THE ROLE OF THE $\beta_2$-INTEGRIN FAMILY ON T CELL SUBSETS

by

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Jillian E. Wohler

MICROBIOLOGY

ABSTRACT

Members of the β2-integrin family of adhesion molecules, CD11a, CD11b, and CD11c, have all been shown to play a role in the pathogenesis of experimental autoimmune encephalomyelitis (EAE). CD11d had yet to be studied in demyelinating disease and its functions remained unclear. We report here that CD11d is the only member of the β2-integrin family of adhesion molecules that fails to protect against the development of EAE. Surprisingly, the EAE studies suggested that CD11a, CD11b, and CD11c were all contributing to T cell activity during disease development by mechanisms beyond the migration of these cells into the CNS. However, the contributions of individual T cell subsets to the overall phenotypes seen were unclear. Earlier studies show that over the course of EAE a higher proportion of γδ T cells express the β2-integrins when compared to αβ T cells. Given this we hypothesized that the β2-integrin family was important to the functions of γδ T cells that contributed to the development of EAE. However, we show here that even though expression is enriched in this T cell subset the β2-integrins do not seem to be required on γδ T cells for disease development. The β2-integrin family has also been implicated in regulatory T cell function and homeostasis. Studies using transfer EAE with CD11a−/− mice have suggested these mice may have regulatory defects. Given this we next investigated the role CD11a plays in regulatory T cell biology. We show here that CD11a−/− mice have reduced Treg populations throughout the secondary lymph tissue and that this reduction may be due to
a reduced capacity to generate peripheral Tregs. We also found that CD11a is critical to Treg function in vitro, but does not seem to be as important in vivo. Importantly CD11a appears to be mediating its immunosuppressive effects independently of interactions with ICAM-1 on target T cells. Overall, the studies presented here provide further evidence that the β₂-integrin family of adhesion molecules functions in many aspects of T cell biology aside from cellular migration and that these functions differ between T cell subsets.
DEDICATION

This work is dedicated to all the family and friends who supported me throughout my graduate studies, especially to my mother Elaine Adams and my husband Matthew Wohler.
ACKNOWLEDGEMENTS

I am thankful for the guidance and support provided by my mentor, Dr. Scott R. Barnum, and the rest of my graduate committee, Drs. Dan Bullard, Pete Burrows, Chander Raman, and Louis Justement. I am indebted to Jane Hu for all the technical help and support throughout my graduate work. I acknowledge the many scientific brainstorming sessions with Kari Dugger, Tom Lowder, and Susan Vogt.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>v</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xi</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Multiple Sclerosis (MS) and Experimental Autoimmune Encephalomyelitis</td>
<td>1</td>
</tr>
<tr>
<td>γδ T Cells and EAE</td>
<td>2</td>
</tr>
<tr>
<td>The role of γδ T Cells in Multiple Sclerosis</td>
<td>6</td>
</tr>
<tr>
<td>Regulatory T Cells and EAE</td>
<td>7</td>
</tr>
<tr>
<td>β2-Integrins: Structure, Function, and Biology</td>
<td>9</td>
</tr>
<tr>
<td>In Demyelinating Disease</td>
<td>12</td>
</tr>
<tr>
<td>Rationale of Dissertation Study</td>
<td>12</td>
</tr>
<tr>
<td>DISRUPTION OF THE β2-INTEGRIN CD11D (αβ2) GENE FAILS TO PROTECT</td>
<td>14</td>
</tr>
<tr>
<td>AGAINST EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS</td>
<td></td>
</tr>
<tr>
<td>γδ T CELLS IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELISIS: EARLY</td>
<td>36</td>
</tr>
<tr>
<td>TRAFFICKING EVENTS AND CYTOKINE REQUIREMENTS</td>
<td></td>
</tr>
<tr>
<td>LFA-1 IS CRITICAL FOR REGULATORY T CELL HOMEOSTASIS AND FUNCTION</td>
<td>66</td>
</tr>
<tr>
<td>CONCLUSIONS</td>
<td>82</td>
</tr>
<tr>
<td>LIST OF REFERENCES</td>
<td>88</td>
</tr>
<tr>
<td>APPENDIX: IACUC APPROVAL</td>
<td>100</td>
</tr>
</tbody>
</table>
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Role of γδ T Cells in EAE</td>
</tr>
<tr>
<td>2</td>
<td>β2-integrin family members: nomenclature and biological functions</td>
</tr>
<tr>
<td>3</td>
<td>The role of the β2-integrin family in EAE</td>
</tr>
<tr>
<td>DISRUPTION OF THE β2-INTEGRIN CD11D (αDβ2) GENE FAILS TO PROTECT AGAINST EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>EAE symptoms in wild-type and CD11d deficient mice</td>
</tr>
<tr>
<td>2</td>
<td>Changes in expression of β2-integrins on T cells and macrophages in CD11d-deficient mice during EAE</td>
</tr>
<tr>
<td>γδ T CELLS IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELISIS: EARLY TRAFFICKING EVENTS AND CYTOKINE REQUIREMENTS</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>EAE in γδ T cell deficient mice reconstituted with wild type γδ T cells</td>
</tr>
<tr>
<td>2</td>
<td>EAE in γδ T cell deficient mice reconstituted with β2-integrin deficient γδ T cells</td>
</tr>
<tr>
<td>3</td>
<td>EAE in γδ T cell deficient mice reconstituted with cytokine deficient γδ T cells</td>
</tr>
</tbody>
</table>
# INTRODUCTION

