"Reflections on Being a Christian Who Enjoys Being a Scientist: (How) Does My Christianity Influence the Practice of My Vocation? How Does My Vocation Impact My Christianity?"

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Messiah College is a Christian college of the liberal and applied arts and sciences. Our mission is to educate men and women toward maturity of intellect, character and Christian faith in preparation for lives of service, leadership and reconciliation in church and society.
Reflections on being a Christian who enjoys being a Scientist:
(How) Does my Christianity influence the practice of my vocation?
How does my vocation impact my Christianity?

An

Alternative Scholarship Essay

Submitted in partial fulfillment of the requirements for

Promotion to the Rank of Professor

by

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Nature and Purpose of this Essay

This Alternate Scholarship Essay is intended to accompany an article by Staveley-O’Carroll and coworkers (29) that was published in The Journal of Immunology and on which I am a coauthor. That study expands upon multiple additional earlier studies (8, 15, 19-22, 24-27) in which I (and others) have investigated CD8+ T lymphocyte induction and recognition for multiple peptide epitopes within the Simian virus 40 large tumor antigen. The latest paper by Staveley-O’Carrol et. al. (29) describes studies performed using Simian virus 40 large tumor antigen epitope I-specific CD8+ cells obtained from transgenic mice which I constructed. The transgenic T cells were used to illustrate how a growing tumor can specifically incapacitate a T cell immune response that should recognize and destroy the tumor. The study additionally shows how proper therapeutic intervention may potentially reverse the tumor-induced peripheral tolerance.

The purpose of this essay is to describe the implicit Christian dimensions of my scientific work. I will reflect on relationships or interactions that I experience between my disciplinary scholarship and how I understand myself as a Christian scholar. In doing so, I will consider two primary interactions. First, the fact that I am an active research scientist means that I collaborate and communicate with secular science professionals; at the same time, I am viewed as a “scientist” by Christian acquaintances who are not scientists. I have found that opinions about Christians held by other professionals in science can challenge my credibility as a scientist, and that opinions about scientists held by believing non-scientists can challenge my credibility as a Christian. Secondly, my self understanding as a Christian does impact on how I view my vocation as a scientist, even though it may not dictate how I actually perform my science on a
daily basis (reasoning, experimentation, reporting). However, my Christian faith does impose limits on experimental strategies, techniques, or reagents I might otherwise employ or condone. Further, my Christian faith allows me to view the biological systems on which I work as a highly complex collection of collaborating molecules through which The Almighty may choose to act in non-quantifiable or non-repeatable ways, and I can view these systems with doxological fascination. It is with such doxological fascination that I view the parallels between the epitope-specific tumor-induced tolerance described in the article coauthored with Staveley-O'Carrol et al. (29) and the way in which morally questionable options can gradually gain a foothold in the life of a believer or within a society.

**As a Christian in Science, I am often misunderstood by scientists who are not Christians, and by Christians who are not scientists.**

Well before the era of opportunities afforded by recent technological advances in the modern sciences of molecular and cell biology, James, the brother of Jesus, reminded first century Jewish Christians that actions often speak more loudly than words. To those who would proclaim the message and benefits of Christ's salvation without personally and consistently showing clear evidence of the life-changing, cleansing work of Christ, James would simply say, "I doubt the authenticity and sincerity of your remarks!" The words of James [especially chapter 2:14 ff; (1)] continue to prompt Christians in today's complex and technologically advanced society to question whether the things we do reflect the faith we profess. Are my actions in both my private and public/corporate/business lives consistent with, or directed by the faith I hold? Do limitations or etiquette unique to the professional world necessarily limit the outward expression of this faith? Do I remember to thank my creator daily for the wonders I discover within the
complexities of His biological world? I think James would suggest that as a professional who does science, my actions in the professional arena should consistently model the Christ-yieldedness that motivates me from within. Toward this goal of consistency, I, like the Apostle Paul, "press on" [Philippians 3:14; (1)].

I consider myself to be a scientist by vocation, and my scholarly efforts have been focused within the disciplines of molecular biology and cellular immunology. The early part of my research career focused on investigating complex aspects of the mechanisms by which simple yeast cells (the bakers yeast, *Saccharomyces cerevisiae*) control the decision to utilize the sugar galactose instead of the energetically preferred substrate glucose. My research focus has since shifted towards understanding how one arm of cellular immunity, namely the CD8+ T lymphocytes, can be harnessed to control murine (and ultimately human) tumors. This includes understanding the cell biology of how epitope targets are recognized by CD8+ T lymphocytes, how the epitopes recognized by tumor-specific CD8+ T lymphocytes can be effectively used to immunize against antigenic tumors, how mutation of these same epitope targets may allow for tumor escape, and how peripheral immunological tolerance mechanisms may influence the success of anti-tumor immunization strategies. I have experienced tension as a result of opinions/perceptions of other science professionals and laypersons while doing science in these areas.

Can an individual be both a committed Christian and a quality scientist (especially a molecular biologist who alters gene sequences as a routine approach to study biology)? In my mind, the answer has always been a resounding "Yes!" As a scientist, I simply want to figure out how a living system works. My methods are observation, reflection, and experimentation.
Hypotheses are conceived and then tested by carefully designed experiments in which limited numbers of variables are carefully altered under controlled conditions. The truly good experiments are those which are designed to rule out competing hypotheses, rather than to simply demonstrate additional phenomena that may be consistent with a favored model.

In their recent publication, *Scholarship and Faith, Enlarging the Conversation*, Douglas and Rhonda Jacobsen broaden the scope of activities that should reasonably be defined as Christian Scholarship to include scholarly activities where the "role of faith will...protrude much less visibly, being only implicit in the motivation, assumptions, and message of the work rather than being explicitly flaunted." The Jacobsens suggest that scholarship that is Christian may be so without appearing outwardly distinct from scholarship that is produced in the same discipline by persons who are not motivated or lead by faith (14). While Christian faith may not change the way a scholar approaches the study of a particular problem, it can add breadth to the implications seen in the results or course of an investigation (10, 12, 13, 32). A scientist of faith has the latitude and responsibility to question how/whether factors beyond the molecules that comprise and control a system may indeed somehow influence its activity even though proper experimental study of the system itself requires exactly the type of simplification, isolation, and control or removal of external variables that would otherwise preclude consideration of such external influences. In a truly objective endeavor, my faith should not confound the empirical processes I use to understand how biological systems work [e.g. immune cell function or transcriptional control in yeast; (12-14)]. It may, however, guide my interest and motivation (9).

Nonetheless, I [and others; (12)] have encountered doubt about my credibility as a Christian and/or a scientist. Often, the question of origins (human origin or the origin or life in
general) may underlie this doubt. Non-scientists and secular scientists often automatically associate the practice of science and the activities of scientists (especially biologists) with automatic accession to an *atheistic* Darwinian view of the origin of all living organisms by unguided common descent from a common original life form. To be a credible scientist, one must hold that the natural world has been generated by random processes which must be viewed as independent of the actions, oversight, or creative will of a supreme being [naturalism; (11, 16)]. Therefore, when a Christian science professional considers that contributions beyond the natural world, which cannot be verified by empirical testing, could have played a role in guiding the process, his logical consistency is considered questionable by professional peers who hold to a strictly naturalistic view. Nonetheless, it is reassuring to me that many committed believers who are also science professionals have, and continue to wrestle with the problem of origins from different perspectives (2, 4, 7, 17, 23, 28, 32). While a growing body of evidence does provide mechanisms whereby the diversity of life on earth *may* be explained, the answer to the question of the actual origin of biological life will probably remain elusive because it was likely a one time event (17).

Are Christian science professionals somehow restricted in their ability to do “pure” science because they rely on logic processes that are limited (or perverted) by an inclination to reach out to or consider the supernatural? After all, how could a Christian who believes in the ability of a supreme creator to bring about and then interact with the diverse natural world ever hope to establish how a natural process works without resorting to simplistic, faith-evoked mechanistic explanations at times when an empirical or natural explanation may remain elusive? Secular scientists may also reasonably question the intellectual consistency or competency of one who agrees that the biblical account of the death and resurrection of Christ
relates a true historical record of how God in a completely human form was killed and the then
returned to life. Perhaps believing scientists (myself included) must simply be willing to live in
a faith-filled tension. Francis Collins, a believer, an internationally respected scientist, and the
former director of The National Center for Human Genome Research (the federally funded
organization that participated in the recently completed human genome sequencing project),
has noted that the bodily resurrection of Christ remains a point about which he (and Christians
in science) will (should) not concede (5). On the other hand, scientists who admittedly study
human origins with the perspective that the Genesis account should be interpreted literally
(order and timing of creation and Noah’s flood) must be cautioned to avoid allowing their faith
to inappropriately narrow the empirical processes needed to interpret geological and cosmic
records (5). My personal response has been to continue to do quality science in a way that
allows experiments to be designed and interpreted in an empirical fashion, and, with an
understanding heart, politely accept the criticisms of my antagonists. In the end I content
myself in knowing that, at this time, no scientist, philosopher, or theologian knows the "how" to
the question of origins, that the biblical record of the resurrection does include the testimony of
multiple witnesses, and that faith would not be faith unless it required holding to unknowns that
cannot be verified using scientific methods.

I have also experienced tension in interactions with Christian non-scientists who are
aware that I am a science professional. A conversation with an advisee who at the time was
considering changing his/her major from a natural science to music illustrates a common
perception about scientists held by many non-scientists. During the course of the conversation,
the question arose (rather abruptly, as though asked out of fear) about how I reconciled being a
Christian in science. The implication was clear. There was, in the mind of the student, the
suspicion that being a scientist was somehow inconsistent with the ability to practice “true” Christianity. As a molecular biologist who studies the natural world inside of a cell or an organism by altering the "natural" structure of the nucleic acid genome, more than once I have been asked by non-scientists whether what I do is “evil”. A red-faced Bishop (also a relative) once apologized after I politely replied that I actually was one of those people who participated in the "evils of recombinant DNA technology." Many non-scientists simply do not appreciate that scrambling of genes and genomes has likely occurred by natural processes (recombination and/or mutation) for as long as organisms have reproduced. Recent analysis of the human genome has shown that humans harbor high numbers of nucleotide sequence regions that appear to be the byproducts of mobile genetic elements similar to retroviruses that have participated in fashioning the modern human genome (5, 18). In fact, analysis of these sequence elements has been used to support to the Darwinian notion that subsets of living creatures do appear to share a common ancestry (5, 6, 17, 32). Countering the incorrect notions about scientists that have been constructed in the minds of Christians who are not scientists (often by sincere Bible teachers in their zeal to support a favored view of the origins question or warn against the immoral use of biotechnology) will continue to require patience and understanding on both sides.

My Christian faith imposes limitations on the technology I may use to do science.

My commitment as a Christian also impacts on how I view and/or carry out my vocation as a scientist. I observe self-imposed limits as to the type of technology that I will use to solve scientific questions. This choice may indeed limit my ability to contribute to a given field of research in the future.
The process of scientific inquiry is intended to uncover new information or provide new understandings of established information. Scientific advancement is often accompanied by the development of new technologies which allow for the new information to be obtained. The healthy cycle of concept, challenge, discovery, and creative re-application drives science. In some cases, however, experimental capabilities provided by conceptual or technological breakthroughs press moral or ethical boundaries. As a Christian in science, should I pursue an avenue of inquiry if the method by which it would be studied may be morally questionable, or if it will inevitably lead to derivative technological gains that from the outset would be used for unethical applications?

Our studies have relied heavily on the ability to generate mice that have been genetically programmed to express either cancer-causing proteins (e.g., SV40 TAg produced by the 501 mice) or a repertoire of T cells that uniformly produce identical TCRs (e.g., epitope I-specific TCR transgenic mice reported in Staveley O'Carrol et al.). Generation of such lines of transgenic mice involves introduction of appropriate recombinant DNA constructs into the single cell embryo or into embryonic stem cells. Viable offspring are then screened to the presence and expression of the inserted transgene, and subsequent breeding is used to achieve appropriate genetic backgrounds for desired experiments.

