Retinoic Acid and Transforming Growth Factor – Beta Control Mucosal Perforin-1 Dependent CD8 CTL Mediated Cytotoxicity by Regulating the Spectrum of Granzyme Expression and CD103 Restriction

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CYTOTOXICITY BY REGULATING THE SPECTRUM OF GRANZYME
EXPRESSION AND CD103 RESTRICTION

By
Louis Edward González

A DISSERTATION

Submitted to the Faculty
of the University of Miami
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

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RETINOIC ACID AND TRANSFORMING GROWTH FACTOR – BETA CONTROL MUCOSAL PERFORIN-1 DEPENDENT CD8 CTL MEDIATED CYTOTOXICITY BY REGULATING THE SPECTRUM OF GRANZYME EXPRESSION AND CD103 RESTRICTION

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Understanding how cytotoxic T cells (CTL) kill is of fundamental importance to the field of immunology. In the current model for how CTL kill target cells there is a critical dependence on lymphocyte-function associated antigen 1 (LFA-1, αLβ2) to tightly bind its ligand, intercellular adhesion molecule 1 (ICAM-1), following T cell receptor (TCR) recognition of cognate antigen (Dustin 2009). Recently, another member of the of integrin family, CD103 (integrin αE) has been characterized and data suggest that it may play an important role in killing ligand bearing targets (Feng, Wang et al. 2002; Gorfu, Rivera-Nieves et al. 2009). CD103 is expressed by lymphocytes of the mucosa, being found on a majority of intraepithelial lymphocytes in the small intestine (~90%)(Cepek, Shaw et al. 1994). Studies of pancreatic islet rejection after allo-transplantation have demonstrated that CD103+ effectors can be found at the site of tissue damage and that CD103−/− animals show delayed allograft rejection.
kinetics (Hadley, Bartlett et al. 1997; Feng, Wang et al. 2002). These data suggest that CD103 may play a role in the execution of CTL function in the mucosa precisely how remains unclear. Despite the understanding that retinoic acid (RA) and TGF-β are found abundantly in the intestinal mucosa, very little evidence exists with regard to their simultaneous effects on CD103. The studies here demonstrate that CD103 expression is rapidly induced by RA and TGF-β in the presence of antigen much more so than either of the two molecules alone. Additionally, previous vaccination studies have illustrated an efficient induction of CD103 on gp96 cross-primed OT-I cells at mucosal sites (Strbo, Pahwa et al. 2010). Results further show that antigen-dependent CTL activation in the presence of RA and TGF-β activate CD103 to participate in killing, which is blocked by antibodies that prevent binding to E-cadherin, CD103’s only known ligand. Moreover, we demonstrate that RA and TGF-β are capable of modulating the expression of granzymes. Notably, susceptibility to killing via CD103 activated cells can be augmented by ectopically expressing E-cadherin in targets that are not normally E-cadherin positive. Given that the only known ligand for CD103, E-cadherin, is highly expressed on the basolateral side of gut epithelium together with the relationship between CD103, RA and TGF-β, we speculate that CD103 may be a provider of immune surveillance function in the gut (Cirulli, Baetens et al. 1994; Le, Yap et al. 1999).
DEDICATION

This work is dedicated to my parents: Luis I. González and Minerva González. Their support and constant badgering love throughout this entire doctoral process was immeasurable. This work is also dedicated to my niece, Gabriella González-Abreu. I want you to know that whatever you choose to do in life, it is not the end of the road that matters most but the journey you undertook to get there.

DEDICACION

Esta obra está dedicada a mis padres: Luis I González y Minerva González. El apoyo y fastidio amor constante a través de este proceso doctoral no se puede calcular. También dedico esta obra a mi sobrina, Gabriella González-Abreu. Quiero que sepas: en lo que desees hacer en esta vida, lo importante no es lograr su éxito si no lo que importa más es el camino que tomaste para lograrlo.
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Heartfelt thanks to Lesley De Armas, Matthew Tsai, and Taylor Schreiber; three Podack Lab members that are great colleagues and even better friends.

Eva Fisher and Auristela Rivera, who are the bedrock of lab life, from baking brownies on birthdays to maintaining 200 cage count mouse colonies, I could not have done all the work without you. Thank you very much.

Natasa Strbo, my “partner in crime” as we have grown to refer to each other, thank you so much for all the scientific as well as personal support over the last few years; I am truly grateful for all you’ve done.

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Lastly, I need to thank three people without whose encouragement I might never have considered applying to graduate school:

- Mahendra Kumar, you helped to reignite my love of science and for that, I am grateful.
- Diana Lopez; you allowed me into your class and opened the possibilities, thank you.
- Sheldon Greer; along with Diana you let me into your class and immediately noticed something I had thought lost, thank you for the early encouragement.
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LIST OF ABBREVIATIONS

CCR9    C-C chemokine receptor type 9
CMA     Concanamycin A
CTL     Cytotoxic T Lymphocyte
CXCR3   Chemokine receptor CXCR3 is a G-protein-coupled receptor in the CXC chemokine receptor family. Other names for CXCR3 are G protein-coupled receptor 9 (GPR9) and CD183.
DDAO    7-Hydroxy-9H-(1,3-Dichloro-9,9-Dimethylacridin-2-One)
DMSO    Dimethyl Sulfoxide
EGTA    Ethylene Glycol Tetraacetic Acid
FACS    Fluorescence Activated Cell Sorting
FBS     Fetal Bovine Serum
GAPDH   Glyceraldehyde 3-phosphate dehydrogenase
GZA     Granzyme A
GZB     Granzyme B
GZD     Granzyme D
GZM     Granzyme M
IEL     Intraepithelial Lymphocytes
IFN-γ   Interferon gamma
IL-2    Interleukin - 2
IMDM    Iscove’s Modified Dulbecco’s Media
| **KLRG1** | Killer cell lectin-like receptor subfamily G member 1 |
| **LFA-1** | Leukocyte Function associated Antigen - 1 |
| **MLN** | Mesenteric Lymph Node |
| **NCBI** | National Center for Biotechnology Information |
| **OT-I** | TCR transgenic mouse specific for the ovalbumin peptide |
| **RA** | Retinoic Acid |
| **RAR** | Retinoic Acid Receptor |
| **RXR** | Retinoid X Receptor |
| **SPL** | Spleen |
| **TCR** | T cell receptor |
| **TGF-β** | Transforming Growth Factor - Beta |
| **TNF-α** | Tumor Necrosis Factor - alpha |
| **Trm** | T resident memory cell |
Chapter 1: Introduction

1.1 Integrins

The term “integrin” was first used by Richard O. Hynes in a 1987 review article for Cell in order to describe a family of related cell-surface receptors that he believed integrated the extracellular environment with the intracellular cytoskeleton (Hynes 1987). Since then, a large body of literature has been generated giving credence to his idea as well as to greatly expand on the role integrins play in cellular interactions. Integrins are a family of heterodimeric proteins consisting of noncovalently associated subunits: one alpha chain and one beta chain. They are restricted to metazoa, having no discernable orthologs in prokaryotes, plants, or fungi (Whittaker and Hynes 2002). Indeed, in vertebrates there is a leukocyte-specific set of integrins (Harris, McIntyre et al. 2000). They are the major receptors for cell adhesion to the extracellular matrix and play an important role in cell-cell adhesions. In addition to these extracellular functions, integrins also transmit signals into the cell via their connections to the cytoskeleton, activating many intracellular signaling pathways (Hynes 2002). This process is termed “outside-in” signaling and these pathways are able to regulate a variety of pathways such as gene expression, proliferation, apoptosis, and differentiation (Abram and Lowell 2009). Conversely, modulation of integrin function can also occur when ligand-binding affinity or receptor clustering is altered due to intracellular
signaling processes and is most often mediated by interactions with the cytoplasmic tails of integrins (Goldfinger and Ginsberg 2004). This process is termed “inside-out” signaling. Since the recognition of integrins as one large family of receptors, there has been great interest in their study (DeSimone, Stepp et al. 1987; Hynes 1987; McEver and Zhu 2010). Integrins have been shown to play important roles in development, disease states like cancer, and importantly immune responses (Schmits, Kundig et al. 1996; Scharffetter-Kochanek, Klein et al. 1998; Huang, Zang et al. 2000; Semmrich 2005; Abram and Lowell 2009; Fooksman, Vardhana et al. 2010). Currently, there are 18 known alpha chains and 8 known beta chains; however, of the 144 possible combinations only 27 pairs have been identified (Figure 1.1.1). The functional state, density, and distribution of cell-surface integrins are regulated by signaling molecules (chemokines, cytokines, lipids, etc.) and by cross-talk from other adhesion molecules (Harris, McIntyre et al. 2000). Each alpha chain and each beta chain is regulated independently and when paired give rise to tissue specificity in lymphocytes and other leukocytes (Plow, Haas et al. 2000). It should be noted that single alpha or beta chains have not been shown to bind ligands, the ligand binding site appears to be established by a region of intersect between the two integrin chains (Goldfinger and Ginsberg 2004). Integrin expression varies greatly by cell type.

Figure 1.1.1. Currently known integrins. Yellow squares are leukocyte-specific. Adapted from www.Biolegend.com/integrin_explorer and Hynes 2002.
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For example, resting T cells can express at least 12 of the 27 known integrins (from the β1, β2 and β7 families) but the profile and relative amounts of the integrins on the T cell surface changes with immune activation and exposure to extracellular signals (Hogg, Laschinger et al. 2003).

The majority of integrins bind ligands that form part of the extracellular matrix: laminins, collagens, fibronectin and other tissue specific extracellular matrix proteins (Figure 1.1.2). These serve to anchor the cell to the basement membrane or adjacent cell, providing structure and rigidity to organs and tissues while simultaneously providing feedback to the cell regarding the surrounding environment. Indeed, during organogenesis, for example, α8β1 (binds nephronectin) is indispensable for the proper formation of kidney while, during adult life, α6β4 (binds keratin filaments) is needed to anchor the skin in place, providing resistance to sheer forces. The leukocyte specific integrin ligands differ from other integrin ligands mainly by being cell surface molecules. The leukocyte integrins (β1 and β7 family members) all bind intracellular adhesion molecule family members (ICAMs, MadCAM, VCAM) as well as additional molecules important to the immune system (CD14, CD23, iC3b and Factor X) with one notable exception: CD103 (αE)/β7. Further, the leukocyte integrins bind promiscuously, each having multiple ligands.

Figure 1.1.2. Ligands of currently known integrins. Yellow squares are leukocyte-specific. Adapted from www.Biolegend.com/integrin_explorer and Hynes 2002.
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This is not the case for CD103/β7, which has only one known ligand, E-cadherin, the prototypical classical cadherin.

1.2 Integrin CD103/αE

What would be named CD103, the integrin αE, was first identified in 1988 by Kilshaw, et al. as a unique cell surface antigen on the intraepithelial lymphocytes of mouse intestine (Kilshaw and Baker 1988). It took several more years for a blocking monoclonal antibody, M290, to be generated, illustrating the importance of the antigen in binding to epithelial cells (Roberts and Kilshaw 1993). Integrin CD103/αE pairs exclusively with β7 integrin to form a functional heterodimer on the surface of cells. The β7 integrin chain, on the other hand, can pair with the α4 integrin to form the mucosal addressin α4β7 (Mad-CAM) (Farstad, Halstensen et al. 1996). The β7 family of integrins is exclusively expressed in leukocytes (Farstad, Halstensen et al. 1996). Each integrin subunit is regulated independently however, and there is competition among different alpha chains for companion beta chains and vice versa (Svensson, Johansson-Lindbom et al. 2008; Kang, Park et al. 2011). αEβ7 has been found on approximately 2% of circulating blood lymphocytes and over 90% of gut intraepithelial lymphocytes (Cepek, Shaw et al. 1994). The term “CD103” is often used to refer to the entire αE-β7 integrin heterodimer as we shall do in this text (Gorfu, Rivera-Nieves et al. 2009).
CD103 is believed to be important for immunity in the gut (Hadley, Bartlett et al. 1997; Lefrancois, Parker et al. 1999; del Rio, Bernhardt et al. 2010; Cerovic, Houston et al. 2012). Although CD103 is not necessary for lymphocyte traffic into the gut, it has been shown to be critical for lymphocyte retention in this tissue (Suzuki, Nakao et al. 2002; Gorfu, Rivera-Nieves et al. 2009). CD103 is preferentially expressed on CD8 T cells in the mucosal lamina propria and in mesenteric lymph node (MLN) T cells where it appears to play a role in the specificity of cytolytic T lymphocytes (CTL) killing ligand bearing targets (Feng, Wang et al. 2002; Gorfu, Rivera-Nieves et al. 2009). CD103 is also expressed on a subset of dendritic cells and on FoxP3+ CD4 T cells believed to be important for controlling intestinal homeostasis and inflammatory responses (del Rio, Bernhardt et al. 2010). Studies of pancreatic islet rejection have demonstrated that CD103+ effector-lymphocytes can be found at the site of tissue damage (Hadley, Bartlett et al. 1997). CD103−/− animals also show delayed islet allograft rejection kinetics (Hadley, Bartlett et al. 1997; Feng, Wang et al. 2002). Our laboratory’s vaccination studies have illustrated an efficient induction of CD103 on gp96 cross-primed OT-I cells at mucosal sites (Strbo, Pahwa et al. 2010). These data and our observations suggest a potential relationship between the expression of CD103, E-cadherin and cytotoxicity in the mucosa. In addition, it is well documented that both RA and TGF-β are abundantly bioavailable in the gut mucosa, being continuously generated by multiple cell types. We
therefore studied the expression and function of CD103 on CD8+ CTL generated in the presence of RA and TGF-β. The data indicate that CD103 controls CD8+ CTL cytotoxicity in the mucosa, which may have important implications for the generation of long lasting immunity to enteric pathogens.

