Yale University
EliScholar – A Digital Platform for Scholarly Publishing at Yale

Yale Medicine Thesis Digital Library | School of Medicine

2000

TGF-[beta] is a crucial counter-regulatory cytokine that inhibits the proliferation and function of CD4+ Th1 cells responsible for colitis inmurine models of Crohn's disease

Andrew S. Resnick
Yale University

Follow this and additional works at: http://elischolar.library.yale.edu/ymtdl

Recommended Citation
Resnick, Andrew S., "TGF-[beta] is a crucial counter-regulatory cytokine that inhibits the proliferation and function of CD4+ Th1 cells responsible for colitis inmurine models of Crohn's disease" (2000). *Yale Medicine Thesis Digital Library*. 3061.
http://elischolar.library.yale.edu/ymtdl/3061

This Open Access Thesis is brought to you for free and open access by the School of Medicine at EliScholar – A Digital Platform for Scholarly Publishing at Yale. It has been accepted for inclusion in Yale Medicine Thesis Digital Library by an authorized administrator of EliScholar – A Digital Platform for Scholarly Publishing at Yale. For more information, please contact elischolar@yale.edu.

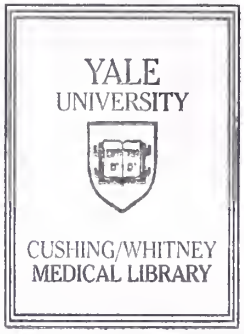
MED
T113
+Y12
6786

TGF-β Is A Crucial Counter-Regulatory Cytokine That Inhibits the Proliferation and Function of CD4+ Th1 Cells Responsible for Colitis in Murine Models of Crohn's Disease

Andrew S. Resnick

YALE UNIVERSITY

2000


YALE
UNIVERSITY
CUSHING/WHITNEY
MEDICAL LIBRARY

Permission to photocopy or microfilm processing of this thesis for the purpose of individual scholarly consultation or reference is hereby granted by the author. This permission is not to be interpreted as affecting publication of this work or otherwise placing it in the public domain, and the author reserves all rights of ownership guaranteed under common law protection of unpublished manuscripts.

Signature of Author

3/23/00

Date

Digitized by the Internet Archive
in 2017 with funding from
The National Endowment for the Humanities and the Arcadia Fund

https://archive.org/details/tgfbetaiscrucial00resn

TGF-β Is A Crucial Counter-Regulatory Cytokine That Inhibits the Proliferation and Function of CD4+ Th1 Cells Responsible for Colitis in Murine Models of Crohn's Disease

A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirements for the Degree of Doctor of Medicine

By

Andrew S. Resnick
Yale University School of Medicine
Class of 2000

YALE MEDICAL LIBRARY

JUL 27 2000

Abstract

TGF-β IS A CRUCIAL COUNTER-REGULATORY CYTOKINE THAT INHIBITS DNA SYNTHESIS AND FUNCTION OF CD4+ TH1 CELLS RESPONSIBLE FOR COLITIS IN MURINE MODELS OF CROHN'S DISEASE. Andrew S. Resnick and Bjorn R. Ludviksson. Mucosal Immunity Section. NIAID. National Institutes of Health. Bethesda, MD (Sponsored by Dr. John Seashore, Department of Surgery, Yale University School of Medicine).

In the late 1980s, it became apparent that under certain experimental conditions, CD4+ T cells would differentiate into either IFN-γ (Th1) or IL-4-producing (Th2) cells. In the past decade, murine models of Crohn's disease have demonstrated that this condition may reflect an over-active Th1 cell response. In several of these models, decreased colonic inflammation has been associated with increased levels of the cytokine, TGF-β. However, it is presently unknown how TGF-β affects the proliferation, cytokine production, or intra-cellular signaling found in Th1 cells.

We primed CD4+ Ova Tg T cells in conditions favoring Th1 differentiation, and also used the previously-established A.E7 Th1 cell line. In both cell lines, TGF-β produced a dose-dependent reduction of DNA synthesis during antigen-specific stimulation. In functional assays we found that TGF-β had a dose-dependent inhibitory effect on IFN-γ production (A.E7: 1 ng/ml TGF-β reduced IFN-γ production by 34%, $p<0.01$; 10 ng/ml reduced production by 61.9%, $p<0.007$. Th1 cell line: 1 ng/ml TGF-β reduced IFN-γ production by 20.9%, $p<0.017$; 10 ng/ml TGF-β reduced production by 30%, $p<0.0004$). However, this counter-regulatory effect of TGF-β was not due to the lack of IFN-γ or IL-2 since addition of these cytokines into the cultures did not reverse the effects of TGF-β. Furthermore, this effect was not attributed to apoptosis since addition of TGF-β did not induce apoptosis of Th1 cells significantly above baseline.

It is known that STAT4 phosphorylation is up-regulated by IL-12 during Th1 cell activation. Therefore, we evaluated whether TGF-β could interfere with IL-12 mediated signaling of Th1 cells. In both the Th1 and A.E7 cell lines, TGF-β significantly down-modulated the phosphorylation of STAT4. Together with the data on DNA synthesis and cytokine production, these experiments suggest that TGF-β regulates the function of fully-differentiated CD4+ Th1 cells by inhibiting the phosphorylation of the intracellular protein, STAT4.

Acknowledgements

I would like to begin by thanking Dr. Bjorn Ludviksson. When I arrived at the NIH for the start of my Howard Hughes Research Scholar year, I had no idea what kind of lab I wanted to work in. I must have sat down and talked with twenty P.I.'s and poured over countless papers, trying to figure out which lab would be the best fit for me. When I spoke with Dr. Warren Strober and Bjorn, I knew that I would like the lab. Bjorn guided me closely in the beginning and allowed me to have a lot of independence by the end. I enjoyed shmoozing with him as much as I did learning how to do immunology research and we had a very fun year, from the lunches at Howard Hughes HQ to those few times when we convinced Ryuta to come to Hooters with us. Bjorn is back in Iceland right now and I hope that I have time to go visit him.

The other members of the Strober lab were instrumental to my success. Dr. Strober gave me the freedom to do whatever I wanted in lab and it made a tremendous difference for me. My goal to become an academic surgeon was confirmed as I was allowed to think for myself, rather than get plugged into someone else's project. Kevin Chua was extremely patient when I needed him to zap my cells for me, and I needed him to do this a lot…Ryuta Nishikomori donated countless materials to my madness and was always a pleasure to talk to, whether it was a lesson in Japanese or a conversation about sports. Tina McIntyre had a desk right around the corner from me and we had a lot of fun together. She not only let me throw internet pies at her but she gave me the use of her car to learn how to drive a stick shift. Ash Jain, Gerd Bouma, Bjorn, and I spent a lot of lunches together and it was nice to know that people in science have a healthy sense of humor.

Yale Medical School is a special place for me and it would not have been the same without Dr. John Seashore. I came to medical school with the idea of becoming a pediatric surgeon and quickly seized the opportunity to secure Dr. Seashore as my clinical tutor. Having a pediatric surgeon as a mentor in medical school was priceless in keeping me focused throughout all the tough times. Our group's long dinners at their house, with discussions on everything from world politics to hospital politics, ethics and just about anything one could think of, will be a lasting memory as I leave New Haven in May. I would especially like to thank him for volunteering to be my thesis advisor at Yale, as he is busy with so many things and still generous with his time.

I would also like to thank Dartmouth College's own Dr. Robert McClung. If I hadn't learned how to do good research, had an amazing experience working with Rob for three years, and met a lot of great people in his lab, I would never have appreciated basic science to begin with. All of Rob's help in preparing my college thesis is still paying off as I write this one.

Finally, I would like to thank everyone in my family, who have continued to support me throughout everything. It seems every step gets more challenging as I move from high school to college, college to medical school, and soon medical school to internship. My parents always encourage me to excel and to choose my own path in life and I am certain that I would not be here if it were not for them.

TABLE OF CONTENTS

Introduction

Author's introductory note

The research completed for this thesis was stimulated by the findings of Dr. Warren Strober and his peers, who found that in their murine models of Crohn's disease (see text), there was an overabundance of inflammatory T cells (Th1 cells) (1). Dr. Strober's group and others have found that when inflammation in these murine models is suppressed by various methods, the cytokine, transforming growth factor beta (TGF-β) is expressed at high levels. In addition, it has been shown that by inhibiting this cytokine, inflammation is restored in the model. Therefore, the hypothesis that was the impetus for this research was that TGF-β inhibits the Th1 cell development responsible for inflammation in the murine models of Crohn's disease. Through experiments measuring DNA synthesis, cytokine production, and intracellular phosphorylation, this hypothesis was tested.

Abbreviations:

A.E7: CD4+ T helper type I cell type
APC: Antigen presenting cell
B10.A: transgenic mouse strain
BALB/c: transgenic mouse strain
CD3: T cell Ligand
CD4: CD4+ T cell Ligand
DNCB: 2,4-dinitrochlorobenzene
EAE: Experimental allergic encephalomyelitis
FCS: Fetal calf serum
GALT: Gut-associated lymphoid tissue
HBSS: Hepes Buffered Saline Solution
HRP: Horseradish peroxidase
IFN-γ: Interferon gamma
IgA: Immunoglobulin A
IL-2$^{-/-}$: IL-2 knockout
IL: Interleukin
MBP: myelin basic protein
NK cell: natural killer cell
OVA: ovalbumin
PBS: phosphate buffered saline
PCC: pigeon cytochrome c peptide
SED: subepithelial dome
STATs: signal transducers and activators of transcription
TCR: T-cell receptor
TGF-β: Transforming Growth Factor Beta
Th1 cell: CD4+ T helper cell type I
Th2 cell: CD4+ T helper cell type II
TNF-α: tumor necrosis factor alpha
TNP: 2,4,6-trinitrophenol

Inflammatory Bowel Disease

Inflammatory bowel disease was first characterized in the late 19th and early 20th centuries. Crohn's disease is primarily a transmural inflammation of the wall of the gastrointestinal tract. The complications of the disease include bowel obstruction, fistulas, and abscess formation (1). Fistulas are a particularly insidious complication and are found in one-third of patients (2). Crohn's disease can occur anywhere in the entire gastrointestinal tract but often involves the terminal ileum. There are "skip lesions," meaning that the areas of inflammation are interspersed with normal areas. Histologically, the lesions in Crohn's disease consist of a dense accumulation of activated T cells and macrophages (1). In some cases, granulomas may form. The clinical course of Crohn's disease is a recurrence of episodes of abdominal pain and symptoms of bowel obstruction. More than 50% of patients will require surgery at some point in their lives. Medical therapy is with immunosuppressant medication. However, neither medical nor surgical treatment is curative, as even with the complete surgical removal of diseased intestine, there is a 50% recurrence rate within ten years (1).

In comparison, ulcerative colitis involves the inflammation only of the mucosa, leading to the formation of ulcers (1). The lesions of ulcerative colitis are nearly always continuous, with no normal areas between inflamed areas of the GI tract. Ulcerative colitis usually begins in the rectum and sigmoid colon and can progress in a proximal direction. Histologically, ulcerative colitis is also different from Crohn's disease. There is a larger variety of cellular infiltrate and

acute inflammatory events, such as the congregation of neutrophils into crypt abscesses, are quite prominent (1). Treatment for ulcerative colitis is the same, with immunosuppressant medication. Depending on the extent of the disease and the presence of any associated dysplastic areas of the colon, surgery is a curative option for patients with ulcerative colitis (1).

Immunology and the GI Tract

The majority of the contact between foreign antigens and the host occur at mucosal surfaces (3). Although most dietary antigens are degraded by the time they reach the small intestine, studies in both rodents and in humans show that some undegraded antigens are absorbed by the gastrointestinal tract and are later found in the systemic circulation (3). The concentration of bacteria in the colon is very high, with over 10^{12} microorganisms found in each gram of stool. The normal flora provides a continuous source of antigenic stimulation to the gut. In order to handle the dietary antigens and the gastrointestinal microbes, the GI tract has an abundance of lymphoid tissue. In fact, there are 10^{12} lymphoid cells per meter of small intestine in the human (3). It has been postulated that the number of antibody-secreting cells in the human gut exceeds the number of these cells elsewhere by severalfold (3).

The gut-associated lymphoid tissue (GALT) system has been extensively studied (4). Lymphoid cells congregate into structures known as Peyer's patches. M cells, which are specialized cells present in the follicle-associated epithelium, transport antigens from the lumen of the gastrointestinal tract into the

subepithelial dome (SED) region of the germinal follicles in the Peyer's patch (4). In this area, the antigens encounter dendritic cells, macrophages, T cells, and B cells. In the GALT system, the B cells stimulated in this manner preferentially produce IgA. After the initial stimulation of IgA-producing B cells in the Peyer's patch germinal center, the B cells leave the Peyer's patch and migrate to draining lymph nodes and then to the lamina propria underlying mucosal surfaces. At this point, the B cells differentiate into plasma cells and function to produce IgA that is ultimately transported into the lumen of the gastrointestinal tract (4).

Parallel to the activation of B cells in the Peyer's patch is the activation of T cells (see Figure 1). It is thought that like B cells, T cells come into contact with dendritic cells in the subepithelial dome (SED) region of the follicles of the Peyer's patch. T cells are activated and can then migrate out of the Peyer's patch and can disseminate to not only the lamina propria of mucosal tissue but also to other lymphoid sites (4).

There are three main immunological responses to the presence of antigen in the gastrointestinal tract (3). The first response is a local, noninflammatory immune response that results in the production of secretory IgA. As discussed above, antigen is transported by M cells into the Peyer's patch, where it is taken up by dendritic cells and presented to B cells, stimulating the production of specific IgA against the antigen.

The second immunological response is a systemic inflammatory response, with the production of antibodies in the serum. This is a rare event and

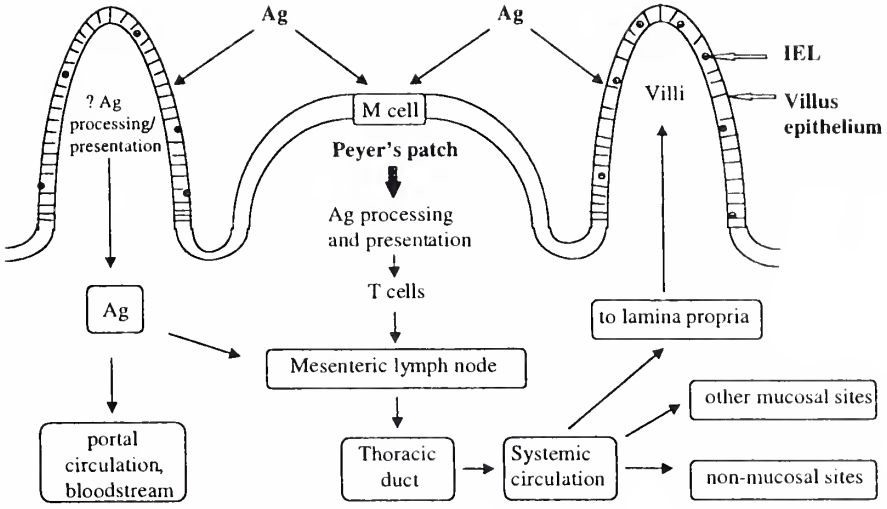


FIGURE 1—T cell movement in the gut-associated lymphoid tissue (GALT). M cells are found in the epithelium near lymphoid follicles called Peyer's patches. The M cells transport antigen from the lumen of the gastrointestinal tract into the lymphoid nodules. There, dendritic cells and macrophages take up antigen and present it to T cells. This activates the T cells, which then migrate out of the Peyer's patch and can travel to both mucosal sites and to the systemic circulation. Adapted from Faria *et al.* (3)

is associated with hypersensitivity reactions to proteins in the diet, or overwhelming inflammatory reactions to bacterial invasion (3).

The final response is a systemic hyporesponsive state known as oral tolerance (3). This response is the most common consequence to the ingestion of an antigen. With a plethora of harmless proteins and microbes present in the gastrointestinal tract, it is apparent why this response should be so dominant. Because of its importance to the understanding of the immune system and its potential utility in the treatment of Crohn's disease and other autoimmune diseases, the theory of oral tolerance is discussed at length below.

Th1 versus Th2 cells

Before discussing the immune cells involved in the pathogenesis of Crohn's disease, it is necessary to define the types of CD4+ T cells involved in this process. There are two types of CD4+ T cells (4), T helper 1 and T helper 2 (Th1 and Th2). It is thought that both Th1 and Th2 cells differentiate from a common precursor cell, the naïve T cell. The process governing the differentiation of naïve T cells into Th1 and Th2 cells is not fully understood at this time. However, it is thought that three main factors contribute to it. Firstly, the cytokine milieu to which the T cell is exposed at the time of antigenic stimulation. Secondly, the nature and quantity of the antigen. Thirdly, the type of antigen presenting cell (APC) involved in the stimulation process (4).