Re-engineering of human embryonic stem cells has been promoted as a means to provide for cellular replacements as a treatment of injury or disease. The therapeutic benefits anticipated from the use of human embryonic stem cells, however, illustrate a timely example of a morally questionable technology. In short, current technologies allow pluripotent stem cells to be harvested from the inner cell mass (ICM, the embryo proper at that stage) of several day old
embryos that have been fertilized in the laboratory. The developmental plasticity of these pluripotent ICM cells should (in theory) allow them to be differentiated into almost any cell type. Therefore the ability to generate and differentiate pluripotent ICM cells in the laboratory represents the potential to generate cells or tissue types needed to replace damaged or degenerated tissues within an adult. The combined use of embryonic stem cell isolation technology and embryonic cloning by somatic cell nuclear transplantation holds out the possibility of generating embryonic replacement cells that are genetically and immunologically matched to the intended recipient. Perfect matching would avoid the otherwise automatic complications arising from immune rejection of the transplanted cells. The moral dilemma is obvious: the embryos from which these stem cells must initially be obtained are several day old human embryos that would have had the potential to develop into human beings if provided with appropriate conditions (womb implantation). The same moral argument applies to embryos that remain unimplanted following standard in vitro fertilization procedures or those that have been generated uniquely for a given recipient by somatic cell nuclear transfer; all represent human zygotes that contain the total complement of genetic and cellular materials required to complete development. If the image of God resides within the zygote after formation of the diploid nucleus (completion of syngamy), then it is morally unacceptable to sacrifice the life of what is from that time an underdeveloped individual to perpetuate the lives of others, regardless of the benefits anticipated from further development of this fledgling technology. More recent advances in iPSC (induced pluripotent stem cell) technology may make the harvesting of pluripotent stem cells from embryos unnecessary (31, 33, 34). Co-transfection with multiple proto-oncogenes may allow donor-derived somatic cells to be reprogrammed to undergo reverse development, reassuming the pluripotent capacities of stem cells derived from the inner cell mass.
of an embryo. If so, such technology would bypass the need to initially generate a totipotent (capable of regenerating the organism) zygote-like individual from which pluripotent (capable of generating any tissue, but not the entire organism) stem cells would later be harvested for therapeutic uses.

My Christian faith adds depth to the meaning and the implications of the science that I do.

As a scientist, I am intrigued by the manner in which the highly complex immune system is controlled with delicate balance. As a Christian, I recognize that a transcendent omnipotent may choose to alter its function in a given individual by means that cannot be measured or repeated under controlled conditions. As a science professional who is a Christian, I regard our increasingly sophisticated understanding of immune system components and their control with doxological fascination, and find in it multiple analogies to aspects of the Christian life that simply would not be evident to an equally dedicated scientist who studied the same system with other than a Christian world view (32).

My Christianity provides me with the privilege and responsibility to view the human immune system as more than the sum of its component molecules and cells. As a scientist, I am intrigued by the manner in which the highly complex immune system is controlled with delicate balance. As a Christian, I am responsible to avoid the reductionist view which would suggest that nothing beyond the molecules comprising the system could impact on its function (in a reductionist view, if an experiment could not be designed to measure “it”, “it” must be considered irrelevant to the system; (11, 32)). Christians in science are called to think beyond their variable-controlled empirical experimental models. As a Christian, I recognize that a transcendent omnipotent may choose to alter immune function within a given individual through
means that could not be measured empirically because it could not be repeated under controlled conditions. What role(s) might the immune system play in instances of miraculous cancer regression? How might attitude and personality combine with faith to influence the function or regulation of the immune system to promote (or frustrate) protective [anti-tumor] immunity? To what extent can (and does) God cause conditions to tip the delicate balance in favor of tumor control vs. tumor progression for His perfect purposes? To an empiricist, such non-repeatable, non-quantifiable interventions must be discounted because they cannot be shown to operate under the variable-controlled, repeatable conditions required to distinguish among alternate explanations. While it is certainly possible for God to intervene by his miraculous hand to cause the disappearance of a life-threatening malignancy, He may choose to do so by miraculous means or by simply orchestrating a chance encounter with another environmental antigen (infectious agent) or allergen that alters the natural balance of immunity already possessed by an individual to favor detection and destruction of the tumor. Therefore, a Christian scientist must entertain the notion that the non-quantifiable can operate in miraculous ways, understand the complexities through which changes could be caused to occur by a perfect orchestration of natural mechanisms, praise God for the outcome as it fits into His perfect will, and wonder how He actually did accomplish the cure.

Finally, as a Christian science professional, I view the complex systems on which I work with doxological fascination (10). I praise God for the immunological wonders he has created for me to study. Moreover, as I study the detailed mechanisms of how the immune system is controlled, it is hard for me to ignore functions and structures that provide life application illustrations. However, the applications I see can only be recognized by a Christian who also understands the complexities of the science. As a science professional, I am privileged to use
scientific components of my discipline to supply illustrations that can enhance understanding of aspects of the Christian life.

The immune system has been considered in a related manner by Brandt (3) as part of an earlier and larger doxological work that focused on multiple aspects of human physiology. Brandt's treatment of the immune system was necessarily simplistic because it was composed a decade before the paradigm-shifting discoveries that have lead to the current understanding of how T lymphocytes are controlled by recognition of MHC-presented peptides. More recently, Story (30) has elaborated in a more sophisticated manner on parallels taken from the guaranteed randomness which contributes to the development of the B lymphocyte immune repertoire and how randomness could be used as an essential agent of a perfect creation.

I point out four interesting parallels that emerge from the newer understanding of the T cell immune system function at the cellular and molecular levels: 1. the phenomenon of tumor-induced peripheral immune tolerance provides an interesting parallel to how sin may slowly accumulate in the life of a believer, or how amoral practices may slowly become accepted within a Christian society (available in an expanded form upon request; this illustration most closely relates to the science presented in the manuscript by Staveley-O'Carrol et al. (29)); 2. the phenomenon of T lymphocyte antigen receptor antagonism by altered peptide ligands nicely parallels the frustration believers may experience when trying to avoid many "good" options while seeking the "best" path or action that is most closely aligned with the will of God; 3. the intracellular processing and cell surface presentation of tumor antigen epitope peptides by MHC class I molecules provides an interesting illustration for the notion that the innermost workings of individuals are not hidden from our omniscient Creator; 4. the realization that the random joining
and selection processes used to generate a vigilant army of T and B lymphocytes (provide for acquired immunity) in each individual is not unlike a "random" process by which an Almighty Creator may have chosen to generate a universe filled with life that could later worship and commune with Him (30).

The essence of the first illustration mentioned above relates to the science presented in the manuscript by Staveley-O'Carrol et al. (29) and is briefly summarized in the following paragraphs. (A more detailed explanation of T cell development and T cell antigen receptor structure and generation is provided in the accompanying appendix.) In short, recognition of foreign peptides by T lymphocytes can trigger effective protective immune responses. The foreign peptides are usually derived from infectious microbes. Therefore, the presence of the foreign, T cell stimulating peptides is usually accompanied by other "danger" signs (e.g. mediators of inflammation) that accompany a microbial infection. In the presence of these danger signs, foreign peptides trigger T cells to respond appropriately in a manner that will destroy the infectious microbe, because the peptides are recognized as foreign under conditions where a threat has been sensed. By contrast, foreign peptides can also be delivered to T lymphocytes in a manner that causes them to instead become tolerant to the presence of the foreign peptide signals. The T cells either become paralyzed, or are eliminated by programmed cell death. This is the manner in which slowly growing tumors may actually destroy immune defenses (tumor antigen-specific T lymphocytes) that should be able to recognize and destroy them. The tumor gradually presents the foreign tumor-derived peptides, but under conditions where additional danger signals (like those that normally accompany a microbial infection) are absent. The manuscript by Staveley-O'Carroll et al. (29) presents results for the tumor induced
peripheral tolerance within populations of SV40 T antigen epitope I-specific transgenic T lymphocyte populations.

An interesting parallel can be drawn between the way in which a developing tumor can induce tolerance to itself and the way by which sin may enter the lives of an otherwise committed Christian individual or a “Christian” society. Believers are supplied with tools to recognize, avoid, and purge sin from their lives. These include God’s written word, the counsel, teaching and example of other believers, and the inner guidance of a God-given spirit and conscience. Nonetheless, Christians do sin (and continue to sin) because they are imperfect, fallible humans living in a world filled with temptations. Actions or omissions that represent a missing of the mark (flagrant disobedience to God’s commands or instructions, or failing to act when called) are routinely experienced by the believer, despite the best of intentions (e.g. Paul’s frustration expressed in Romans 7). The cycle of temptation, failure, and forgiveness is all too familiar to many believers (myself included). Believers do experience success in recognizing and avoiding sin. Like the immune system, the wrongs that appear to be obviously wrong can be readily recognized and avoided. Like a microbial infection, the absurdity of many temptations and their obvious consequences automatically trigger many danger flags that prompt us to avoid them.

There are numerous examples of “little” things that routinely confront us and, like the tumor peptides that are presented in the absence of the danger signals accompanying infection, can deaden our normal defensive reactions to sin. Initial choices that appear to be small, morally neutral or “white lie” choices may represent the beginning of a sinister continuum. Over time, flawed or misguided rationalization replaces proper reasoning, and reduces sensitivity to God’s
leading in that area. Peripheral, tumor-induced tolerance exerts its affects on individual T cells, but the insensitivity of growing numbers of individual T cells is ultimately borne out by at by the inability to detect a tumor-specific response at the level of the total T cell population. Christians can succumb to sin on an individual level, or fail to speak out as members of a society that is being misled. As morally questionable practices take hold within a society, is it not easier for a society to take subsequent steps down the sinister continuum (whatever it is) because increasing numbers of individuals who might otherwise oppose an activity fail to recognize the wrong or are no longer convicted to speak out against it? The “wrong” may be similar to things to which the society has become accustomed. It becomes easier to simply not respond with the crowd. Therefore, tumor-induced tolerance in the SV40 T antigen system provides a compelling parallel to the manner in which sin can take hold in the life of the individual believer or within a Christian society.

**Final thoughts**

In this alternate scholarship essay I have described several ways in which I have experienced interaction between my vocation and my Christian faith. I see that my Christian faith does influence the way I am perceived by other scientists and by non-scientists. My Christian faith compels me to set limits on the technologies that I might employ to do research. My Christian faith allows me to acknowledge that God may choose to work in non-repeatable ways in his creation through the marvelous and complex immune system provided to humans. Finally, my Christian faith allows me to see interesting and new ways in which my science can illustrate biblical principles. My vocation as a researcher in molecular biology and cellular immunology and my life as a Christian are inextricably intertwined.
References


Figure 1 (Mylin). T cells (top) use a two subunit (α and β) antigen receptor to recognize (dock with) peptides (jagged line) presented by (bound to) major histocompatibility (MHC) molecules displayed on the surface of host antigen presenting cells (bottom). Vertical lines extending from the peptide backbone (zig zag line) indicate amino acid side chains important for MHC binding or TCR contacts. The surface of the TCR that makes "distinguishing" contacts with the key peptide side chains is coded for by primarily by the D and J regions and the end of the variable region (V). Alpha chains do not contain a D region segment.
**Figure 2 (Mylin).** Recombination (somatic) at special TCR alpha and beta chromosomal loci must occur for a T cell to "build" the two genes needed to encode its antigen receptor (TCR). Only the enlarged beta locus is illustrated. The process is irreversible because the DNA between the joined segments is lost. Perfect joining is required for the joined segments to code for the full subunit.

\[ J = \text{Joining segments (~12)} \]

\[ V = \text{Variable region Segments (~30)} \]

\[ D = \text{Diversity segments (~4)} \]

\[ C = \text{Constant segments (2; identical)} \]

"Successfully recombined VDJ – C TCR beta gene

T cell expressing a functional TCR

Gene expression (transcription, splicing, translation)

Folds, is paired with a partner subunit, and the pair are transported to the membrane surface of the T cell"
Figure 3 (Mylin). Steps and Anatomic Locations of T cell Development.