## DISRUPTION OF THE $\beta_2$-INTEGRIN CD11D ($\alpha_D\beta_2$) GENE FAILS TO PROTECT AGAINST EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

1. The clinical course of MOG-induced EAE in wild-type mice and CD11d$^{-/}$ mice is identical .................................................................30

2. Leukocyte subsets in spinal cord of control and CD11d$^{-/}$ mice with EAE are identical..................................................................................31

3. Intracellular levels of TNF-$\alpha$ and IFN-$\gamma$ are identical in spinal cord-derived CD4$^+$ T cells from wild-type and CD11d$^{-/}$ mice with active EAE........32

4. CD11d$^{-/}$ T cells proliferate comparably to wild-type T cells ..................33

5. The cytokine repertoire of CD11d$^{-/}$ T cells is not significantly altered compared to wild-type T cells during EAE .........................................................34

# $\gamma\delta$ T CELLS IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS: EARLY TRAFFICKING EVENTS AND CYTOKINE REQUIREMENTS

1. Bioluminescent imaging of early trafficking events of $\gamma\delta$ T cells during active EAE........................................................................................................59

2. Bioluminescent imaging of $\gamma\delta$ T cells in the brain and spinal cord during EAE .................................................................60

3. Bioluminescent imaging of wild type and LFA-1$^{-/}$ $\gamma\delta$ T cells during EAE ...........................................................................................................61

4. The clinical course of active EAE in $\gamma\delta$ T cell$^{-}$ mice reconstituted with wild type or LFA-1$^{-/}$ $\gamma\delta$ T cells is comparable to that of control mice ...............62
IFN-γ and TNF-α produced by γδ T cells is critical to the clinical disease severity in active EAE .................................................................63

LFA-1 IS CRITICAL TO REGULATORY T CELL HOMEOSTASIS AND FUNCTION

1 LFA-1−/− Mice Have Reduced Frequencies of Regulatory T Cells .......................78
2 LFA-1 Deficiency Prevents Peripheral Treg Generation
   From CD4+CD25− T Cells ........................................................................79
3 LFA-1−/− Tregs Fail to Suppress CD4+ Effector Cell Proliferation .................80
4 LFA-1−/− Tregs Suppress Colitis Development Comparably
   to Wild Type Tregs ...............................................................................81
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS</td>
<td>Multiple Sclerosis</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental Autoimmune Encephalomyelitis</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>Tregs</td>
<td>Regulatory T cells</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>Foxp3</td>
<td>Forkhead box P3</td>
</tr>
<tr>
<td>LAG3</td>
<td>Lymphocyte-activating gene 3</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T lymphocyte antigen 4</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule 1</td>
</tr>
<tr>
<td>OND</td>
<td>Other neurological disorders</td>
</tr>
<tr>
<td>WC1</td>
<td>Workshop Cluster-1</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
</tbody>
</table>
INTRODUCTION