Once differentiated, Th1 and Th2 cells have different functions. Th1 cells produce the cytokines interleukin-2 (IL-2), interferon-γ (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukin-3 (IL-3), and granulocyte-macrophage colony stimulating factor (GM-CSF). Th2 cells produce interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-10 (IL-10), interleukin-13 (IL-13), interleukin-3 (IL-3), and GM-CSF (4). Each type of CD4+ T cell produces different autocrine growth factors, with Th1 cells producing IL-2 and Th2 cells producing IL-4. Th1 cells are responsive more to macrophages than B cells, whereas Th2 cells are more responsive to B cells than to macrophages. When involved in inflammatory reactions, Th1 cells induce the proliferation of predominantly neutrophils, while Th2 cells induce the proliferation of predominantly eosinophils (4).

Crohn's Disease and the Immune System

The study of the immunoregulatory pathways that might be involved in Crohn's disease was not begun until the early 1960s (5). The first studies trying to link the immune system with inflammatory bowel diseases were done by Kirsner, who created several animal models of mucosal inflammation (5). In a review article by Strober *et al.*, two groups of murine models (see Figure 2) are described (5). These models are extremely useful because they not only provide insight into the immunological aberrations found in inflammatory bowel disease, but can also be used to create potential immunological treatments for the disease (1).

The first group consists of "spontaneous" or "induced" models in which the inflammatory cytokine, IL-12 (interleukin-12), drives the mucosal inflammation (Figure 2) (5). Inflammatory cytokines, such as IL-12, are mediators that result in an influx of lymphocytes, leading to inflammation. Th1 cells, which are T cells that have differentiated to produce inflammatory cytokines such as IFN-γ and TNF-α, are produced. The classic model for this type involves injecting the compound, 2,4,6-trinitrophenol (TNP)-substituted protein plus adjuvant into IL-$2^{-/-}$ mice (6). The histology shows a massive lymphocyte and macrophage infiltration of the colon wall. Similar to Crohn's disease in humans, the inflammation is present in the full thickness of the wall. In both this murine model and in Crohn's patients, the lymphocytes found in the sites of inflammation are mostly Th1 cells. It has been proposed that Crohn's disease represents an inadequate secretion of the counter-regulatory (i.e., inhibitory) and anti-inflammatory cytokine, transforming growth-factor beta (TGF-β) (1). In the IL-$2^{-/-}$ mice injected with TNP, there is a failure of TGF-β production after injection with TNP (6). It has been proposed that the model is similar to Crohn's disease because the IL-$2^{-/-}$ mice given TNP develop a Th1 response that is not appropriately counter-regulated by TGF-β (6).

The second category of model for inflammatory bowel disease is the Th2 cell model. In contrast to Th1 cells, Th2 cells are differentiated T cells that secrete the cytokine, IL-4, and stimulate antibody production from B cells, but do not produce inflammatory cytokines. The T-cell receptor knockout mouse represents the classic form of this model. A newer model in this category is

rectally-administered oxazolone. Although the two models seem very different, in both, the mechanism is a Th2-mediated process that results in the production of IL-4. The inflammation in the gastrointestinal tract is superficial. The histologic picture is similar to that seen in human ulcerative colitis (5).

It has been proposed by many that inflammatory bowel disease represents an imbalance between Th1 and Th2 cell development. In other words, because of a combination of genetics and the environment, patients with Crohn's disease have an overactive Th1 cell response, whereas patients with ulcerative colitis have an overactive Th2 cell response (5). Evidence for the role of genetics is provided by the fact that the incidence of both Crohn's disease and ulcerative colitis shows a strong familial pattern (1). Evidence for the role of the environment is provided by the fact that the incidence of Crohn's disease is increased in urban environments (1).

Characterization of the imbalance of Th1 and Th2 cells in humans with Crohn's disease is provided by Fuss *et al.*, who isolated CD4+ T cells from intestinal lesions in patients with both Crohn's disease and ulcerative colitis (1). The CD4+ T cells from Crohn's patients produced twice as much interferon-γ (IFN-γ) compared to CD4+ T cells from controls or patients with ulcerative colitis (1). Fuss also isolated CD4+ T cells from the peripheral circulation of patients with Crohn's disease and showed that these T cells secreted three times as much IFN-γ as did the peripheral T cells from controls or patients with ulcerative colitis (1). Additional studies showed that the intestinal T cells from Crohn's patients also secreted less IL-4 and IL-5 than T cells from controls. The cytokine

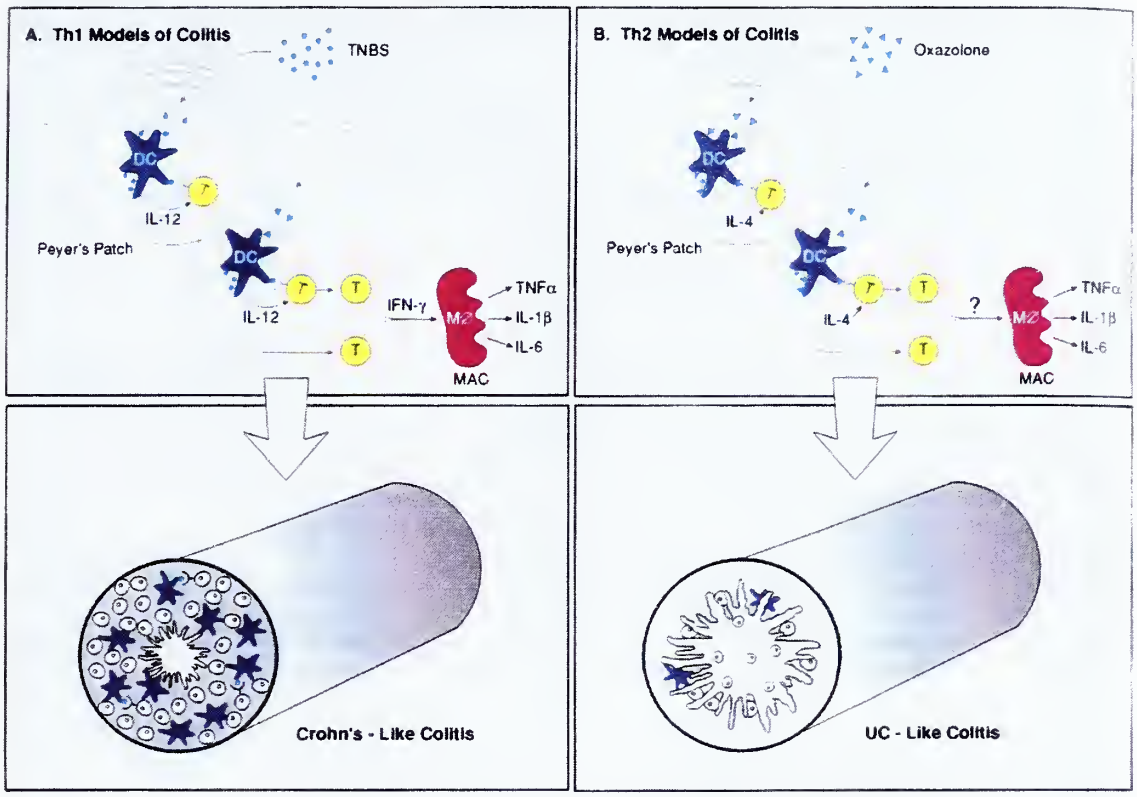


FIGURE 2—There are two categories of murine models of inflammatory bowel disease. The first group is made up of Th1 models, such as the TNBS colitis model depicted in the figure. The inflammatory lesion is characterized by increased levels of IFN-γ. The second group is made up of Th2 models, such as the oxazolone model above. In this group, the inflammatory lesion is characterized by increased levels of IL-4. Used with permission from Strober *et al.*, 1998.

profile of T cells from Crohn's lesions (increased interferon-γ and decreased IL-4 and IL-5 production) is consistent with Th1 cells. In contrast, the cytokine profile of T cells isolated from ulcerative colitis lesions (decreased production of interferon-γ and increased production of IL-5) is partially consistent with a Th2 imbalance, although there is no elevated IL-4 present (2).

With evidence that the lesions found in patients with Crohn's disease have an overactive Th1 cell population, Neurath *et al.* did a series of experiments to try to show that IL-12, which is the key stimulant of Th1 cell activation, is found in greater amounts in the lesions of patients with Crohn's disease (1). It is known that IL-12 is produced by antigen-presenting-cells (APCs) when they interact and stimulate Th1 cells (1). Neurath *et al.* showed that inflamed tissue from patients with Crohn's disease stained positive for IL-12, whereas tissue from controls and from patients with ulcerative colitis did not (1).

The above information is the foundation for the model that Crohn's disease represents an overactive stimulation of Th1 cells. The mucosa of the gastrointestinal tract is exposed to an initiating antigenic stimulus. Antigen-presenting cells produce an increased level of IL-12, which stimulates a dysregulated, over-active Th1 cell response. The Th1 cells secrete increased levels of interferon-γ, which in turn, activates macrophages to secrete inflammatory cytokines, such as TNF-α, IL-6, and IL-1β, which results in inflammatory lesions in the gastrointestinal tract. The cycle is self-perpetuating, because high levels of TNF-α and interferon-γ further stimulates the activation of antigen-presenting cells and the secretion of additional IL-12 (1).

Many questions remain, however. Does this represent an under-activation of counter-regulatory T cells? Is there a deficiency of counter-regulatory cytokines? What are the counter-regulatory cytokines? What effect do they have on the pathways involved in the activation of Th1 cells? The bridge between the knowledge of the immunology of Crohn's disease and the knowledge of the counter-regulatory elements involved in T cell suppression is derived from the ancient concept of oral tolerance.

Crohn's Disease and Oral Tolerance

It has been known for centuries that one can alter the immune system through a mechanism known as oral tolerance. Research in the field of oral tolerance has demonstrated that anti-inflammatory cytokines are produced by counter-regulatory T cells in response to the ingestion of various antigens (7). Because Crohn's disease may represent a deficiency in the production of counter-regulatory T cells and anti-inflammatory cytokines, oral tolerance is therefore very important in the understanding of, and possibly could have a therapeutic role in the treatment of, inflammatory bowel disease.

The study of the balance of Th1 and Th2 cells really pre-dates any knowledge of their existence, as the concept of oral tolerance was described in the nineteenth century, when it was first recognized that by ingesting an immunogenic compound, it was possible to manipulate the immune system and to reduce or preclude a systemic reaction to that same compound at a later time

(8). In fact, the terminology was quite different, since none of these concepts were known at the time. However, there was a report commenting on the use of oral administration of Rhus leaves to Native American children to prevent sensitization to poison ivy. Dakin wrote, "some good meaning, mystical, marvelous physicians, or favoured ladies with knowledge inherent, say the bane will prove the best antidote, and hence advise the forbidden leaves to be eaten, both as preventive and cure to the external disease." (3). This is quite extraordinary, considering that there was no such knowledge of a B cell, T cell, or antibody at the time.

In 1911, Wells fed hen's egg proteins to guinea pigs and discovered that the guinea pigs were resistant to anaphylaxis when later challenged systemically with these egg proteins (9). In 1946, Chase fed guinea pigs the compound 2,4-dinitrochlorobenzene (DNCB) and demonstrated that the animals had decreased skin reactivity to the compound after ingesting it orally (10). Indeed, only 3% of animals given oral DNCB showed a high skin hypersensitivity response to the same compound, compared to 74% of control animals.

What mechanism is there in the gastrointestinal tract to account for the "oral tolerance" discovered so long ago? The gut-associated lymphoid tissue (GALT) consists of Peyer's patches, or lymphoid nodules, epithelial cells found in villi, and intraepithelial lymphocytes and lymphocytes found in the lamina propria (4). Inside Peyer's patches, there are both T and B lymphocytes, macrophages, dendritic cells, and germinal centers containing B lymphocytes (11). The Peyer's patches are associated with M cells, which take up antigen from the gut and

transfer it to antigen-presenting cells in the Peyer's patches. It has been known for some time that the major antibody-mediated response that arises in the GI tract is that of IgA B cells. If the Peyer's patches are the site of both the induction of oral tolerance and/or an antigen-specific antibody response, there must be some mechanism in this location to differentiate between the two (11). Further complicating the picture is the knowledge that there is more than one mechanism for oral tolerance. In fact, it has been shown that low doses of antigen favor "active suppression," which involves the generation of suppressive cells in the Peyer's patches, whereas high doses of antigen favor clonal anergy or deletion of responsive T cells (see Figure 3) (11).

So, we know that there are important regulatory events that take place in the Peyer's patches. These events determine the ultimate response to ingested antigens, whether these antigens are harmless *Escherichia coli* bacteria, food products, or cholera toxins. It was not known until recently what elements in the Peyer's patches were responsible for the "active suppression" taking place in this site.

TGF-β is a crucial counter-regulatory cytokine involved in oral tolerance

In 1992, Weiner *et al.* demonstrated that TGF-β played an important role in this process (12). Using their experimental allergic encephalomyelitis (EAE) model of multiple sclerosis in mice, they successfully induced oral tolerance to myelin

basic protein (MBP) and showed that this reduced symptoms of EAE (12). They demonstrated that after giving oral MBP to mice *in vivo*, there was reduced proliferation of splenic T cells *in vitro*. Therefore, they postulated that suppressive cells from the Peyer's patches must be inhibiting splenic T cells. In addition, they showed that by adding anti-TGF-β antibodies *in vitro* to splenic cell cultures, they could reverse the "oral tolerance" induced by MBP in a dose-dependent fashion (12). When they tried feeding various peptides to mice, they showed that there were increased levels of TGF-β found in splenic supernatants *in vitro* when they induced oral tolerance in the animals with any number of peptides, as long as the same peptide was used *in vitro*. In other words, if they administered oral MBP to the mice, they could measure increased TGF-β production from *in vitro* splenic cultures only if they stimulated the cultures with MBP. TGF-β producing T cells must be activated in response to the oral antigen, most likely in the Peyer's Patches, and then must be able to migrate to peripheral lymph nodes and to the spleen, where secondary stimulation in an antigen-specific manner activates these suppressor T cells to secrete TGF-β in this location. Furthermore, *in vivo* administration of anti-TGF-β antibody could reverse the oral tolerizing effects of MBP on clinical symptoms of EAE (12).

Several human trials have arisen from research on oral tolerance. Groups are using oral peptides of various sorts to treat many illnesses. Oral bovine myelin protein is being tested for the treatment of multiple sclerosis, chicken type II collagen is being tested for the treatment of rheumatoid arthritis, bovine s-antigen is in trials for treatment of uveoretinitis, and human insulin peptides are

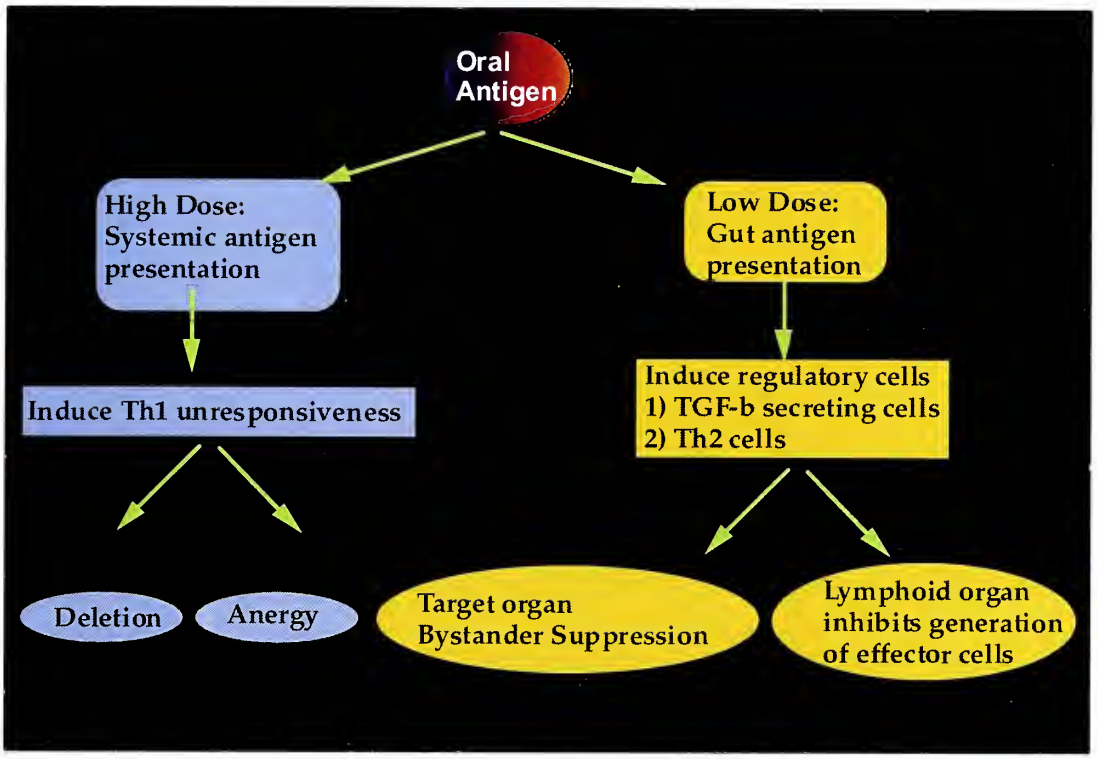


FIGURE 3—In this model, the presence of antigen in the gastrointestinal tract can lead to different responses by the host organism, depending on the concentration of antigen. A high concentration of antigen leads to the clonal deletion or global inhibition of T cells that recognize the antigen. However, when a low dose of antigen is present in the GI tract, counter-regulatory cells are produced that include TGF-β-secreting cells and Th2 cells, which actively inhibit the activation and proliferation of T cells that recognize the antigen. Adapted from Friedman *et al.,* 1994.

being tested for treating type I diabetes (13). In all trials, the theory is the same. By administering an antigen orally, suppressive T cells will be formed in the Peyer's patches, which will then migrate to peripheral lymph nodes and suppress an immunological response to the same antigen (13).