New, immature T cells are produced by division of stem cells

Bone marrow

Migrate

Thymus

Recombination attempts to form bet a subunit gene

Survive if successful; recombination attempts to form alpha subunit gene

Positive Selection: Expression of “functional” receptor (alpha and beta subunit genes are both "good") and co-receptor (CD8 or CD4) that can dock with MHC molecules allows survival

Negative Selection: T cells whose receptor binds to tightly to an MHC-peptide combination found in the thymus are triggered to die.

Periphery: lymph nodes, spleen, etc. T cells wait for antigen(s) & activating signals

Exit and Migrate
Appendix: Lessons from Cell Mediated Tumor Immunity, T Lymphocyte Development and Mechanisms of Peripheral Immune Tolerance

Did God really design the human immune system to prevent cancer? What role(s) might the immune system play in miraculous cancer regression? How might attitude, personality, faith or other environmental factors influence the function or regulation of the immune system to promote or frustrate protective [anti-tumor] immunity? Because cancer cells are immunologically similar to normal host tissues, will it be possible to trigger anti-tumor responses that will recognize and effectively destroy cancer while leaving normal tissues unharmed? Can these insights be translated into generally effective anti-cancer vaccines? How can the immune system be manipulated to achieve these beneficial outcomes without inducing debilitating or lethal autoimmunity? Answering such questions will require a better understanding of our complex and delicately balanced acquired immune system.

My motivation as a Christian in science has been to understand the basic mechanisms that control immune detection/recognition of cancer with the hope that such an understanding can be used to ultimately develop cures for this dreaded disease. Understanding how to properly overcome peripheral immune tolerance will be crucial to designing successful strategies to induce anti-tumor immunity in tumor-bearing individuals where the tumor itself interferes with immune function. As a Christian in science, I have the unique ability to understand the complex science and to derive useful life applications from the structures, pathways, and activities that I study. The organization and function of the immune system provides several such illustrations. I suggest that the phenomenon of tumor-induced peripheral immune tolerance provides an interesting parallel to how sin may slowly accumulate in the life of a believer or how amoral
practices may slowly become accepted within a Christian society.

The human body and its “trainable” immune system are highly complex and fascinating. We often take for granted an ever-present army of silent sentinel molecules and cells that notice infection and initiate an effective and timely response that will lead to the removal of infectious organisms. For example, we learn by experience that common “routine” viral infections such as the common cold or influenza will usually be only a memory (albeit, an unpleasant one) within two weeks. During that same episode, our immune system has “learned” the identity of a particular strain of influenza virus, and will use this “knowledge” to more effectively recognize and defend against it when encountered later in life. 

The same immune system that protects us from external infectious agents can also aid in controlling cancer. Cancer represents the result of improperly controlled cellular proliferation. Unchecked multiplication of a few altered cells leads to their accumulation as tissue masses in inappropriate anatomic locations and disrupts or prevents normal or essential tissue or organ function. Its causes (environmental insult or errors of nucleic acid metabolism) remind us that, as complex living creatures, we are subject to the results of inappropriate genetic alterations that can upset otherwise delicately balanced systems that regulate cell division and the capacity to build or repopulate tissues (embryonic development or homeostasis). Even though there are multiple stages through which abnormal cells must progress to produce a cancer, the process nonetheless ultimately represents proliferation from one (or a very few) initially altered cells. Genetic alterations (mutations) will often result in the production of altered protein sequences that the immune system should recognize as foreign. The important difference between a cancer and an infectious agent (virus), though, is that the cancer develops slowly, and develops from
within. In spite of its potential for morbid consequences and the presence of altered protein sequences, the immune system accepts the cancer largely as "self" tissue rather than as a foreign invader or danger.\(^2\)

Cellular immunity is especially well suited to detect and control cancer. The cellular immune system is an arm of the immune system that relies on lymphocytes which bear specialized antigen sensory receptors to detect and destroy infectious agents (Figure 1). These sensors (the TCR, antigen-specific T cell receptor will be described later) have the capacity to recognize the presence of "foreign" amino acid chains. The presence of a foreign amino acid chain(s) may indicate the presence of an infectious microbe, or a subtle change to the amino acid sequence of a protein that has resulted from a mutation to the host DNA.

T lymphocytes of the cellular immune system are usually called upon to recognize dramatic differences such as those that occur during a viral infection when multiple foreign viral proteins are produced within a limited number of host cells during the multiplication of the virus. If a virus contacts you (someone sneezes on you), but cannot multiply within you (the virus particles land on your face, but never gain access to the moist lining inside of your nose), it does not pose a threat (you do not get a cold). Viruses must enter host cells to multiply. To multiply, the virus must use the host cell to produce the proteins from which new virus particles will be constructed. T cells can recognize host cells that have been corrupted due to intracellular multiplication of the virus. T cells have the unique ability to detect evidence of the new viral proteins that are being produced inside of the infected host cells by viewing parts of the proteins that are presented on the host cell surface before the virion emerges. Breakdown products of the newly made virus proteins are taken from the inside of the infected host cell to its surface where
they can be detected by the sensory receptors of T cells. This provides external markers on host cells in which the virus has begun to replicate. Because T cells can identify which of the host cells have been infected with a virus, the corrupted host cells can be eliminated before they can produce more virus particles. Halting the production of new virus particles stops the spread of the virus to other uninfected host cells, and allows for its ultimate removal from the host. Due to the large number of viral proteins produced during the multiplication of a virus and the fact that the viral proteins are unlike proteins normally produced by the host cells, viruses are relatively easy for the immune system to detect.

Nonetheless, the immune system can be trained to detect more subtle alterations. For example, the sensing receptor on an individual T lymphocyte may be triggered by the presence of a single change in an otherwise normal host cell protein's amino acid sequence. A single amino acid change in turn can result from the simple change of one nucleotide in a gene. Such a mutation may represent the first step towards cancer formation if the mutation changes the function of a protein that normally controls cell division or adhesion. As with multiplying viruses, T cell recognition of the altered host cell protein can occur even if a protein altered by gene mutation remains hidden inside of the [cancerous] cell that produces it. Peptide-presenting proteins known as major histocompatibility (MHC) molecules constantly sample peptide byproducts produced by the intracellular breakdown of cellular proteins (pieces of proteins are called peptides; peptides are made of strings of amino acids) and present them on the surface of the cell (Figure 1). MHC molecules present peptides derived from both normal and abnormal cellular proteins, including those produced by cancer cells. MHC molecules are expressed by virtually all cells, and therefore represent a system by which production of altered proteins within all individual cells of the host can be monitored by a constantly moving army of T
lymphocytes. Once broken down and presented on the outside surface of the cell, altered peptides from the infectious agent or altered tumor cell protein(s) are available to be recognized by the specialized antigen receptor of a unique T lymphocyte (Figure 1). Recognition will cause the T lymphocyte to release molecules that can destroy the abnormal cell presenting the altered protein segment. Thus, the T lymphocytes should be able to “surgically” remove/destroy the abnormal cancer cells.

If abnormal proteins that contain cancer-promoting mutations cannot hide inside of abnormal host cells, why do T lymphocytes fail to find and destroy cancer as it develops? (How is it possible for otherwise good Christians to take on obviously destructive lifestyles or habits that clearly do not glorify God?) To understand this, one must first appreciate how a diverse population of potentially useful T lymphocytes is developed (this happens in a unique way in each person) and why the T cells produced by this process are poised to recognize obvious “threats” from the outside while at the same time they have been “trained” to ignore “self” peptide fragments. Unfortunately, mechanisms that are used to “train” T cells to avoid harming the normal immune “self” can also prevent them from responding forcefully subtle changes that can indicate the seeds of a growing cancer.

How T cells are produced, and why, as a diverse population, they represent a set of diverse TCR specificities that can recognize a wide variety of infectious agents but at the same time ignore host peptides is at the heart of how T cells can remain silent in the face of subtle changes that should trigger their activity. T lymphocytes utilize a special surface receptor protein known as the T lymphocyte antigen receptor (TCR) to recognize peptides (foreign or abnormal protein fragments; Figure 1). A TCR can only recognize a peptide when it is properly
presented on the surface of a host cell by an MHC molecule. *(Think of a teacher holding out one brail flash card in each hand for inspection by a blind student who can only feel the bumps when properly positioned in and presented by the hand of the teacher.)* The role of a T cell is to look at (contact, touch) host cell MHC molecules, determine whether or not a host cell is displaying evidence of an abnormal protein, consider whether other evidence supports that the host is in danger, and respond accordingly. 4 The TCRs expressed on the entire population of T cells in an individual represent a wide variety of structurally related, but distinct molecules. Different TCR structures will “look for” different targets. Each distinct TCR is structurally suited to recognize only a limited variety of related amino acid sequences (peptides). Humans (and mice) are not born with enough genes to encode this wealth of different TCRs. Instead, the T cell immune system produces the diverse number of different TCR-encoding genes needed to allow for the recognition of a wide variety of distinct peptides. Each T cell generates the pair of genes that will encode its own unique TCR through what appears to be random recombination events (a mix and match genetic procedure) that it undergoes while developing in the thymus (Figures 2 & 3). Recombination (cutting and rejoining of a distinct region of the chromosome) brings two or three pieces of TCR-encoding DNA fragments into a properly ordered and perfect alignment. *(Imagine how a group of people might create a variety of different ice cream sundaes if provided with a variety of different ice cream flavors and toppings.)* The random mix and match TCR gene construction procedure is dangerous for the T cell. If two attempts to generate genes needed to encode a useful receptor fail, the developing cell dies. As a T cell goes through attempts to make a useful receptor gene, its success is gauged by whether or not the TCR it has produced can make “meaningful” interactions with MHC molecules on neighboring cells in the thymus (Figure 3, “Positive Selection”). *(After you have made your first sundaes, you ask your*
If so, the T cell has produced a potentially useful receptor (TCR). The “testing” interactions that the newly derived TCR makes with other cells in the thymus are not as “good” or “strong” as the interaction that it will ultimately make when it detects a peptide from an infectious agent, but the interactions are “good enough” to confirm that the receptor can at least dock with MHC molecules. *(Because everyone is busy making or looking at sundaes, they just give a quick “yes” without closely inspecting your creation.)* Therefore, the T cells that survive the early steps of development in the thymus are those that have successfully constructed what appear to be functional TCR-encoding genes by the random recombination/assembly process.

