S100A8 and S100A9 expression in this model that the inflamed TPA-treated keratinocytes would be expressing significantly higher levels of S100A8 and S100A9 than the normal keratinocytes. Therefore one would expect to see a noticeable increase in S100 mRNA transcript between ST and DT groups for inflamed skin over normal skin. As this increase in difference in not observed, I would hypothesize that a majority of the S100A8 and S100A9 are immune cell derived.

It is not clear whether TGFβ itself or S100A8 and S100A9 are primarily responsible for recruiting MPO^+ cells to the epidermis. However, since TGFβ is induced first, it is likely to be responsible for an initial recruitment of MPO^+ cells. At this point, TGFβ and the MPO^+ cells may cause inflammation in the keratinocytes, leading to their upregulation of S100A8 and S100A9. This upregulation would then amplify recruitment of MPO^+ cells and give rise to the positive feedback mechanism.

Alternatively, TGFβ induction may influence the keratinocytes to upregulate S100A8 and S100A9, which then go on to recruit MPO^+ cells. In this scenario, little MPO^+ cells are recruited

before S100A8 and S100A9 are expressed. Based on the idea that S100A8 and S100A9 are mostly immune cell derived, I would hypothesize that TGFβ is responsible for the early recruitment of MPO^+ cells, while in the later inflammatory response S100A8 and S100A9 are the major contributors to MPO infiltration due to the positive feedback-based expansion. What remains in question though, and what the order of events hinges on, is whether TGFβ is inducing MPO cells or the keratinoctyes to increase S100A8 and S100A9.

One limitation of this study to be noted was the fact that occasionally mice in the doxycycline treated DT groups would become ill. These mice presented typically with a lethargic and apathetic demeanor, as well as alopecia and a swollen appearance of the skin over their whole bodies. Although the K5rTA system is designed to localize TGFβ overexpression to the epidermis, this is possibly due to a systemic increase of TGFβ. TGFβ overexpressed in the epidermis may enter into the blood stream due to increased vasodilation associated with inflammation in the skin, leading to a more systemic illness. The reason for this occasional observation of systemic sickness could be due perhaps either to genetic hypersensitivity or high consumption of the doxycycline feed.

Conclusion

The results of this study confirm a link between TGFβ and induction of inflammation, further elucidating one mechanism of such induction. This comes in the context of chronic inflammation as a known promoter for neoplasia. Therefore, it is important to evaluate TGFβ as a tumorigenic cytokine, although known for holding diverse immunomodulatory and inflammatory roles. More specifically, it was found that overexpression TGFβ is able to induce increased levels of S100A8 and S100A9, MPO^+ cell recruitment to the epidermis, and epidermal thickness in both normal and chronically inflamed skin. These effects could be seen after only four days of TGFβ induction, and were amplified over time in normal skin. The importance of this study comes from the elucidation of a pathway by which TGFβ supports hyperplasia and subsequent tumor development. Understanding the complex mechanistic nature of neoplasia is vital in directing future therapeutic efforts. Future courses of this research could focus on the mechanism through which TGFβ upregulates S100A8 and S100A9 expression, and how longer-term TGFβ induction influences chronically inflamed skin.

References

1. Li, Ming O., Y. Y. Wan, S. Sanjabi, A. L. Robertson, and R. A. Flavell. 2006. Transforming Growth Factor-β Regulation of Immune Responses. *Annu. Rev. Immunol.* 24:99-146.

2. Talyor, A. W. 2009. Review of the activation of TGF-β in immunity. *J. Leukocyte Biol.* 85: 29-33.

3. Yoshimura, A., Y. Wakabayashi, and T. Mori. 2010. Cellular and molecular basis for the regulation of inflammation by TGF-b. *J. Biochem.* 147:781-792.

4. Glick, A. B., A. B. Kulkarni, T. Tennenbaum, H. Hennings, K. C. Flanders, M. O'Reilly, M. B. Sporn, S. Karlsson, and S. H. Yuspa. 1993. Loss of expression of transforming growth factor β in skin and skin tumors is associated with hyperproliferation and a high risk for malignant conversion. *Proc. Natl. Acad. Sci. U S A.* 90:6076-6080.

5. Liu, X., V. Alexander, K. Vijayachandra, E. Bhogte, I. Diamond, and A. Glick. 2001. Conditional epidermal expression of TGFβ1 blocks neonatal lethality but causes a reversible hyperplasia and alopecia. *Proc. Natl. Acad. Sci. U S A.* 98:9139-9144.

6. Li, A. G., D. Wang, X. Feng, and X. Wang. 2004. Latent TGFb1 overexpression in keratinocytes results in a severe psoriasis-like skin disorder. *EMBO J.* 20:1770-1781.

7. Mohammed, J., A. Ryscavage, R. Perez-Lorenzo, A. Gunderson, N. Blazanin, and A. B. Glick. 2010. TGFb1-Induced Inflammation in Premalignant Epidermal Squamous Lesions Requires IL-17. *J. Invest. Dermatol.* doi:10.1038.

8. Bachman, K. E., B. Park. 2005. Duel nature of TGFβ signaling: tumor suppressor vs. tumor promoter. *Curr. Opin. Oncol.* 17:49-54.

9. Gebhardt, C., J. Nemeth, P. Angel, and J. Hess. 2006. S100A8 and S100A9 in inflammation and cancer. *Biochem. Pharmacol.*

Academic Vita

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Education

Schreyer Honors College of The Pennsylvania State University, University Park, PA

- Bachelor of Science in Immunology and Infectious Diseases
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Undergraduate Research Assistant in Viral Biology Laboratory at The Pennsylvania State University, University Park, PA; April 2008-January 2009, November 2009-Present

- Worked independently completing Honors Thesis with focus in Immunology
- Contributed data to two individual post-doctoral research papers pending publication in the field of immunology
- Gained proficiency in laboratory techniques such as western blot analysis, PCR and real-time PCR, DNA and RNA extractions, and blood serum analysis

Laboratory Technician in Viral Biology Laboratory at The Pennsylvania State University, University Park, PA; May 2010-September 2010

- Managed mouse room for eleven person laboratory via weaning, extracting DNA, and genotyping mice

Activities

- Volunteer in Emergency Room and Patient Floors at Mount Nittany Medical Center 2-3 times each month; June 2007-Present
- Member of Atlas Thon team at The Pennsylvania State University; August 2007-Present
- Member of Reformed University Fellowship at Penn State; August 2007-Present
  - Serve on Ministry Leadership Team: Determine future direction of ministry program and lead weekly Bible studies; 2009-2011 academic years
- Deep Six Racing Team; 2005-2008
  - Founder and Team Captain
- Proficient in the Spanish language

References

- Academic and personal references available upon request