TGF-β, A Multi-faceted Peptide

TGF-β plays an important role in oral tolerance but it is not limited to this. There are at least three closely-related isoforms of TGF-β found in the human, with 70-80% homology among the isoforms (14). TGF-β1 is located on 19q13, TGF-β2 on 1q41, and TGF-β3 on 14q24. TGF-β is synthesized as a large precursor with an amino-terminal pro-domain and a mature carboxy-terminal subunit (15). The amino-terminal domain contains a signal sequence and a latency-associated protein, or LAP. Active TGF-β is a homodimer composed of 2 disulfide-linked mature subunits that are cleaved from the LAP.

There are 3 types of TGF-β receptors, types I-III (15). The functional receptor complex is a tetramer of two Type I and two Type II receptors, which are constitutively-active serine/threonine kinases. The two types of Type III receptors, betaglycan and endoglin, are proteoglycans that are involved in stabilization but not signaling (16). The initial step is the binding of TGF-β to the Type II receptor. Bound TGF-β is then recognized by the Type I receptor, which is recruited into the complex. The Type I receptor is then phosphorylated by the

Type II receptor and this propagates a signal to various intracellular targets called Smads (16). The TGF-β receptor complex phosphorylates Smad2 or Smad3, which then form a complex with Smad4, resulting in translocation of the complex to the nucleus. Type III receptors are believed to be binding proteins. Betaglycan is a membrane-anchored proteoglycan and has been shown to bind all three isoforms of TGF-β with high affinity and to facilitate the binding of TGF-β to the Type II receptor (16). The role of betaglycan is most evident with TGF-β2. Endoglin is the second kind of Type III receptor and has been shown to bind TGF-β1 and TGF-β3 but unlike betaglycan, it does not bind TGF-β2 (16).

There are 3 main biological activities of TGF-β (17). However, it is important to keep in mind that one confusing characteristic of TGF-β is its bifunctional nature (17). The first activity is the inhibitory effect that TGF-β has on the growth of most types of cells. The cytokine inhibits the cycling of epithelial cells in G1 phase via the inhibition of the cyclin dependent kinase-2/cyclin complex (15). However, TGF-β stimulates the growth of certain cell types, including Schwann cells, osteoblasts, and chondrocytes (17). The second activity is the immunosuppressive effect of TGF-β, which results from its anti-proliferative properties on both B and T cells. However, TGF-β stimulates production of IgA antibodies, possibly playing a role in IgA nephropathy, a renal glomeruloproliferative disease. The third activity is the enhancement TGF-β has on the deposition of extracellular matrix components, such as collagens, fibronectin, tenascin, glycosaminoglycans, and proteoglycans.

TGF-β is important in wound repair, as might be expected due to its effects on cellular proliferation and extracellular matrix formation (17). A high concentration of TGF-β1 is found in circulating platelets, where it plays a role as a chemoattractant for monocytes, neutrophils, and fibroblasts. In fact, local administration of TGF-β1 accelerates the healing of cutaneous wounds. However, excess TGF-β1 has been shown to cause formation of scar tissue. Some preliminary studies have shown that by giving antibodies to TGF-β1 and TGF-β2, together with active TGF-β3, one can enhance wound repair without scar formation (17). A potential use for TGF-β is in nerve injuries in the central nervous system. After a brain injury, TGF-β1 is expressed and causes the formation of scar tissue. The regeneration of the damaged nerve is impossible if scar tissue has formed. It might be feasible in the future to manipulate the levels of the various TGF-β isoforms in order to prevent CNS scar formation and allow nerve re-growth after damage.

The nitric oxide cascade, which is involved in neurotransmission, immunological tissue injury, and host defense against bacterial invasion, is suppressed by TGF-β1 (17). It has been shown that TGF-β1 inhibits the formation of nitric oxide in macrophages, bone marrow cells, cardiac monocytes, smooth muscle cells, and retinal pigment epithelial cells.

TGF-β is of obvious importance during infection (14). *Trypanosomas cruzi* triggers the activation of the TGF-β signaling pathway. This blocks the activation of macrophages by IFN-γ, thereby diminishing the oxidative response. Similarly,

infection of macrophages by *Leishmania* induces the production of TGF-β. Neutralizing antibodies to TGF-β can halt disease in a murine model.

Because solid tumors require the formation of a vascularized stroma, TGF-β is quite important in the progression of cancer (17). TGF-β stimulates the production of stromal elements, such as fibronectin. A high level of TGF-β stimulates stromal expansion, allowing the tumor to proliferate, and also results in immunosuppression, thereby inhibiting the immune response to the tumor. In some cancers, the loss of the type II TGF-β receptor has been correlated with the loss of growth inhibition. In some forms of leukemia, the impairment of the T cell response and the suppression of lymphokine-activated killer cells is linked to uncontrolled expression of TGF-β.

As might be expected, TGF-β is involved in inflammatory diseases (17). In rheumatoid arthritis, increased levels of TGF-β are found in the synovial fluid in affected joints. In fact, intra-articular injection of TGF-β in rodents leads to synovial erythema, whereas intra-articular injection of anti-TGF-β antibody can reverse synovial inflammation. However, systemic administration of TGF-β reverses the synovial inflammation as well. If TGF-β is mainly immunosuppressive, why, then, is its presence in joints correlated with inflammation? Why does systemic TGF-β reverse this inflammation? One theory is that localized administration of TGF-β1 enhances inflammation by increasing leukocyte adhesion and infiltration via its chemoattractive properties for inflammatory cells. Systemic administration might reverse this process because

TGF-β1 initially encounters the capillary endothelium, where it decreases endothelial cell expression of adhesion molecules (17).

Some other inflammatory diseases associated with TGF-β include uveitis, glomerulonephritis, atherosclerosis, and autoimmune disease (17). In uveitis, low levels of TGF-β are found. In glomerulonephritis, TGF-β might act by increasing IgA production, leading to IgA nephropathy, or by increasing the proliferation of smooth muscle in the glomerulus. In atherosclerosis, a lack of TGF-β might allow the proliferation of smooth muscle cells in the atherosclerotic lesion. In autoimmune disease, such as Multiple Sclerosis, there are increased levels of TGF-β secreted by cultured blood cells from patients during regression of exacerbations.

Because TGF-β is associated with many cellular processes and biological activities, a knockout mouse was made for each isotype of this cytokine (17) (18). The first few experiments using TGF-β1$^{-/-}$ mice demonstrated that the mice could be grouped into two phenotypes. One phenotype was death in mid-gestation due to defects in yolk sac vasculogenesis and hematopoiesis (18). The second phenotype was death at three weeks after birth due to multi-system inflammation (18). Interestingly, it was noted that the vascular defects found in TGF-β1$^{-/-}$ mice were similar to lesions found in patients with Hereditary Hemorrhagic Telangiectasia (HHT) (18). The varying, but inevitably fatal, effects of the knockout condition were initially explained by the presence of maternal TGF-β, which was thought to rescue some of the embryos (18). In more recent studies, Akhurst has shown that it is the genetic background of the mice that

determines whether the mouse will die during embryogenesis or shortly after birth (18).

Due to the loss of another role of TGF-β1, that is, the inhibition of progression of thymocytes from the double-negative stage to the double positive stage ($CD4^-CD8^{lo}$ to $CD4^+CD8^+$) during T cell development, the knockout mouse has increased numbers of thymic double-positive thymocytes (14). However, there is actually decreased cellularity in the thymus due to decreased numbers of thymic precursors, whereas the peripheral lymph nodes and spleen have increased CD4+ cells with an activated phenotype. In the knockout, the T cells are continuously proliferating, expressing mRNA for IL-2, IL2R, IL-6, and IL-10. There are IgG autoantibodies found, glomerular immune complex deposition, and widespread vasculitis (14).

TGF-$\beta2^{-/-}$ and TGF-$\beta3^{-/-}$ knockout mice have not been as well studied, compared to the TGF-$\beta1^{-/-}$ mice discussed above. TGF-$\beta2^{-/-}$ mice all die around the time of birth and demonstrate a wide range of developmental defects, including disruptions of the cardiac, lung, craniofacial, limb, vertebral, eye, ear, and urogenital systems (19). TGF-$\beta3^{-/-}$ knockout mice die within 20 hours of birth, due to aberrant development of the lungs and palate (17). This isotype has been implicated in type II pneumocyte development.

The Role of TGF-β As A Counter-Regulatory Cytokine in Crohn's Disease

Recent work by the Strober lab has shown TGF-β to be of great importance as a counter-regulatory cytokine (21). In the IL-2 knockout mouse model, mice develop colitis and autoimmunity when kept in a non-sterile environment. In a sterile environment, the IL-$2^{-/-}$ mice, but not the control mice, develop colitis when immunized with various antigens. When the inflamed areas of the colon were isolated, it was discovered that they were $CD4^{+}$, IFN-γ-secreting T cells. When anti-CD3 antibody (αCD3) was given either alone or with the colitis-inducing antigen, surprisingly, the mice remained healthy. Anti-CD3 antibody binds CD3, which is an important cofactor in the presentation of antigen to the T-cell receptor (TCR) (20). This stimulates T cells directly, in a non-antigen-specific manner. Why would this result in the prevention of colitis? Perhaps, anti-CD3 antibody was stimulating counter-regulatory T cells, which would inhibit the colitis-producing T cells. The lab showed that, in fact, TGF-β was produced by lymphocytes after addition of anti-CD3 antibody (21). There was a decrease in IFN-γ production, hinting that the Th1 response that caused colitis in the mice was inhibited by TGF-β in those mice given anti-CD3 antibody. To prove this, they administered anti-TGF-β antibody *in vivo* and showed that this reversed the protective effect of anti-CD3 antibody. They therefore concluded that TGF-β was involved in protection against a Th1-driven inflammatory response (21). Furthermore, by giving the mice neutralizing

antibodies to IL-12, there was an enhanced oral tolerance found. Clearly, TGF-β is affecting Th1 cell differentiation or proliferation in a counter-regulatory manner.

The question that followed was how this occurred. How did TGF-β affect naive T cells in the gut? How did it affect Th1 cells? Did TGF-β act by altering the intracellular signaling pathways, which therefore altered the pattern of cytokine expression? Or, did TGF-β directly alter the pattern of cytokine expression, resulting in an altered cellular growth pattern? It is necessary to examine the effects of TGF-β on expression of cytokines associated with Th1 cell activation (IFN-γ, IL-12) and Th2 cell activation (IL-4, IL-10), in addition to its effects on DNA synthesis and expression of intracellular proteins.

If one is to understand the interaction between TGF-β, IL-2 production, and Th1 development, it is necessary to examine the cytokine pathways involved. As seen in Figure 4, these cytokines signal through signal transduction elements called JAKs and STATs (22). JAKs are protein tyrosine kinases coupled to cytokine receptors. JAK1, JAK2, and TYK2 are ubiquitously expressed, whereas JAK3 is highly expressed only in activated lymphoid and myeloid cells (22). STATs are "signal transducers and activators of transcription." There are seven family members, most of which are activated by a few different cytokines. In addition, most cytokines activate multiple STATs. Ligand binding induces dimerization of the STAT proteins. Following phosphorylation, STATs detach from the receptor complex and translocate to the nucleus. As shown in the figure, IL-12 induces phosphorylation of STAT4 in activated T cells.

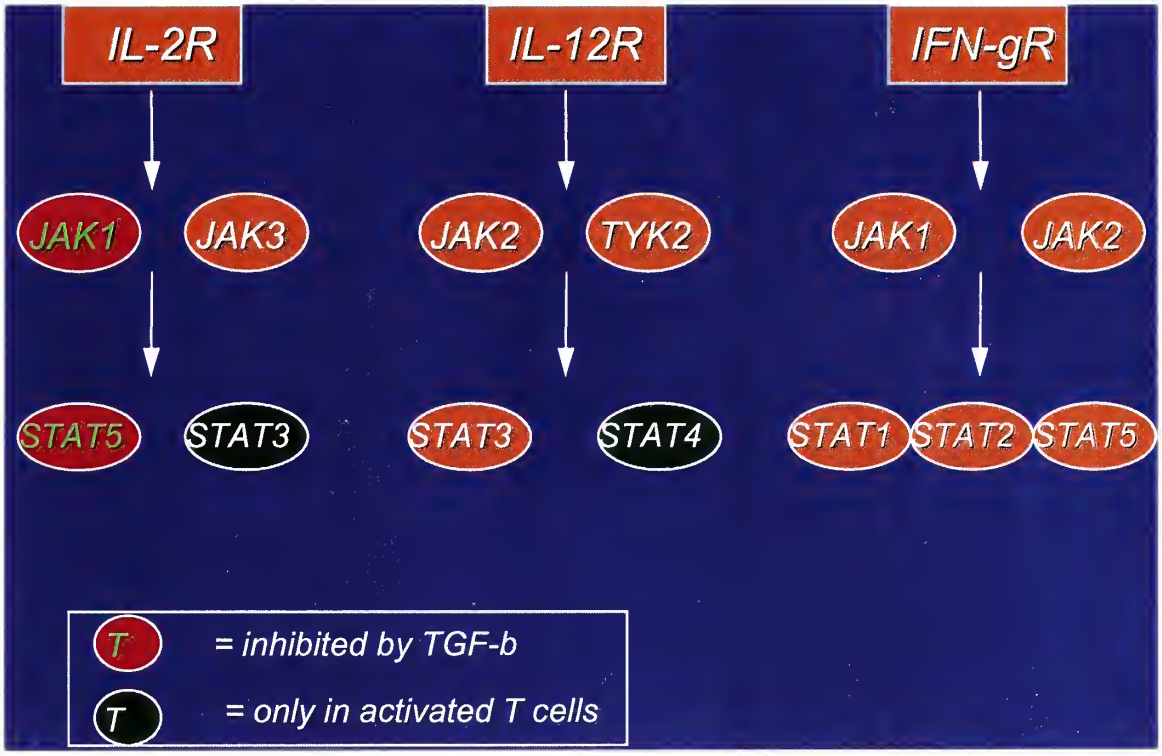


FIGURE 4: JAKs and STATs are intracellular proteins involved in the signal transduction pathways of the cytokines IL-2, IL-12, and interferon-γ. STAT4 is phosphorylated only in T cells that have been activated by IL-12. Figure based on information from Lamont *et al.* (24) and O'Shea (25).

Schmitt *et al.* published some preliminary data on the interaction of TGF-β and Th1 cells (23). They showed that TGF-β inhibited production of IFN-γ by IL-12-stimulated, naive CD4+ T cells and inhibited IL-12-induced Th1 cell development from naive T cells. One hypothesis that follows from the preceding discussion and the results by Schmitt is that if IL-12 activates the Th1 pathway and that TGF-β is a counter-regulatory cytokine that inhibits this activation, perhaps it is acting through down-regulation of STAT4 phosphorylation.