A second important screening event must occur before a T cell can leave the thymus. This second screening step (Figure 3, “Negative Selection”) is needed to weed out (destroy) those T cells that have by chance produced a TCR that recognizes a host peptide. The “mix and match” generation of the TCR genes during T cell development in the thymus is a stochastic (random) process. The T cell is provided with a finite number of short genetic sequences from which it produces its needed TCR genes. *(Remember, you were told to make an ice cream sundae, provided with multiple ice cream flavors and toppings, but the recipe for the ice cream sundae was not specified. You just tried to make a sundae that was eatable and looked good to busy neighbors. Many combinations and arrangements were acceptable.)* Therefore, many T cells assemble TCRs capable of recognizing foreign peptides, but many also assemble TCRs that can recognize normal host peptides. These potentially self-reactive T cells must be eliminated, or they could recognize and destroy normal host cells after leaving the thymus. As the T cells migrate through the thymus toward the egress points, each TCR (one T cell makes only one type of TCR) is tested to see if it interacts “strongly” with MHC molecules present on other cells it
contacts in the thymus. If so, the “good” interaction at this secondary stage indicates that the T cell can recognize a self-peptide (on an MHC molecule) that is normally produced a host thymus cell. Such T cells are therefore considered to be auto-reactive. If allowed to leave the thymus, they could be triggered into action when they recognize the same peptide on another normal host cell in the periphery (locations outside of the thymus are referred to as being peripheral). T cells that appear to be auto-reactive are triggered to go through apoptosis (programmed cell death). *(A sundae checker at the doorway to the dining hall looks at all sundaes. Individuals who have made sundaes containing the combination of strawberry topping, cashew nuts and chocolate ice cream are not permitted to leave the serving area. These unfortunate T cells are instead instructed to die.)* By requiring that each developing T cell construct a unique and functional TCR receptor (by assembling novel TCR receptor genes through a stochastic process of somatic recombination), and then discarding those T cells that appear to interact too strongly with host MHC molecules (strongly recognize some self peptide in a host MHC), each T cell that leaves the thymus should expresses a functional receptor that should be useful for detecting a non-host or foreign peptide structure. Thus a diverse repertoire of functional T cells has been produced from which the majority of auto-reactive T cells have been removed.

The random “mix and match” process of TCR gene construction followed by the two-fold selection process provides the fundamental basis for immune tolerance by T cells. Similar random construction with subsequent disposal of self-reactive clones accounts for tolerance in the other major humoral arm of the immune system that uses antibodies and B lymphocytes. Due to this and other mechanisms, the immune system as a whole is largely tolerant of the host, and has been “trained” to ignore host structures by culling of auto-reactive lymphocyte clones. Nonetheless, some auto-reactive T cell clones do appear to survive thymic selection for two
reasons. First, it is likely that all host proteins are not expressed at sufficient levels in the thymus to trigger death of all auto-reactive T cells. Second, post-developmental peripheral changes in host protein expression such as those that occur during puberty or lactation may not be mirrored within the thymus. Therefore, additional safety mechanisms must operate outside of the thymus. The result of these additional control mechanisms that occur outside of the thymus is known as peripheral tolerance.

Upon completing thymic development and selection, individual T cells migrate from the thymus into the peripheral lymphatic system where they await encounter with MHC-peptide complexes that their respective TCRs will recognize (Figure 3). If a T cell that has left the thymus does recognize an MHC-presented peptide, this implies that the peptide it has detected is foreign, and therefore the peptide provides evidence of infection or abnormality. Recognition implies that the cell presenting the peptide is infected or cancerous and should be destroyed before it can produce more infectious virus particles.

With such an intricately tuned system producing a vast repertoire of dedicated sentinel T cells that should be poised to recognize almost any invader or cellular protein abnormality, why can cancer cells that produce altered proteins proliferate undetected? (How is it possible for otherwise faithful Christians to ultimately develop destructive lifestyles or habits that clearly do not glorify God?) A key reason seems to be that the cancer develops slowly and without the trauma and additional diffusible molecular “danger” signals that routinely accompany an infection. When bacteria or viruses infect human tissues, their replication invariably leads to the death of some host cells and to the recruitment of ubiquitous phagocytic leukocytes that, unlike T cells, serve as the initial defenses against infectious invaders. The actions of these primary
immune defenders and the accompanying tissue trauma lead to the production of chemical signals that a T cell needs in addition to recognition of an appropriate peptide-MHC combination to trigger it into become fully activated. The opposite result occurs if a T cell efficiently recognizes a peptide-MHC combination in the absence of the additional “danger” signals. TCR recognition in the absence of additional reinforcing danger signals triggers the T cell to undergo programmed cell death (apoptosis) or to become functionally paralyzed. Both T cell death and paralysis appear to be involved in maintaining normal peripheral tolerance. As a result, peripheral tolerance mechanisms designed to control auto-reactive T cells that may remain following thymic selection may also prevent the unaided immune system from the early elimination of tumor cells that express altered peptides. Because the changes in the early tumor are subtle, and are not accompanied by a variety of additional warning or danger signals, the relevant T cells that should have been able to recognize and destroy the developing tumor cells are instead gradually eliminated or silenced. Consequently the subtle immunological differences between the tumor and normal host cells are not exploited, and the tumor continues to grow and increases its tolerogenic influence.

Multiple parallels can be drawn between the cellular immunology described above and elements of Christian belief. Here I will comment on how I see a distinct parallel between the way in which a developing tumor can induce tolerance to itself and the way by which sin may enter the lives of otherwise committed Christians (individuals or a society). Believers are supplied with tools to recognize, avoid, and purge sin from their lives. These include God’s written word, the counsel, teaching and example of other believers, and the inner guidance of God-given spirit and conscience. Nonetheless, Christians do sin (and continue to sin) because they are imperfect, fallible humans living in a world filled with temptations. Actions or
omissions that represent a missing of the mark (flagrant disobedience to God’s commands or instructions, or failing to act when called) are routinely experienced by the believer, despite the best of intentions (e.g. Paul’s frustration expressed in Romans 7). The cycle of temptation, failure, and forgiveness is all too familiar to many believers (myself included). Believers do experience success in recognizing and avoiding sin. Like the immune system, the wrongs that appear to be obviously wrong can be readily recognized and avoided. For example, the temptation to take a neighbor’s newer car or a juicy-looking apple from a well-stocked and under-monitored fruit stand along a crowded sidewalk are immediately dismissed because they are recognized as clearly being wrong. Like a viral infection, the absurdity of these temptations and their obvious consequences automatically trigger many danger flags. Conscience and good sense recognize the impropriety of such behavior. The desire to act is dismissed without a second thought.

The preceding general description of T lymphocyte tolerance can be more specifically illustrated by the immunological responses to CD8+ T lymphocyte epitopes within the Simian virus 40 large tumor antigen (SV40 T ag). The relationship between peripheral T cell tolerance and the endogenous development of antigenic T antigen-induced tumors parallels how recurrent, subtle temptations/failures may gain a foothold over time or how initial actions in morally gray areas may ultimately lead to sustained patterns of wrong behavior. For the T antigen epitopes, delivery of the same antigenic epitope peptide (a peptide that normally provokes and is the target for a CTL immune response) by different methods can result in dramatically different T cell responses. The epitope I peptide of the SV40 T ag protein is highlighted in the attached manuscript by Staveley O’Carrol et al.
The SV40 T ag protein can cause normal cells to grow indefinitely (cancer) when a gene encoding it is expressed continuously within them. Therefore, the SV40 T ag protein is useful as a model for studying how a tumor-activating protein can cause cellular immortalization and tumorigenesis. Epitope I is a 10 amino acid long peptide contained within the SV40 T ag (residues 206-215 within the 708 amino acid SV40 T ag protein). When the T ag protein is produced in host cells, the epitope I peptide is liberated (excised or "processed") from the T antigen protein and is presented on MHC class I molecules at the surface of the T ag-expressing host cell where it can be recognized by CD8+ T lymphocytes that express an epitope I-reactive TCR. Normal healthy mice contain T cells that will recognize the epitope I peptide. When injected into a normal murine host by immunization procedures, cells expressing the epitope I peptide effectively trigger a T cell response against the epitope I peptide. In fact, tumorigenic T antigen immortalized cells fail to form tumors when injected into healthy mice. The recipient mouse recognizes the otherwise genetically matched tumor cells as abnormal due to the T antigen protein and epitope peptides they produce, and destroys them. Under these conditions, the preformed T cell defenders detect the epitope I peptide (and other T antigen peptides) under conditions where the presence of the foreign peptide and reinforcing secondary signals confirm that it is clearly foreign and is a legitimate target. Large numbers of T cells that recognize the peptide (danger) become activated. The threat is obvious, readily recognized, and the response is quick and appropriate.

T cells in SV40 T ag transgenic 501 mice behave differently. In 501 mice, the SV40 T ag protein is expressed within the mouse from an artificially inserted gene that is present within the mouse at birth. As far as the immune system of the 501 mouse knows, the T ag gene and its protein product should be present. The control region of the T ag gene used in the 501 mouse
model gradually turns on production of the T ag in only a subset of cells (limited to certain tissue types), after the animal has been born. 501 mice ultimately develop tumors in many tissue types where the T ag protein is expressed. Under these conditions, a majority of the T cells in the mouse are produced and undergo the two stage thymic selection process in the absence of the T ag protein or its epitope I peptide. Nonetheless, epitope I-reactive T cells are difficult to detect in 501 mice. In fact, experiments designed to illustrate the tolerogenic effects of the T ag epitope peptide I are routinely done by adding extra epitope I-reactive T cells to the mouse in question to increase their numbers so that they can be easily detected following immunization experiments. The results of such experiments reveal that the continuous, low-level presence of the epitope I peptide in these mice has a detrimental rather than activating effect on the added epitope I-reactive T cells. Instead of causing them to become activated and destroy host tumor cells that produce the T ag, the presence of the endogenously produced epitope I peptide paralyzes the epitope I-specific T cells. The epitope I-specific T cells gradually become ineffective. While some of the epitope I-specific T cells do give evidence that they have "seen" the epitope I peptide, they no longer respond properly to the presence of this signal that indicates cancer ahead. In the 501 model, the target peptide has been delivered in small, continuous doses and is not accompanied by additional danger signals. Ultimately, continued presence of the epitope I peptide removes or silences the epitope I-specific defender T cells, and the animal develops lethal T ag expressing tumors.

There are numerous examples of "little" things that routinely confront us and can deaden our normal defensive reactions to sin: that first time you realized that you could "creatively" calculate an income tax return without consequence; an abundance of sexually suggestive advertisements in legitimate magazines that gradually desensitize the reader to their presence;
busyness (even Church-related) or selfish patterns that reduce sensitivity to needs around us; the temptation to spend increasing amounts of time in self-absorbed entertainment. The reader may think of additional examples. Initial choices that appear to be small, morally neutral or “white lie” choices may represent the beginning of a sinister continuum. Over time, flawed or misguided rationalization replaces proper reasoning, and reduces sensitivity to God’s leading in that area.

Peripheral tolerance exerts its affects on individual T cells, but the insensitivity of growing numbers of individual T cells is ultimately borne out by at by the inability to detect an epitope I-specific response at the level of the total T cell population. As morally questionable practices take hold within a society, is it not easier for a society to take subsequent steps down the sinister continuum (whatever it is) because increasing numbers of individuals who might otherwise oppose an activity fail to recognize the wrong or are no longer convicted to speak out against it. The wrong may be similar to things to which the society has become accustomed. It becomes easier to simply go with the crowd. (How many times does traffic on the interstate actually travel at or below the posted speed limit?) Issues related to the sanctity of life and the impending cloning and embryonic stem cell revolution impress me as one such danger.

Hopefully by this point, the reader has not become overwhelmed by the immunology and cell biology I have attempted to explain in my attempt to draw parallels between an immunological phenomenon, peripheral T cell tolerance, and the way in which sin may enter and gain hold within the life of a committed believer (or society). As mentioned above, I am a scientist who studies anti-tumor T cell responses using model murine systems in which the epitope targets are defined. As a Christian, I understand how easily sinful failure can cloud what
should be the victorious joy of a life in Christ. The parallels I see as a Christian who has been trained as a scientist caution me to scan my own life and practices that Christians around me seem to accept without question (or of which they are not aware) with renewed urgency.

Footnotes for Appendix.

1. Although a disturbing number of young parents now question the safety of childhood vaccination, as a population we do collectively benefit from this practice because it has virtually eliminated epidemics of infectious diseases (e.g. diphtheria, measles, or polio) that were problematic less than a century ago.