Multiple Sclerosis (MS) and Experimental Autoimmune Encephalomyelitis (EAE)

Multiple Sclerosis is an autoimmune disease in which the myelin sheaths of neurons in the central nervous system (CNS) are targeted by cells of the immune system. This disease affects approximately 2.5 million individuals worldwide and symptoms of the disease can vary widely from numbness and tingling to loss of coordination, vision, cognition and paralysis. This disease is characterized by the formation of demyelinating lesions containing inflammatory cell infiltrates in the brain and spinal cord [1]. Experimental autoimmune encephalomyelitis (EAE) mimics the classical pathology seen in MS and is currently used as the animal model to study autoimmune demyelinating disease. EAE is characterized by inflammatory cell infiltration into the CNS, which is followed by demyelination and the development of ascending paralysis. It is well established that T cells contribute to the development and progression of EAE and MS (reviewed in [1]). EAE is considered to be a CD4\(^+\) T cell mediated disease, classically attributed to T\(_{H1}\) type cells, but more recently thought to be driven by T\(_{H17}\) cells [2-5]. The relative contribution of the proportionally smaller T cell subsets, such as \(\gamma\delta\) T cells and regulatory T cells (Tregs), to the development and regulation of demyelinating disease remains unclear. Deletion of individual T cell subsets by gene targeting methods has clearly demonstrated that \(\alpha\beta\) and \(\gamma\delta\) T cells contribute in unique ways to the pathogenesis of EAE [1, 6, 7], while the absence of Tregs results in exacerbated disease [8-11].
Deletion of cell subsets is a relatively blunt approach and does not address subtle mechanistic differences utilized by each cell type that contributes to the pathogenesis or remission of disease. Here, we examine the unique roles of a single family of adhesion molecules, the $\beta_2$-integrins, and a limited set of cytokines that set the stage for EAE development within the context of these T cell subsets. Our model proposes that $\gamma\delta$ and regulatory T cells contribute to the development of demyelinating disease through the use of a specific $\beta_2$-integrin and cytokine repertoire to reach the CNS and modulate disease events.

$\gamma\delta$ T Cells and EAE

$\gamma\delta$ T cells are an important link between innate and adaptive cellular immunity. These cells develop from lymphocyte precursors at an early stage in T cell development, under the influence of IL-7 and Sox13 and do so in a fashion independent of T cell receptor (TCR) signaling [12]. In contrast to $\alpha\beta$ T cells, $\gamma\delta$ T cells represent a minor portion of the T cell population (1-5%; although they can expand to 50% of the T cell population [13]) and are perhaps the least understood T cell subset. The ligands for these cells remain elusive. However, the absence of CD4 or CD8 on most $\gamma\delta$ T cell subsets, combined with crystal structure data suggests that $\gamma\delta$ T cells recognize antigen in a MHC-independent manner and that phosphoantigens are strong candidate ligands (reviewed in [16, 19, 20]). $\gamma\delta$ T cells uniquely express WC1 molecules (workshop cluster 1), a group of transmembrane glycoproteins that are members of the scavenger receptor cysteine-rich family, which may also function in antigen recognition [21, 22]. The rearrangement of $\gamma\delta$ T cell antigen receptor genes results in the generation of functional $\gamma\delta$ subsets based on the
usage of specific Vγ or Vδ gene segments. These resulting γδ T cell subsets are further divided based on their levels of TCR diversity and tissue-specific homing. For example, tissue-specific γδ T cells generally (but not always) have restricted TCR diversity and are found in the epithelial layers of organs or in the mucosa. These cells provide protection from infection in the skin, lung, intestine, oral cavity and genitourinary tract. γδ T cells are also found in blood and lymphoid tissues (reviewed in [14-16]). Although it has been suggested that a subset of γδ T cells are present in the normal mouse CNS, no other group including ours has reported similar findings [17]. γδ T cells are thought to play an important role in many aspects of the host immune response depending on their location. For example γδ T cells in the skin (also termed dendritic epithelial T cells) produce growth factors required for tissue repair, while γδ T cells in the gut (also known as intraepithelial lymphocytes) are often cytolytic and produce cytokines and chemokines to regulate local immune responses to infection (reviewed in [15, 16, 23]). γδ T cells have also been shown to play important roles in tumor regression, systemic infections, and in modulating inflammation and adaptive immune responses [18, 24]. More recently it has become clear that the mechanism of γδ T cell modulation of immune responses is, in part, dependent on developmental competition and interdependence with αβ T cells and NK cells [25-27].