Future Treatments of Crohn's Disease Will Be Based on The Knowledge of the Immunology of the Disease

It has recently been shown that macrophages isolated from sites of inflammation in the gastrointestinal tracts of patients with Crohn's disease produce greatly increased amounts of IL-12. T cells in these lesions have been shown to be mostly Th1 cells, producing IFN-γ and TNF-α (5). STAT4, an intracellular protein that is activated in the IL-12-driven Th1 cell activation pathway, is phosphorylated to greater levels in T cells isolated from patients with Crohn's disease (5). Recent work with the murine model of colitis has demonstrated that administration of anti-IL-12 antibody can prevent, or even reverse, the inflammation. A project is currently underway to create a modified anti-IL-12 antibody from mice with regions of the antibody containing human sequences and to use this in clinical trials in humans (5).

Regardless of the etiology of Crohn's disease, if the model of Th1 cell dysregulation is assumed to be correct, one could target any step in the Th1 cell activation pathway as a potential treatment for the disease. TNF-α is a major cytokine "player" in the model. It has two major roles, the first of which is its function as a pro-inflammatory cytokine that directly mediates mucosal inflammation (1). The second is its ability to keep the Th1 cell activation pathway going, as TNF-α, together with interferon-γ, synergize to activate IL-12 production by antigen-presenting cells (1). In murine models of Crohn's disease, systemic administration of anti-TNF-α antibodies decreases the amount of colitis present in the gastrointestinal tract (1). In addition, blocking the transcription of TNF-α by treating mice with intrarectal antisense oligonucleotides of NF-κB (NF-κB is necessary for the transcription of TNF-α) is more effective than corticosteroids in the treatment of mice with symptoms of Crohn's disease (1). Human trials of anti-TNF-α antibody produced excellent clinical responses with few side effects (2) (26) (27). In one multi-center, double-blind, randomized, controlled trial, Infliximab (anti-TNF-α antibodies) achieved a 38-55% success rate in the closure of all fistulas (2). However, the negative side to the manipulation of the immune system is that this drug has been shown to induce a monocytopenia (28) and has been associated with lymphoma in four patients (29).

Another method to try to abrogate the Th1 response in patients with Crohn's disease is to try to inhibit IL-12 directly. IL-10 is known to inhibit the synthesis of IL-12, whereas anti-IL-12 antibodies would inhibit the function of IL-

12 (1). One group is conducting trials with administration of IL-10, whereas another group is trying to create human anti-IL-12 antibodies (30).

Crohn's disease clearly represents a dysregulation of the immune system. With the decades-old, brute-force treatment of the disease with general immunosuppressant medication being slowly replaced by newer, custom-tailored cytokine therapies designed to directly target the imbalance found in Crohn's disease, it is clear that research is going in the right direction and that increased knowledge of the regulation of Th1 cells is needed. In order to design more specific, efficacious treatments for Crohn's disease, while decreasing the side effects caused by anti-immune therapy, it is necessary to find out what the counter-regulatory pathways are that do not function properly in patients with Crohn's disease.

The field of oral tolerance has yielded the information that TGF-β is produced by suppressive T cells. TGF-β is known to inhibit the Th1 cell pathway but little is known about the mechanism of this action. Does it decrease the proliferation of cells when stimulated with antigen and antigen-presenting cells? Does it alter the cytokines that are produced by the T cells? Does it have an effect on any of the intracellular signaling pathways found in Th1 cells? These questions were asked in the series of experiments described below.

STATEMENT OF PURPOSE:

Preliminary data from several laboratories have suggested that the cytokine, TGF-β, is a crucial counter-regulatory cytokine that can suppress the function of Th1 cells. The purpose of this thesis is to:

1) Quantify the effects of TGF-β on CD4+ Th1 cell DNA synthesis as a reflection of its effects on cellular proliferation

2) Determine the effects of TGF-β on CD4+ Th1 cell cytokine production

3) Search for an intracellular signaling target for the action of TGF-β on Th1 cells.

Methods

All experiments were conducted by the author of this thesis in the laboratory of Dr. Warren Strober, Mucosal Immunity Section, LCI, NIAID, National Institutes of Health, Bethesda, MD. All media was prepared by NIH laboratory unless otherwise stated.

Media

All cell media was made from RPMI with the following added: 10% heat-inactivated Fetal Calf Serum (Whittaker), 100 U/ml penicillin (courtesy of Kevin Chua, NIH, Bethesda, MD), 100 µg/ml streptomycin, 75 mM Hepes, 5% NCTC-109 (Life Technologies, NY) 0.05 mM 2-ME, 2 mM L-glutamine.

Naive T cell isolation

In order to isolate and eventually stimulate *in vitro* CD4+ T cells from murine spleens, mice were chosen based on their ability to respond to a specific antigen. Transgenic mice were used (clone D011.10) so that the TCR (T-cell receptor) would recognize a specific peptide that could be provided to cell cultures *in vitro* after the T cells were isolated. In this case, the TCR recognized a peptide fragment (corresponding to amino acids 323-339) of the protein, ovalbumin (henceforth referred to as "OVA") when presented in conjunction with a specific MHC ligand, IA^d. The genetic background of the mice was BALB/c. All mice were maintained by the NIH Twinbrook II Animal Care Facility. 4-8 week old female mice were used for all experiments in order to maintain consistency throughout all experiments. Intact murine spleens were removed using sterile technique. HBSS solution and a mortar were used to homogenize the splenic cells, which were passed through a 100 µM nylon filter (Falcon, Franklin Lakes, NJ) into a 50 ml conical tube in order to remove any undissolved splenic debris. The filtrate was spun at 300 G for 10 minutes. Supernatant was discarded and the cellular pellet was washed with 50 ml HBSS and spun again under the same conditions. After removing the supernatant once again, 2-3 ml ACK lysis buffer (Biofluids, Rockville, MD) per spleen was used to re-suspend the pellet. This

lysis preferentially degrades erythrocytes during the first 2 minutes after addition. During this time, the suspension was thoroughly mixed via pipeting up and down rigorously. After 2 minutes, the 50 ml tube was filled with HBSS and centrifuged, again under the same conditions. After removing the supernatant, the pellet was again washed with 50 ml HBSS and re-spun. At this time, the cells were counted and re-suspended in HBSS with 1% FCS (fetal calf serum) at a concentration of 30 x 10^6 cells per 0.4 ml.

In order to remove cells other than the T cell population of interest, negative selection was performed using biotinylated antibodies and streptavidin-coated magnetic beads. In this protocol, antibodies against undesirable cells are added to the cell culture. In this case, because CD4+ T cells were the target for purification, antibodies against CD8+ T cells and non-T cells were used. All antibodies are covalently linked to biotin. Magnetic beads that are covalently linked to streptavidin are mixed with the cell cultures. In theory, only those cells that have been targeted by the biotinylated antibodies will bind to the magnetic beads. A magnet is used to separate the beads from the cells that are still free in solution (CD4+ T cells), which are then separated.

In these experiments, only CD4+ T cells were desired. To remove granulocytes, macrophages, NK cells, and B-1 cells, biotin anti-CD11b antibody (Pharmingen, CA) was added. To remove CD8+ T cells, biotin anti-CD8a antibody (Pharmingen, CA) was added. To remove B cells, biotin anti-B220 antibody (Pharmingen, CA) was added. Biotin anti-I-A^d antibody (Pharmingen, CA) was added to deplete MHC Class II antigen. All antibodies were added at concentrations of 12 µg per 30 x 10^6 T cells. The cells were kept at 0°C on ice for 30 minutes, at which point they were washed twice with HBSS containing 1% FCS. At this point, the cells were re-suspended in 400 µl HBSS with 1% FCS per 30 x 10^6 T cells. 100 µl of previously-washed streptavidin-conjugated Dynal beads (Dynal, Oslo, Norway) at a concentration of 350 µl beads per 30 x 10^6 T cells were added to an eppendorf tube and placed next to a magnet for 1 minute. Supernanant was removed and an equal volume of PBS with 0.1% BSA was added. Again, the supernatant was removed. Following this, the beads were re-

suspended in 100 µl PBS/0.1% BSA for 30 minutes. The T cells and beads were placed on ice for 30 minutes, after which time 1.5 ml HBSS/1% FCS was added per 30 x 10^6 T cells. Finally, the tube was placed on a magnet for 5 minutes. In theory, the streptavidin binds to the biotin linked to the antibodies, which are bound to CD8+ T cells and non-T cells, but not to CD4+ T cells. The supernatant, containing only CD4+ T cells, was removed and saved. 2 ml HBSS/1% FCS was added to the beads and the tube placed next to the magnet. The supernatant was saved again, washed, and re-suspended at a final concentration of 0.5 x 10^6 T cells per ml.

Antigen presenting cell (APC) isolation

BALB/c and B10.A mice (used in A.E7 cultures, see below) were maintained by the NIH Animal Care Facility. 4-12 week old female mice were used for all experiments (see above). Spleens were removed in sterile fashion. Splenic cells were homogenized with a mortar and HBSS solution, after which they were passed through a 100 µM filter (Falcon, Franklin Lakes, NJ) into a 50 ml conical tube in order to remove any undissolved splenic debris. The cells were lysed to remove erythrocytes and washed as described above in the naïve T cell isolation protocol.

For T-cell-depleted APCs only: Antibodies reactive to T cell markers were then added: anti-Thy1.2, GK1.5, and anti-Ly2.2 antibodies (kindly provided by Dr. Robert Seder) were all added at a concentration of 1 ml per spleen. The cells and antibodies were kept on ice for 30 minutes and then washed once with HBSS. A pre-warmed complement cocktail was then added. This contained (per spleen) 0.5 ml complement (Gibco Life Technologies), 1.5 ml HBSS, 10 µl 1 M Hepes, 50 µl MAR12.5 (provided by Dr. Robert Seder). The cells and complement cocktail were incubated at 37°C for 30 minutes to lyse any T cells present. Following the incubation, the dead cells were removed by Percoll gradient (40 and 100% Percoll).

For both T-cell-depleted and non-T-cell-depleted APCs, cells were irradiated at 3000 rads, and washed 2-3 times (to remove any free radicals produced during the irradiation procedure). T-cell-depleted APCs were re-suspended at 5 x 10^6 cells/ml and used for experiments (in which the APC:T cell ratio was 10:1), while non-T-cell-depleted APCs were re-suspended at 10 x 10^6 cells/ml (and thus a 20:1 ratio of APCs to T cells) and used to expand the initial cultures for use in experiments.

Th1 and Th2 cultures

When first setting up cultures, 1.0 ml each of T cells (0.5 x 10^6 cells/ml) and APCs (10 x 10^6 cells/ml) were plated in each well in 24-well plates. The ratio of 20:1 APCs to T cells was chosen to maximize the growth of T cells in culture in order to expand the desired population of cells. This ratio was based on prior experiments comparing multiple ratios. For all cells, 20 µl of 300 mM ovalbumin peptide (Peptide Synthesis Facility, NIAID, NIH, Bethesda, MD) were added to each 2 ml culture. Several investigators have shown that naïve T cells will differentiate into a Th1 phenotype when cultured in media containing IL-12 and anti-IL-4 antibody, whereas they will differentiate into a Th2 phenotype when cultured in media containing anti-IL-12 antibody and IL-4 (31). For Th1 cells, 10 U/ml IL-12 and 10 µg/ml anti-IL-4 antibody were added to each 2 ml culture. For Th2 cells, 3 µg/ml anti-IL-12 antibody and 200 U/ml IL-4 were added to each 2 ml culture.

On day 2-3, when the T cells appeared activated and crowded, the 2 ml cultures were expanded to 6 ml in 6-well plates. Cytokines were added at the same concentrations, with the addition of 10 units/ml of IL-2 to both Th1 and Th2 cultures. Until using the cultured cells, the cultures were expanded or media changed as necessary.

A.E7 cell culture:

A.E7 cells are an established Th1 cell line borrowed from the laboratory of Dr. Michael Lenardo. This is a cell line originally isolated from transgenic mice

that have a T-cell receptor that recognizes the peptide, PCC (pigeon cytochrome c). These mice are in a different genetic background than the BALB/c mice used in the above experiments. Rather, these mice have a B10.A genetic background and must be stimulated with antigen presenting cells isolated from the spleens of B10.A mice. It has been shown by Dr. Lenardo's lab and others that when stimulated with peptide and antigen presenting cells, these cells proliferate and produce interferon-γ selectively, thus qualifying the cell line as Th1 (32), (33). In order to passage the cells, the following protocol was used. On Day 0, 10 x 10^6 A.E7 cells and the [non-T-cell-depleted] APCs from one B10.A murine spleen (irradiated at 3000 rads) were cultured in a volume of 30 ml in a flask. 100 µl of 1500 µm pigeon cytochrome c peptide (Peptide Synthesis Facility, NIAID, NIH, Bethesda, MD) were added to the flask for optimal stimulation. After 48 hours, the culture was spun in a Percoll gradient (40% and 100%) and the dead cells (mostly APCs) were removed. The viable cells were then cultured in 150 ml of fresh media with 100 units/ml IL-2. Between days 12-24, the A.E7 cells were re-stimulated and the cycle repeated.

Cell culture during experiments:

0.5 ml of T cells at 0.5 x 10^6 cells/ml (either freshly-isolated "naive" CD4+ cells or cultured Th1, Th2, or A.E7 cells) and 0.5 ml of APCs at 5 x 10^6 cells/ml (T-cell-depleted) were added to 24-well plates. These concentrations were selected to optimize growth of T cells. In the initial cultures to expand the population of T cells, a 20:1 ratio of APCs (not T-cell-depleted) to T cells was used. In the experimental cultures, a 10:1 ratio of T-cell depleted APCs to T cells was found to be optimal. For the initial expansion of freshly-isolated CD4+ T cells, ovalbumin peptide or pigeon cytochrome c (for A.E7 cultures) was added to each well, to make the final concentration of antigen either 3.0 µM (ovalbumin) or 5.0 µM (pigeon cytochrome c). For Th1, Th2, or A.E7 experiments, 1/10th the concentration of peptide was added to each well. For those experiments in which TGF-β was used, TGF-β1 (Genzyme) was diluted in PBS and added to wells at a final concentration of 10 ng/ml, 5 ng/ml, 1 ng/ml, or 0.1 ng/ml.

DNA synthesis (Thymidine Uptake) Assay

This assay is commonly used in immunologic research as an estimate of cellular proliferation. 100 µl each of T cells (0.5×10^6 cells/ml) and APCs (T-cell-depleted cells at 5×10^6 cells/ml) were added in triplicate to 96-well plates. Ovalbumin and pigeon cytochrome c were added at levels consistent with the experiments described above. TGF-β was added at various concentrations, consistent with the experiments above. 8 hours before measuring the DNA synthesis, 1 µCi of [^{3}H] thymidine was added and the counts measured with a Beta-Plate reader.

IFN-γ ELISA

The basic premise of an ELISA assay (Enzyme-linked immunosorbent assay) is that antibodies to the target molecule are linked to an enzyme that catalyzes a colorimetric assay. In this case, antibodies are first linked to the bottom of a 96-well microtiter plate. Samples are added to the wells and the target molecule binds to the antibodies. Next, secondary antibodies are added that are covalently linked to biotin. Horseradish peroxidase-streptavidin (HRP-streptavidin) is then added. The biotin linked to the secondary antibodies (which are now bound to the target molecule) binds to streptavidin and the peroxidase catalayzes a colorimetric reaction when substrate buffer is added. Therefore, the amount of color seen in the wells is a reflection of the amount of enzyme present, which in turn, is a reflection of the amount of bound secondary antibody present, which is determined by the amount of target molecule present in the well.

Day 1:

After 48 hours of culture, the experimental cell cultures were spun for 10 minutes at 300 G and the supernatants were collected and frozen at -20 °C. At a later

time, the supernatants were thawed for use in the ELISA assay. Wells of a 96-well flatbottom Immulon plate (Dynatech Lab, Inc. - cat. # 011-010-3850) were coated with 50 µl of a 2 µg/ml solution of anti-IFN-γ antibody in carbonate buffer. The plates were kept at 4°C overnight.