1.3 Lymphocyte traffic in the gut

Circulating naïve lymphocytes leave the blood through high endothelial venules, entering secondary lymphoid tissue in the search for cognate antigen. If no antigen is encountered, they exit the lymphatic tissue via efferent lymphatic vessels, returning to systemic blood circulation (Miyasaka and Tanaka 2004). The naïve lymphocyte is then free to repeat the process, capable of randomly entering any secondary lymphoid organ. On average a naïve lymphocyte can enter one lymph node per day, though is modulated based on inflammatory states and infection (Abbas, Lichtman et al. 2011).

Upon antigen encounter in secondary lymphoid tissue, however, the pattern of migration is profoundly changed (von Andrian and Mempel 2003). Activated lymphocytes proliferate and differentiate, becoming imprinted by the microenvironment that activated them. In the gut mucosa, the Peyer’s Patch (PP) is one such location (Mora, Bono et al. 2003; Johansson-Lindbom, Svensson et al. 2005) (Figure 1.3.1). Lymphocytes activated in PPs acquire expression of molecules like CCR9 and the integrin α4β7, which bind the mucosal chemokine CCL25 and the mucosal
Figure 1.3.1. Lymphocyte traffic in the gut. Naïve lymphocytes (NL) enter the Peyer’s patch through high endothelial venules (HEV) where they can encounter cognate antigen and are primed for mucosal homing (acquire α4β7, CCR9 expression); afferent lymphatics carry cells to the mesenteric lymph node (MLN) for further maturation and eventual return to systemic circulation via the thoracic duct. Activated lamina propria lymphocytes (LPL) can now preferentially enter the lamina propria through permissive HEV, where some acquire CD103 expression and become intraepithelial lymphocytes (IEL).

addressing MadCAM-1, respectively. These mucosal immunoblasts drain into the mesenteric lymph node (for further maturation) and then onto the thoracic duct where they make their way back to systemic circulation (Salmi and Jalkanen 2005). Upon return to the mucosa, they preferentially enter the intestinal lamina propria via interactions between MadCAM-1.
and α4β7, which are further potentiated by CCR9/CCL25 ligand-receptor binding (Miles, Liaskou et al. 2008; Gorfu, Rivera-Nieves et al. 2009). A subset of these lymphocytes will traverse the lamina propria moving towards the gut epithelium. These intraepithelial lymphocytes (IEL) embed themselves between gut epithelial cells via CD103 interactions with E-cadherin, generating a lymphocyte population that is characteristic of many sites along the gut mucosa (Schon, Arya et al. 1999). It is believed that the majority of CD103 expressing IELs become permanently anchored between the intestinal epithelia, forgoing their circulatory capability (Figure 1.3.1). PP can thus be thought of as a primary inductive site for naïve lymphocytes in the intestinal mucosa and lamina propria as secondary target organs for activated lymphocytes. Moreover, while lymphocytes activated in one part of the gut show a preference for homing back to that same anatomical location, the homing pattern is not absolute. Evidence suggests that mucosally activated cells (e.g. by gp96-Ig vaccination) are able to migrate to other immunologic compartments (both systemic and mucosal) (Mora, Cheng et al. 2005; Strbo, Pahwa et al. 2010).

### 1.4 E-cadherin

E-cadherin (also known as epithelial cadherin, uvomorulin, or CD324) is a type I classical cadherin encoded by the Cdh1 gene (in both mouse and humans) and was first identified in 1977 (Kemler, Babinet et al. 1977).
Classical cadherins are typified by five cadherin ectodomains, numbered EC1 through EC5 and a conserved cytoplasmic tail that associates with the catenin family of cytoplasmic proteins (Takeichi 1995). Like all classical cadherins, E-cadherin is a calcium-dependent cell surface glycoprotein that mediates cell-cell adhesion in tissues. It dimerizes on the cell surface and goes on to form trans-homophilic clusters, establishing adherens junctions (Figure 1.4.1). It has been studied extensively since its
discovery, its role in embryonic development and cancer being most often highlighted (Katagiri, Watanabe et al. 1995; Spencer, Eastham et al. 2007; Stemmler 2008; van Roy and Berx 2008). In both these areas of study it is the cadherin-cadherin homotypic interaction that is of primary interest. Importantly, homotypic interactions occur only with cells expressing the same cadherin (Nose, Nagafuchi et al. 1988). Indeed, during development as cells segregate and differentiate, the pattern of expressed cadherins also changes to match developing tissue type (Takeichi 1991). The first cadherins identified were named according to the tissue in which they were first found P-cadherin, R-cadherin, N-cadherin, and E-cadherin (placental, retinal, neuronal and epithelial). In early development E-cadherin (along with N-cadherin) is essential for proper gastrulation and neurulation (Stemmler 2008).

In postnatal life, E-cadherin is expressed in epithelial cells and is responsible for the formation of adherens junctions at the plasma membrane and tissues. E-cadherin has been found on pancreatic islets and is highly expressed on the basolateral side of gut epithelium (Cirulli, Baetens et al. 1994; Le, Yap et al. 1999). It has been shown experimentally that the ectodomain (EC1) of E-cadherin is what confers specific binding capability. Using genetic methods to swap EC1 domains from N- and E-cadherins, Nose et al demonstrated that binding specificity could be altered, while others have shown that beads coated with ectodomain fragments are capable of supporting calcium-dependent
aggregation (Nose, Tsuji et al. 1990; Paradies and Grunwald 1993). While the ectodomain alone creates mechanical adhesion, physiological adhesion requires involvement of the entire molecule. Subsequently, the cytoplasmic tail of E-cadherin was found to play a functional role in adhesion. Several members of the catenin family (e.g. α- and β-catenin) have been described as associating with the conserved cadherin cytoplasmic tail (Ozawa, Ringwald et al. 1990). Specifically, β-catenin has been shown to bind the cadherin cytoplasmic tail with α-catenin binding to β-catenin (Hulsken, Birchmeier et al. 1994). α-catenin then mediates interactions with the actin cytoskeleton (Rimm, Koslov et al. 1995). Deletion of the cadherin cytoplasmic tail or of the catenin binding site disrupts otherwise stable cadherin mediated aggregation of cells in tissue culture (Nagafuchi and Takeichi 1988; Ozawa, Ringwald et al. 1990). While catenins are essential to adhesion, other intracellular signaling proteins (e.g. Formin, Vinculin, and p120 catenin) have been shown to associate with cadherins, though their roles in adhesion remains unclear (Reynolds, Daniel et al. 1994; Brady-Kalnay, Rimm et al. 1995). Cadherin-based adhesion is a dynamic process. Several lines of evidence suggest that adherens junctions at epithelial surfaces are regularly and rapidly recycled in clathrin-dependent processes (Le, Yap et al. 1999; Delva and Kowalczyk 2009). Moreover, the state of adherens junctions can be modulated by exposure to a variety of extracellular signals, cellular or mechanical in nature. For example, in the intestinal epithelium, changes in
tissue organization, cell death, and division can lead to stresses that are readily relieved by changing the number, length or strength of adherens junctions (Cavey, Rauzi et al. 2008; Baum and Georgiou 2011). These data clearly indicate that E-cadherin plays a central role in the maintenance of proper tissue organization and function, giving structural/mechanical support while simultaneously providing information on the state of the surrounding environment. It should not be surprising then, that the downregulation of E-cadherin has been linked to increased metastasis and tumor invasiveness in gastric cancers (Becker, Atkinson et al. 1994; Katagiri, Watanabe et al. 1995; van Roy and Berx 2008). Cells that have lost a) the ability to sense their surroundings and b) receive inhibitory signals from neighboring cells are capable of uninhibited proliferation and pose a risk to the highly organized structures of the body.

Importantly, E-cadherin is the only known ligand for CD103 and the only known example of a cadherin acting as a ligand for an integrin. We have previously highlighted the role CD103 (as well as other integrins) plays in targeting lymphocytes to the intestinal mucosa, acting as both marker and potential mediator of effector function. Since E-cadherin is also a signaling platform, however, it is possible that CD103 binding could modulate epithelial cell function (either directly or indirectly). This might be readily accomplished, indirectly, by cytokines or chemokines produced by resident IEL or, directly, by signals generated by E-cadherin/CD103 interactions.
1.5 Retinoic Acid

Retinoic acid (RA) is a metabolite of retinol otherwise known as vitamin A, a low molecular weight primary alcohol. For humans and other mammals, vitamin A is considered an essential nutrient, consumed in the diet (Blomhoff, Green et al. 1991). Scientific interest in retinol and its metabolites has increased dramatically over the last 20 years. Specifically, interest in RA and its effects on the developing and adult nervous system, the immune system, and obesity have been notable (Iwata, Hirakiyama et al. 2004; Maden 2007; Svensson, Johansson-Lindbom et al. 2008; Berry and Noy 2009; Allie, Zhang et al. 2013). Its central role in a zebrafish model of hindbrain development was described by White et al. in 2007 (White, Nie et al. 2007). In the adult nervous system, RA is involved in responding to nerve damage, which initiates regeneration. In rat sciatic nerve tissue, all three isoforms of the retinal dehydrogenase (RALDH) enzymes are detectable. These enzymes generate RA from retinal precursors. Dorsal root ganglia, motor neuron cell bodies, and Schwann cells all express isoforms of retinoic acid receptors (RAR). After peripheral nerve injury RA synthesis is increased as are the levels of RAR isoforms, leading to neurite outgrowth (Maden 2007). RA administration has been shown to lead to weight loss in obese mice. The mechanism by which this occurs is not yet fully appreciated but recent evidence suggests that RA induces the upregulation of its nuclear receptors in adipocytes (a lineage which downregulates RAR upon differentiation) and shifts metabolism by
increasing target genes that regulate lipid and sugar processing (Berry and Noy 2009). Immunologically, in recent years, RA has become recognized as having pronounced effects on immune cell differentiation, survival and effector function. RA has been shown to inhibit the Th1 program in CD4 T cells, decreasing IFN-γ production, suppressing T-bet and IL-12Rβ2 while simultaneously enhancing the Th2 program, increasing IL-4 production, c-maf and GATA-3 (Iwata, Eshima et al. 2003). CD8 T cell effector and memory phenotypes are also influenced by RA signaling. Absent RA signaling leads to a decrease in highly differentiated short-lived effector cells and an increase in the memory-
precursor effector cell population, which causes enhancement of the central memory pool. In addition, effector function was somewhat impaired with granzyme B and IFN-γ production diminished during the acute phase of an immune response (Allie, Zhang et al. 2013). In B cells, RA not only induces a gut-homing phenotype but also synergizes with cytokines to promote the production of IgA in gut-associated lymphoid tissues (GALT) (Mora, Iwata et al. 2006). RA’s effects are not limited to the adaptive immune system. In NK cell lines, RA was able to suppress cytotoxicity in a dose dependent fashion, reducing the amount IFN-γ and granzyme B while having no effect on Perforin-1 (Li, He et al. 2007). In broader terms, RA is widely accepted as a gut-imprinting molecule. It increases the expression of α4β7 and CCR9, generating lymphocytes that preferentially home to GALT upon recirculation and activation (Iwata, Hirakiyama et al. 2004; Hammerschmidt, Ahrendt et al. 2008; Svensson, Johansson-Lindbom et al. 2008). Importantly, it is found abundantly in the gut, home to the mucosal immune system (Figure 1.5.1). In the gut, RA is made from retinal precursors by retinal dehydrogenases of which there are three isoforms (RALDH1, RALDH2, and RALDH3) (Napoli 2012). All three isoforms can be found expressed at high levels in the intestine, stomach, and gut-draining lymph nodes (Frota-Ruchon, Marcinkiewicz et al. 2000; Molenaar, Knippenberg et al. 2011). RALDH1 is highly expressed by the epithelial cells of the stomach, small intestine, and large intestine; whereas the other two isoforms have been found expressed in gut lymph
node stroma and gut dendritic cells (Hammerschmidt, Ahrendt et al. 2008). Moreover, control of retinal dehydrogenase enzyme synthesis appears to be directly controlled by dietary vitamin A (Molenaar, Knippenberg et al. 2011). The relative abundance of both precursor and converting enzyme, localized to the gut mucosa then, leads to a microenvironment rich in RA. It should not be surprising that mammals have evolved mechanisms to take advantage of this enrichment, generating a system that effectively targets immune responses to a well-defined and highly specialized compartment. For example, RA and TGF-β (TGF-β is discussed in the next section) exert differential effects on the transcription of CD103 as well on its partnering beta chain (β7). RA upregulates the α4 integrin, the only binding partner of β7 and subsequently a direct competitor with CD103 (Mora, Cheng et al. 2005). TGF-β, on the other hand, antagonizes α4 upregulation in addition to promoting the transcription of CD103 and β7 chain mRNA (Kilshaw and Murant 1991). The interplay then, between RA and TGF-β signals help to determine the balance of CD103/β7 and α4β7 on the cell surface. (Farstad, Halstensen et al. 1996; Gorfu, Rivera-Nieves et al. 2009). In addition, RA has been shown to up-regulate other gut homing receptors on CD8, CD4, B, and natural Treg (nTreg) cells (Mora, Bono et al. 2003; Iwata, Hirakiyama et al. 2004; Mora, Iwata et al. 2006; Siewert, Menning et al. 2007). Apart from cell surface markers RA has been shown to enhance peripheral Treg (pTreg) frequency, influence effector/effector memory CTL differentiation, inhibit Th17 cells in vitro, and
inhibit the cytotoxicity of NK cells (Li, He et al. 2007; Mucida, Park et al. 2007; Allie, Zhang et al. 2013).