Experimental autoimmune encephalomyelitis is a T cell-mediated autoimmune disease of the central nervous system that shares many features of the human disease multiple sclerosis (MS) [1, 28, 29]. One of the hallmark features of EAE is cellular infiltration of the brain and spinal cord by a number of different immune cells, including macrophage, dendritic cells, and lymphocyte subsets including B and T cells [1, 29]. The function of γδ T cells in EAE remained controversial for some time (see Table 1 for overview).
Undoubtedly the contradictory nature of these findings for γδ T cells in EAE is due, at least in part, to the use of different rodent species and strains and technical approaches (see Table 1). In addition, comparison of such studies can be complicated by differences in the specificity, avidity, and biological half-life of the different antibody clones used to deplete γδ T cells. Nevertheless, the consensus of all the studies cited below, particularly those using δ-chain−/− mice (and thus γδ T cell deficient mice), is that γδ T cells make an important contribution to the pathogenesis of EAE. Although these results indicate that γδ T cells contribute to disease, they do not address fundamental questions regarding the kinetics of their trafficking, adhesion molecule requirements for trafficking, or effector functions including cytokine production that contribute to disease development.

Table 1. Role of γδ T Cells in EAE

<table>
<thead>
<tr>
<th>Gene Deficiency/Treatment</th>
<th>Effect in EAE</th>
<th>Species/strain</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-γδ T cell Mab (clone GL3)</td>
<td>Protection</td>
<td>Mouse/SJL</td>
<td>Rajan et al., 1996</td>
</tr>
<tr>
<td>α chain-KO mice</td>
<td>Protection</td>
<td>Mouse/SJL/NOD</td>
<td>Elliott et al., 1996</td>
</tr>
<tr>
<td>Anti-γδ T cell Mab (clone UC7–13D5)</td>
<td>Exacerbation</td>
<td>Mouse/B10PL</td>
<td>Kobayashi et al., 1997</td>
</tr>
<tr>
<td>Adoptive transfer in δ-chain KO mice</td>
<td>No effect</td>
<td>Mouse/C57BL/6</td>
<td>Clark and Lignenheld, 1998</td>
</tr>
<tr>
<td>Anti-γδ T cell Mab (clone V65)</td>
<td>No effect</td>
<td>Rat/Lewis</td>
<td>Matsumoto et al., 1998</td>
</tr>
<tr>
<td>Active EAE in δ-chain KO mice</td>
<td>Protection</td>
<td>Mouse/C57BL/6</td>
<td>Spahn et al., 1999</td>
</tr>
<tr>
<td>Anti-γδ T cell Mab (clone UC7–13D5)</td>
<td>Protection</td>
<td>Mouse/C57BL/6</td>
<td>Dandekar and Perlman, 2002</td>
</tr>
</tbody>
</table>

To determine potential mechanisms whereby γδ T cells contribute to EAE, several groups have examined cytokine and chemokine production during the course of EAE. Rajan and colleagues reported significantly reduced levels of several pro-inflammatory cytokines and chemokines, in mice depleted of γδ T cells by antibody treatment, compared
to untreated controls [30, 31]. Although this approach was informative, it has significant limitations in that γδ T cell effector functions in the course of EAE can only be inferred rather than directly determined. For example, in γδ T cell-depleted mice, the expression of several cytokines and chemokines including interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6, IL-12 and others, was reduced. However since spinal cord homogenate was used to quantitate cytokine mRNA levels in these studies, it is unclear which cell types produced (or didn’t produce) these cytokines, a concern that extends to γδ T cell-sufficient control mice. Other studies have shown that γδ T cells producing IFN-γ and IL-4 are significantly more numerous during EAE [32]. Similar results were reported by Gao and colleagues, however in this study it was shown that in both the spleen and CNS of normal mice, there were substantial numbers of CD3+ cells and γδ T cells secreting both IFN-γ and IL-4 [33]. Since the CNS of normal mice is usually devoid of any lymphocyte subset, and particularly of γδ T cells, the meaning of these results is unclear. Recent studies have indicated that γδ T cells act in an antigen-independent fashion to modulate cytokine production (IL-12 and IFN-γ) and thus the early effector phase of the immune response in EAE [34, 35]. Cheng and colleagues have shown that γδ T cells are able to present MOG35-55 peptide to naïve T cells, further raising the possibility that these cells may play a role in disease initiation [24]. It is evident that γδ T cells contribute to the pathogenesis of EAE; however the mechanisms behind this contribution are still unclear.
The role of γδ T cells in Multiple Sclerosis