Day 2:

Plates were washed twice with PBS/Tween (500 ml PBS/250 µl Tween 20) in order to decrease any protein-protein interactions. Next, the wells were blocked with filtered (0.45 µm) 3% BSA in PBS (Sigma Bovine Albumin Cat. # A-7030) at 200 µl/well for 1 hour at 37°C. After one hour, the plates were washed twice with PBS/Tween. Standards were then added by serial dilution, in final concentrations ranging from 1000 U/ml to 7.8 U/ml. Samples were plated at 100 µl/well, usually with a 2-10 fold dilution. The plates were then incubated for 2 hours at 37°C. Following incubation, the plates were washed 4 times with PBS/Tween. 100 µl of secondary antibody was added at 2 µg/ml in 3% BSA/PBS solution and the plates were incubated for 1 hour at 37°C. Following this incubation, the plates were washed 6 times with PBS/Tween. Next, a 1:1000 avidin-peroxidase solution (HRP-Streptavidin by ZYMED) in PBS was added and incubated for 25 minutes at 37°C. The plates were washed 8 times with PBS/Tween. 100 µl substrate buffer was added and the plates were read by a Dynatech MR500 plate reader. Substrate buffer was made by combining 24.3 ml 0.1 M citric acid, 25.7 ml 0.2 M Na_2PO4, and 50 ml dH_2O. One 15 mg O-phenylenediamine dihydrochloride tablet (Sigma #4664) was added per 30 ml solution. The substrate buffer was completed with the addition of a 30% hydrogen peroxide solution at 1:1000 dilution.

IL-4 ELISA

Day 1:

The protocol for the IL-4 ELISA is similar to the above description of the IFN-γ ELISA assay except for the IL-4 ELISA, a three-day procedure was used. On the

first day, the wells of a 96-well flatbottom Immulon plate (Dynatech Lab, Inc. - cat. # 011-010-3850) were coated with 100 µl of a pre-aliquoted solution of anti-IL-4 antibody in PBS. The plates were kept at room temperature overnight.

Day 2:
The wells were blocked with filtered (0.45 µm) 3% BSA in PBS (Sigma Bovine Albumin Cat. # A-7030) at 200 µl/well for 1 hour at room temp. After one hour, the plates were washed three times with PBS/Tween (500 ml PBS/250 µl Tween 20). 50 µl of 3% BSA/PBS were added to each well. Standards were then added by serial dilution, in final concentrations ranging from 862 pg/ml to 13.5 pg/ml, with the last pair of wells lacking any IL-4. Samples were plated at 50 µl/well. The plate was covered and left at room temperature overnight.

Day 3:
Following incubation, the plates were washed 3 times with PBS/Tween. One aliquot of detecting antibody was obtained from the -80°C freezer and diluted in 11 ml 3% BSA/PBS solution, of which 100 µl were added per well. The plates were incubated for 1 hour at room temperature. Following this incubation, the plates were washed 3 times with PBS/Tween. Next, 100 µl of a 1:1000 avidin-peroxidase solution (HRP-Streptavidin by ZYMED) in PBS was added and incubated for 30 minutes at room temperature. The plates were washed 3 times with PBS/Tween. 100 µl substrate buffer was added and the plates were read by the Dynatech MR500 plate reader.

STAT4 Western Assay

The principle of immunoblotting is as follows. The cells are lysed carefully and proteins are selected out by adding proteinase inhibitors to the buffer. The protein mixture is loaded onto a polyacrylamide gel and electrophoresed to separate the proteins by molecular weight. The two-dimensional gel is then placed beside nitrocellulose paper and electrophoresis is used to transfer the

proteins from the gel to the paper. Enzyme-labeled antibodies are added to a solution that is then used to bathe the nitrocellulose paper. The antibodies bind to proteins on the nitrocellulose paper and enzyme substrate then reveals the presence of bands of proteins, where the antibodies have bound to the paper.

Day 1:

The protocol for the first day of this assay involves lysing the cells in a buffer that will protect the intracellular proteins from degradation. Samples were washed twice with cold PBS solution and spun at 10,000 rpm. Supernatants were discarded. RIPA solution contains 100 ml PBS, 1 g IGEPAL (Sigma, #630), 0.5 g 0.5% sodium deoxycholate (Sigma), and 1 ml 10% SDS solution. To make lysate buffer, the following proteinase inhibitors (courtesy of Dr. Bjorn Ludviksson) were added to RIPA: 10 µl/ml 100 mM PMSF, 1 µl/ml Apoprotinin, and 10 µl/ml 100 mM sodium orthovanadate. 1 ml of lysate buffer was added to each cell sample following the two washes. Samples were incubated at 4°C on a shaker for 30 minutes. Next, they were centrifuged at 15-20,000 rpm for 30 minutes. In order to prevent warming of each sample, the supernatants were transferred to a 1 ml tube previously cooled on dry ice, and then stored in the -80°C freezer for at least 12 hours. By the end of this part of the experiment, all non-protein cellular elements should have been degraded.

Day 2:

Samples were thawed at 0°C and were not allowed to warm past this temperature. 1 µg rabbit IgG (Santa Cruz Technologies) was added to each sample, followed by 20 µl of Agarose conjugate (Santa Cruz Technologies). In this step, all cellular elements that might bind non-specifically to rabbit immunoglobulin are removed. The samples were spun at 2500 rpm for 5 minutes at 4°C. The supernatant was collected. 1 µg rabbit anti-mouse STAT4 antibody (Santa Cruz Technologies) was added to each sample and incubated for 1 hour at 4°C in a rocker. 20 µl agarose conjugate was added to the samples and incubated at 4°C on a rocker overnight. The agarose conjugate binds to the anti-

mouse STAT4 antibody, which presumably is bound to any STAT4 protein present.

Day 3:

The samples were washed 4 times by spinning at 2500 rpm for 5 minutes per wash, and replacing the supernatant with RIPA buffer. Following the fourth wash, the pellets were re-suspended in 40 µl electrophoresis buffer (450 µl sample buffer (Novex) + 450 µl PBS + 100 µl 2-ME) and boiled for 90 seconds. 15 µl were loaded into a pre-made gel (Novex) and run at 125 V for 90 minutes. The protein was transferred to a nitrocellulose membrane (Novex 0.45 µm) by electrophoresis at 25 V for 2 hours. The membrane was blocked with Blotto B (PBS with 2% BSA) without Tween at 4°C overnight on a rocker.

Day 4:

The membrane was incubated for one hour at room temperature in HRP (Horseradish peroxidase)-conjugated anti-phosphotyrosine antibody solution (200 ng/ml Py99 - Santa Cruz, sc-7820) diluted in Tris-buffered saline with 0.05% Tween (to inhibit non-specific protein interactions) and 2% BSA. Anti-phosphotyrosine antibodies were used because it has been well documented that STAT4 is phosphorylated by a tyrosine kinase during activation (24) (25). Following incubation, the membrane was washed twice in Tris-buffered saline with 0.05% Tween, each wash with 15 ml buffer for 7 minutes. This step removes all unbound antibody. The nitrocellulose paper was then placed next to film (Kodak) in a cartridge and the film was developed in an automated Kodak machine. After measuring the level of phosphorylated STAT4 protein on the gel, the nitrocellulose paper was stripped and incubated with HRP-conjugated anti-STAT4 antibody solution. These results demonstrated that there was equal protein loading of STAT4 in all experimental and control groups (data not shown)

Statistical Analysis Methodology:

Each experiment was performed in triplicate and thus n=1 reflects three separate wells cultured under the same conditions. All Th1 and A.E7 experiments were repeated six times (n=6) and thus reflect data from 18 wells. For the Th2 cells, the DNA synthesis assay was repeated six times but the cytokine assay was performed only one time. The STAT4 Phosphorylation assay was performed several times but due to protein loading variation, only the one experiment presented in the results section was used because in this experiment, there was equal protein loading in all conditions. All mean values and p values were calculated using Microsoft Excel.

Results

A. Effects of TGF-β on DNA synthesis

The first set of experiments was conducted to see what effect TGF-β would have on the DNA synthesis of a known Th1 cell line called A.E7. In the above experiments, the mean levels of DNA synthesis (thymidine uptake) were Control (117907 cpm), 1.0 ng/ml TGF-β (108020 cpm), 10 ng/ml TGF-β (98213 cpm). When expressed as a percent of the control level of DNA synthesis (thymidine uptake) (See Figure 5), 1.0 ng/ml TGF-β inhibited DNA synthesis by 8.4% (91.6% of control, p=0.031) while 10.0 ng/ml TGF-β inhibited DNA synthesis by 16.7% (83.3% of control, p=0.026).

In order to verify whether the effect TGF-β had on Th1 DNA synthesis could be generalized to Th1 cells with different genetic backgrounds and MHC class types, the same experiments were performed in Th1 cells created in the laboratory. Using mice bred in a BALB/c genetic background, CD4+ T cells were isolated from murine spleens by negative selection. These cells were stimulated with peptide (ovalbumin) and antigen-presenting cells and then cultured in conditions favoring Th1 cell development (IL-12 and anti-IL-4 antibody). In the above experiments, the mean levels of DNA synthesis (thymidine uptake) were Control (34016 cpm), 1.0 ng/ml TGF-β (21904 cpm), 10.0 ng/ml TGF-β (16378 cpm). When expressed as a percent of the control level of DNA synthesis (see Figure 5), 1.0 ng/ml TGF-β inhibited DNA synthesis by 35.6% (64.4% of control,

A.E7 Th1 Cells (B10.A background)	Mean % DNA synthesis, vs. control (n=6)	St Dev	P value
control	100	0	
1.0 ng/ml TGF-β	91.6	5.13	0.031
10.0 ng/ml TGF-β	83.3	9.81	0.026
Th1 Cells (BALB/c background)	**Mean % DNA synthesis, vs. control (n=6)**	**St Dev**	**P value**
Control	100	0	
1.0 ng/ml TGF-β	64.4	0.71	0.002
10 ng/ml TGF-β	48.1	9.19	0.01

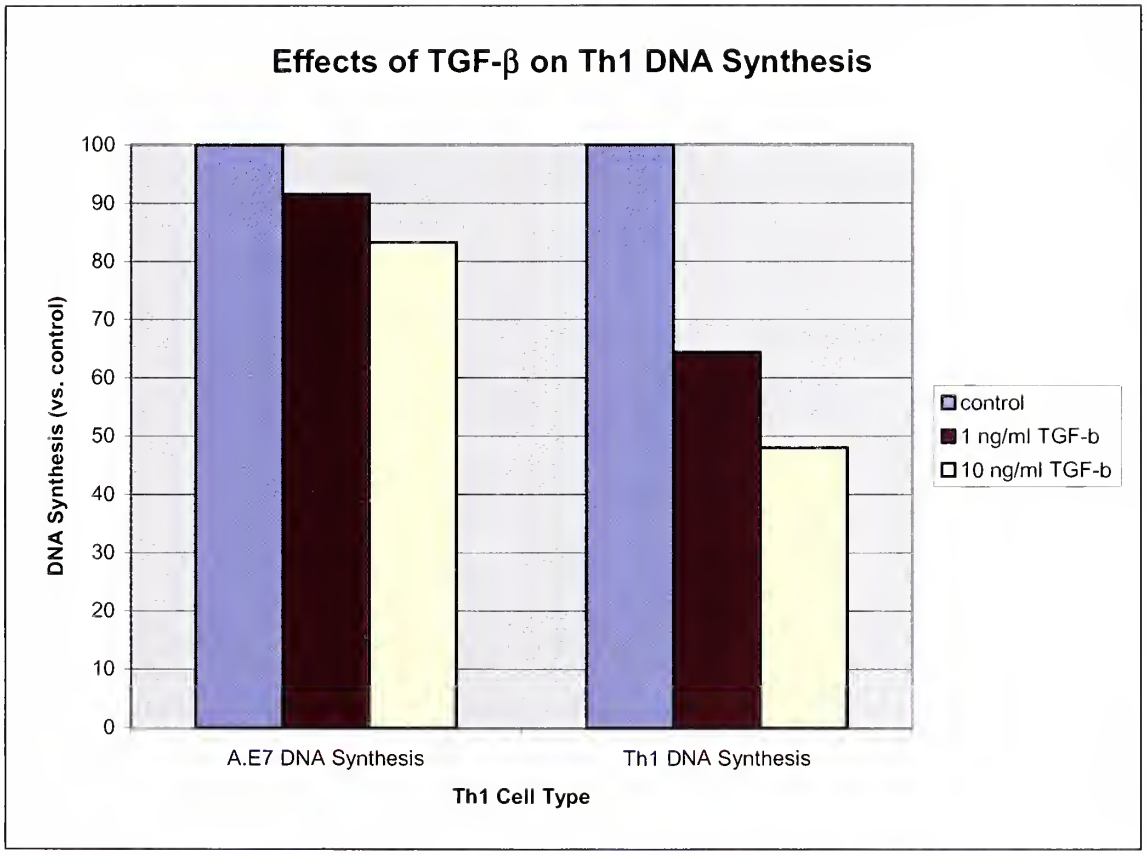


TABLE 1 & FIGURE 5: A.E7 CD4+ T cells are a known T cell line of the Th1 phenotype. The cell line was passaged every three weeks and re-stimulated with pigeon cytochrome c peptide (PCC) and antigen-presenting cells from B10.A spleens. For use in experiments, A.E7 CD4+ T cells were stimulated with antigen-presenting cells (10:1 APC:T cell ratio) and PCC in media-containing IL-12 and anti-IL-4 antibody. CD4+ Th1 cells from the BALB/c background were created by removing spleens from OVA TCR-transgenic mice in a BALB/c genetic background. CD4+ T cells were isolated by negative selection and were stimulated with antigen-presenting cells (20:1 APC:T cell ratio) and albumin peptide. Th1 cells were created by culturing CD4+ T cells with IL-12 and anti-IL-4 antibody. For use in experiments, the Th1 cells were stimulated with ovalbumin peptide and a 10:1 ratio of APCs. TGF-β was added to make the final concentration in cell cultures either 0 ng/ml, 1 ng/ml, or 10 ng/ml. After 48 hours of culturing cells in media, [3H] thymidine was added for 8 hours and uptake detected by a beta plate reader. In both cell types, TGF-β was found to inhibit DNA synthesis in a dose-dependent manner.

p=0.002) while 10.0 ng/ml TGF-β inhibited DNA synthesis by 51.9% (48.1% of control, p=0.01).

If TGF-β inhibits the DNA synthesis of differentiated Th1 cells, what effect does it have on naïve, undifferentiated CD4+ T cells? Naïve CD4+ T cells were isolated as in previous experiments, by using negative selection to limit the cell population to CD4+ T cells and then stimulating the cells with antigen (ovalbumin) and antigen-presenting cells. No Th1-favoring (IL-12, anti-IL-4 antibody) or Th2-favoring (IL-4, anti-IL-12 antibody) cytokines or antibodies were added to the media. In the above experiments, the mean levels of DNA synthesis (thymidine uptake) were Control (59295.5 cpm), 1.0 ng/ml TGF-β (83439.2 cpm), 10.0 ng/ml TGF-β (66745.5 cpm). When expressed as a percent of the control level of DNA synthesis (see Figure 6), 1.0 ng/ml TGF-β increased DNA synthesis by 40.7% (p=0.013) while 10.0 ng/ml TGF-β increased DNA synthesis by 12.6% but was not statistically significant (p=0.113). Here, we see a response that is not straightforward. While the addition of a low concentration of TGF-β has a profound stimulatory effect on DNA synthesis, it appears as though high levels of TGF-β starts to have less of an effect. Although the p value for the 10 ng/ml concentration of TGF-β group is above 0.05, in each of six experiments, 10 ng/ml TGF-β results in decreased DNA synthesis, compared to the 1 ng/ml TGF-β group. This suggests that TGF-β might have a bi-modal action on naïve T cells. Similar effects have been noted with TGF-β by others (16) (18).

In order to see what effect TGF-β would have on the Th2 population of CD4+ T cells, similar experiments to those conducted with Th1 cells were

performed. CD4+ T cells were purified from murine spleens as described above. These cells were stimulated with peptide and antigen-presenting cells and cultured in media favoring the differentiation of the T cells to the Th2 cell phenotype (IL-4, anti-IL-12 antibody). In the above experiments, the mean levels of DNA synthesis (thymidine uptake) were Control (29455 cpm), 1.0 ng/ml TGF-β (53972 cpm), 10.0 ng/ml TGF-β (55940 cpm). When expressed as a percent of the control level of DNA synthesis (see Figure 6), 1.0 ng/ml TGF-β increased DNA synthesis by 83% ($p=0.06$) while 10.0 ng/ml TGF-β increased DNA synthesis by 90% ($p=0.88$). Contrary to the results seen when TGF-β was added to naïve T cells, the stimulatory effect of TGF-β on the DNA synthesis of Th2 cells is not bimodal. Therefore, the stimulatory effect of TGF-β appears maximal at 1 ng/ml for Th2 cells.