Lastly, the production of RA or its exogenous supplementation is meaningless without a cellular means to sense and respond to its presence. RA binds to two families of nuclear receptors: (1) the RA receptor (RAR) alpha, beta, and gamma and (2) the retinoid X receptor (RXR) alpha, beta, and gamma (Chambon 1996). Both families of nuclear receptors are expressed in T cells as well as other immune cells, though it has been shown that the RAR receptor family, and not the RXR family of receptors, is primarily responsible for conveying the effects of RA into cells (Iwata, Eshima et al. 2003; Iwata, Hirakiyama et al. 2004). Mice deficient in RAR receptors have impaired homing and effector responses to antigens (Svensson, Johansson-Lindbom et al. 2008). The cells of the immune system are then capable of responding to RA in the mucosa in much the same way that chemokines and cytokines modulate their behaviors. That is to say, RA signals are integrated along with all other environmental queues in order to coordinate a niche dependent and appropriate response to an immunological stimulation.

1.6 Transforming Growth Factor -β

Transforming growth factor beta (TGF-β) signaling is surprisingly canonical given the vast array of developmental and behavioral programs that it influences (Figure 1.6.1). It can influence gene expression in a wide
array of cell types, from neuroblasts and myoblasts to vascular cells and epithelium (Massague 2012). Mediated by SMAD intermediaries, TGF-β also plays an important role in the immune system (Konkel and Chen 2011). It is an essential factor in the de novo induction of FoxP3 and the generation of antigen specific CD4+ Treg cells and has been shown to be important in the differentiation of Th17 cells (Chen, Jin et al. 2003; Mangan, Harrington et al. 2006). Importantly, TGF-β has been shown to influence CD8 CTL function by inhibiting IL-2 production, granzymes,
Perforin-1, and even proliferation and survival (Heath, Murphy et al. 2000; Thomas and Massague 2005; Das and Levine 2008). Further, its effects are not limited to T cells as TGF-β plays a major role in the production of mucosal IgA by promoting class switch recombination and by providing support to other cell types that support IgA production (Mora, Iwata et al. 2006; Cerutti and Rescigno 2008; Cong, Feng et al. 2009). It is found abundantly in the gut, home to the mucosal immune system, where it is produced by cells of the intestinal epithelium, CD4+ FoxP3+ Treg cells, and CD103+ DCs (Figure 1.6.2) (Barnard, Warwick et al. 1993; Frota-Ruchon,
Marcinkiewicz et al. 2000; Coombes, Siddiqui et al. 2007; Hammerschmidt, Ahrendt et al. 2008). Indeed, it is accurate to say that the gut is an environment in which all immune responses occur in the presence of TGF-β. Given the amount of data highlighting the inhibitory effects of TGF-β on the immune system, it is within reason to call it an immunosuppressive cytokine. Yet, to paint TGF-β with such a singular brush would be dismissive of its pluripotency in the gut since it serves to directly or indirectly promote and inhibit responses to antigens: essentially, it plays a key role in keeping the gut mucosa neither totally regulatory nor inflammatory.

As was mentioned previously, TGF-β and RA exert differential effects on the transcription of CD103 as well on its partnering β7 chain increasing the expression of both transcripts upon activation (Farstad, Halstensen et al. 1996; Gorfu, Rivera-Nieves et al. 2009). Individually, RA and TGF-β have received attention as important modulators of immunity in recent years but very little has been done to highlight the effects these molecules exert simultaneously (Das and Levine 2008; Hammerschmidt, Ahrendt et al. 2008; Svensson, Johansson-Lindbom et al. 2008; Allie, Zhang et al. 2013). One recent paper by Takahashi, et al nicely illustrates the effects that both RA and TGF-β can have on T helper cells. Only when both molecules are present is microRNA miR10-a expressed; targeting BCL-6 and thus influencing the fate program of exposed cells (Takahashi, Kanno et al. 2012). Given the abundance of evidence indicating that RA
and TGF-β are present in continuous and measureable levels in the gut mucosa it seems reasonable to investigate them jointly, perhaps being able to shed some light on the complexity, and sometimes seeming confounding nature, of immunity in the intestinal mucosa.

1.7 Cytotoxicity

Understanding the rules by which cytotoxic CD8+ T cells (CTL) kill is critically important to immunology. The ability of CTL to kill is dependent on cell-cell adhesion (Gromkowski, Heagy et al. 1983). The current model for how CTL kill target cells is dependent on lymphocyte-function associated antigen 1 (LFA-1, αLβ2) tightly binding its ligand intercellular adhesion molecule 1 (ICAM-1) after the T cell receptor (TCR) recognizes cognate antigen (Dustin 2009). Cells displaying activating peptide MHC (pMHC) trigger cognate TCR signaling that leads to firm cell adhesion (Dustin 2009). This adhesion is central to the delivery of cytotoxic effector molecules like Perforin-1 and granzymes (Dustin and Depoil 2011). Canonically, LFA-1 and its ligand, ICAM-1, provide this adhesion function (Abram and Lowell 2009). As previously discussed, there is mounting evidence regarding the importance of the integrin CD103 in the gut (Schon, Arya et al. 1999; Suzuki, Nakao et al. 2002; Liu, Anthony et al. 2011). It is now well established that CD103 serves to mark a variety of cell types as being gut-resident: DCs, CD4 cells, CD8 cells, and B cells can all be induced to use CD103 (and other mucosal homing molecules)
to enter and anchor in the gut. At least in the case of CD103+ DCs and FoxP3+ CD4+ cells, the evidence suggests these cells function in a regulatory capacity, promoting the induction of other regulatory cells and immunoglobulins or themselves acting as suppressors (Chen, Jin et al. 2003; Coombes, Siddiqui et al. 2007; Cerutti and Rescigno 2008). What then becomes of the CD8 CTL? Is a cytotoxic T cell generated under conditions typifying a gut mucosal microenvironment as capable at providing effector function when compared to one generated under more typically inflammatory conditions? We have observed in our own gp96Ig vaccination studies an efficient induction of CD103+ CD8+ T cells within the IEL compartment (after intraperitoneal injection), capable of responding to in-vitro antigenic stimulation, producing Th1 type cytokines (Strbo, Pahwa et al. 2009). Are induced cells like these using CD103 as singularly as an anchor or is it participating in CTL killing?

1.8 Specific Aims

**Hypothesis: CD103 expressed on antigen specific CD8+ IEL provides immune surveillance function**

Work published by others correlates pancreatic islet allograft rejection with the presence of CD103+ CD8 effector cells at the site of tissue destruction. CD103/- knockout mice further strengthen these findings by exhibiting delayed rejection kinetics. Our own studies find an accumulation of antigen specific CD103+ CD8 cells in the gut after
gp96IgOVA intraperitoneal vaccination that are unable to kill E.G7 tumor cells in vitro. Since E-cadherin, the only known ligand for CD103, is found on the epithelial cells of pancreatic islets (in addition to being expressed abundantly in the gut epithelia) and is not expressed by E.G7 tumor cells we propose that CD103 plays a role in CD8 CTL killing. Similar to LFA-1 (αLβ2)/ICAM interactions that provide for tight binding and permit delivery of cytotoxic effector molecules after encounter with cognate antigen, CD103 binding to E-cadherin would tightly tether CD8 CTL to an epithelial cell. This interaction generates sentinel cells at the site of insult, ready to kill upon antigen re-encounter.

Specific Aim 1: Examine the expression of CD103 and LFA-1 in splenic or MLN cells activated under antigen/IL2/RA/TGF-β conditions versus antigen/IL-2 conditions

- What is the frequency of CD103 expression on the surface of naïve OT-I from the MLN and spleen?
- How does the expression of CD103 change upon activation under antigen/IL-2/RA/TGF-β conditions versus antigen/IL-2 conditions?
- Do CD103+ cells express similar gut-homing molecule profiles versus CD103- cells?

Specific Aim 2: Examine the ability of CD103+ CTL to kill e-cadherin expressing targets versus LFA-1 expressing targets.
- Do CD103+ cells generated under antigen/IL2/RA/TGF-β conditions versus antigen/IL2 conditions have comparable killing potential?
- Are the cells generated under antigen/IL2/RA/TGF-β conditions versus antigen/IL2 conditions able to kill E-cadherin expressing targets when LFA-1 and CD103 are blocked?
- Does the killing observed under antigen/IL2/RA/TGF-β conditions versus antigen/IL2 conditions occur in a perforin dependent manner? If not perforin, then how is killing mediated?
- Does the addition of E-cadherin to target cells make them susceptible to killing by CD103+ cells?
- Do individual CD8 T cells kill by CD103 or LFA-1 dependent lysis?
Chapter 2: Results and Discussion

2.1 CD103 expression in OT-I from the MLN and SPL increases upon activation with antigen and is boosted by Retinoic Acid and TGF-β

The integrin CD103, also known as αE, pairs exclusively with the β7 integrin chain to form a functional heterodimer on the surface of cells (Gorfu, Rivera-Nieves et al. 2009). Each subunit is regulated independently and there is competition among different alpha chains for companion beta chains and vice versa (Svensson, Johansson-Lindbom et al. 2008; Kang, Park et al. 2011). Retinoic Acid (RA) and TGF-β have differential effects on the transcription of CD103 as well on its partnering beta chain (β7) (Farstad, Halstensen et al. 1996; Gorfu, Rivera-Nieves et al. 2009). Before evaluating the contribution of CD103 to cytotoxic T cell lymphocyte (CTL) mediated cytotoxicity, we examined the effects of IL-2 and antigenic stimulation in the presence or absence of RA and TGF-β on CD103 expression on T cell receptor (TCR) transgenic CD8 OT-I cells. The frequency of naïve CD103+ OT-I from mesenteric lymph node (MLN) was significantly higher when compared to spleen (SPL) (Figure 2.1.1 A). There was no statistical difference in absolute number of SPL or MLN OT-I (Figure 2.1.1 B). Freshly isolated MLN or SPL cells from TCR transgenic mice (OT-I) were placed into culture in the presence of IL-2 with or without antigen, RA and TGF-β. Increased surface expression of CD103 could be
accomplished after 7 days of culture with TGF-β alone, however, without antigenic stimulation the frequency of live CD8+ cells was very low (less than 5%) (data not shown). Robust expansion of CD8 cells plus early up regulation of CD103 was absolutely dependent on antigenic stimulation indicating TCR signaling (Figure 2.1.2 A - D). In spleen OT-I IL-2 plus antigen increased CD103 from 8% to 58%, in MLN OT-I from 12% to 34%.

Detection of cell surface CD103, in the presence of RA and TGF-β, was maximal (~90%) by day 4 and then remains constant on almost all OT-I cells. In the presence of antigen the combination of IL-2, RA and TGF-β mediated the highest level of CD103 up-regulation in both spleen (from 8% on day 0 to 87% on day 4) and MLN OT-I (from 12% on day 0 to 91% on day 4) (Figure 2.1.3). The absolute number of CD8+ CD103+ OT-I cells
Figure 2.1.2. CD103 expression in OT-I from the MLN and SPL increases upon activation with antigen and is boosted by Retinoic Acid and TGF-β. Frequency of CD103+ CD8+ T cells from (A and B) SPL OT-I and (C and D) MLN OT-I cultured in combinations of RA (100 nM), TGF-β (10ng/ml), IL-2 (50U/ml) and OVA peptide (10 nM) on days 1, 4, and 7. N = 6 or 7 independent experiments.
from both SPL and MLN also continuously increased in the presence of antigen and IL-2 with or without RA and TGF-β. By contrast, the absolute number of CD8+ CD103- did not increase over time in OVA plus IL2 conditions whereas the absolute number of CD8+ CD103- cells rose in

![Pie charts showing CD103 expression in SPL and MLN OT-I cells.](image)

**Figure 2.1.3.** CD103 expression is maximal and plateaus by day 4 in the presence of Retinoic Acid and TGF-β. Pie charts represent the mean expression level of CD103+ CD8+ CTL in SPL OT-I and MLN OT-I on days 1, 4, and 7. N=6 independent experiments.
OVA, IL-2, TGF-β, and RA conditions (Figure 2.1.4). Based on these observations we used OVA, IL-2, RA and TGF-β and 4 days of activation as our experimental treatment for maximal CD103 induction and OVA and IL2 as control condition yielding about 50% CD103+ T cells.

Figure 2.1.4. Absolute count of CD8+ CD103+ and CD8+ CD103- cells. CD8+ CD103+ cells increase steadily over the seven day observation period regardless of growth conditions. By contrast, CD8+ CD103- cells only increase in number in conditions containing RA and TGF-β. N = 3 independent experiments.
Since CD103+ OT-I cells are detectable in both naïve SPL and MLN preparations it was important to determine whether CD8+ CD103+ OT-I cells are generated de novo from CD8+ CD103- cells or expand from a pre-existing pool of CD8+ CD103+ OT-I T cells. In order to test this, MLN OT-I and SPL OT-I from naïve mice were freshly isolated and stained with antibodies against CD8α and CD103. Gating on lymphocytes by forward scatter and side scatter properties, we sorted for CD8+ CD103- cells, as depicted by the gates drawn in the “Pre-Sort” panels of figure 2.1.5. Post-sort analysis demonstrates that depletion of CD8+ CD103+ cells was robust. The cells were then placed into culture with OVA and IL-2 with and without RA and TGF-β. On days one, four, and seven cells were harvested and stained with antibodies against CD8, CD3, and CD103. Gating on OT-I cells, we observe that CD103+ CD8+ cells can be detected by day one and remain highest in conditions supplemented with RA and TGF-β (Figure 2.1.5). We conclude that CD103+ CD8+ T cells can arise from a CD103- population as a consequence of antigenic stimulation and are maintained by the presence of RA and TGF-β.