As previously mentioned, γδ T cells are found throughout a variety of tissues including skin, mucosa, secondary lymphoid organs and blood [15, 16, 36-39]. Initial studies examining γδ T cells in MS readily found these cells in chronic active lesions along with CD4 and CD8 T cells and macrophages [40-46]. However, shortly after this report, a study by Perrella et al. [47], suggested no role for γδ T cells in MS based on low numbers in CNS infiltrates compared to other neurological disease (OND) controls. This study is of limited value however, as the sample sizes of the groups analyzed were very small and no functional markers were assessed. Subsequently it was shown that during acute MS, γδ T cells of an oligoclonal nature were preferentially expanded compared to γδ T cells from patients with chronic MS or OND [41, 43, 48-52]. It has been shown that γδ T cells produce higher levels of cytokines and chemokines and express higher levels of cytokine receptors in the CSF of MS patients [49, 50, 53]. For example the production of the chemokine RANTES is increased, but lower levels of its receptor CCR5 are expressed, suggesting repeated activation of γδ T cells by autoantigens [50]. Importantly, recent studies suggest that γδ T cells may be cytotoxic to oligodendrocytes through an NKG2D-mediated mechanism [54-56]. Although the data to date suggest that γδ T cells may contribute to the pathogenesis of MS, we know little to nothing regarding their infiltration kinetics or role in modulation of CNS inflammation and demyelination relative to αβ T cells during disease development or in the proposed MS subtypes [57]. It is clear from the present state of knowledge, that the role of γδ T cells in demyelinating disease remains a significantly understudied topic.
Regulatory T cells and EAE

Regulation of the immune system is critical to maintaining peripheral tolerance. This tolerance prevents auto-reactive T cells, normally present in the T cell repertoire, from causing damage to self-tissues [58]. Tolerance is a dynamic process that can be achieved through various routes including, anergy, ignorance, and deletion. A critical mechanism in maintaining tolerance is the suppression of auto-reactive T cells by regulatory T cells [59]. Treg cells can be differentiated from naïve and effector T cells by the unique cellular markers they express such as CD4, CD25, and forkhead box P3 (Foxp3), a transcription factor critical to the developmental program of the Treg lineage. Ablation of this transcription factor in mice results in the failure to generate a Treg population and the spontaneous development of severe organ specific auto-immunity [60, 61]. Regulatory T cells are capable of repressing naïve and effector T cell responses at various stages of activation including initial activation, clonal expansion, differentiation into effector lineages, and effector functions at the site of inflammation [62-70]. These cells have been found to have exquisite specificity as to which stage of activation and effector functions to control [71, 72]. Given this, the site of suppression, cytokine milieu, and antigen stimulation may all dictate what suppressive mechanisms regulatory T cells utilize.

Many mechanisms of suppression have been ascribed to Tregs. To date suppression can occur through cell-to-cell contact, release of soluble mediators, or competition for growth factors [59]. Cell-to-cell contact inhibition may be mediated through many cell surface molecules such as; membrane bound transforming growth factor-β (TGF-β), Fas, lymphocyte-activating gene 3 (LAG3), and cytotoxic T lymphocyte
antigen 4 (CTLA-4) [73-76]. Tregs may also use direct cell contact to deliver cytotoxic or immunosuppressive mediators [77]. The release of soluble mediators such as adenosine, IL-10, TGF-β, and IL-35 is important to the maintenance of tolerance [78-84]. Another mechanism important for suppression is the competition for cytokines. Tregs effectively out compete effector T cells for growth factors resulting in cytokine deprivation-induced apoptosis of the effector T cells [85]. These mechanisms are not exclusive and most likely Tregs utilize a combination of strategies to maintain tolerance.