Naïve CD4+ T cells (BALB/c background)	Mean % DNA synthesis, vs. controls (n=6)	St Dev	P value
control	100	0	
1.0 ng/ml TGF-β	140.7	19.7	0.013
10.0 ng/ml TGF-β	112.6	15.6	0.113
Th2 cells (BALB/c background)	**Mean % DNA synthesis, vs. controls (n=6)**	**St Dev**	**P value**
Control	100	0	
1.0 ng/ml TGF-β	183	46.36	0.06
10.0 ng/ml TGF-β	190	42.10	0.88

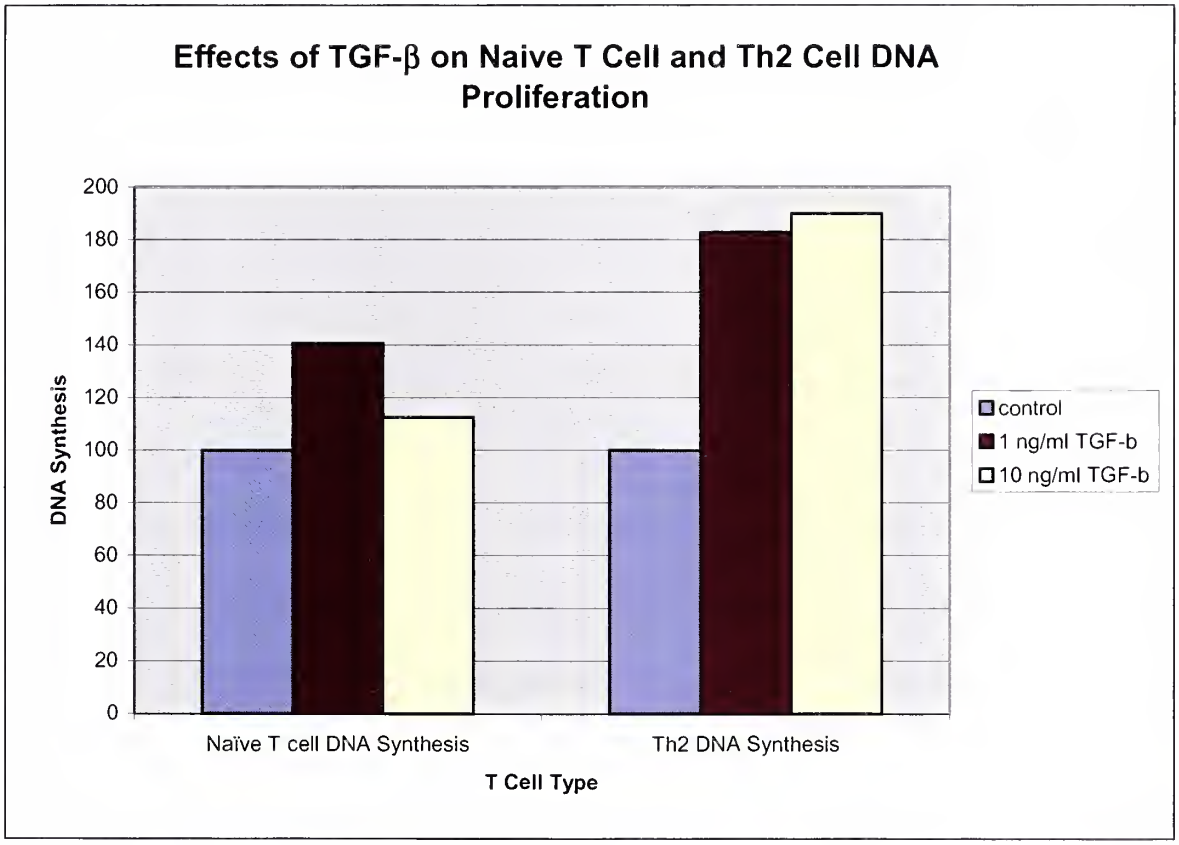


TABLE 2 & FIGURE 6: CD4+ T cells were isolated from the spleens of OVA TCR-transgenic mice in a BALB/c genetic background by negative selection, and were stimulated with antigen-presenting cells (20:1 APC:T cell ratio) and ovalbumin peptide during initial expansion of cell cultures. Th2 cells were created by adding IL-4 and anti-IL-12 antibody to the culture media. During experiments, ovalbumin and APCs (10:1 APC:T cell ratio) were added to the naïve or Th2 cells. TGF-β was added to cultures to make the final concentration 0 ng/ml, 1 ng/ml, or 10 ng/ml. After 48 hours of culture with TGF-β, [^{3}H] thymidine was added for 8 hours and uptake was detected with a beta plate reader. TGF-β stimulates Th2 DNA synthesis at both 1 ng/ml and 10 ng/ml concentrations, whereas it has a biphasic effect on freshly-isolated, naïve CD4+ T cells.

B. Cytokine Production

Once again, the known Th1 cell line, A.E7, was used in these experiments. The cells were stimulated with peptide and antigen-presenting cells and cultured in 96-well microtiter plates. ELISA was used to detect the concentration of interferon-γ in the supernatants of the cell cultures. In the above experiments, the mean values were control (2816 U/ml), 1 ng/ml TGF-β (1860 U/ml), and 10 ng/ml TGF-β (1073 U/ml). When expressed as a percentage of the control value of the concentration of interferon-γ (see Figure 7), 1.0 ng/ml TGF-β inhibited the production of interferon-γ by 34% (66% of the control value, p=0.01) while 10.0 ng/ml TGF-β inhibited the production of interferon-γ by 61.9% (38.1% of control, p=0.007).

As in the earlier experiments, BALB/c Th1 cells were produced by isolating CD4+ T cells from murine spleens, stimulating their growth with antigen-presenting cells and peptide, and culturing in conditions favoring the differentiation of the cells towards the Th1 cell phenotype. The cells were cultured in 96-well microtiter plates and ELISA was performed to measure the concentration of interferon-γ in the supernatants of the cell cultures. In the above experiments, the mean values were control (1271 U/ml), 1 ng/ml TGF-β (1006 U/ml), 10 ng/ml TGF-β (890 U/ml). Expressed as a percentage of the concentration of interferon produced by control cells (see Figure 7), 1.0 ng/ml TGF-β inhibited the production of interferon by 20.9 (79.1% of control, p=0.017) while 10.0 ng/ml TGF-β inhibited the production of interferon by 30% (70% of

A.E7 Th1 Cells (B10.A background)	Mean % IFN-γ production, vs. control (n=6)	St Dev	P value
control	100	0	
1.0 ng/ml TGF-β	66.0	17.6	0.01
10.0 ng/ml TGF-β	38.1	23.1	0.007
Th1 Cells (BALB/c background)	Mean % IFN-γ production, vs. control (n=6)	St Dev	P value
Control	100	0	
1.0 ng/ml TGF-β	79.1	8.5	0.017
10.0 ng/ml TGF-β	70.0	5.0	0.004

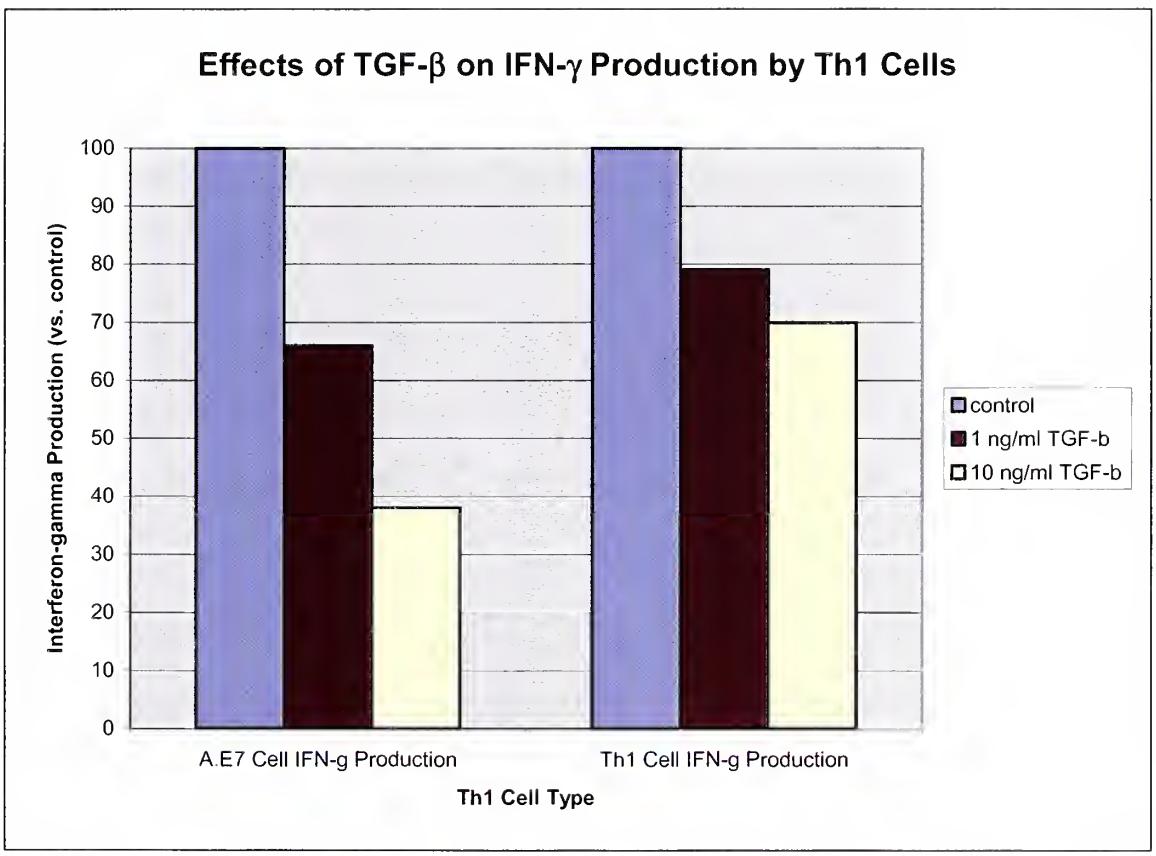


TABLE 3 & FIGURE 7: A.E7 CD4+ T cells are a known T cell line of the Th1 phenotype. The cell line was passaged every three weeks and re-stimulated with pigeon cytochrome c peptide (PCC) and antigen-presenting cells from B10.A spleens. For use in experiments, A.E7 CD4+ T cells were stimulated with antigen-presenting cells (10:1 APC:T cell ratio) and PCC in media-containing IL-12 and anti-IL-4 antibody. CD4+ Th1 cells from the BALB/c background were created by removing spleens from OVA TCR-transgenic mice in a BALB/c genetic background. CD4+ T cells were isolated by negative selection and were stimulated with antigen-presenting cells (20:1 APC:T cell ratio) and albumin peptide. Th1 cells were created by culturing CD4+ T cells with IL-12 and anti-IL-4 antibody. For use in experiments, the Th1 cells were stimulated with ovalbumin peptide and a 10:1 ratio of APCs. TGF-β was added to make the final concentration in cell cultures either 0 ng/ml, 1 ng/ml, or 10 ng/ml. After 48 hours of culture with TGF-β, ELISA was performed on culture supernatants to detect IFN-γ. TGF-β inhibits the production of IFN-γ by the two types of Th1 cells in a dose-dependent manner.

control, p=0.004). Again, there is a dose-dependent inhibition of Th1 cell function seen with the administration of TGF-β.

While the main cytokine produced by Th1 cells is interferon-γ, the major cytokine produced by Th2 cells is IL-4. In order to see what effect TGF-β would have on the function of Th2 cells, CD4+ T cells were isolated from murine spleens, stimulated with antigen-presenting cells and peptide, and then cultured in conditions favoring the differentiation towards the Th2 cell phenotype (IL-4, anti-IL-12 antibody). These results are preliminary, as only one set of experiments was performed. Expressed as a percentage of the concentration of IL-4 produced by control cells, 1 ng/ml TGF-β decreased the production of IL-4 by 9% while 10 ng/ml TGF-β decreased the production of IL-4 by 7%. These slight decreases in IL-4 production are statistically insignificant, given the n=1. However, from this initial data on three culture wells in one experiment, it appears that TGF-β does not induce a large decrease in Th2 cell function similar to the inhibition seen of Th1 cell function.

IL-4 Production in Th2 Cells	% IL-4 Production vs. controls (n=1)
Control	100
1 ng/ml TGF-β	91
10 ng/ml TGF-β	93

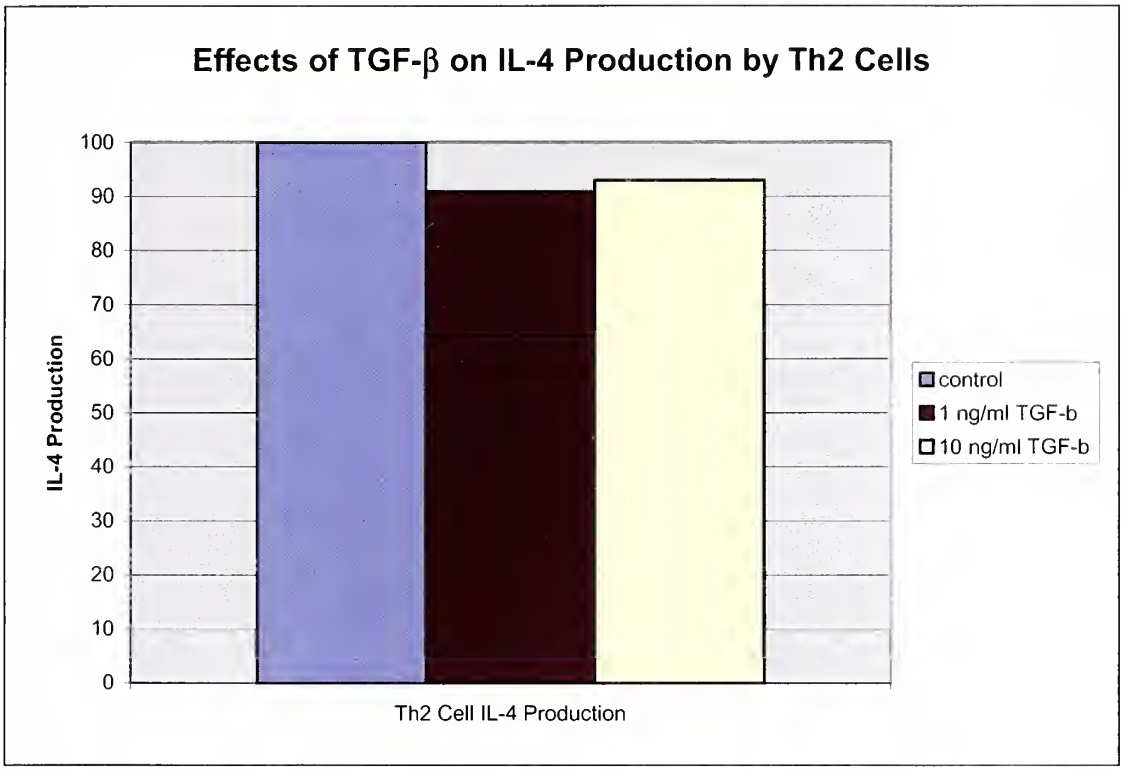


TABLE 4 & FIGURE 8: CD4+ T cells were isolated from the spleens of OVA TCR-transgenic mice in a BALB/c genetic background by negative selection, and were stimulated with antigen-presenting cells (20:1 APC:T cell ratio) and ovalbumin peptide during initial expansion of cell cultures. Th2 cells were created by adding IL-4 and anti-IL-12 antibody to the culture media. During experiments, ovalbumin and APCs (10:1 APC:T cell ratio) were added to the naïve or Th2 cells. TGF-β was added to cultures to make the final concentration 0 ng/ml, 1 ng/ml, or 10 ng/ml. After 48 hours of culture with TGF-β, ELISA was performed on culture supernatants to detect IL-4. TGF-β does not significantly affect production of IL-4 by Th2 cells.

C. STAT4 Phosphorylation

As discussed in the introductory section, IL-12 induces the phosphorylation of STAT4 as part of its intracellular signaling cascade. Therefore, detection of a phosphorylated band corresponding to STAT4 on a Western blot demonstrates that the Th1 cell line has been activated by IL-12. Before performing this experiment, it is necessary to demonstrate that the Th1 cell lines are indeed IL-12 responsive in order to avoid a false negative result. A.E7 cells, demonstrated to be of the Th1 cell phenotype, were used in these experiments. IL-12 responsiveness was demonstrated by the increase in DNA synthesis caused by the addition of IL-12 to the media of A.E7 cells.