Figure 2.1.5. CD103 Expression occurs de novo in the presence of retinoic acid and TGF-β. SPL OT-I (top half) and MLN OT-I (bottom half) from naïve mice were made into single cell suspensions and stained with antibodies against CD8 and CD103, then sorted by FACS for CD8+ CD103- cells and placed into culture with or without RA and TGF-β. On days, one, four, and seven cells were harvested and stained with antibodies against CD3, CD8, and CD103; analyzed by FACS for the expression of CD103. GFP, expressed by all cells, was used as a marker for discrimination between live and dead cells.
2.2 RA and TGF-β promote an effector/effector memory phenotype

RA and TGF-β have been previously reported to influence the phenotypes of effector cells (McKarns and Schwartz 2005; Das and Levine 2008; Allie, Zhang et al. 2013). It has also been reported that bioactive forms of both RA and TGF-β exist abundantly in the gut (Barnard, Warwick et al. 1993; Molenaar, Knippenberg et al. 2011). We have shown above that antigen, IL-2, RA and TGF-β up-regulate surface expression of CD103 on OT-I from SPL and MLN to maximal levels (~95%) by day four of culture. We next examined the effects of TGF-β and RA on the expression of the memory markers CD62L, CD44, CD127, and CCR7 on OT-I MLN and SPL over four days of activation.

2.2.1 Memory phenotype of CD103 positive cells

The majority of freshly ex vivo isolated and CD8+ CD3+ CD103+ spleen OT-I (66%) and MLN OT-I (74%), express CD62L and are negative for CD44, which identifies them as naïve T cells. The markers CCR7 and CD127 (IL-7Rα) are found on (30%) of naive OT-I cells in the spleen and 40% in MLN (Figure 2.2.1). By day 1 of culture both CD103+ SPL OT-I and CD103+ MLN OT-I with or without RA and TGF-β are mostly of effector/effector memory phenotype (42%-90%) (Figure 2.2.2, leftmost column). As the co-culture continues through days two and three, cells generated in the absence of RA and TGF-β acquire phenotypic
characteristics of central memory cells while those cultured with RA and TGF-β remain predominantly effector/effector memory (Figure 2.2.2, middle; right columns). Co-culture of SPL OT-I with IL-2 and OVA generates a predominantly (58%) central memory phenotype by day 4 of culture, while the majority (87%) of spleen OT-I supplemented with RA and TGF-β express an effector/effector memory phenotype by day 4 of culture. Stimulation of CD103+ MLN OT-I with IL-2 and OVA for four days generates central memory cells (62%). Addition of RA and TGF-β in
Figure 2.2.2. RA and TGF-β promote an effector/effector memory phenotype during culture. MLN OT-I and SPL OT-I were made into single cell suspensions, placed into culture with media containing OVA and IL-2 or OVA, IL-2, TGF-β, and RA. Cells were harvested on days one, two, and three then stained with antibodies against CD3, CD8, CD103, CD62L, and CD44. FACS plots shown are gated on GFP+ CD3+ CD8+ CD103+ cells. SPL OT-I (top half) and MLN OT-I (bottom half) show effector/effector memory skewing by day two. Representative of four independent experiments.
contrast induces effector/effector memory markers on 83% of the cells (Figure 2.2.3). The overall effect of co-culture with RA and TGF-β over time is depicted graphically in figure 2.2.4. RA and TGF-β, appear to enhance the effector memory phenotype on both MLN OT-I and SPL-OT-I in culture when compared to cells cultured in OVA plus IL-2 alone, which exhibit a reduced proportion of effector/effector memory cells and
increasing central memory cells.

2.2.2 Memory phenotype of CD103 negative cells

Much like their CD103+ counterparts, freshly ex vivo CD103- spleen OT-I (81%) and MLN OT-I (85%), are naïve T cells by CD62L and CD44 expression. Both naïve CD103- spleen OT-I and CD103- MLN OT-I express CD127 (IL-7Rα) to a similar degree when compared to naïve CD103+ spleen OT-I and MLN OT-I (100% positive for all cells). However, CCR7 expression is reduced in CD103- effector/effector memory SPL OT-I when compared to CD103+ effector/effector memory SPL OT-I (40% vs.
A similar reduction in CCR7 is observed for in CD103- effector/effector memory MLN OT-I when compared to CD103+ effector/effector memory MLN OT-I (30% vs. 19%) (Figure 2.2.1 vs. 2.2.5). The memory phenotype of CD103- MLN OT-I and SPL OT-I co-cultured with OVA plus IL2 and OVA, IL2, TGF-β, and RA matches that of the previously observed CD103+ MLN OT-I and SPL OT-I on days one through three (Figure 2.2.2 vs Figure 2.2.6). Analysis of day 4 CD103- MLN OT-I and CD103- SPL OT-I memory markers reveal a similar pattern to that seen with day 4 CD103+ cells: coculture with RA and TGF-β.
Figure 2.2.6. RA and TGF-β promote an effector/effector memory phenotype. MLN OT-I and SPL OT-I were made into single cell suspensions, placed into culture with media containing OVA and IL-2 or OVA, IL-2, TGF-β, and RA. Cells were harvested on days one, two, and three then stained with antibodies against CD3, CD8, CD103, CD62L, and CD44. FACS plots shown are gated on GFP +CD3+ CD8+ CD103- cells. SPL OT-I (top half) and MLN OT-I (bottom half) show effector/effector memory skewing by day two. Representative of four independent experiments.
preserves an effector/effector memory phenotype (Figure 2.2.7). It should be noted that in conditions containing RA and TGF-β the frequency of CD103- cells from either the MLN or SPL is very low (1% to 11%), which makes the analysis of phenotype on these cells difficult. However, it does appear that the overall pattern of central memory versus effector/effector memory phenotype in CD103 negative cells. MLN and SPL were made into single cell suspensions and stained with antibodies against CD3, CD8, CD103, CD62L, CD44, CD127, and CCR7. FACS plots shown are gated on CD3+ CD8+ CD103- cells with sub-gating as indicated. The frequency of effector/effector memory cells from day 4 SPL OT-I from OVA and IL-2 cultures and SPL OT-I from OVA, IL-2, RA, and TGF-β cultures are shown on the left; from day 4 MLN OT-I on the right. It should be noted that in cultures containing RA and TGF-β the frequency of CD103- cells on day 4 ranged from 11% to 1%.
memory distribution is similar to that of CD103 positive cells, as summarized in figure 2.2.4.

Our data, support recent findings that highlight the role of RA in the formation of effector/effector memory CD8 cells (Allie, Zhang et al. 2013). The effects of RA and TGF-\(\beta\) would appear to exert themselves on the total CD8+ CTL population, regardless of CD103 expression. The observation that a subset (~50%) of the CD103+ OT-I effector memory cells are CCR7 negative suggests that they are unable to migrate out of the location where they originally encountered antigen. Taken together with the memory marker profile of RA and TGF-\(\beta\) cultured cells it would indicate that during a gut initiated immune response a subset of responding cells will likely retain a greater degree of plasticity (proliferative potential?) and be less able to enter secondary lymphoid organs, generating a local sentinel pool.

2.2.3 Proliferation and memory in CD103 positive cells

In order to examine cellular proliferation and its relationship to memory formation, MLN OT-I and SPL OT-I were stained with the proliferation-tracking dye lauryl-dimethylamine N-oxide (DDAO). The majority of freshly isolated CD103+ MLN OT-I and SPL-OT-I were observed to be phenotypically naïve and highly labeled with DDAO (Figure 2.2.8). On day one the majority of CD103+ MLN OT-I and SPL OT-I, under antigen plus IL-2 and antigen plus IL-2, RA and TGF-\(\beta\) conditions, were
effector/effector memory; DDAO dilution suggests two to three divisions had already occurred (Figure 2.2.9). By day 4 of culture CD103+ SPL OT-I in antigen and IL-2 express a central memory phenotype. In contrast, the majority of cells cultured in antigen, IL-2, RA and TGF-β express an effector/effector memory phenotype (Figure 2.2.10; top left half). CD103+ SPL OT-I central memory cells cultured with or without RA and TGF-β proliferate equally well under either condition. However, effector memory SPL OT-I cells raised in the presence of IL-2 proliferate only modestly when compared to those raised in the additional presence of RA and TGF-β (Figure 2.2.10; top right half). The CD103+ MLN OT-I cells cultured with antigen, IL2, RA and TGF-β remained effector/effector memory cells, as previously observed, with 45% of the cells having a central memory
phenotype. CD103+ MLN OT-I cultured with antigen and IL2 on the other hand were mostly of central memory phenotype with only ~4% of the cells expressing effector/effector memory markers (Figure 2.2.10; bottom left half). CD103+ MLN OT-I central memory cultured with antigen and IL2 proliferated many more times than the central memory cells cultured with antigen, IL2, RA and TGF-β. CD103+ MLN OT-I effector/effector memory cells cultured under antigen and IL-2 conditions proliferate to a lesser extent than those cultured under antigen, IL-2, RA and TGF-β conditions (Figure 2.2.10; bottom right half). The observations described above are summarized in Figure 2.2.11. Summarizing the data in this manner.
Figure 2.2.10. RA and TGF-β do not inhibit proliferation in CD103+ cells. SPL OT-I from day four cultures are shown on the top half and MLN OT-I on the bottom half. SPL OT-I and MLN OT-I were made into single cell suspensions and stained with antibodies against CD3, CD8, CD103, CD62L, CD44, and DDAO, a lipophilic cell-tracking dye. FACS plots shown are gated on CD3+ CD8+ CD103+ cells and the DDAO dilution histograms, indicating cell divisions, are gated on either central memory or effector memory populations as indicated.
Figure 2.2.11. Summary of memory phenotype and proliferation for CD103 positive (top) SPL and (bottom) MLN. Each cell represents a discrete population of DDAO positive cells. The size of the cell indicates the level of proliferation dye within the population. Thus, larger cells have proliferated less often than smaller cells. Cells cultured in RA and TGF-β proliferate to a greater degree than those cultured in OVA plus IL-2 alone.
highlights the proliferative enhancement that RA and TGF-β provide to antigen specific T cells

2.2.4 Proliferation and memory in CD103 negative cells

The majority of freshly isolated CD103- MLN OT-I and SPL-OT-I were also observed to be phenotypically naïve and highly labeled with DDAO (Figure 2.2.12). On day one the majority of CD103- MLN OT-I and SPL OT-I, under antigen plus IL-2 and antigen plus IL2, RA and TGF-β conditions, were effector/effector memory; comparison to their CD103+ counterparts finds the effector/effector memory phenotype equal between them (Figure 2.2.8 vs. 2.2.12). DDAO dilution of the CD103-
The effector/effector memory subset suggests two to three cell divisions occurred for both MLN OT-I and SPL OT-I (Figure 2.2.13). Examination of the memory phenotype of CD103- SPL OT-I and MLN OT-I on day 4 shows a pattern of CD62L and CD44 distribution similar to that of CD103+ SPL OT-I and MLN OT-I, wherein RA and TGF-β promote an effector/effector memory phenotype as previously observed (Figure 2.2.10 vs 2.2.14). Similarly, the dilution of DDAO among central memory and effector/effector memory populations matches that previously observed with CD103+ SPL OT-I and MLN OT-I; one notable exception being the central memory MLN cultured with OVA plus IL-2, which appear to have divided more than their CD103+ counterparts. There do not appear to great differences in proliferative capacity by CD103+ versus CD103- cells,
Figure 2.2.14. RA and TGF-β promote an effector/effecter memory phenotype in CD103- cells. CD103- SPL OT-I from day four cultures are shown on the top half and CD103- MLN OT-I on the bottom half. SPL OT-I and MLN OT-I were made into single cell suspensions and stained with antibodies against CD3, CD8, CD103, CD62L, CD44, and DDAO, a lipophilic cell-tracking dye. FACS plots shown are gated on CD3+ CD8+ CD103- cells and the DDAO dilution histograms, indicating cell divisions, are gated on either central memory or effector memory populations as indicated.
when comparing across the same culture conditions (Figure 2.2.11 vs. 2.2.15). McKarns et al demonstrated in 2005 that TGF-β alone could have
profound suppressive effects on CD8 proliferation (McKarns and Schwartz 2005). We have not observed this in our RA and TGF-β containing cultures, perhaps that RA may act to counterbalance the previously reported suppressive effects of TGF-β alone.

2.2.5 Transcription factor profile of CD103 positive cells

We examined the transcription factor profile of our day four cultured cells by quantitative real time PCR. Two sets of transcription factors have been described to play roles in the genetic fate of CTL (Sheridan and Lefrancois 2011; Kaech and Cui 2012). Eomesdermin (EOMES) and T-bet are a pair of transcription factors that have critical roles in the formation of effector and effector memory function (Intlekofer, Takemoto et al. 2007; Joshi, Cui et al. 2007; Banerjee, Gordon et al. 2010; Pipkin, Sacks et al. 2010). BCL-6 and B lymphocyte induced maturation protein 1 (BLIMP1; also known as PRDM1) are a second pair of antagonistic transcription factors that act as genetic fate switches promoting self-renewal or terminal effector function, respectively (Gong and Malek 2007; Kallies, Xin et al. 2009; Pipkin, Sacks et al. 2010).