2. In immune system terminology, host = self = molecules and cells that should be part of the human anatomy; foreign structures found on or produced by infectious agents are considered foreign or non-self, and are referred to as antigens because they provoke a response by the immune system.

3. MHC molecules are also the proteins that prevent tissue transplantation between unrelated individuals because they are expressed by essentially all nucleated cells, differ between unrelated individuals, and trigger cause a rapid inappropriate cross-reactive triggering of T lymphocyte destructive activities against the newly transplanted vasculature.

4. There are actually two different subsets of T cells (CD4+ and CD8+) that function in different roles in immune responses to vial infections. For the sake of simplicity I am ignoring those differences, because presentation on cell surface MHC molecules is required for recognition of a peptide by the TCR of either subset.
In Vivo Ligation of CD40 Enhances Priming Against the
Endogenous Tumor Antigen and Promotes CD8+ T Cell
Effector Function in SV40 T Antigen Transgenic Mice1

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The ability to initiate and sustain CD8+ T cell responses to tumors in vivo is hindered by the development of peripheral T cell tolerance against tumor-associated Ags. Approaches that counter the onset of T cell tolerance may preserve a pool of potentially tumor-reactive CD8+ T cells. Administration of agonist Ab to the CD40 molecule, expressed on APCs, can enhance immunization approaches targeting T lymphocytes in an otherwise tolerance-prone environment. In this report, the effects of anti-CD40 administration on priming of naive CD8+ T cells against an endogenous tumor Ag were investigated. Line 501 mice express the SV40 large T Ag oncogene as a transgene from the α-amylase promoter, resulting in the development of peripheral CD8+ T cell tolerance to the H-2Dk-restricted immunodominant epitope I of T Ag by 6 mo of age, before the appearance of osteosarcomas. We demonstrate that naive epitope I-specific TCR transgenic (TCR-I) T cells undergo peripheral tolerance following adoptive transfer into 6-mo-old 501 mice. In contrast, administration of agonistic anti-CD40 Ab led to increased expansion of TCR-I T cells in 501 mice, the acquisition of effector function by TCR-I T cells, and the establishment of T cell memory. Importantly, this enhanced priming effect of anti-CD40 administration did not require immunization and was effective even if administered after naive TCR-I T cells had encountered the endogenous T Ag. Thus, anti-CD40 administration can block the onset of peripheral tolerance and enhance the recruitment of functionally competent effector T cells toward an endogenous tumor Ag. The Journal of Immunology, 2003, 171: 697–707.

Due to their specificity and potent effector function, CD8+ T cells are an attractive target population for immunotherapeutic approaches to cancer. Many tumor-associated Ags recognized by CD8+ T cells have now been identified and represent nonmutated self-Ags that can serve as the focus for vaccination approaches to cancer (1, 2). Several mechanisms have been identified, however, that might limit the effectiveness of targeting self-reactive T cells, including deficiencies in the T cell repertoire due to negative selection of potentially tumor Ag-reactive T cells (3–6) as well as regulatory mechanisms that downmodulate immune responses to self-Ags in the periphery (7). In particular, recent evidence suggests that the recognition of peripheral self-Ags by naive CD8+ T cells leads to activation, expansion, and subsequent deletion of these self-reactive T cells to maintain tolerance to self-Ags in the periphery (8). Thus, strategies that protect naive tumor-reactive T cells from destruction or lead to the expansion and maintenance of effector and memory T cells within the tumor-bearing host will be critical for designing immunotherapeutic approaches to cancer.

Mice that develop spontaneous tumors due to the transgenic expression of oncogenes provide realistic models to assess immunotherapeutic strategies for potential use in the treatment of human cancer. Expression of the large tumor Ag (T Ag)4 from SV40 as a transgene in mice leads to the development of spontaneous tumors (9, 10). This oncogenic activity stems from the ability of the T Ag to inactivate the tumor suppressor proteins Rb and p53 as well as initiate cell cycle progression (11). In addition, T Ag serves as the target of a strong CTL response that can lead to the rejection of T Ag expressing tumors (12). Immunization of C57Bl/6 mice with wild-type T Ag leads to the development of CTL specificity for these three immunodominant epitopes: designated epitope I (T Ag residues 206–215), epitope II/III (T Ag residues 223–231), and epitope IV (T Ag residues 404–411) (13–15). Epitopes I and II/III are H-2-Dk restricted, whereas epitope IV is H-2-Kd restricted. Expression of T Ag as a transgenic also leads to the onset of either central or peripheral CD8+ T cell tolerance to the T Ag epitopes, depending on the site and timing of transgene expression (4, 16–19). Line B6/Sv129 (H-2b) transgenic mice express T Ag under the α-amylase promoter, resulting in the expression of T Ag in the salivary glands and bone (20, 21). Although neoplasia is not detected in the salivary glands, T Ag expression in bone leads to the development of osteosarcomas that can metastasize to the liver and lung. The expression of T Ag as a self Ag in 501 mice leads to the progressive loss of CTL responses to the T Ag epitopes (19). In particular, loss of CTL responsiveness to T Ag epitope I is tightly associated with the onset of T Ag expression in 501 mice.

4 Abbreviations used in this paper: T Ag, large tumor Ag; DC, dendritic cell; LN, lymph node; NP, nucleoprotein; Fia, influenza virus; HA, hemagglutinin; rVV, recombinant vaccinia virus; TCR-I, epitope I-specific TCR transgenic.

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Immunization of 501 mice at 6 mo of age, when T Ag is expressed at high levels in the salivary glands (21), failed to induce epotope-specific CTL, although immunization of these mice at 3-4 mo of age resulted in the successful recruitment of epotope-specific CTL (19). Whether the loss of epotope-specific CTL activity in line 501 mice is due to the peripheral deletion of reactive CD8+ T cells or the induction of unresponsiveness is unknown.

The presence of potentially autoreactive cells in peripheral lymphoid organs can result in either ignorance of cognate Ag, unless a response is initiated by exogenous immunization (22-24), or Ag encounter resulting either in an immune response (25, 26) or the induction of tolerance (19, 27-30). It has become increasingly clear that the nature of the immune response to peripheral Ag is largely determined by the characteristics of the APC (31). Both T cell activation and the induction of peripheral T cell tolerance can be mediated through recognition of Ag on bone marrow-derived APCs, which are most likely dendritic cells (DCs) (28, 32-34). Recent evidence suggests that tissue-resident DCs with an immature phenotype acquire Ag from the surrounding tissues during noninflammatory conditions (35) and migrate to secondary lymphoid organs where an encounter with Ag-specific T cells can result in tolerance to these self-Ags. The amount of self-Ag expressed in the periphery may be one factor that determines whether a naive T cell encounter with APCs results in tolerance or ignorance, as recent studies indicate that high levels of endogenous Ag can lead to the induction of T cell tolerance (36, 37).

Recent approaches to immunotherapy have focused on modifying the maturation status of APCs such that T cell encounter with APCs leads to full activation of naive T lymphocytes. One promising approach has been the engagement of CD40 on the APC via soluble CD40 ligand or agonist anti-CD40 Abs. CD40 on APCs is naturally engaged by the ligand CD154 (CD40 ligand) on activated CD4+ T cells, resulting in APC activation and differentiation (38). Ligation of CD40 leads to changes in the APC phenotype, including increased surface expression of MHC molecules and costimulatory receptors and the production of high levels of the T cell-stimulating cytokine IL-12 (39-41). In addition, CD40 ligation induces the migration of APCs to the secondary lymphoid tissue (42) where they can engage naive T cells. Ligation of CD40 in vivo following the injection of agonistic anti-CD40 Abs can substitute for CD4+ T cell help in the priming of naive CD8+ T cells, suggesting that T cell help may be mediated primarily via CD40 ligand-CD40 interactions with APCs (43-45). Several recent reports have demonstrated that the injection of agonistic anti-CD40 Abs can prevent or delay the onset of peripheral T cell tolerance (46-48). In addition, injection of mice with anti-CD40 in combination with specific immunization can lead to enhanced immunity to transplantable tumors (46, 47, 49, 50) or spontaneous tumors expressing a neo-Ag (51). Thus, anti-CD40 ligation may result in both the preservation of the host self-reactive T cell repertoire and enhanced expansion of tumor-reactive T cells in vivo.

In this report, the fate of naive T Ag epitope-i-specific TCR transgenic (TCR-I) CD8+ T cells was determined after exposure to the endogenous T Ag in 501 T Ag transgenic mice. The results indicate that epitope-specific CD8+ T cells proliferated and developed an activated phenotype in lymph nodes (LNs) draining areas of T Ag expression; however, these cells failed to acquire effector function. In vivo treatment with anti-CD40 Ab resulted in enhanced expansion of epitope-i-specific T cells and promoted the acquisition of effector function in the absence of exogenous immunization. Importantly, this effect was observed even if anti-CD40 treatment was administered after exposure of the epitope-i-specific T cells to the endogenous T Ag and led to long-term maintenance of epitope-i-specific CD8+ T cells. Thus, in vivo CD40 ligation results in preservation and expansion of tumor-reactive and functional effector T cells that might be targeted for subsequent immunotherapy of tumors.

Materials and Methods

Mice

Male C57BL/6 (H-2b) mice (4-6-wk-old) were purchased from The Jackson Laboratory (Bar Harbor, ME), maintained in isolator cubicles at the animal facility of the Milton S. Hershey Medical Center, Hershey, PA and routinely used between the ages of 7 and 12 wk. Line C57BL/6-Tg(amy1tg)501Kvw, or 501, mice express full-length SV40 T Ag as a transgene from the α-amylose promoter and have been previously described (20, 21). The 501 mice were bred and maintained at the animal research facility of the Milton S. Hershey Medical Center. B6.SJL mice were purchased from Taconic Farms (Germantown, NY) and bred and maintained at the animal research facility of the Milton S. Hershey Medical Center. All animal studies were performed in accordance with guidelines established by the Pennsylvania State University College of Medicine Animal Care and Use Committee under an approved protocol.

Cloning of the epitope-i-specific TCR from CTL clone Y-1

TCR sequences for both the α- and β-chains were derived from the epitope-i-specific CTL clone Y-1 (52). To determine the nucleotide sequence of the combining regions for each subunit, total RNA was extracted from clone Y-1 cells using Trizol reagent (Sigma-Aldrich, St. Louis, MO) and reverse transcribed with avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) utilizing an oligo(dT) reverse primer. The TCR α-chain combining region was amplified by PCR using an antisense 3' constant region oligonucleotide (5'-CAAGAGATTTTCAACTGTTTG3'; Ref. 53) and a 5' variable region Vα3-chain sense oligonucleotide (5'-GGCTCTGGACAGGCTTGGCAGCAGCAGCAGGCGGCG3'; Ref. 53) while the TCR β-chain combining region was amplified using the reverse antisense constant region oligonucleotide (5'-GGTGGGTTTCACTGTTGGCAGCAGCAGCAGGCGGCG3'; Ref. 53) and a 5' sense Vβ7 oligonucleotide (5'-GGGGAAGGGAAGAGCTCAGTCTGTTTAACAC3'; Ref. 53) were used in combination. Sequence analysis of the TCR α-chain sequence revealed Jβ1.4 use while analysis of the TCR β-chain sequence revealed Jβ1.4 use. Accordingly, 3' antisense genomic primers corresponding to intron sequences flanking the respective TCRα and TCRβ J regions (Jα1.1, 5'-TGCCCGGCGTCGGTTCTATCCTCAACACAC3'; Ref. 56) and Jβ1.4, 5'-AAAGGATCCGCCGCGTCGGTTCTATCCTCAACACAC3'; Ref. 54) were used in combination and, respectively, with 5' sense primers corresponding to 5' noncoding sequences of Vα3.1 (5'-TGGCCCGGCGTCGGTTCTATCCTCAACACAC3'; Ref. 55) or Vβ7 (5'-CAAGGAGCAGGGAAGAGCTCAGTCTGTTTAACAC3'; Ref. 55) to amplify the genomic sequences from Y-1 cell-derived DNA and incorporate restriction endonuclease cleavage sites at the ends of each product (5' XbaI site; 5' KpnI site). Amplified genomic VDJ fragments were subcloned into PUC19 as blunt-ended fragments, the nucleotide sequences were verified, and the fragments were excised by restriction digestion and ligated into the appropriately digested TCR α- or β-expression cassette plasmids (pBoss and pBess, respectively, obtained from Dr. D. Mathis, Institute de Genetique et de Biologie Moleculaire et Cellulaire, Strasbourg, France; Ref. 57). Recombinant cassette vectors containing the appropriate Y-1 TCR α- and β-chain fragments were identified by restriction digestion analysis, and the presence of the proper V(D)J inserts verified by PCR amplification. Plasmids were purified by cesium chloride gradient centrifugation and digested (α, SalI; β, KpnI) to liberate a fragment bearing the respective subunit expression cassettes, which were isolated from agarose gels using the QiAEXII method (Qiagen, Valencia, CA) and eluted directly into microinjection buffer. Fragment solutions were stored at -80ºC.