Table 5/Figure 9 demonstrates the IL-12 responsiveness of the A.E7 Th1 cell line. IL-12 caused a mean 62% increase in the DNA synthesis of A.E7 cells ($p<0.05$). Therefore, these cells are highly responsive to the presence of IL-12, a major growth factor for Th1 cells. A Western blot was performed (Figure 10), fixing the intracellular protein from the A.E7 cell line onto a membrane and then staining with antiphosphotyrosine antibodies. Because it is the activated, or phosphorylated, form of STAT4 that is found at high levels in IL-12-stimulated Th1 cells, it is this form of STAT4 that could potentially be inhibited by TGF-β. Anti-phosphotyrosine antibodies were used to detect the presence of the phosphorylated form of STAT4 in the Th1 cells. A control was performed that demonstrated equal protein loading of STAT4 (including both the phosphorylated and non-phosphorylated forms) in all groups (data not shown). In the control group (neither IL-12 nor TGF-β present),

A.E7 Experiments	Control	IL-12
1	8275	13990
2	8261	12112
3	8290	13829
4	9237	13310
Mean	8216	13310
St Dev	481	850
P value		0.006

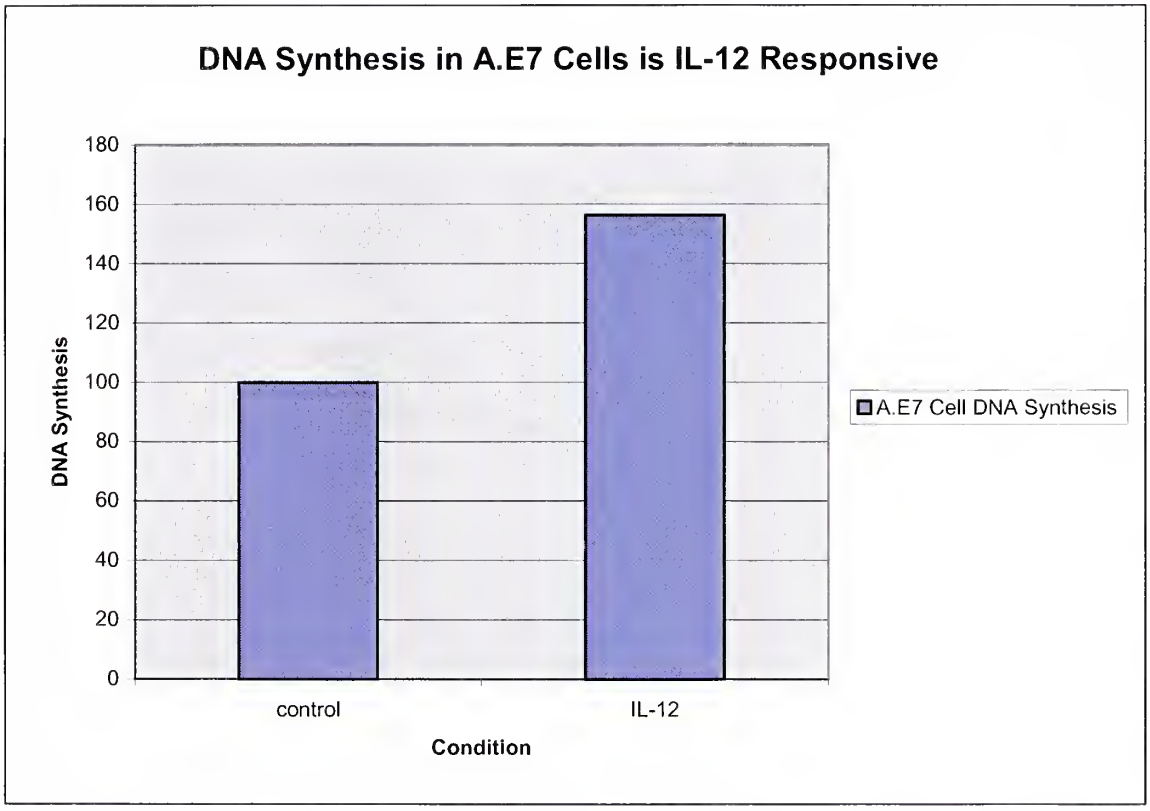


TABLE 5 & FIGURE 9: A.E7 CD4+ T cells are a known T cell line of the Th1 phenotype. The cell line was passaged every three weeks and re-stimulated with pigeon cytochrome c peptide (PCC) and antigen-presenting cells from B10.A spleens. For the use in experiments, A.E7 CD4+ T cells were stimulated with antigen-presenting cells (10:1 APC:T cell ratio) and PCC in media-containing IL-12 and anti-IL-4 antibody. The cells were washed after 48 hours and cultured with and without 10 U/ml IL-12. Cells cultured with IL-12 underwent DNA synthesis that was 62% greater than cells cultured without IL-12, verifying that the cells were IL-12-responsive.

there was no phosphorylated band corresponding to the size of STAT4 (95 kD). In the IL-12 group, there was a phosphorylated 95 kD band, corresponding to phosphorylated STAT4 protein. The addition of TGF-β to the culture media results in the inhibition of any phosphorylation of this band. Anti-STAT4 antibody was used to demonstrate the presence of a STAT4 band for all experimental conditions. The level of total STAT4 protein was equal in all groups (data not shown).

This experiment was performed several times but due to protein loading and cell culture difficulty, only one experiment was performed in which there was equal STAT4 protein present in all conditions. It is from this one experiment that the Western blot shown below was taken.

TGF-β Inhibits Phosphorylation of STAT4

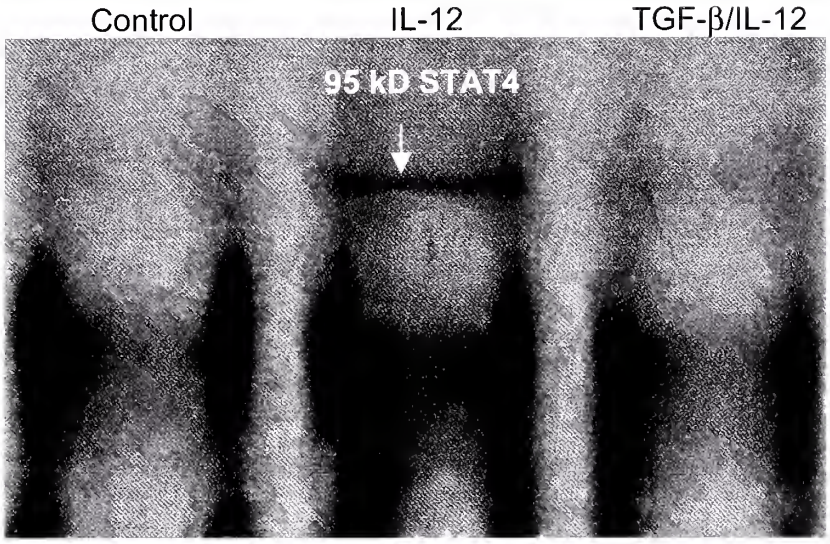


FIGURE 10: A.E7 CD4+ T cells are a known T cell line of the Th1 phenotype. The cell line was passaged every three weeks and re-stimulated with pigeon cytochrome c peptide (PCC) and antigen-presenting cells from B10.A spleens. For the use in experiments, A.E7 CD4+ T cells were stimulated with antigen-presenting cells (10:1 APC:T cell ratio) and PCC in media-containing IL-12 and anti-IL-4 antibody. Cells were cultured for 48 hours and lysed after a 6-hour incubation in 10 ng/ml TGF-β. Th1 cells were incubated in media containing 10 ng/ml TGF-β for six hours and then transferred to media containing 10 U/ml IL-12 for another 20 minutes. Cells were then lysed. Immunoprecipitation for STAT4 protein, followed by incubation with anti-phosphotyrosine antibodies was performed. STAT4 protein weighs 95 kD and can be seen on the Western blot above only for those cells exposed to IL-12 with no prior exposure to TGF-β, demonstrating that TGF-β inhibited the IL-12-induced phosphorylation of STAT4 (n=1). A control was performed using anti-STAT4 antibody, verifying that equal levels of STAT4 were present for all conditions (data not shown).

Discussion

Murine models of inflammatory bowel disease suggest that the pathogenesis of Crohn's disease is an overly-active Th1 cell response in the gut-associated lymphoid tissue, or GALT (5). Crohn's disease, then, can be depicted as an auto-immune disease in which a combination of genetics and the environment results in the dysregulation of the immune system. An antigen, that would be harmless to most, is transported by the M cell into the Peyer's patch. There, it is presented to both B cells and T cells, stimulating an immune response. As demonstrated by Fuss *et al.*, CD4+ T cells isolated from the lesions of patients with Crohn's disease produced twice as much interferon-γ when compared to CD4+ T cells from patients with ulcerative colitis and from controls (1). These T cells also produced less IL-4 and IL-5 than did T cells from controls. These experiments demonstrate that patients with Crohn's disease have increased numbers of CD4+ T cells consistent with Th1 cells.

IL-12 is a key stimulant of Th1 cells. Neurath *et al.* demonstrated that, in fact, IL-12 was indeed present in lesions found in patients with Crohn's disease, whereas it was not present in tissue taken from control patients (1). Therefore, there are two independent lines of experimentation that support the theory that the pathogenesis of Crohn's disease is an over-active Th1 cell response.

It is known from studies of oral tolerance that TGF-β is a crucial counter-regulatory cytokine. Weiner *et al.* performed several key experiments that demonstrated the fact that low doses of oral myelin basic protein fed to mice with EAE resulted in a suppression of the Th1 response and an improvement in

symptoms (3). This group made the key discovery that CD4+ T cells isolated from the Peyer's patches of mice after oral administration of an antigen were increased. Moreover, they provided evidence that suppressor T cells must then migrate to the murine splenic lymph nodes because when CD4+ T cells were isolated from spleens, administration of the same antigen to these *in vitro* T cells resulted in the production of TGF-β (12).

Knowledge of the importance of TGF-β as a counter-regulatory cytokine, in addition to several experiments that supported the fact that patients with Crohn's disease appeared to have an overactive Th1 cell response, led to the hypothesis that these patients might have a defect in one of the regulatory pathways involved in Th1 cell activation. It was known that TGF-β inhibited Th1 cell function but little was known regarding the exact nature of its effects.
In order to quantify the effects of TGF-β on Th1 cell development, several experiments were performed, as described above. The first question was what effect TGF-β had on the DNA synthesis of stimulated CD4+ T cells.

Two different Th1 cell lines were used for these experiments, one in a BALB/c background and the other in a B10.A background. In both cell lines, TGF-β inhibited DNA synthesis in a dose-dependent manner. For the A.E7 cells, 1 ng/ml TGF-β inhibited the DNA synthesis of cells by 8.4%, whereas 10 ng/ml inhibited DNA synthesis by 16.7%. In the BALB/c Th1 cell line, 1 ng/ml TGF-β decreased DNA synthesis by 35.6%, while 10 ng/ml inhibited DNA synthesis by 51.9%. It is apparent in both cell lines that TGF-β inhibited the incorporation of thymidine into DNA. Presumably, this is a reflection of cellular proliferation and

thus, TGF-β had a profound, dose-dependent effect on the growth of the Th1 cells. Knowing that TGF-β administration reversed colitis in murine models of Crohn's disease, and that anti-TGF-β antibodies can restore colitis in these mice, it is clear that this cytokine has profound effects on inflammation and the Th1 cells responsible for it.

Although TGF-β inhibited the growth of previously-differentiated Th1 cells, it had a different effect on naive CD4+ T cells and on Th2 cells. Naïve CD4+ T cells were isolated from murine spleens in the same manner as the Th1 cells. However, they were cultured in the absence of IL-12 or anti-IL-4 antibody and represented truly undifferentiated CD4+ T cells. In these naive cells, 1 ng/ml TGF-β enhanced DNA synthesis by 40.7%, whereas 10 ng/ml TGF-β enhanced DNA synthesis by 12.6%. In other words, there is a positive effect of low doses of TGF-β, while with increasing levels after this point, we see an enhancement of DNA synthesis that begins to decrease in strength. Clearly, TGF-β affects Th1 cells in a very different way than it affects naïve T cells. The bimodal effect of TGF-β has been noted by others and demonstrates the complexity of this cytokine's actions (18).

Similar experiments were performed to see what effect TGF-β would have on the DNA synthesis of differentiated Th2 cells. Weiner's model of oral tolerance predicts that TGF-β should inhibit the cell proliferation, and thus DNA synthesis, of Th1 cells, but should increase that of Th2 cells. In fact, our data support this. CD4+ T cells were again isolated from the spleens of mice in a BALB/c background. In comparison to the Th1 cell line, which was stimulated

with IL-12 and anti-IL-4 antibody, the Th2 cell line was stimulated with IL-4 and anti-IL-12 antibody, which has been shown to create a CD4+ T cell line that has the characteristics of Th2 cells (1)(27). When TGF-β was added to the Th2 cell cultures, 1 ng/ml of TGF-β increased DNA synthesis by 83%, whereas 10 ng/ml TGF-β increased DNA synthesis by 90%. In comparison to the Th1 cell lines, the Th2 cells demonstrated a positive response to the administration of TGF-β. If the mouse model of Crohn's disease suggests that the pathogenesis of the illness reflects a regulatory pathway that has gone awry, leading to an imbalance between Th1 and Th2 cells, then this result makes much sense. Decreased levels of TGF-β would activate the function of Th1 cells, while at the same time inhibit the function of Th2 cells.

These DNA synthesis studies demonstrate that TGF-β affects different populations of T cells in different ways. When oral antigen is presented to T cells in the Peyer's Patches, we know that TGF-β production activates a population of T cells that can suppress a systemic response. From these studies, we know that TGF-β inhibits the growth of Th1 cells in a dramatic fashion and also enhances the expansion and the function of other types of T cells. This provides an explanation for the biological induction of oral tolerance.

Interferon-γ production is a sign of Th1 cell activation. Therefore, measuring levels of this cytokine is another way of looking at the effects of TGF-β on the phenotype of T cells. In the A.E7 cell line, 1 ng/ml TGF-β inhibited IFN-γ production by 30%, while 10 ng/ml TGF-β inhibited production by nearly 50%. In the BALB/c Th1 cells, 1 ng/ml TGF-β inhibited IFN-γ production by nearly 20%,

while 10 ng/ml inhibited production by 30%. Because these two different Th1 cell lines are from different genetic backgrounds, as previously discussed, this shared effect demonstrates that TGF-β has important inhibitory effects on cytokine production from Th1 cells. Because the activation of Th1 cells leads to inflammation, it is no surprise that by inhibiting their DNA synthesis and interferon-γ production, one finds that increased levels of TGF-β are associated with decreased levels of inflammation in both murine models of Crohn's disease and *in vitro*.

The above data support the idea that TGF-β inhibits both the DNA synthesis and the function of Th1 cells. What effect might TGF-β have on the function of Th2 cells? These cells do not make IFN-γ, but do produce abundant levels of IL-4. Contrary to its effect on Th1 cell cytokine production, TGF-β does not seem to have a strong effect on the function of Th2 cells. IL-4 production is decreased very slightly by the administration of TGF-β, with 1 ng/ml TGF-β inhibiting cytokine production by 8% and 10 ng/ml TGF-β inhibiting production by 6%. These values are not statistically significant and really do not represent a decrease in the function of Th2 cells.

The initial studies demonstrate that TGF-β has a great inhibitory effect on both the DNA synthesis of, and cytokine production of, Th1 cells. In comparison, TGF-β actually stimulates the DNA synthesis of both naïve and Th2 cells and has no real effect on the cytokine production of Th2 cells. The question that follows is where in the pathway of DNA synthesis and cytokine production is TGF-β having an effect.

Close examination of the cytokine pathways (see Figure 4) yields a possible answer to the above question. It is known that IL-12 stimulates naïve T cells to differentiate into Th1 cells. In addition, the experiments above demonstrate that Th1 cells cultured in media containing IL-12 show decreased DNA synthesis and decreased cytokine production when TGF-β is added to the media. Therefore, TGF-β is able to overcome the stimulatory effects of IL-12. It is known that two intracellular proteins, STAT3 and STAT4, are phosphorylated during the signal transduction cascade precipitated by the stimulation of Th1 cells with IL-12 (25). Of the two intracellular proteins, only STAT4 is specifically phosphorylated solely when the T cells are activated by IL-12. There is no phosphorylated STAT4 present when the Th1 cells are not activated. Therefore, perhaps, TGF-β inhibits phosphorylation (and therefore activation) of STAT4, which would account for its Th1 cell suppressive activities.