FACS sorted CD103+ OT-I from either the MLN or SPL cultured under conditions containing RA and TGF-β express much higher levels of EOMES than do cells cultured in only Ag and IL-2 by day four. In addition, these cells have no detectable T-bet; indicating a skewing towards the
effector memory phenotype (Figure 2.2.16; top row). BCL-6 is found in both SPL and MLN from either set of culture conditions, and as expected BLIMP1 is not readily detected, (Figure 2.2.16; bottom row). OT-I from either MLN or SPL cultured with Ag and IL-2 exhibit higher levels of BCL-6, indicating a stronger skewing towards central memory like characteristics.

Taken together, the memory phenotype, proliferation, and transcription factor profile data suggest that RA and TGF-β promote the generation of effector memory cells over that of central memory. These effector memory cells have greater proliferative capacity (by DDAO
dilution) as well as increased self-renewal potential (greater BCL6 vs. BLIMP1). These effects are on total CD8+ T cells and do not segregate based on CD103 expression. Previous findings in the literature indicate that TGF-β alone induces cell cycle arrest in effector/effector memory cells and that RA is needed for efficient production of effector/effector memory cells (McKarns and Schwartz 2005; Das and Levine 2008; Allie, Zhang et al. 2013). Our findings suggest that, in combination, TGF-β and RA efficiently generate effector/effector memory cells capable of proliferating many times. RA may therefore, help overcome the cell cycle effects attributed to TGF-β. A subset (~40%-50%) of the CD103+ OT-I effector memory cells are CCR7 negative which implies that they would be unable efficiently home to secondary lymphoid organs. These findings then would support the notion that during a gut initiated immune response a subset of responding cells can retain a greater degree of plasticity and be unable to exit the environment that created them, generating a localized pool of antigen-experienced sentinels.

2.3 Co-culture with RA and TGF-β increases gut homing markers

Gut homing markers CD103, CCR9, α4β1 and α4β7 are regulated differentially by RA and TGF-β (Kang, Park et al. 2011). Intestinal lamina propria cells, for example, more abundantly express TGF-β when compared to surrounding tissue whereas RA is abundantly secreted by DC of the intraepithelial compartment (Barnard, Warwick et al. 1993;
Molenaar, Knippenberg et al. 2011; Napoli 2012). These gradients of RA and TGF-β give rise to effector cells with dynamic patterns of surface gut homing molecules. We examined MLN and SPL OT-I under our culture conditions in order to determine if similar patterns are induced in-vitro.

Figure 2.3.1. Co-Culture with RA and TGF-β increases gut homing markers on CD103+ SPL OT-I. Flow cytometry of CD3+ CD8+ CD103+ SPL OT-I (A-D) cells stained with antibodies against LFA-1(A), α4β1 (B), α4β7 (C), and CCR9 (D). Day 0 (black bars), day 4 OVA/IL-2 (orange bars), and day 4 OVA/IL-2/RA/TGF-β (green bars) depicted. A-I = OVA/IL-2; A-I-R-T = OVA/IL-2/RA/TGF-β. N=3 independent experiments.
Freshly isolated (day 0) CD103+ MLN OT-I and CD103+ SPL OT-I each express equal frequencies of LFA-1 and the gut homing markers α4β1, α4β7 and CCR9 (99-100%, 39-42%, 1.5-1.7%, 60-63%, respectively (Figure 2.3.1 and 2.3.2 black bars). LFA-1 is constantly

![Image of Flow Cytometry Graphs]

Figure 2.3.2. Co-Culture with RA and TGF-β increases gut homing markers on CD103+ MLN OT-I. Flow cytometry of CD3+ CD8+ CD103+ MLN OT-I (A-D) cells stained with antibodies against LFA-1(A), α4β1 (B), α4β7 (C), and CCR9 (D). Day 0 (black bars), day 4 OVA/IL-2 (orange bars), and day 4 OVA/IL-2/RA/TGF-β (green bars) depicted. A-I = OVA/IL-2; A-I-R-T = OVA/IL-2/RA/TGF-β. N=3 independent experiments.
expressed on the cell surface of all lymphocytes, regardless of culture condition (Figure 2.3.1A and 2.3.2A) (Abram and Lowell 2009).

On the other hand, on day four CD103+ MLN OT-I and CD103+ SPL OT-I exhibit an increase in α4β1 and α4β7 expression as a result of antigen stimulation in the presence of IL-2 with or without RA/TGF-β supplementation (Figure 2.3.1B, 2.3.1C, 2.3.2B, and 2.3.2C). CCR9 is significantly increased only in CD103+ SPL OT-I (Figure 2.3.1D). Day 4 CD103+ SPL OT-I cultured in antigen, IL-2, RA, and TGF-β show a significant increase in the frequency of α4β1 cells over day 0 (Figure 2.3.1B) while the increase in CD103+ MLN OT-I is not statistically significant (Figure 2.3.2B). Day 4 CD103+ SPL OT-I cultured under any of the two conditions also significantly increases their expression of the gut homing integrin α4β7, increasing from 1.6% on day 0 to 37% (antigen and IL-2) and 91% (antigen, IL-2, RA, and TGF-β), respectively (Figure 2.3.1C). CD103+ MLN OT-I, on the other hand, significantly increases α4β7 expression only when compared to day 0 or the antigen and IL-2 conditions (Figure 2.3.2C). CCR9 expression significantly increases in CD103+ SPL OT-I cultured in antigen, IL-2, RA and TGF-β containing conditions only (Figure 2.3.1D) whereas it is unchanged in CD103+ MLN OT-I (Figure 2.3.1D). We can illustrate all of the cell surface marker

Figure 2.3.3. RA and TGF-β program gut homing in CD103 positive cells. Cells from either MLN or SPL acquire an abundance of gut entry molecules upon activation in the presence of RA and TGF-β whereas cells without RA and TGF-β maintain a balance of systemic and gut entry molecules.
Gating on CD103+ T cells

SPL

IL-2, OVA
RA, TGF-β

MLN

IL-2, OVA
RA, TGF-β

systemic + gut

gut

Surface Marker Legend

αEβ7 (CD103)  α4β7  α4β1  αLβ2 (LFA-1)  CCR9

Naive T cell  Effector/Effector Memory T cell  Central Memory T cell
changes in Figure 2.3.3; allowing us to readily see that surface marker
distribution segregates by the cell culture conditions in which they were
grown. That is to say, CD103+ cells cultured in RA and TGF-β acquire a

![Figure 2.3.4](image)

**Figure 2.3.4.** Co-Culture with RA and TGF-β increases some gut homing
markers on CD103- SPL OT-I. Flow cytometry of CD3+ CD8+ CD103- SPL
OT-I (A-D) cells stained with antibodies against LFA-1(A), α4β1 (B), α4β7
(C), and CCR9 (D). Day 0 (black bars), day 4 OVA/IL-2 (orange bars), and
day 4 OVA/IL-2/RA/TGF-β (green bars) depicted. A-I = OVA/IL-2; A-I-R-T =
OVA/IL-2/RA/TGF-β. N=3 independent experiments.
strongly gut-tropic phenotype whereas those cultured OVA plus IL-2 cells maintain a balance between systemic and gut entry molecules.

Among the gut homing markers CD103, it can be argued, is at the end of the line. Its binding partner, E-cadherin localizes cells exercising this interaction within the intraepithelial space, the “last stop” before the lumen of the gut. We then, also examined the CD103- population in our culture system in order to determine if, despite not having CD103, they upregulated other gut markers. Freshly isolated (day 0) CD103- MLN OT-I and CD103- SPL OT-I each express equal frequencies of LFA-1 and the gut homing markers \(\alpha_4\beta_1\), \(\alpha_4\beta_7\) and CCR9 (99-100%, 28-33%, 2.2-5%, 54-49%, respectively (Figure 2.3.4 and 2.3.5 black bars). LFA-1 is constantly expressed on the cell surface of all lymphocytes, regardless of culture condition (Figure 2.3.4A and 2.3.5A) (Abram and Lowell 2009). Day 4 CD103- MLN OT-I and CD103- SPL OT-I do not exhibit an increase in \(\alpha_4\beta_1\) across any of the culture conditions (Figure 2.3.4B and 2.3.5B). On the other hand, \(\alpha_4\beta_7\) expression, as a result of antigen stimulation in the presence of IL-2 and antigen with or without RA/TGF-\(\beta\) supplementation, increases slightly, though significantly (Figure 2.3.4C and 2.3.5C). CCR9 is significantly increased in both CD103- SPL OT-I and CD103- MLN OT-I (Figure 2.3.4D and 2.3.5D). Day 4 CD103- SPL OT-I and CD103- MLN OT-I cultured under any of the culture conditions do not significantly increase in the frequency of \(\alpha_4\beta_1\) cells over day 0 (Figure 2.3.4B and 2.3.5B). Only day 4 CD103- SPL OT-I and CD103- MLN OT-I
cultured under antigen, IL-2, RA, and TGF-β conditions significantly increase their expression of the gut homing integrin α4β7 over day 0 cells;

Figure 2.3.5. Co-Culture with RA and TGF-β increases some gut homing markers on CD103- MLN OT-I. Flow cytometry of CD3+ CD8+ CD103- MLN OT-I (A-D) cells stained with antibodies against LFA-1(A), α4β1 (B), α4β7 (C), and CCR9 (D). Day 0 (black bars), day 4 OVA/IL-2 (orange bars), and day 4 OVA/IL-2/RA/TGF-β (green bars) depicted. A-I = OVA/IL-2; A-I-R-T = OVA/IL-2/RA/TGF-β. N=3 independent experiments.
increasing from 5% to 18% and 2% to 14% respectively (Figure 2.3.4C and 2.3.5C). CCR9 expression significantly increases in CD103- SPL OT-I and CD103- MLN OT-I over day 0 in the presence of IL-2 and antigen with or without RA/TGF-β supplementation (Figure 2.3.4D and 2.3.5D).

Summarizing the surface marker changes (Figure 2.3.6) it is apparent that the absence of CD103 also coincides with a lack of gut-homing integrins. CCR9, which mediates chemotaxis towards CCL25 (expressed in the small intestine), is significantly increased. This would suggest that while these cells are not pre-disposed to home to the gut they are not barred from entering the compartment.

These data indicate that CD103+ OT-I cells increase their expression of gut homing markers upon antigenic stimulation and that co-culture with RA and TGF-β serves to further refine their gut homing markers. These data further suggest that, while lacking homing capacity to the intraepithelial compartment, CD103- OT-I cells do have the potential of trafficking to the lamina propria of the colon and small intestine. They are then, not barred from passing through the gut, but perhaps from just establishing long-term residency within the IEL compartment.

Figure 2.3.6. RA and TGF-β do not influence gut tropism on CD103 negative cells. Cells from either MLN or SPL acquire an abundance of gut entry molecules upon activation in the presence of RA and TGF-β whereas cells without RA and TGF-β maintain a balance systemic and gut entry molecules.
Gating on CD103-T cells

**Surface Marker Legend**
- **αEβ7 (CD103)**
- **α4β7**
- **α4β1**
- **αLβ2 (LFA-1)**
- **CCR9**

---

**SPL**
- **++** IL-2, OVA
- **++** RA, TGF-β

**MLN**
- **++** IL-2, OVA
- **++** RA, TGF-β

**Systemic**
2.4 Effector molecules of OT-I cells

2.4.1 Effector molecules of CD103+ OT-I from mesenteric lymph node and spleen

CD8 T cells are known to express effector molecules including Perforin-1 and granzymes in response to cognate antigen stimulation (Reiner 2001; Szabo, Sullivan et al. 2003). In addition, other inflammatory molecules, like IFN-γ and TNF-α can also be induced by antigenic stimulation (Reiner and Seder 1999; Marzo, Klonowski et al. 2005). It is also known that TGF-β can exert an anti-inflammatory influence on these effector molecules despite having a role in the inhibition of Th2

![Figure 2.4.1. Kinetics of IFN-γ and TNF-α expression in CD103+ OT-I from SPL by FACS. Intracellular cytometric analysis of CD3+ CD8+ CD103+ SPL OT-I from days 0, 1, 4, and 7 of culture with antigen and IL-2 (black squares) or antigen, IL-2, RA, and TGF-β (grey circles). IFN-γ is depicted on the left graph; TNF-α on the right graph. N= 3 independent experiments.](image-url)
development (Gorelik, Fields et al. 2000; Das and Levine 2008). Further, recent findings in the literature describe a role for RA signaling in the differentiation of CD8 T cells (Allie, Zhang et al. 2013). In short, both molecules can function as immune regulators. Indeed there is recent evidence that the two molecules can cross-talk (Mucida and Cheroutre 2007). We examined the expression of cytotoxic effector proteins in MLN and SPL OT-I following activation by antigen and IL-2 or antigen, IL-2, RA and TGF-β.

\[\text{CD103}^+\text{MLN OT-I}\]

![Figure 2.4.2. Kinetics of IFN-γ and TNF-α expression in CD103+ OT-I from MLN by FACS. Intracellular cytometric analysis of CD3+ CD8+ CD103+ MLN OT-I from days 0, 1, 4, and 7 of culture with antigen and IL-2 (black squares) or antigen, IL-2, RA, and TGF-β (grey circles). IFN-γ is depicted on the left graph; TNF-α on the right graph. N= 3 independent experiments.}

SPL OT-I and MLN OT-I activated under either set of conditions showed similar levels and kinetics of IFN-γ and TNF-α staining throughout the observation period (Figure 2.4.1 and 2.4.2). In contrast, CD103+ MLN
OT-I expressed strikingly lower levels of granzymes B when, RA and TGF-β were added compared to the conditions containing antigen and IL-2 alone; furthermore, this difference was not observed in the SPL OT-I cultures (Figure 2.4.3).