Generation of T Ag TCR-I mice

Purified Y-1 TCR α- and β-chain expression cassettes were combined before injection. Microinjection of fertilized embryos by B6D2F1/J mice was performed as previously described (58). The presence of the α- and β-transgene(s) in chimeras was determined at 4 wk of age by PCR analysis of tail-derived DNA using the following primer pairs: Vα3.1 chain sense, 5'-TCCCTCAACCTCGGAAGAAGC3'; Jα1.1 chain anti-sense, 5'-TGGCCCGGCGTCGGTTCTATCCTCAACACAC3'; Vβ7 chain sense, 5'-AAAGGATCCGCCGCGTCGGTTCTATCCTCAACACAC3'; and Jβ1.4 chain anti-sense, 5'-AAAGGATCCGCCGCGTCGGTTCTATCCTCAACACAC3'. Amplification of the corresponding 180- and 200-bp fragments from genomic
DNA was diagnostic for the presence of the transgenes. Amplification of a 430-bp segment of the p53 gene using previously described primers served as a control for the integrity of the DNA (4, 59). Expression of the transgene products was confirmed by staining lymphocytes from various lymphoid tissues with TCRβ7-specific mAb (BD Pharmingen, San Diego, CA), anti-TCRα3.1 antibodies (generously provided by Drs. B.-C. Slim and N. Gascoigne, The Scripps Research Institute, La Jolla, CA) (60) and Db/ 
epitope I tetramer (61). Two founder lines were derived and designated as lines 416 and 422. Line 416 mice were maintained by backcrossing transgenic positive males with C57BL/6 females followed by screening for the presence of the transgene by PCR analysis as described below. Line 416 mice have been backcrossed to C57BL/6 mice for over 10 generations.

Cell lines and media

B6/K-Tagl cells were derived by transformation of C57BL/6 primary kidney cells with plasmid pLM506-G(DC-1), which encodes a T Ag variant containing alanine substitutions of residues N227, F408, and N493. Plasmid pLM506-G(DC-1) was constructed by the Altered Sites oligonucleotide-directed mutagenesis procedure (Promega) using as a template ssDNA derived from pSelect-ESV (14, 61). Because residues N227, F408, and N493 constitute critical anchor residues within epitopes II/II, IV, and V, respectively, alanine substitution effectively abrogates both the immunogenicity and antigenicity of epitopes II/III, IV, and V (data not shown). Cells were maintained in DMEM supplemented with 100 U penicillin/ml, 100 μg streptomycin/ml, 100 μg kanamycin/ml, 2 mM L-glutamine, 10 mM HEPES buffer, 0.075% (w/v) NaHCO3, and 5% FBS. All lymphocyte manipulations were performed using complete RPMI 1640 medium, which contained 100 U penicillin/ml, 100 μg streptomycin/ml, 2 mM L-glutamine, 10 mM HEPES buffer, 50 μM 2-ME, and 10% FBS.

Synthetic peptides and viruses

Peptides were synthesized at the Macromolecular Core Facility of the Milton S. Hershberg Medical Center by Fmoc chemistry using a peptide synthesizer (9050 Milligen PepSynthesizer; Millipore, Bedford, MA). Peptides were dissolved in DMSO and diluted to the appropriate concentration with RPMI 1640 medium. Peptides used in these experiments correspond to SLY40 T Ag epitope I (SAINNYAQKL) and influenza virus (Flu) nucleoprotein (ND) 366-374 (ASNNEMMTM). The recombinant vaccinia virus (rVV) encoding T Ag epitope I as a minigene, rVV-I, has been previously described (28).

Flow cytometric analysis of lymphocyte populations by surface and intracellular cytokine staining

Ex vivo staining of lymphocytes was performed on single-cell suspensions obtained from cervical LNs and spleens, as previously described (61), that had been depleted of RBCs by centrifugation over a Ficoll-Plaque (American Biosciences, Piscataway, NJ) gradient according to the manufacturer's specifications. Production and characterization of the H-2-D7/7 Ag epitope I (Db/I) and H-2-D7/7 influenza virus NP epitope 366-374 (Db/Flu) tetramers (Tet) and surface staining of lymphocytes with MHC tetramers and primary conjugated Abs was performed as previously described (61). The following Abs were purchased from BD Pharmingen: FITC-PE, or CyChrome-labeled anti-CD8α (clone 53-6-7), PE-labeled mouse anti-mouse CD45.1 (clone A20), FITC-labeled rat anti-mouse CD44 (clone IM7), FITC-labeled hamster anti-mouse CD69 (clone HI1.2F3), FITC-labeled rat anti-mouse TCRβ7 (clone TR310), unlabeled rat anti-mouse CD16/32, and FITC-labeled rat anti-mouse IFN-γ (clone XMG1.2). The rabbit anti-mouse Tac.1 anti-TIA (60) was used in conjunction with a secondary goat anti-rabbit-FITC conjugate (BD Pharmingen). Cells were washed, fixed with 2% paraformaldehyde in PBS, and analyzed using a FACScan flow cytometer (BD Biosciences, San Jose, CA). Data were analyzed and prepared using CellQuest software (BD Biosciences) or FlowJo software (Tree Star, San Carlos, CA). In some cases, the percentage of CD8+ cells that stained specifically with Db/I Tet was determined by subtracting the percentage of CD8+ Db/I Flu Tet- cells from the percentage of CD8+ Db/I Tet+ cells within the same population. Intracellular cytokine staining was performed using the Cytofix/Cytoperm kit (BD Pharmingen) according to the manufacturer's specifications and as previously described (61). Where indicated, the percentage of CD8+ cells that express intracellular IFN-γ was calculated by subtracting the percentage of cells that do not produce IFN-γ following exposure to an unrelated peptide from those that stained for IFN-γ after stimulation with T Ag epitope I peptide.

Adoptive transfers, anti-CD40 treatment, and immunizations

Six-month-old C57BL/6 and 501 mice received adoptive transfers with RBC-depleted lymphocytes derived from spleens and LNs of line 416 TCR-I mice. Recipients were injected i.v. in the tail vein with lymphocytes derived from line 416 mice containing 5 × 10⁶ clonotypic TCR-I CD8+ T cells, as determined by staining with Db/I epitope I tetramer, in 200 μl HBSS. Mice received 100 μg of purified agonist anti-CD40 Ab FGK45 (62) or polyclonal rat IgG (Sigma-Aldrich) by i.p. injection at the indicated times. Some mice were injected i.p. with 3 × 10⁶ B6/K-Tagl cells or with 1 × 10⁶ PFU of the indicated rVV.

In vivo proliferation assay

For in vivo proliferation assays (63, 64), RBC-depleted spleen cells from line 416 mice were resuspended at 1 × 10⁶/ml in PBS/0.1% BSA and

FIGURE 1. Line 501 mice are tolerant to T Ag epitope I. Six-month-old C57BL/6 or 501 mice were vaccinated with 1 × 10⁶ PFU rVV-I. Nine days later, spleens were harvested and lymphocytes stained with anti-CD8a and either Db/I epitope I tetramer (Db/I Tet) or a control tetramer constructed using the unrelated D3-restricted epitope NP 366-374 from influenza virus (Db/Flu Tet). The upper right quadrant represents epitope I-specific CD8+ T cells. The percentage of CD8+ T cells specific for epitope I is indicated.

FIGURE 2. Characterization of line 416 TCR-I transgenic mice. A, Predicted amino acid sequence for the combining regions of the TCR α- and β-chains derived from CTL clone Y-1 used to develop TCR transgenic line 416. B, Staining of spleen cells from control C57BL/6 or TCR-I transgenic line 416 mice using the indicated Abs or Db/I Tet. The gated regions in the lower right panel indicate the CD8+ Db/I Tet+ and CD8+, Db/I Tet- populations, and the corresponding histograms indicate the level of TCRβ7 expression. The percentage of cells within each gate is indicated. The lower left histogram indicates the percentage of CD8+ TCRβ7+ spleen cells that stain positively for TCRα3.1.
labeled with 5 μM 5- and 6-CFSE (Molecular Probes, Eugene, OR) for 10 min at 37°C. Cells were then washed three times in PBS, resuspended in HBSS, and injected i.v at a dose of 5 × 10^6 clonotypic TCR-I T cells per mouse. After 8 days, spleens and LNs from recipient mice were harvested, and the intensity of CFSE staining was measured among CD8^+ Db/I Tet^+ cells.

**In vivo cytotoxicity assay**

Six-month-old 501 and C57BL/6 mice were injected with 5 × 10^6 clonotypic TCR-I T cells. After 14 days, RBC-depleted spleen cells from B6/SJL-congenic mice were pulsed with either 1 μM T Ag epitope I peptide or the control Flu NP 366–374 peptide for 90 min at 37°C, 5% CO2. Excess peptide was removed with five washes and cells were resuspended at 1×10^7 cells/ml in PBS/0.1% BSA. Epitope-I pulsed cells were labeled with 5 μM CFSE (CFSEhigh cells) and Flu NP peptide-pulsed cells were labeled with 0.5 μM CFSE (CFSElow cells) for 10 min at 37°C. Labeling reactions were terminated by the addition of ice-cold PBS followed by two washes in PBS. Mice received an i.v injection containing a mixture of 2.5 × 10^6 CFSEhigh cells and 2.5 × 10^6 CFSEhigh cells in 200 μl HBSS. After 6 h, spleens from recipient mice were harvested, and CFSEhigh CD845.1^+ and CFSElow CD45.1^+ cells were quantitated by flow cytometry.

**Results**

Endogenous T Ag epitope I-specific CD8^+ T cells are undetectable in 6-mo-old 501 mice

We have shown previously that 3-mo-old 501 mice immunized with a RVV-encoding epitope I as a minigene induces CTL specific for T Ag epitope I, whereas 6-mo-old 501 mice failed to develop detectable levels of epitope I-specific CTL, even after in vitro expansion (19). Because detection of epitope I-specific CTL required both expansion and the development of effector function, it remained to be determined whether the endogenous cells were deleted, failed to proliferate in response to immunization against epitope I, or proliferated but failed to acquire effector function. To examine whether a nonfunctional population of epitope I-specific CD8^+ T cells accumulated in 6-mo-old 501 mice following immunization against epitope I, MHC class I tetramers (Db/I Tet) were utilized to detect the presence of epitope I-specific CD8^+ T cells directly ex vivo. Nine days after vaccination with RVV-I, C57BL/6 mice developed epitope I-specific CD8^+ T cells (1.5% of all CD8^+ cells) detectable by ex vivo staining with Db/I Tet, while epitope I-specific CD8^+ T cells were undetectable in 501 mice (Fig. 1). Thus, 501 mice failed to accumulate detectable numbers of endogenous epitope I-specific CD8^+ T cells following specific immunization.