MS generally presents as a relapsing remitting disease course indicating, that at least initially, individuals have some capacity to limit inflammatory episodes. Regulatory T cells isolated from patients with MS do have suppression defects when compared to Tregs isolated from control individuals, however the frequency of Tregs is not altered in MS patients [86-91]. Understanding how Tregs contribute to recovery from MS episodes and how they are altered in MS patients may lead to improved therapies for the disease. This possibility has been studied extensively in EAE in relation to disease resistance and recovery. Studies have shown that transfer of Tregs isolated from the periphery of naïve mice, from the CNS of mice with EAE, or from the periphery of mice with EAE can prevent onset and progression of EAE [8, 10, 92, 93]. Further evidence that Tregs are critical to the maintenance of tolerance preventing the development of EAE was generated using transgenic mice with T cell receptors specific for myelin basic protein. When these mice were crossed onto the RAG1<sup>−/−</sup> background depleting them of Tregs they developed spontaneous autoimmune disease; which could be prevented by reconstituting the Treg populations [94, 95]. Depletion of Treg cells using anti-CD25 antibodies has also been shown to increase susceptibility to or to exacerbate EAE in various mouse strains [9, 10,
Recent studies have found that Tregs enter the CNS during EAE and are at the highest frequencies during remission of the disease [10, 98-100]. Together this data indicate that Tregs are critical in the maintenance of peripheral tolerance that prevents the development of EAE and dysregulation of these cells can lead to an exacerbation of the disease.

\[\beta_2\text{-Integrins: Structure, Function and Biology in Demyelinating Disease}\]

The \(\beta_2\)-integrins are a leukocyte-specific family of cellular adhesion molecules composed of four heterodimers: CD11a/CD18 (LFA-1, \(\alpha_\ell\beta_2\)), CD11b/CD18 (MAC-1, CR3, \(\alpha_M\beta_2\)), CD11c/CD18 (p150, 95, CR4, \(\alpha_X\beta_2\)), and CD11d/CD18 (\(\alpha_D\beta_2\)) (Figure 1).

<table>
<thead>
<tr>
<th>CD11a</th>
<th>CD11b</th>
<th>CD11c</th>
<th>CD11d</th>
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<tr>
<td>(\alpha_L), LFA-1</td>
<td>(\alpha_M), Mac-1, CR3</td>
<td>(\alpha_X), p150,95, CR4</td>
<td>(\alpha_D)</td>
</tr>
</tbody>
</table>

**Ligands**
- CD11a: ICAM-1/3
- CD11b: C3bi, CHOs ICAM-1, ECM
- CD11c: C3bi, ICAM-1/2, VCAM-1, ECM
- CD11d: VCAM-1, ICAM-3

**Expression**
- CD11a: Leukocytes
- CD11b: Myeloid cells, T cell subsets
- CD11c: Myeloid cells, DCs, T cell subsets
- CD11d: Myeloid cells, T, B and NK cells

**Functions**
- CD11a: Co-stimulation, Trafficking
- CD11b: Phagocytosis, Trafficking, Tolerance
- CD11c: Phagocytosis, Trafficking
- CD11d: Phagocytosis? Trafficking?

\[\text{Figure 1: Structure of the } \beta_2\text{-integrin family. Each of the four members contains a unique } \alpha\text{-chain (CD11a-d) and shares a common } \beta\text{-chain (CD18).}\]
β2-integrins bind a variety of ligands including, but not limited to, proteolytic fragments of C3, extra-cellular matrix proteins, various carbohydrates and members of the immunoglobulin-gene super family such as intercellular adhesion molecule 1 (ICAM-1). On the plasma membrane of unstimulated cells integrins adopt a closed conformation, which is inaccessible for binding. Shifting to an open conformation, where the receptors are able to bind ligand, requires activation of the integrin through “inside-out signaling.” This activation process is rapid and can be initiated through chemokine, cytokine, or TCR signaling [101, 102]. Once activated, the β2-integrin family members participate in a number of functions including, cellular trafficking, phagocytosis, and T cell activation.