Granzyme A and Granzyme B are the most studied members of the granzymes family (Barry and Bleackley 2002). The family is large, however, with many members, having both overlapping and non-redundant functions (Barry and Bleackley 2002; Grossman, William J., Revell et al. 2003). Given the differential regulation of granzymes B and A during a CTL response and the wide variety of expression patterns within the granzyme family, we examined the expression of a panel of different
Figure 2.4.4. Day 4 granzymes of CD103+ SPL OT-I by real time PCR. CD3+ CD8+ CD103+ SPL OT-I were sorted from day 0 and day 4 cultures. RNA was isolated from sorted cells and cDNA made. Real time PCR with probes for GZM M, GZM A, and GZM D was performed and fold change over day 0 was calculated using the delta-delta CT method. GAPDH was used as housekeeping gene. Black bars represent expression data from OVA/IL-2 cells and gray bars represent expression data from OVA/IL-2/RA/TGF-β cells. Student’s t test p values are shown in red. N= 3 independent experiments.

granzymes (Grossman, William J., Revell et al. 2003). Quantitative real-time PCR was performed using probes detecting granzyme M, granzyme A, and granzyme D. In CD103+ SPL OT-I granzyme M is equally
upregulated by IL2 with or without RA and TGF-β while Granzyme A was equally and modestly down regulated under either condition. Granzyme D is potently down regulated in SPL OT-I by IL-2 and this effect is largely

Figure 2.4.5. Day 4 granzymes of CD103+ MLN OT-I by real time PCR. CD3+ CD8+ CD103+ MLN OT-I were sorted from day 0 and day 4 cultures. RNA was isolated from sorted cells and cDNA made. Real time PCR with probes for GZM M, GZM A, and GZM D was performed and fold change over day 0 was calculated using the delta-delta CT method. GAPDH was used as housekeeping gene. Black bars represent expression data from OVA/IL-2 cells and gray bars represent expression data from OVA/IL-2/RA/TGF-β cells. Student’s t test p values are shown in red. N= 3 independent experiments.
neutralized by the addition of RA and TGF-β (Figure 2.4.4). In CD103+ MLN OT-I granzyme M and granzyme A are significantly highly upregulated by antigen, IL-2 than by antigen, IL-2 plus RA and TGF-β.

![Perforin-1 induction in CD103+ SPL OT-I](image)

Figure 2.4.6. Perforin-1 induction in CD103+ SPL OT-I. SPL OT-I were made into single cell suspensions and cultured for 7 days in Ag/IL-2 and Ag/IL-2/RA/TGF-β. (A) On days 0, 1, 4, and 7 cells were harvested and sorted for GFP+ CD3+ CD8+ CD103+ cells. Cells were then lysed and prepared for western blot, probing for Perforin-1 with GAPDH as loading control. (B) Densitometric analysis of blot is shown using ImageJ to quantify pixel intensity. Ag/IL-2 (black squares) and Ag/IL-2/RA/TGF-β (grey circles) are depicted; dotted line represents background level of signal. All lanes were normalized to GAPDH.
Granzyme D on the other hand is equally down regulated under either condition (Figure 2.4.5). These findings show that CD103+ MLN OT-I induce granzyme A message preferentially over CD103+ SPL OT-I and

Figure 2.4.7. Perforin-1 induction in CD103+ MLN OT-I. MLN OT-I were made into single cell suspensions and cultured for 7 days in Ag/IL-2 and Ag/IL-2/RA/TGF-β. (A) On days 0, 1, 4, and 7 cells were harvested and sorted for GFP+ CD3+ CD8+ CD103+ cells. Cells were then lysed and prepared for western blot, probing for Perforin-1 with GAPDH as loading control. (B) Densitometric analysis of blot is shown using ImageJ to quantify pixel intensity. Ag/IL-2 (black squares) and Ag/IL-2/RA/TGF-β (grey circles) are depicted; dotted line represents background level of signal. All lanes were normalized to GAPDH.
that OT-I of either origin contain comparable amounts granzyme M message. The data suggest that RA and TGF-β in MLN OT-I preferentially induce granzyme M and down-regulate granzyme D with little effect on granzyme A. The major effect of RA and TGF-β in activated SPL OT-I is antagonism of down regulation of granzyme D by IL-2 alone.

In order to detect the induction of Perforin-1 protein in cultured cells, protein lysates were prepared on days zero, one, four and seven from sorted CD103+ SPL OT-I and MLN OT-I. Western blots were performed and membranes probed for Perforin-1 and GAPDH, as loading control. Densitometric analysis illustrates the induction of Perforin-1 protein over the seven-day observation period relative to day 0 (Figure 2.4.6 and 2.4.7). Perforin-1 protein was maximal on day four under both sets of culture conditions in both CD103+ SPL OT-I and CD103+ MLN OT-I. Perforin protein appeared to be lower in CD103+ SPL OT-I cultured in antigen, IL-2, RA and TGF-β when compared to cells cultured in antigen and IL-2 alone, however that difference was not readily observed in CD103+ MLN OT-I. These data suggest that cells from both sets of culture conditions are able to induce perforin robustly, even in the presence of RA and TGF-β, upon recognition of cognate antigen. TGF-β alone has been shown to inhibit the development of CTL from naïve T cells in addition to having suppressive effects on other facets of CD8 T cell development (Ranges, Figari et al. 1987; Gorelik and Flavell 2002). There is not a great deal of literature concerning the role of RA in the development of CD8
CTL. One recent paper by Allie et al however, characterizes the impact of RA on the development of CD8 CTL effector function (Allie, Zhang et al. 2013). Moreover, while Perforin-1 is not specifically examined in this work it bears mentioning that in the absence of RA signaling there is a decrease in Granzyme B+ IFN-γ producing CD8 cells, suggesting that RA plays a role in developing CTL function, which might influence Perforin-1 as well.

2.4.2 Effector molecules of CD103 negative OT-I from mesenteric lymph node and spleen

Because the expression of CD103 was not absolute, we decided to analyze the effector molecules of the CD103- fraction of cultured OT-I MLN and SPL. Gating on CD3+ CD8+ CD103- SPL OT-I, the expression of IFN-γ and TNF-α was examined by intracellular cytokine staining and FACS analysis over the seven-day culture period. IFN-γ is significantly increased in OVA plus IL-2 cultured cells when compared to OVA, IL-2, RA and TGF-β cells on day four (94% vs 52%, respectively), remaining significantly higher through day seven (Figure 2.4.8, left). TNF-α, on the other hand, increased for both sets of culture conditions to a similar extent by day one and remained equally elevated throughout the observation period (Figure 2.4.8, right). CD3+ CD8+ CD103- MLN OT-I showed a similar trend, where IFN-γ expression was significantly increased by day four of culture in OVA plus IL-2 compared to OVA, IL-2, RA and TGF-β cells (89% and 22%, respectively). Despite the decrease in IFN-γ+ cells
on day seven, OVA plus IL-2 culture conditions generated a significantly greater frequency of IFN-γ positive cells than did OVA, IL-2, RA and TGF-β cells (33% vs 4%) (Figure 2.4.9, left). TNF-α expression in MLN OT-I also followed the same trend observed for SPL OT-I: an increase in positive cells on day one, remaining equally positive throughout the observation period (Figure 2.4.9, right). Results presented earlier examining the expression of granzyme B (GZB) in CD3+ CD8+ CD103+ SPL OT-I showed no differences in GZB levels between OVA plus IL-2 compared to OVA, IL-2, RA and TGF-β cultured cells (Figure 2.4.3, right). Gating on CD3+ CD8+ CD103- SPL OT-I, however, we observe significantly lower GZB expression in OVA, IL-2, RA and TGF-β cultured
cells as early as day one, continuing through the end of the observation period (Figure 2.4.10, left). CD3+ CD8+ CD103- MLN OT-I reveal a similar expression pattern when compared to their CD103+ counterparts: GZB expression is greatly reduced in OVA, IL-2, RA, and TGF-β cultured cells (Figure 2.4.10, right).

Taken together these data suggest that RA and TGF-β have no effect on TNF-α production, regardless of the compartment generating the effectors. It further appears that RA and TGF-β exert a dampening effect on IFN-γ production in CD103- OT-I cells since this was not observed in the CD103+ population (Figures 2.4.1, 2.4.2, 2.4.8, and 2.4.9). CD103
expression, then offers a measure of protection to CTL in the gut, allowing them to retain cytotoxic potential in a generally immunosuppressive environment.

2.5 SPL and MLN OT-I kill in a perforin-dependent manor

Canonical CD8 T cell cytotoxicity depends on functional Perforin-1 and granzyme molecules (Reiner 2001). The H+ATPase concanamycin A (CMA) has been described as a potent inhibitor of Perforin-1 mediated cytotoxicity by preventing acidification of the perforin-containing vacuole and promoting Perforin-1 degradation (Kataoka, Shinohara et al. 1996). In addition, the calcium chelator, ethylene glycol tetraacetic acid (EGTA) has
also been shown to inhibit Perforin-1 by preventing the calcium-dependent polymerization of Perforin-1 in the membrane (Grossman, W. J., Verbsky et al. 2004). We examined the ability of MLN and SPL OT-I generated with or without RA and TGF-β to kill antigen-loaded targets in a Perforin-1 dependent manner.

SPL and MLN OT-I were plated in round bottom 96 well microtiter plates for four days in media containing OVA and IL-2 or OVA, IL-2, RA and TGF-β. On day four the effector cells were harvested and pre-incubated for two hours in complete media supplemented with CMA, EGTA or vehicle control (DMSO). After washing, the effectors were plated

Figure 2.5.1. Concanamycin A inhibits perforin-1 dependent SPL OT-I killing of antigen-loaded CMT93 cells. Day 4 SPL OT-I from (A) OVA/IL-2 and (B) OVA/IL-2/RA/TGF-β cultures were pre-treated with concanamycin A for 1 hour before being placed into a 4-hour 51chromium cytotoxicity assay with ova pulsed, chromium labeled, CMT93 cells at the effector to target ratios indicated. Student’s t-test was performed for each time point (* indicates p< 0.05). N= 6 independent experiments.
at varying ratios with the chromium$^{51}$ labeled H2b+ gut epithelial cells (CMT93) pulsed with OVA (target cells) for four hours.

<table>
<thead>
<tr>
<th>SPL OT-I</th>
<th>Ag/IL-2</th>
<th>Ag/IL-2/RA/TGF-β</th>
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<tbody>
<tr>
<td>A.</td>
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Figure 2.5.2. EGTA inhibits Perforin-1 dependent SPL OT-I killing of antigen-loaded CMT93 cells. Day 4 SPL OT-I from (A) OVA/IL-2 and (B) OVA/IL-2/RA/TGF-β cultures were pre-treated with EGTA for 1 hour before being placed into a 4-hour 51chromium cytotoxicity assay with ova pulsed, chromium labeled, CMT93 cells at the effector to target ratios indicated. Student's t-test was performed for each time point (* indicates p< 0.05). N= 6 independent experiments.

CMA blocked the cytotoxicity of SPL OT-I activated in both antigen and IL-2 or antigen, IL-2, RA and TGF-β culture conditions when compared to vehicle control (Figure 2.5.1). EGTA also blocked the cytotoxicity of SPL OT-I activated in both antigen and IL-2 or antigen, IL-2, RA and TGF-β when compared to control (Figure 2.5.2). Next, we examined MLN OT-I, blocking cytotoxicity using CMA. CMA blocked the cytotoxicity of SPL OT-I activated under both sets of culture conditions when compared to vehicle control (Figure 2.5.3). Lastly, we observed that
EGTA also blocked the cytotoxicity of MLN OT-I activated under both sets of culture conditions when compared to control (Figure 2.5.4). Based on these observations we conclude that CTL killing by OT-I from either the MLN or the SPL cultured in the presence of RA and TGF-β is dependent on Perforin-1.

### 2.6 RA and TGF-β activate CD103 for CTL killing of target cells

The ligand for CD103 is E-cadherin which is highly expressed on the surface of the gut epithelia including the intestinal epithelial cell line CMT93 (Figure 2.6.1) (Le, Yap et al. 1999). CD103+ T cells are thought to
home and permanently reside as IEL in the epithelial compartment of the gut by anchoring CD103 to E-cadherin (Gorfu, Rivera-Nieves et al. 2009).

Further, CD103+ T cells have been found at sites of allograft rejection in transplantation models, suggesting a role for CD103 in cytotoxicity (Feng, Wang et al. 2002). We tested the role of CD103 and LFA-1 expression on cognate OT-I cytotoxicity on E-cadherin positive and negative target cells.