A Western blot was performed to detect whether the presence of phosphorylated STAT4 is affected by the addition of TGF-β to activated T cells. As seen in Figure 10, there is a 95 kD band present, corresponding to the presence of phosphorylated STAT4 protein, when IL-12 is added to cultures of activated Th1 cells. This band is not present in naïve T cells (data not shown). When TGF-β is added to the cultures of activated Th1 cells, the 95 kD band disappears. Controls were performed to verify that the total level of STAT4 (both the phosphorylated and un-phosphorylated forms) were equal in all conditions. However, these results are preliminary, as there has been only one successful experiment and the results are not statistically significant.

Using all of the results above, a model begins to come to light (see Figure 11). TGF-β inhibits the DNA synthesis of Th1 cells, but not that of naïve T cells or Th2 cells. TGF-β strongly inhibits the production of interferon-γ from Th1 cells, but has no real effect on the production of IL-4 by Th2 cells. Clearly, TGF-β has profound effects on the DNA synthesis and function of Th1 cells that are very different from its effects on other CD4+ T cells. The data support the idea that TGF-β exerts its effect on Th1 cells by inhibiting the signal transduction cascade downstream of IL-12. Thus, the addition of IL-12 is insufficient to overcome the effects of TGF-β. TGF-β results in decreased DNA synthesis and decreased interferon-γ production in Th1 cells. According to this model, un-phosphorylated STAT4 protein is unable to translocate to the nucleus and have Th1-like effects. Because different signal transduction cascades are involved in the DNA synthesis of, and function of, naïve and Th2 cells, TGF-β has different effects on these cells.

The proposed model is only one possibility. TGF-β might affect more than one pathway in Th1 cells. Because a controlled experiment evaluating the effect of TGF-β on the phosphorylation of STAT4 in Th1 cells was performed only once, this must be repeated in the future. However, even if this result was statistically significant, there are other alternative explanations for the effects of TGF-β. Rather than inhibit the phosphorylation of STAT4, it might inhibit another protein in the signal transduction cascade involved in Th1 cell activation. Future experiments could be performed to further delineate the role of TGF-β in the inhibition of Th1 cell activation. By inhibiting the STAT4 protein directly, one

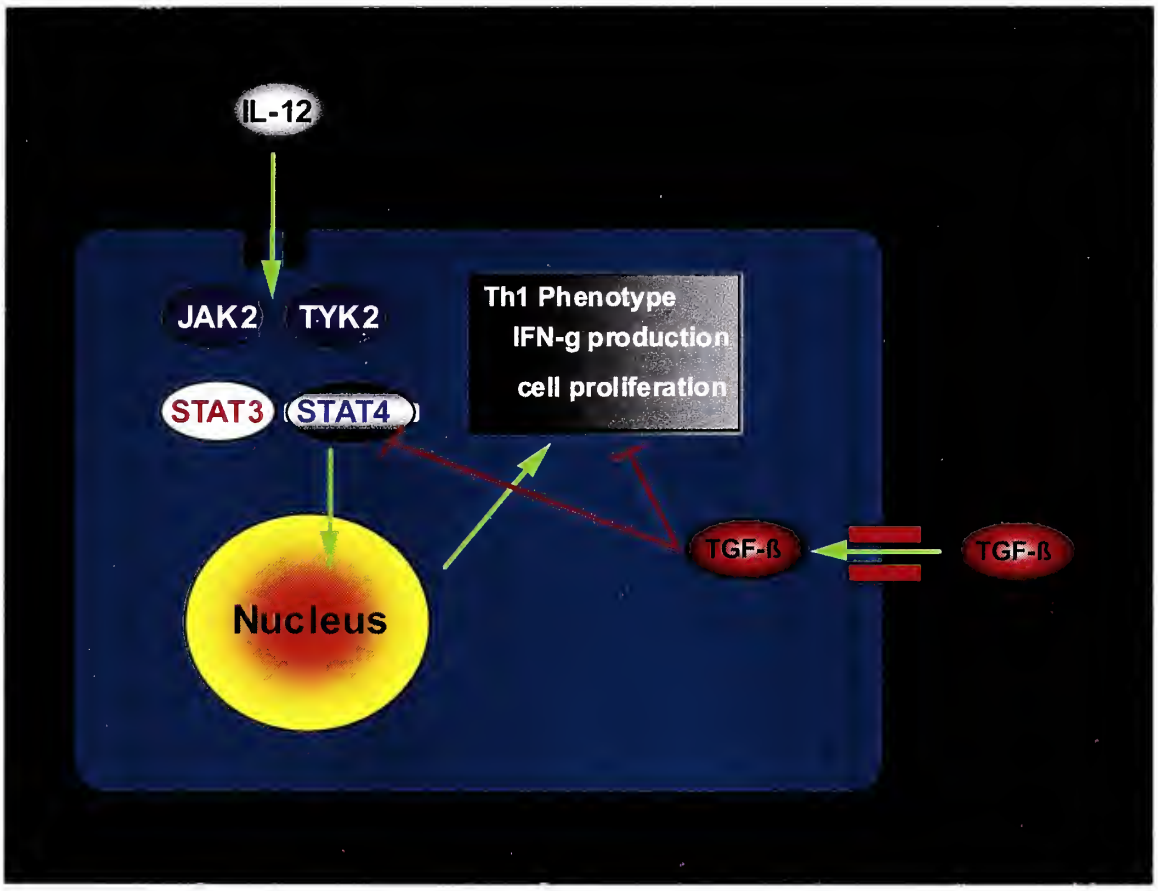


FIGURE 11: Proposed model for IL-12-induced signaling pathways in Th1 cells. TGF-β inhibits STAT4 phosphorylation, thereby inhibiting Th1 DNA synthesis and IFN-γ production.

could see if this would have the same effect as TGF-β. This might be performed through the use of antibodies or anti-sense oligonucleotides of STAT4.

Beginning with the knowledge that TGF-β is an important counter-regulatory cytokine that is produced by suppressor T cells, a model has been created that accounts for all of the effects of TGF-β on Th1 cells. Translating this knowledge into newer, more specific treatments for diseases like Crohn's disease is the next important step. Crohn's disease is an autoimmune disease of unknown etiology. Murine models of Crohn's disease support the hypothesis that the illness reflects an imbalance between Th1 and Th2 cells, with a larger proportion of the former leading to inflammation in the gastrointestinal tract. Examination of tissue from humans with the disease also supports this theory.

Trials with medications that target the IL-12-induced Th1 cell activation pathway have yielded good results thus far, further supporting the model. TNF-α, which not only has a direct effect as an inflammatory cytokine but also activates the production of IL-12 by antigen-presenting cells (1), has been one major target. Administration of anti-TNF-α antibodies decreases the amount of inflammation in murine models of Crohn's disease and has shown promising early results in human trials (26) (27) (28). NF-κB, which is an intracellular protein that is necessary for the transcription of TNF-α, has been another target. Treatment of mice with intrarectal antisense oligonucleotides of NF-κB has been effective in alleviating symptoms of colitis in the murine model (1).

Both anti-TNF-α and antisense NF-κB are treatments that inhibit the production of IL-12 and act much more specifically than general

immunosuppressants, like corticosteroids. However, the model above suggests that there might be a treatment that is still more specific for inhibiting the Th1 pathway. STAT4 is an intriguing target. Administration of antisense oligonucleotides of STAT4 could selectively inhibit Th1 cells and reduce inflammation in Crohn's disease. Because STAT4 is phosphorylated only in activated Th1 cells, perhaps the phosphorylation of STAT4 would be a similar target in the development of a specific treatment for Crohn's disease. Both of these methods should specifically inhibit Th1 DNA synthesis and cytokine production, leaving the Th2 pathways responsible for antibody production alone.

Several key experiments are necessary to further understand the effects of TGF-β and the model of Th1 cell activation. How does TGF-β affect the other signaling elements involved in the IL-12-induced signal transduction cascade? What effects does TGF-β have on the signal transduction elements involved in the activation of Th2 cells or naïve T cells? If Crohn's disease reflects an imbalance of Th1 and Th2 cells, what exactly is the defect? Is it the same in every patient or are there multiple sites at which the system can become dysregulated? All of these questions need to be answered in order to design treatment modalities that can correct the defect or defects in the regulation of the T cell activation and differentiation pathways. With antibodies, gene therapy, antisense oligonucleotides, or other new modalities, the treatment of Crohn's disease will become more specific, will have fewer side effects, and will eliminate the need for harmful, generalized immunosuppressant medications.

VI. References

1. Strober, W., Ludviksson, B.R., and Fuss, I.J. 1998. The pathogenesis of mucosal inflammation in murine models of inflammatory bowel disease and Crohn disease. *Ann. Int. Med.* 128(10): 848-56.

2. Present, D.H., Rutgeerts, P., Targan, S., Hanauer, S.B., Mayer, L., *et al.* 1999. Infliximab for the Treatment of Fistulas in Patients with Crohn's Disease. *N. Engl. J. Med.* 340: 1398-405.

3. Faria, A.M. and Weiner, H.L. 1999. Oral tolerance: mechanisms and therapeutic applications. *Advances in Immunology*. 73: 153-264.

4. Kelsall, B.L. and Strober, W. 1996. Host Defenses At Mucosal Surfaces. In *Clinical Immunology: Principles and Practice*. R. Rich, T. Fleisher, B. Schwartz, W. Shearer, and W. Strober, editors. Boston: Mosby. 299-332.

5. Strober, W., Fuss, I.J., Ehrhardt, R.O., Neurath, M., Boirivant, M., and Ludviksson, B.R. 1998. Mucosal Immunoregulation and Inflammatory Bowel Disease: New Insights from Murine Models of Inflammation. *Scand. J. Immunol.* 48: 453-458.

6. Ludviksson, B.R., Ehrhardt, R., and Strober, W. 1997. TGF-β Production Regulates the Development of the 2,4,6-Trinitrophenol-Conjugated Keyhole Limpet Hemocyanin-Induced Colonic Inflammation in IL-2-Deficient Mice. *J. Immunol.* 159: 3622-3628.

7. Marth, T., Strober, W., and Kelsall, B.L. 1996. High Dose Oral Tolerance in Ovalbumin TCR-Transgenic Mice: Systemic Neutralization of IL-12 Augments TGF-β Secretion and T Cell Apoptosis. *J. Immunol.* 157: 2348-2357.

8. Dakin, R.1829. Remarks on a cutaneous affection produced by certain poisonous vegetables. *Am. J. Med. Sci.* 4: 98-100.

9. Wells, H.G. 1911. Studies on the chemistry of anaphylaxis. Experiments with isolated proteins, especially those of the hen's egg. *J. Infect. Dis.* 8: 147-171.

10. Chase, M.W. 1946. Inhibition of Experimental Drug Allergy by Prior Feeding of the Sensitizing Agent. *Proc. Soc. Exper. Biol. & Med.* 61: 257-259.

11. Friedman, A. and Weiner, H.L. 1994. Induction of anergy or active suppression following oral tolerance is determined by antigen dosage. *Proc. Natl. Acad. Sci. USA.* 91: 6688-6692.

12. Miller, A., Lider, O., Roberts, A.B., Sporn, M.B., and Weiner, H.L. 1992. Suppressor T cells generated by oral tolerization to myelin basic protein suppress both *in vitro* and *in vivo* immune responses by the release of transforming growth factor β after antigen-specific triggering. *Proc. Natl. Acad. Sci. USA.* 89: 421-425.

13. Weiner, H. 1994. Oral Tolerance. *Proc. Natl. Acad. Sci. USA.* 91: 10762-10765.

14. Letterio, J.L. and Roberts, A.B. 1997. TGF-β: A Critical Modulator of Immune Cell Function. *Clinical Immunology and Immunopathology.* 84(3): 244-250.

15. Cox, D.A. and Maurer, T. 1997. Tranforming Growth Factor-β. *Clinical Immunology and Immunopathology.* 83(1): 25-30.

16. Massague, J. 1998. TGF-β Signal Transduction. *Annu. Rev. Biochem.* 67: 753-91.

17. Lawrence, D.A. 1996. Transforming growth factor-β: a general review. *European Cytokine Network.* 7(3): 363-374.

18. Bonyadi, M., Rusholme, S.A., Cousins, F.M., Su, H.C., Biron, C.A., Farrall, M., and Akhurst, R.J. 1997. Mapping of a major genetic modifier of embryonic lethality in TGFβ1 knockout mice. *Nature Genetics.* 15: 207-211.

19. Sanford, L.P., Ormsby, I., Gittenberger-de Groot, A.C., Sariola, H., Friedman, R., et al. 1997. TGFb2 knockout mice have multiple developmental defects that are non-overlapping with other TGFb knockout phenotypes. *Development.* 124: 2659-2670.

20. Janeway, C.A., and Travers, P. 1996. *Immunobiology: The Immune System in Health and Disease.* 2nd ed. Garland Publishing. New York.

21. Ehrhardt, R.O., Ludviksson, B.R., Gray, B., Neurath, M., and Strober, W. 1997. Induction and Prevention of Colonic Inflammation in IL-2-Deficient Mice. *J. Immunol.* 158: 566-573.

22. Bacon, C.M., Cho, S.S., and O'Shea, J.J. 1996. Signal Transduction by Interleukin-12 and Interleukin-2: A Comparison and Contrast. *Annals New York Academy of Sciences.* 795: 41-59.

23. Schmitt, E., Hoehn, P., Huels, C., Goedert, S., Palm, N., Rude, E., and Germann, T. 1994. T helper type 1 development of naïve CD4+ T cells requires the coordinate action of interleukin-12 and interferon-γ and is inhibited by transforming growth factor-β. *Eur. J. Immunol.* 24: 793-798.

24. Lamont, A.G. and Adorini, L. 1996. IL-12: a key cytokine in immune regulation. *Immunology Today*. 17(5): 214-217.

25. O'Shea, J.J. 1997. Jaks, STATs, Cytokine Signal Transduction, and Immunoregulation: Are We There Yet? *Immunity*. 7: 1-11.

26. Targan, S.R., Hanauer, S.B., van Deventer, S.J., Mayer, L., Prersent, D.H., and Braakman, T. 1997. A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor-alpha for Crohn's disease. *N Engl J Med*. 337: 1029-35.

27. Stack, W.A., Mann, S.D., Roy, A.J., Heath, P., Sopwith, M., and Freeman, J. 1997. Randomized controlled trial of CDP571 antibody to tumor necrosis factor-α in Crohn's disease. *Lancet*. 349: 521-4.

28. Hawkey, C.J., and Stack, W.A. 1998. Chimeric Monoclonal Antibody cA2 to Tumor Necrosis Factor (alpha) for Crohn's Disease. *N. Engl. J. Med.* 338(5): 333.

29. Bickston, S.J. 1998. Treatment of Crohn's Disease at the Turn of the Century. *N. Engl. J. Med.* 339(6): 401-402.

30. Van Deventer, S.J., Elson, C.O., and Fedorak, R.N. 1997. Multiple doses of intravenous interleukin 10 in steroid-refractory Crohn's disease. Crohn's Disease Study Group. *Gastroenterology*. 113: 383-9.

31. Seder, R.A., Gazzinelli, R., Sher, A., and Paul, W.E. 1993. Interleukin 12 acts directly on CD4+ T cells to enhance priming for interferon γ production and diminishes interleukin 4 inhibition of such priming. *Proc. Natl. Acad. Sci. USA*. 90: 10188-10192.

32. Hecht, T.T., Longo, D.L., and Matis, L.A. 1983. The relationship between immune interferon production and DNA synthesis in antigen-specific, MHC-restricted T cell lines and clones. *J. Immunol.* 131(3): 1049-1055.

32. Combadiere, B., Reis e Sousa, C., Germain, R.N., and Lenardo, M.J. 1998. Selective Induction of Apoptosis in Mature Lymphocytes by Variant T Cell Receptor Ligands. *J. Exp. Med.* 187(3): 349-355.

HARVEY CUSHING / JOHN HAY WHITNEY
MEDICAL LIBRARY

MANUSCRIPT THESES

Unpublished theses submitted for the Master's and Doctor's degrees and deposited in the Medical Library are to be used only with due regard to the rights of the authors. Bibliographical references may be noted, but passages must not be copied without permission of the authors, and without proper credit being given in subsequent written or published work.

This thesis by Andrew S Resnick has been used by the following persons, whose signatures attest their acceptance of the above restrictions.

NAME AND ADDRESS	DATE

YALE MEDICAL LIBRARY
3 9002 01107 1827