TCR-I T cells accumulate in cervical LNs but fail to acquire effector function following transfer into 501 mice

To develop a system in which the fate of naive epitope I-specific CD8^+ T cells could be monitored in 501 mice, a mouse line expressing the TCR α- and β-chains of the T Ag epitope I-specific

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**FIGURE 3.** Activation and expansion of TCR-I T cells in 501 mice in the absence of effector function. A, Five million clonotypic TCR-I cells from line 416 TCR transgenic mice were transferred into 6-mo-old 501 or C57BL/6 mice. At 7 and 14 days after transfer, T cells isolated from the spleen and cervical LNs were stained with anti-CD8 and either a T Ag epitope I MHC tetramer (Db/I Tet) or a control tetramer (Db/Flu Tet). Results were quantitated by flow cytometric analysis. The data are representative of three mice per group and the experiment was repeated twice with similar results. The percentage of CD8^+ T cells that stained positive with each tetramer is indicated in the upper right quadrant of each
CTL clone Y-1 was derived. (Fig. 2A). Two founder lines were identified and designated lines 416 and 422. Characterization of line 416 is described in this report. Expression of the transgene products in each line was detected by immunofluorescence staining with Db/Tet, and Abs specific for the transgenic TCR Vα3.1 and Vβ chains. Analysis of spleen cells from line 416 mice revealed that ~94% of the CD8+ T cells were specific for T Ag epitope I (Fig. 2B), although 100% of the CD8+ T cells expressed TCRVβ7. (Fig. 2B). This discrepancy was explained by the finding that ~87% of the TCRVβ7+ CD8+ T cells coexpressed the transgenic TCRVα3.1 chain (Fig. 2B, left histogram), consistent with the percentage of CD8+ T cells that stained positive with the Db/Tet. The specificity of the rabbit anti-Vα3.1 soro is indicated by the lack of positive staining on CD8+, TCRVβ7+ T cells in C57BL/6 spleen cells (Fig. 2B, left histogram). The CD8+ TCR-I T cells derived from line 416 mice were determined to be functional as in vitro stimulation of line 416 spleen cells with T Ag transformed cells induced epitope I-specific CTL (data not shown).

The fate of naïve TCR-I T cells exposed to endogenous T Ag was monitored following transfer of line 416 spleen cells into 6-mo-old 501 or C57BL/6 mice. Seven and fourteen days after adoptive transfer, spleen and cervical LN were harvested from recipient mice and the number of epitope I-specific CD8+ T cells was quantitated by staining with Db/Tet (Fig. 3A). As a negative control, parallel cell samples were stained with the control tetramer, Db/flu. In C57BL/6 recipient mice, ~1.6% of the CD8+ T cells in the spleen and LNs were specific for epitope I as indicated by staining with Db/Tet. By contrast, epitope I-specific T cells represented on 8% of the CD8+ cells in the LNs draining the salivary glands in 501 mice. The frequency of epitope I-specific CD8+ T cells detected in the spleens of 501 and C57BL/6 mice were similar at day 7. The frequencies of TCR-I cells detected in the cervical LN and spleen remained relatively stable from days 7 to 14 after adoptive transfer into both 501 and C57BL/6 mice. Thus, TCR-I cells accumulate predominantly in the cervical LNs draining this site of T Ag expression in 501 mice.

To determine whether the accumulating TCR-I T cells remained ignorant of endogenous T Ag or had encountered T Ag in the periphery of 501 mice, re-isolated TCR-I T cells were stained for surface expression of CD44 and CD69. These markers are upregulated on CD8+ T cells following Ag encounter. Of the epitope I-specific CD8+ T cells (CD8+, Dp11 Tet+) that accumulated in the cervical LNs in 501 mice, 78.5% expressed increased levels of CD44 and 54.4% expressed CD69 (Fig. 3B). In contrast, only 20% and 10% of the Db/epitope I negative-staining CD8+ T cells (CD8+, Dp11 Tet-) expressed CD44 and CD69, respectively. TCR-I cells recovered from C57BL/6 mice maintained a naive phenotype as revealed by low surface expression of both CD44 and CD69. Thus, the TCR-I cells that accumulated in LNs draining areas of T Ag expression in 501 mice displayed a phenotype consistent with Ag encounter.

To determine whether Ag encounter by TCR-I T cells in 501 mice resulted in the acquisition of effector function, CD8+ T cells re-isolated from 501 mice were tested for their ability to produce IFN-γ in response to epitope I. Freshly isolated cervical LN or spleen cells were stimulated in vitro with epitope I peptide or a control peptide (Flu NP 366-374) in the presence of brefeldin A for 6 h and then stained for accumulation of intracellular IFN-γ. As expected, cells re-isolated from C57BL/6 mice that were never exposed to T Ag failed to produce IFN-γ in response to epitope I peptide (Fig. 3C). Lymphocytes isolated from 501 mice, which developed an Ag experienced phenotype in LNs draining sites of T Ag expression (Fig. 3B), also failed to produce significant amounts of IFN-γ (Fig. 3C). Although 8-9% of CD8+ T cells from the cervical LNs of 501 mice were positive by tetramer staining (Fig. 3A), <1% of the CD8+ T cells from these same LNs were capable of producing IFN-γ after stimulation with epitope I peptide. Thus, although TCR-I T cells accumulated in LNs draining the site of T Ag expression and developed an activated phenotype in 501 mice, they failed to acquire effector function.

**TCR-I T cells exposed to endogenous T Ag respond inefficiently to subsequent immunization**

To determine whether naïve TCR-I T cells exposed to the endogenous T Ag in 501 mice are compromised in their ability to respond to T Ag epitope I in vivo, 6-mo-old 501 and control C57BL/6 mice were given adoptive transfers with 5 x 10^6 clonotypic TCR-I T cells, rested for 2 wk and then immunized with rVV-I. Representative mice from each group were sacrificed at day 14, before immunization, to determine the frequency of TCR-I T...
FIGURE 5. Treatment of 501 mice with anti-CD40 Ab enhances proliferation and induces effector function in TCR-I T cells. A, Five million clonotypic TCR-I cells were transferred into 6-mo-old 501 mice that also received 100 μg of purified FGK45 or rat IgG on the day before and the day after cell transfer. Seven days after transfer, lymphocytes were isolated from the spleen and cervical LN's and CD8⁺ T cells were stained with Db/epitope I tetramer (Db/I Tet) or stained for the production of IFN-γ following 6 h of in vitro stimulation with epitope I peptide. The number of epitope I-specific CD8⁺ T cells was quantitated by FACS analysis. The percentage of CD8⁺ T cells specific for epitope I is indicated in the upper right quadrant. (Figure legend continues)
cells present at this time point. TCR-I T cells were present at a frequency of 1%-2% of CD8+ T cells in both the spleen and cervical LN's of C57BL/6 recipients (Fig. 4B). As shown previously (Fig. 3), while the frequency of TCR-I T cells present in the spleen of 501 mice at day 14 postransfer was similar to that detected in C57BL/6 mice, increased numbers of TCR-I T cells accumulated in the cervical LN's of 501 mice at this time point (Fig. 4A).

Immunization of C57BL/6 mice with rVV-I resulted in a dramatic expansion of TCR-I T cells (∼10-fold) in both the spleen and LN's by day 9 postimmunization (Fig. 4B). It should be noted that the endogenous epitope I-specific CD8+ T cell response in C57BL/6 mice represents ∼1.5% of the CD8+ T cells by 9 days postimmunization with rVV-I (see Fig. 1). In contrast, TCR-I T cells expanded ∼2–3-fold in the spleens of 501 mice with no apparent increase in the frequency of TCR-I T cells in the cervical LN's (Fig. 4A). Low levels of TCR-I T cells were detected in unimmunized 501 and C57BL/6 mice at this same time point, although the percentage of TCR-I T cells detected in 501 mice had apparently decreased by 25 days postadoptive transfer. These results indicate that exposure of naive TCR-I T cells to the endogenous T Ag in 501 mice compromises their ability to respond to subsequent immunization, consistent with the development of tolerance to T Ag epitope I.

Expansion of effector TCR-I T cells following conditioning of 501 mice with activating anti-CD40 Ab

Professional APCs can be stimulated to increase their Ag-presenting activity and immunogenic potential by ligation of their CD40 receptor (39, 45, 65). This is mediated through engagement of CD154, a TNF family member expressed on activated CD4+ T cells, platelets, and mast cells (38). The engagement of CD40 by CD154 can be replaced by the administration of the FGK45 agonistic Ab to CD40, bypassing the requirement for CD4+ T cell help in the activation of naive CD8+ T cells (43, 44). Thus, the ability of FGK45 administration to promote the acquisition of effector T cell function among adoptively transferred TCR-I T cells following transfer into 501 mice was determined.

TCR-I cells were transferred into 501 mice treated with FGK45 or an isotype-matched control Ab. After 7 days, TCR-I cells isolated from cervical LN's and spleens were quantitated by staining with Dbi/Tet and epitope-I peptide-induced IFN-γ production. Line 501 mice treated with FGK45 accumulated 2- and 3-fold more TCR-I cells in cervical LN's and spleens, respectively, compared with mice that received control Ab (Fig. 5A). Importantly, treatment with FGK45 resulted in an increase in the fraction of TCR-I T cells that produced IFN-γ in response to epitope-I peptide. The ratio of IFN-γ-producing cells to Dbi/epitope-I tetramer-positive cells in the LN's was 0.6 in FGK45-treated mice compared with 0.18 in control Ab-treated mice. Thus, treatment with FGK45 led to enhanced accumulation of TCR-I T cells in 501 mice that displayed increased effector function.

We next determined whether the increased accumulation of TCR-I T cells within the lymphoid organs of FGK45-treated 501 mice could be explained by increased proliferation of TCR-I T cells. Line 416 spleen cells were labeled with CFSE before transfer into 501 or C57BL/6 mice. CFSE is a stable fluorescent dye that decreases in intensity by approximately one-half with each cell division, due to equal partitioning into daughter cells, and can therefore be used to measure the rate and extent of cell proliferation. Over 70% of the CD8+, Dbi/Tet-1 T cells recovered from the cervical LN's and spleen of 501 mice that received FGK45 had lost the CFSE label by 8 days postransfer, indicating that the majority of accumulating cells had undergone more than seven divisions (Fig. 5B). In contrast, only a small fraction (3%) of TCR-I cells isolated from the cervical LN's of 501 mice given control Ab had divided enough times to lose the CFSE stain, with most cells having undergone three to five cell divisions. Similarly, only 22% of the TCR-I T cells re-isolated from the spleen of control Ab treated 501 mice had undergone more than seven divisions and lost CFSE fluorescence. Thus, in vivo ligation of CD40 resulted in an increase in the rate of TCR-I T cell proliferation (Fig. 5B) as well as an increase in the total number of TCR-I T cells that accumulated in the secondary lymphoid organs (Fig. 5A). TCR-I cells transferred into C57BL/6 mice only proliferated if the mice were challenged with rVV-I, which resulted in the proliferation of all TCR-I T cells more than seven cell divisions and a corresponding loss of the CFSE label (Fig. 5B).