Total SPL and MLN OT-I cells were cultured for four days in 96 well round bottom microtiter plates in media supplemented with OVA and IL-2 or OVA, IL-2, RA and TGF-β. After four days, the effectors were harvested and used for chromium-release cytotoxicity assays. CMT93 target cells

Figure 2.5.4. EGTA inhibits Perforin-1 dependent MLN OT-I killing of antigen-loaded CMT93 cells. Day 4 MLN OT-I from (A) OVA/IL-2 and (B) OVA/IL-2/RA/TGF-β cultures were pre-treated with EGTA for 1 hour before being placed into a 4-hour 51chromium cytotoxicity assay with ova pulsed, chromium labeled, CMT93 cells at the effector to target ratios indicated. Student's t-test was performed for each time point (* indicates p< 0.05). N= 6 independent experiments
were treated with citric saline, labeled with chromium$^{51}$ and pulsed with OVA for one hour. Effectors and targets were plated in 96 well round-bottom microtiter plates in a standard four-hour chromium release cytotoxicity assay with or without blocking antibodies to LFA-1 and CD103 or control IgG. The concentrations of blocking antibody for LFA-1 and CD103 used in each assay were determined from previously published experimental data (Gromkowski, Heagy \textit{et al.} 1983; Karecla, Bowden \textit{et al.} 1995).

SPL OT-I cultured with antigen and IL-2 lysed CMT93 cells to equal degrees in the presence of anti-CD103 blocking antibody and control.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{CMT93_Cells}
\caption{CMT93 cells express high levels of E-cadherin. CMT93 cells were treated with citric saline, counted, and stained with an isotype control or anti-E-cadherin antibodies for 30 minutes. Citric saline was used because E-cadherin is cleaved from the cell surface by trypsin. The cells were then washed and analyzed via FACS. Cells in the live gate are depicted.}
\end{figure}
Addition of anti-LFA-1 blocking antibody, alone or in combination with anti-CD103 completely blocked cytotoxicity indicating that it is LFA-1 dependent (Figure 2.6.2A). In contrast, anti-LFA-1 only partly (~50%) inhibited killing of CMT93 targets by SPL OT-I cultured with antigen, IL-2, RA and TGF-β. Anti-CD103 notably, and for the first time, demonstrated direct killing inhibition of CMT93 targets by RA/TGF-β induced SPL-OT-I by ~50% (Figure 2.6.2B). The combination of anti LFA-1 and anti CD103 completely blocked cytotoxicity for CMT targets by RA/TGF-β induced SPL OT-I (Figure 2.6.2B).

![Figure 2.6.2 RA and TGF-β license CD103 on SPL OT-I CTL killing of CMT93 target cells. Day 4 SPL OT-I from (A) OVA/IL-2, (B) OVA/IL-2/RA/TGF-β cultures were co-incubated with control IgG (circles), anti-CD103 (squares), anti-LFA-1 (up triangle), and anti-CD103/anti-LFA-1 (down triangle) antibodies throughout a standard 4-hour 51chromium cytotoxicity assay with ova pulsed, chromium labeled, CMT93 cells at the effector to target ratios indicated. N= 3 independent experiments.](image)
Cytotoxicity of MLN OT-I cultured with antigen and IL-2 for CMT93 targets was completely inhibited by either anti-LFA-1 or anti-CD103 (Figure 2.6.3A). MLN OT-I similar to SPL OT-I cultured with antigen, IL-2, RA and TGF-β exhibited partial block of cytotoxicity by either anti-LFA-1 or anti-CD103. The combination of both antibodies blocked cytotoxicity entirely (Figure 2.6.3B). Taken together these data suggest that RA and TGF-β, in addition to inducing CD103 expression, also act to activate CD103 to participate in CTL killing. In addition, it appears that the licensing of CD103 serves to add it to the CTL killing machinery not

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**Figure 2.6.3 RA and TGF-β license CD103 on MLN OT-I CTL killing of target cells.** Day 4 MLN OT-I from (A) OVA/IL-2, (B) OVA/IL-2/RA/TGF-β cultures were co-incubated with control IgG (circles), anti-CD103 (squares), anti-LFA-1 (up triangle), and anti-CD103/anti-LFA-1 (down triangle) antibodies throughout a standard 4-hour 51chromium cytotoxicity assay with ova pulsed, chromium labeled, CMT93 cells at the effector to target ratios indicated. N= 3 independent experiments.
replace existing mechanisms, since killing is only partially abrogated by its blockade, as is the case with SPL derived CTL. The blockade of killing in MLN OT-I by anti-CD103 under antigen and IL-2 conditions was unexpected. It suggests that MLN OT-I have altered killing activity when compared to SPL OT-I under the same conditions. It is possible that differences in the microenvironments where the cells resided (systemic vs. mucosal) has led to an altered signaling modality for CD103, though this would need to be examined further.

2.7 E-cadherin increases susceptibility to lysis by CD103 expressing cells

The EL4 murine tumor cell line does not natively express E-cadherin and neither does the ova-transfected descendant cell line E.G7 (Figure 2.7.1). Others have previously shown, using standard chromium$^{51}$-release cytotoxicity assays, that OT-I cells can efficiently lyse E.G7’s while parental EL4’s are unaffected (Dalyot-Herman, Bathe et al. 2000). In order to test the hypothesis that CD103 can increase the susceptibility of E-cadherin-bearing targets we ectopically express E-cadherin in E.G7 cells. Using the published sequencing data from NCBI to design primers, we cloned E-cadherin from CMT93 cells into the pcDNA3.1-Zeo expression vector. E.G7 cells were transfected, maintained in selection media, and screened by FACS for expression of E-cadherin (Figure 2.7.1). We term these E-cadherin high transfectants E.G7$^{Ecad}$ cells.
We previously established that co-culture with RA and TGF-β licensed CD103 to kill ligand-bearing targets in both MLN OT-I and SPL OT-I cultured cells. Here, total SPL and MLN OT-I cells were cultured for four days in 96 well round bottom microtiter plates in media supplemented with OVA, IL-2, RA and TGF-β. After four days, the effectors were harvested and used for chromium-release cytotoxicity assays. Approximately 87% of the SPL OT-I cells were CD103+ and 94% of the MLN OT-I cells were CD103+ on day 4 of culture (Figure 2.1.2). EL4, E.G7 and E.G7\textsuperscript{Ecad} were labeled with chromium and placed into cytotoxicity assays with the culture generated effectors at various effector-target ratios. As expected, day four SPL OT-I did not lyse EL4 cells to any
Figure 2.7.2. E-cadherin increases susceptibility to lysis by CD103 expressing cells only when they are licensed by RA and TGF-β. Day 4 (A) SPL OT-I and (B) MLN OT-I from OVA/IL-2/RA/TGF-β cultures; day 4 (C) SPL OT-I and (D) MLN OT-I from OVA plus IL-2 cultures were co-incubated with EL4 (triangles), E.G7 (circles) and E.G7Ecad (squares) expressing cells in a standard 4-hour chromium-release cytotoxicity assay at the effector to target ratios indicated. N= 6 independent experiments. * indicates p<0.05 from a one-way ANOVA followed by a Bonferroni post-hoc test.
appreciable level. E.G7 cells, on the other hand, were efficiently lysed by OT-I. Addition of CD103 to E.G7 (E.G7^{Ecad}) significantly increased specific lysis of target cells (Figure 2.7.2A). MLN OT-I exhibited a similar pattern of lysis. OVA, IL-2, RA and TGF-β co-cultured MLN OT-I did not lyse EL4 cells. EG.7 cells were lysed to approximately the same extent SPL OT-I were (~20%). E.G7^{Ecad}, however, were lysed to a much greater (and significant) degree (Figure 2.7.2B). Moreover, when SPL OT-I and MLN OT-I that were cultured under conditions containing OVA plus IL-2 alone were placed into a similar chromium release assay we see that despite measureable levels of CD103 (~57% and ~35% for SPL and MLN, respectively; Figure 2.1.2) there is no enhancement of killing when E-cadherin is ectopically expressed in E.G7 cells (Figure 2.7.2C and 2.7.2D). Taken together these data indicate that CD103 interacts with E-cadherin to kill target cells under conditions containing RA and TGF-β.

2.8 Summary & Discussion

We have observed that RA and TGF-β exert phenotypic effects on CD8 CTL, regardless of splenic or mesenteric origin or CD103 status. RA and TGF-β alter the mosaic of cell surface homing molecules, increasing the gut tropism of the cell. The surface memory markers of cells from RA and TGF-β conditions show a clear skewing of the CTL population to that of effector memory while those that are cultured without RA and TGF-β have an even distribution of effector memory and central memory cells.
Memory Phenotype

<table>
<thead>
<tr>
<th></th>
<th>Ag/IL-2</th>
<th>Ag/IL-2/RA/TGF-β</th>
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<tr>
<td>OT-I</td>
<td></td>
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<tr>
<td>Spleen</td>
<td>CM/EM</td>
<td>EM</td>
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<td>Mesenteric Lymph Node</td>
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Gut Homing Phenotype

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Granzyme Utilization

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Perforin-1 Content

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<td>Mesenteric Lymph Node</td>
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Figure 2.8.1. Summary of OT-I CTL properties from RA and TGF-β cultures. Memory phenotype determined by FACS analysis of CD62L, CD44, CD127 and CCR7 staining pattern. Gut homing phenotype calculated by assessing statistically significant differences in the frequency of CCR9, α4β7, α4β1, and CD103 expressing cells between day 0 cells and day 4 cells from OVA plus IL-2 and OVA, IL-2, RA, and TGF-β cultures. Granzyme utilization determined by FACS analysis and real time PCR. Perforin-1 content determined by quantification of western blot using ImageJ software.

(Figure 2.8.1). We have also found that CD103+ CTL made under conditions containing RA and TGF-β have high levels of the transcription factor EOMES when compared to CD103+ CTL made under conditions
lacking RA and TGF-β. CD103+ CTL, regardless of how they are generated, have very little expression of the transcription factor T-bet. In addition, we can informally assess the molecular programming of memory and effector function by calculating the ratio of the master regulators T-bet to EOMES. Performing such a calculation with our CD103 positive CTL reveals that those cells made with RA and TGF-β skew much more towards a memory fate than those made in conditions lacking RA and TGF-β, which skew towards an effector fate (Figure 2.8.3). We have also studied two additional antagonistic transcription factors which act as genetic fate switches for B and T cells are BLIMP1 and BCL-6 (Crotty, Johnston et al. 2010). We have observed BCL-6 as the dominant transcription factor in both MLN and SPL, regardless of culture conditions, indicating that the cells are geared toward a memory program. The downregulation of BLIMP1 and upregulation of BCL-6 further serve to refine the transcriptional landscape of the generated cells. Both OVA plus IL-2 and OVA, IL-2, RA, and TGF-β cultured cells have decreased BLIMP1 on day four of culture indicating they do not have terminal effector properties (Figure 2.2.16). Instead, they have upregulated BCL-6, which controls self-renewal potential and central memory characteristics (Figure 2.2.16). Even so, MLN OT-I and SPL OT-I cultured in OVA plus IL-2 increase their BCL-6 message significantly more than those cultured in OVA, IL-2, RA, and TGF-β, confirming the previous observation that cells co-cultured with RA and TGF-β are more effector memory in behavior.
Taken together, the transcription factor and surface marker profile of the RA and TGF-β cultured cells indicates that effector memory-like cells with self-renewing potential have been generated.

Cells cultured with RA and TGF-β display on their surfaces gut homing molecules like CCR9, α4β7, and α4β1 to a much greater degree than those that have not been so cultured (Figure 2.8.1). This is not to imply, however, that RA and TGF-β are strictly required for entry into the gut mucosa. Even though SPL OT-I and MLN OT-I cultured in OVA plus IL-2 do not possess the same gut entry potential, they do have the ability to enter the lamina propria of the mucosa thus being able to circulate through the compartment.

Granzymes are a large family of proteases that provide cytotoxic effector function for CD8 cells. The most widely studied of these is granzyme B but, as we have shown, its utilization is not universal among CTL. Notably, MLN OT-I cultured under OVA, IL-2, RA, and TGF-β containing conditions do not employ granzyme B, rather they use granzymes M and A (Figure 2.8.1). This may suggest an underlying intrinsic difference between the CTL of the mesenteric lymph node and that of the spleen when killing in the gut. Perforin-1, the pore-forming protein of the adaptive immune system, can be detected readily by western blot in all of the cells, regardless of the culture condition (Figure 2.8.1). This observation demonstrates, that in combination, TGF-β and RA
do not induce the immuno-suppressive phenotype commonly reported in the literature for TGF-β alone (Gorelik, Fields et al. 2000; Heath, Murphy et al. 2000; McKarns and Schwartz 2005; Thomas and Massague 2005).

Our study has indicated, at least in the case of CD8 CTL, that CD103 plays a role in the execution of CTL function. When activated in the presence of RA and TGF-β antigen specific CTL are able to greatly upregulate CD103 expression and, importantly, use the integrin to kill specific targets. Its blockade is able to abrogate some, but not all,

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<td>(αLFA-1/αCD103)</td>
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<td>---------------------</td>
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<tr>
<td>Spleen</td>
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Figure 2.8.2. Summary of OT-I CTL killing activity. CTL killing was assessed by chromium release assay as previously described in the materials and methods. Maximal killing for each experimental culture condition was set by killing level achieved in the condition with no blocking antibodies (+/-) present. The character “Ø” indicates no killing activity observed.
Perforin-1 dependent killing, suggesting that CD103 is added to the CTL armament (in addition to LFA-1) instead of replacing existing mechanisms (Figure 2.8.2). We further demonstrate that CTL generated under systemic versus mucosal conditions respond differently to the same antigenic challenge. While both types of CTL are able to kill effectively, they do so by employing different mechanisms, a finding that merits further study.

Previous work reported in the literature has clearly shown the presence of CD103+ CTL at sites of allograft rejection, where E-cadherin, the ligand for CD103 is abundantly expressed by host epithelia (Feng, Wang et al. 2002; El-Asady, Yuan et al. 2005). In addition, published work where CD103 is specifically blocked by antibody treatment has ameliorated the severity of tissue destruction during GVHD (Kilshaw and
Higgins 2002). Studies like these have established an association between CTL activity and CD103 expression but have not demonstrated CD103 role in cytotoxicity. We have shown that CD103 expression is increased as part of the normal activation program under in-vitro inflammatory settings regardless of where the CTL originate from (spleen versus mesenteric lymph node). This makes sense, when considering the broader immunological response to antigen exposure: CTL activated in distant systemic compartments upregulate the components needed to travel to sites of local inflammation. In the case of the mucosa, receptors for entry into the lamina propria are upregulated early during activation and, as cells encounter increasing gradients of RA and TGF-β, CD103 expression increases. Likely due to the overlap of homing receptor expression patterns for the gut, expression of CD103 has been shown to not be required for entry into the intestinal microenvironment (El-Asady, Yuan et al. 2005). CD103 expression is needed however for long-term retention in these tissues (Schon, Arya et al. 1999; El-Asady, Yuan et al. 2005). Indeed, increasing evidence suggests that once T cells enter the intraepithelial compartment of the gut mucosa a subset of them never migrate out, becoming instead so-called tissue resident memory (Trm) cells. Trm cells have been defined by characteristic phenotypic markers that suggest these cells have been activated, migrated into tissue, and lost the ability to extravasate from said tissue (Sheridan and Lefrancois 2011) (Kaech and Cui 2012). Taken together with our findings, this suggests that
CD103 in the gut does more than just function as an anchor. Once bound to E-cadherin, it is able to provide a sentinel function, keeping the CTL at the ready; awaiting only the detection of cognate antigen for killing activity to be employed. We have found that CTL made under conditions containing RA and TGF-β express similar cell surface markers to Trm cells, suggesting a pathway by which these cells are generated.

CD103, most commonly, has been used as a marker for gut resident lymphocytes; with respect to CD4 T cells and DC subsets it has been used to mark tolerogenic populations. We show that on CD8 CTL CD103 acts as more than just a marker. CD8 T cells that have previously encountered antigen at a site of mucosal priming (i.e. Peyer’s Patch) are permissive to reentry into the gut lamina propria due to acquisition of gut homing molecules, like CCR9, α4β7 and CD103. CD103 would then become activated by exposure to RA and TGF-β in the surrounding microenvironment. Gradients of RA and TGF-β promote migration toward the basolateral side of gut epithelia where E-cadherin is highly expressed. Intercalation between epithelial cells via CD103/E-cadherin interactions leads to the establishment of a sentinel pool of lymphocytes that are readily able to exert cytotoxic effector function upon re-encounter with cognate antigen (Figure 2.8.4). Upon activation in the presence of RA and TGF-β, then, CD103 becomes an armament of CTL effector function, helping to deliver granzymes in a Perforin-1 dependent manner.
The model described in figure 2.8.4 is readily expanded to other organs that are bounded by epithelial cells expressing E-cadherin (e.g. skin, lung, pancreatic islets). It is known that skin and lung epithelial cells express E-cadherin, in addition to being microenvironments where RA and TGFβ are produced (Redfern and Todd 1992; Gold, Jussila et al. 2000;
Malpel, Mendelsohn *et al.* 2000; Bartram and Speer 2004). It is easily conceivable then, that by changing the priming conditions to enrich for skin-homing or lung-homing molecules the immune system would be able to redirect CD103+ CD8 CTL to those sites, establishing sentinel populations throughout epithelial cell border areas.
Chapter 3: Materials and Methods

3.1 Mice

C57BL/6 OT-I mice were obtained from Dr. M. Bevan (University of Washington School of Medicine, Seattle, WA) and crossed with green fluorescent protein (GFP) transgenic mice obtained from Jackson Laboratory (Bar Harbor, ME) to produce OT-I/GFP mice. Mice were used at 6–12 weeks of age and were maintained in pathogen-free conditions at the University of Miami animal facilities. All animal use procedures were approved by the University of Miami Animal Care and Use Committee.

3.2 Reagents

Retinoic Acid (Sigma-Aldrich; St. Louis, MO) was made into solution and sterile filtered (.2 μm pore) at 3mg/ml, which is 10mM, and stored at -20°C. Retinoic Acid (RA) was used at a final concentration of 100nM in all experiments. Concanamycin A (CMA) (Sigma-Aldrich; St. Louis, MO) was dissolved in DMSO at a concentration of 1.25M (25X) and used a final concentration of 50mM (1X) in complete media. EGTA (Sigma-Aldrich; St. Louis, MO) dissolved in Iscove’s Modified Dulbecco’s Media (IMDM) at a concentration of 100mM (25X) and used a final concentration of 4mM (1X) in complete media. TGF-β was obtained from Peprotech (Rocky Hill, NJ); a stock solution prepared at 20ng/μl in 0.2% sterile BSA/PBS and used at a final concentration of 10 ng/ml in all
experiments. IFN-γ was obtained from Peprotech (Rocky Hill, NJ); a stock solution prepared at 100 μg/ml in 0.2% sterile BSA/PBS and used at a final concentration of 100 ng/ml in experiments as indicated. IL-2 was obtained Hoffman-LaRoche; a stock solution of 200,000 IU/ml prepared in IMDM and used at a final concentration of 50 IU/ml in all experiments. SIINFEKL peptide (AnaSpec; Fremont, CA) stock solution was prepared at 1 mM in sterile PBS and used at final concentration of 10 nM in all experiments. DDAO (7-Hydroxy-9H-(1,3-Dichloro-9,9-Dimethylacridin-2-One) was obtained from Life Technologies (Grand Island, NY); first dissolved in DMSO, in the dark, to make a 1 mM stock solution and used at a final concentration of 5 μM in IMDM.

3.3 DDAO staining

MLN OT-I and SPL OT-I were made into single cell suspensions, counted and washed twice in serum-free IMDM. They were stained with the proliferation-tracking dye lauryl-dimethylamine N-oxide (DDAO) according to manufactures instructions. Lyophilized DDAO was resuspended in sterile, tissue culture grade DMSO to make a 1 mM solution. Then, cells were suspended in serum-free IMDM to a final concentration of 10^6 cells/ml and DDAO added to a final concentration of 5μM. Labeling cells were kept at in a 37°C/5% CO2 incubator for 10 minutes and then an equal volume of 20%FBS in IMDM was added for an additional 15 minutes of incubation. Cells were harvested by pelleting in a
centrifuge at 300 x g for 10 minutes. DDAO OT-I cells were placed into culture under conditions containing antigen and IL-2 or antigen, IL-2, RA and TGF-β. On days one, four, and seven the cells were harvested and stained for cell surface makers CD8, CD3, CD103, CD62L, and CD44, and analyzed by flow cytometry.

### 3.4 Antibodies and Flow cytometry

CD103 blocking antibody (clone M290) was obtained from BD Pharmingen (San Jose, CA) and LFA-1 blocking antibody (clone M17/4) was obtained from BioLegend (San Diego, CA). Antibodies against CD8a, CD3, CD103, CD62L CD44, CD29, CD49d, LFA-1, α4β7 CD127, KLRG1, CXCR3, CCR7, GZB, IFN-γ and TNF-α were obtained from Biolegend (San Diego, CA). Anti-Perforin-1 for western blotting was obtained from eBioscience (San Diego, CA). All samples were acquired on the BD Fortessa with FACSDiva software. Analysis was performed on either FACSDiva or FlowJo software.

### 3.5 Intracellular cytokine staining

Total SPL and MLN cells from OT-I mice were plated in round bottom 96 well microtiter plates and cultured for seven days with antigen and IL-2 or antigen, IL-2, RA and TGF-β. The cells were harvested on days zero, one, four and seven; incubated for four hours with GolgiPlug, stained for surface markers to identify CD3+ CD8+ CD103+ cells,
permeabilized for 30 minutes and then stained for intracellular proteins granzyme B, IFN-γ and TNF-α. All samples were acquired on a BD Fortessa Flow Cytometer within two hours of staining.

3.6 Chromium Release Cytotoxicity Assay

CTL effectors were harvested on day 4 of culture and pre-incubated for 1 hour with 10 mM CMA, 4mM EGTA, media, or media plus vehicle control in a 37° incubator as indicated by the experiment. Target cells were harvested and labeled with fresh ⁵¹Chromium or fresh ⁵¹Chromium plus SIINFEKL peptide for one hour, as indicated by experimental design. All cells were washed extensively in dPBS and plated in 96 round bottom plates with or without indicated antibodies for four hours. Plates were centrifuged at 226 x g for 5 minutes and 100 μl of supernatant was collected and counted in a Wallac 1460 Beckman Coulter gamma counter (Miami, FL) for one minute per tube.

3.7 Western Blotting

CTL from SPL and MLN were cultured in Ag/IL2 or Ag/IL2/RA/TGF-β for 7 days; day 0, 1, 4, and 7 CD103+ CD8+ CTL were FACS sorted and protein lysates made via lysis in ice-cold Laemmli sample buffer supplemented with protease inhibitor cocktail (Laemmli 1970) (Roche Diagnostics, Germany). Samples were subsequently denatured by boiling for 5 min in the presence of 2-ME and Laemmli sample buffer (2% SDS,
2.5% glycerol, 15 mM Tris [pH 6.8]) and separated by electrophoresis on 4–15% SDS-PAGE. SDS-PAGE gel was transferred to nitrocellulose membranes for analysis. The membrane was treated with Tween 20 and TBS. Perforin was detected with anti-Perforin mAb (2 μg/ml; eBioscience), HRP-coupled secondary Ab (1:1000 dilution; Jackson ImmunoResearch), and the SuperSignal West Pico chemiluminescent substrate (Thermo Scientific). Anti-GAPDH (0.5 μg/ml; Biolegend) was used as loading control and detected using the previously described method. Densitometry analysis of immunoblot assay signals was accomplished with ImageJ software (NIH; Bethesda, MD).

3.8 Real-Time PCR

CTL from SPL and MLN were cultured in Ag/IL2 or Ag/IL2/RA/TGF-β for 4 days; day 0 and 7 CD103+ CD8+ CTL cells were FACS sorted and RNA was made using the Qiagen RNeasy kit (Germantown, MD); cDNA was made using the QuantiTect reverse transcription (RT) kit (Qiagen, Germantown, MD). Granzyme M, A and D were amplified with Applied Biosystems TaqMan Gene Expression Assays (Life Technologies). Message for the glyceraldehyde 3-phosphate dehydrogenase (GAPDH)-encoding housekeeping gene was amplified as an internal normalization control. All assays were performed in triplicate for each RNA sample.
3.9 Cell Culture

CMT93, E.G7, EL4, E.G7\textsuperscript{Ecad} cells were maintained in Iscove's modified Dulbecco's medium containing 10% fetal bovine serum, 1 μg/mL gentamicin, and the appropriate antibiotics at the indicated concentrations: G418 (1 μg/mL) and Zeocin (100 μg/mL) at 37° C incubator at 5% CO\textsubscript{2}. 100 ng/ml of murine IFN-γ was added to CMT93 cells the day before cytotoxicity assays.

OT-I cells were made by physical disruption of MLN and SPL from mice 6-8 weeks old. Red blood cells in the spleen were lysed by treatment with ACK lysis buffer. Cells were washed, counted, and resuspended at 10\textsuperscript{6} cells/ml of media and plated in round bottom 96 well tissue culture dishes for up to 7 days. Ag/IL-2 conditions contained 10nM SIINFEKL peptide and recombinant IL-2 at 50 IU/ml. Ag/IL2/RA/TGF-β conditions contained 10nM SIINFEKL and 50 IU/ml IL-2 plus 100 nM RA and TGF-β at 10 ng/ml.

3.10 Cell Lines

The murine intestinal epithelial cell line, CMT93 and the mouse lymphoblastoma cell line EL4 were obtained from the American Type Culture Collection (Manassas, VA). E.G7 cells were generously provided by Dr. M. Bevan(Moore, Carbone et al. 1988). EG7 cells were further transfected with pcDNA3.1-Zeo(+) vector containing the cDNA for E-cadherin cloned from the murine CMT93 cell line.
3.11 Cloning

CMT93 cells were grown to confluency in a T-175 tissue flask using 10% FBS/IMDM. Confluent CMT93 cells were incubated for 7 minutes with 0.05% Trypsin/EDTA solution in order to detach the cells from the flask. The Trypsin/EDTA solution was inactivated with FBS containing media and centrifuged for 10 minutes at 300 x g. Total RNA was made using the Qiagen RNeasy kit (Germantown, MD); cDNA was made using the QuantiTect reverse transcription (RT) kit (Qiagen, Germantown, MD). PCR primers for murine E-cadherin were designed using VectorNTI (Life Technologies) and using the accession sequence BC098501. The primers were 5'-ATGGGAGCCCGGTGCCGCAG - 3' and 3'-GTCGTCCTCACCACCGCCGTACA -5'. The PCR product identity was confirmed by sequencing and alignment back to the accession sequence. The PCR product was cloned into the pcDNA3.1+ ZEO (Life Technologies) mammalian expression vector and transfected into E.G7 cells using Lipofectamine transfection reagent (Life Technologies). Transfected cells were selected in complete media containing zeocin. Expression of E-cadherin was confirmed by FACS analysis.

3.12 Statistical Analysis

Student’s t-test and One-way ANOVA with Bonferroni post-hoc test were employed to analyze data as appropriate to the experiment.
Graphpad Prism software and SPSS (IBM; Armonk, NY) (San Diego, CA) were used to perform statistical calculations.
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