Although these results indicated that FGK45 treatment could promote the acquisition of T cell effector function if administered before the transfer of TCR-I T cells into 501 mice, a more realistic scenario was whether FGK45 treatment could promote the accumulation of effector T cell populations that had been previously exposed to the tolerant environment of 501 mice. Thus, the effect of administering FGK45 Ab 7 days postransfer of TCR-I T cells was determined. This corresponds to a time point when TCR-I T cells had expanded in 501 mice, but failed to develop effector function (Fig. 3A). This approach resulted in the accumulation of similar numbers of TCR-I T cells in the draining LN's by day 14 postadoptive transfer whether or not FGK45 was administered before transfer of TCR-I T cells or at 7 days postransfer (Fig. 5C, tetramer analysis, compare day -1, +1 to day 7, 8). By comparison, significantly more TCR-I T cells accumulated in the spleens of 501 mice if FGK45 was administered before cell transfer (62.4 vs 26.4% of CD8+ T cells). This difference was less apparent by day 21 postransfer. The ratio of IFN-γ-producing cells to Dbi/Tet CD8+ T cells also was reduced at day 14 in mice that received delayed treatment with FGK45 (LN, 0.31; spleen, 0.51) compared with mice pretreated with FGK45 (LN, 0.54; spleen, 0.96). This discrepancy was not apparent at day 21, perhaps reflective of the stable memory T cell pool. These results indicate that FGK45 treatment remained effective in promoting the acquisition of T cell function if administered after exposure of naive TCR-I T cells to the tolerant environment of 501 mice and established a stable memory T cell population capable of producing IFN-γ in response to Ag.

Some FGK45-treated 501 mice that received TCR-I T cells at 6 mo of age were monitored for the long-term maintenance of
epitope I-specific T cells. Three of five mice retained detectable levels of epitope I-specific CD8$^+$ T cells in the peripheral blood up to 1 year postadoptive transfer, ranging from 3–18% of CD8$^+$ T cells (data not shown). Analysis of lymphocytes derived from spleen and cervical LNs with MHC tetramers revealed similar frequencies of epitope I-specific CD8$^+$ T cells in these mice. In addition, a proportion of these epitope I-specific CD8$^+$ T cells were capable of producing IFN-γ in response to epitope I peptide stimulation in vitro (data not shown). Thus, FGK45 treatment is associated with the long-term survival of TCR-I T cells in 501 mice.

**TCR-I T cells from anti-CD40-treated 501 mice have in vivo effector function**

A classic function of a CD8$^+$ T cell is to lyse a target cell expressing its cognate Ag. To test the ability of TCR-I cells that encountered endogenous T Ag in 501 mice to destroy target cells in vivo, the fate of epitope I peptide-pulsed spleen cells was monitored following injection into 501 mice previously given TCR-I T cells. Target cells were obtained from congenic B6/SJL mice, which express the CD45.1 molecule that allowed the donor cells to be distinguished from the recipient CD45.2-expressing lymphocytes by staining with anti-CD45.1 mAb. B6/SJL spleen cells were pulsed with either T Ag epitope I peptide or influenza NP 366–374 peptide. Epitope I-pulsed cells were subsequently labeled with a high concentration of CFSE while NP366–374 pulsed cells were labeled with a 10-fold lower concentration of CFSE. These cells were subsequently mixed in equal proportions and 5 × 10⁴ total cells were injected into 501 or C57BL/6 mice that had previously received TCR-I cells 14 days earlier with or without FGK45 treatment. After 6 h, spleens were harvested and analyzed for the presence of CD45.1$^+$, CFSE$^+$ cells. As shown in Fig. 6, epitope I-pulsed (CFSEhigh) cells were undetectable in the spleens of 501 mice that were treated with FGK45, whereas the low CFSE-labeled cells pulsed with the control peptide remained. In contrast, equal proportions of high and low CFSE-labeled CD45.1 lymphocytes were detected in the spleens of 501 mice given control Ab, indicating the absence of significant epitope I-specific lytic activity. Control C57BL/6 recipients maintained both high and low CFSE-labeled populations of target cells, demonstrating that FGK45 treatment in the absence of endogenous T Ag does not lead to activation of the TCR-I T cells. In contrast, epitope I-pulsed target cells were eliminated in TCR-I T cell recipient C57BL/6 mice that were previously immunized against epitope I. These results demonstrate that FGK45 treatment of 501 mice leads to the activation of adoptively transferred TCR-I T cells that develop potent in vivo epitope-specific lytic activity.

**Discussion**

Recent studies have indicated that the loss of CD8$^+$ T cell responsiveness to parenchymal Ags in the periphery is initiated by the cross-presentation of these self-Ags on bone marrow-derived APCs, such as DCs, to naive T cells (8, 34). CD8$^+$ T cell recognition of endogenous Ag results in the modulation of surface molecules indicative of T cell activation, followed by T cell proliferation (28, 66). These T cells, however, fail to obtain effector function and are typically deleted within a period of a few weeks (28, 30, 66). This scenario represents a major drawback for immunotherapeutic strategies to cancer that target tumor-associated self-Ags recognized by CD8$^+$ T cells. The results presented in this report define a system in which the onset of peripheral CD8$^+$ T cell tolerance to an endogenous tumor Ag can be readily observed. Naïve CD8$^+$ T cells specific for T Ag epitope I encountered the endogenous T Ag after transfer into 501 mice, resulting in the up-regulation of activation markers and T cell proliferation, but failed to acquire effector function. That the TCR-I T cells became functionally compromised and did not simply maintain a naive phenotype was evidenced by their lack of responsiveness to subsequent immunization against epitope I. In addition, TCR-I T cells disappeared from the peripheral lymphoid organs of 501 but not C57BL/6 mice around 4 wk postadoptive transfer, consistent with a deletion mechanism of tolerance induction.

Several approaches have been proposed to block or reverse the onset of peripheral T cell tolerance for the purpose of salvaging potentially therapeutic T cells within the host T cell repertoire. Because the role of the APC is central for complete activation of naïve CD8$^+$ T cells, triggering APC function via ligation of the CD40 receptor has received recent attention (67). This approach was shown to block the onset of peripheral T cell tolerance in vivo if agonistic anti-CD40 Ab was administered along with specific immunization (46–48, 68). The ability of anti-CD40 ligation to lead to the priming of CD8$^+$ T cells against endogenous self-Ags or preexisting tumors, however, has yielded a different set of results. Keld et al. (50) demonstrated that the administration of anti-CD40 Ab into tumor-bearing mice in the absence of specific immunization led to the enhanced deletion of tumor-specific CD8$^+$ T cells. More recently, Hernandez et al. (69) found that administration of anti-CD40 enhanced the proliferation of TCR transgenic CD8$^+$ T cells specific for an influenza virus hemagglutinin (HA) epitope in HA transgenic mice, but failed to result in the induction of effector function. In contrast to these studies, we demonstrate that administration of FGK45 into 501 mice resulted in increased...
proliferation of TCR-I T cells within the secondary lymphoid organs as well as the acquisition of effector functions, including the ability to destroy T Ag epitope I-expressing target cells in vivo. Although the basis for this difference is not readily apparent, some possible explanations may include differences in the antigenic systems utilized, different levels of endogenous Ag expressed, or differences in the route of delivery or amount of anti-CD40 Ab utilized. Hernandez et al. (69) demonstrated that CD8+ T cell effector function could be induced in HA-specific CD8+ T cells if activated HA-specific CD4+ T cells were coinjected or if IL-12 was coadministered with the anti-CD40 Ab. These results suggested that additional signals, such as cytokines, were required for the full activation and subsequent acquisition of effector functions by naive HA-specific TCR transgenic CD8+ T cells in HA transgenic mice. One possible explanation for the success of CD40 ligation alone to lead to the acquisition of effector function among naive TCR-I T cells is that the CD4+ T cell compartment might not be tolerant in 501 mice, in contrast to the HA transgenic mice, such that additional signals could be provided by the endogenous CD4+ T cells in 501 mice to either the APCs or the TCR-I T cells. Preliminary results in 501 mice, however, indicate that the presence of CD4+ T cells is not required for anti-CD40 administration to promote the acquisition of effector function by TCR-I T cells (data not shown). An alternative explanation is that the TCR-I T cells might not require additional signals other than those delivered by CD40-induced APCs. Future studies will assess the phenotype of CD40-induced APCs from 501 mice to determine whether these APCs alone are fully capable of activating naive TCR-I T cells.

Differences in the level of Ag expression or the antigenic properties of the Ag systems investigated might also explain the observed differences. Previous investigations assessing the ability of H-2Kb-restricted, T Ag epitope specific TCR transgenic CD8+ T cells to respond to endogenous T Ag expressed from the insulin promoter demonstrated that transgenic expression of the full-length T Ag (70), but not a nontransforming truncated protein (26), led to the induction of autoimmunity. These results suggested that the oncogenic process initiated by full-length T Ag might increase the priming of autoreactive T cells, while expression of a nontransforming mutant T Ag resulted in immunologic ignorance (26). Thus, the full-length T Ag might be capable of providing additional signals to the APCs or other immune effector cells, such that CD40 ligation alone results in full activation of the naive CD8+ T cells in 501 mice.

A few studies have indicated that the administration of anti-CD40 Ab can lead to a more rapid onset of tolerance (50, 71). Our analysis indicates that TCR-I T cells exposed to the endogenous T Ag for one week remained responsive to the activating effects of anti-CD40 administration. Whether these T cells will remain responsive for longer periods of time is unknown, but our data indicate that at least some of the T cells can be rescued by anti-CD40 administration. One potential difference between our study and those that showed anti-CD40-induced deletion of responsive T cells is that the mice in previous studies were given multiple injections of anti-CD40 Ab over the course of the experiment vs two doses of FGK45 at 1-day intervals administered in this study. Mauri et al. (71) assessed the effects of anti-CD40 ligation on the development of collagen-induced arthritis following repeated injections of anti-CD40 into collagen type II-treated animals. These authors found that repeated anti-CD40 administration decreased the onset of disease severity and skewed the T cell response from Th type 1 to type 2. They suggested that anti-CD40 treatment might skew the dominant APC from DCs to B cells, which favor a Th type 2 response rather than a Th type 1 response. Thus, repeated injections of anti-CD40 might be detrimental for maintaining CD8+ T cell responses, suggesting that the timing of anti-CD40 administration needs to be carefully assessed.

One important aspect of the present study is the finding that administration of anti-CD40 led to the establishment of a stable memory population of functional TCR-I T cells on a systemic level. High levels of TCR-I T cells were detected in 501 mice by day 21 postadoptive transfer regardless of whether the mice were treated with anti-CD40 at the time of TCR-I T cell transfer or 1 wk later. In addition, a significant proportion of these T cells maintained the ability to produce IFN-γ upon stimulation in vitro. We also found that these T cells could be detected in the peripheral blood of anti-CD40 treated 501 mice up to 1 year following adoptive transfer, ranging from 3 to 18% of the total CD8+ T cells. This finding is particularly intriguing in the light of recent studies that demonstrated that CD4+ effector T cells and CD8+ memory T cells remained susceptible to the effects of peripheral tolerance to self-Ag in HA transgenic mice (72, 73). We have yet to determine whether the TCR-I long-term memory T cells in 501 mice can effectively control tumor progression in 501 mice or maintain the ability to destroy epitope I-expressing target cells in vivo. These memory T cells, however, retained the ability to produce IFN-γ following in vitro stimulation (data not shown). Thus, effector T cells generated in the presence of anti-CD40 treatment establish a stable memory T cell pool that might later be tapped for immunotherapy of developing tumors.

In conclusion, the results presented in this study provide encouraging support for the use of anti-CD40 treatment to generate significant CD8+ T cell responses to tumor-associated self-Ags. Clearly the conditions for anti-CD40 treatment must be carefully evaluated for each particular Ag system to provide maximal stimulatory responses and prevent accelerated deletion of responding T cells. Key to this approach is the presence of a population of T cells capable of responding to the target Ag, which might consist of the residual endogenous T cell response, or be derived from adoptive transfer. Thus, in cases where T cell precursors are limiting, the use of specific immunization or in vivo expansion to generate increased numbers of effector T cells in combination with in vivo anti-CD40 treatment might be desirable.

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References


cells, or vaccinia virus recombinants expressing full-length T antigen or epitope minigenes. J. Virol. 74:6922.