With the exception of CD11d/CD18, all the β2-integrins have established roles in the trafficking of phagocytic cells and/or lymphocytes under normal and inflammatory conditions [103-107]. All family members are differentially expressed on a wide variety of leukocytes including macrophage, neutrophils, dendritic cells, NK cells, B cells, and T cells [108, 109]. It is important to note that basal expression of the β2-integrins is remarkably distinct for naive αβ and γδ T cells [110]. CD11b/CD18 and CD11c/CD18 are also well characterized with respect to their functions in macrophage and neutrophil-mediated phagocytosis of invading pathogens and in clearance of apoptotic cells [111-114]. CD11b is also implicated in antigen-specific T cell-mediated oral tolerance [115]. CD11d/CD18 is the least well characterized member of this family of proteins however, given its known ligand specificity of the Ig-supergene family (see table 2), it is likely to be involved in trafficking of leukocytes, however a role in phagocytosis remains possible [106, 116-118].
In addition to roles in trafficking and phagocytosis, there is growing evidence for a β2-integrin mediated role in T cell functions. It is now well established that CD11a/CD18 plays an important role in the formation of the immunological synapse in

Table 3. The role of the β2-integrin family in EAE

<table>
<thead>
<tr>
<th>Gene Deficiency/Treatment</th>
<th>Effect in EAE</th>
<th>Species/Strain</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11a</td>
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<tr>
<td>Anti-CD11a Mab</td>
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<td>Mouse/C57Bl/6</td>
<td>Wang, et al., 2007</td>
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<tr>
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<tr>
<td>CD11d</td>
<td>CD11d−/−</td>
<td>No effect</td>
<td>Adams, et al., 2007</td>
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</table>

and out of the CNS [119-123] and in T cell activation [124-127]. Aside from CD11a/CD18, there is evidence that other members of the β2-integrin family may play a role in modulating T cell function and the development of EAE (see table 3). For example, we have shown that deletion of CD11a, CD11b or CD11c significantly delays and attenuates EAE [128-130]. Protection from EAE in these mice is not due only to
reduced cellular infiltration, there are differences in T cell proliferation and cytokine production by β2-integrin-deficient T cells. For example, CD11b-deficient T cells have poor antigen-specific responses in EAE, but elevated proliferation when activated through the TCR [129]. In contrast, CD11c-deficient T cells proliferate to a greater extent in antigen-specific assays [128]. Surprisingly, when WT encephalitogenic T cells mice were transferred into CD11a deficient recipients they generated a more severe disease than when transferred into WT recipients, suggesting regulatory defects in these mice [130]. Given this it is clear that the β2-integrins play a larger role on T cells than previously thought.

Rationale of Dissertation Study

We first set out to complete the characterization of the β2-integrin family in EAE. To do this we examined the role CD11d/CD18 on T cells during EAE using CD11d⁻/⁻ mice. We found that CD11d/CD18 is the only member of the family that does not have a significant role on T cells during the development of EAE [131]. Once the biology of the family was characterized on T cells as a whole during EAE, we further pursued the functions of the β2-integrin family in subsets of T cells first focusing on γδ T cells during disease development. Previous studies from our lab indicated that a higher proportion of γδ T cells express the β2-integrins when compared to αβ T cells, and that this expression increases during EAE [110]. Given this, we were led to examine whether this expression was critical to the functions of γδ T cells important for driving disease pathogenesis. We found that, despite their enhanced expression, the β2-integrins were not required for γδ T cells to contribute to EAE development. However, the production of IFN-γ and TNF-α
was found to be vital to the role γδ T cells play in the progression of EAE. Another T cell subset we chose to focus on was regulatory T cells, due to a recent study from Marski and colleagues in which CD18-deficient Tregs were found to be non-functional [132]. EAE studies from our lab using CD11a−/− mice suggested a possible regulatory defect in these mice ([130] and unpublished observations). In further examining the role of CD11a on Tregs we found that CD11a−/− mice have reduced frequencies of Tregs in the periphery, which may be due to defects in peripheral induction of Tregs in the lymph nodes. Also CD11a−/− Tregs are non-functional in vitro and this appears to be independent of interactions with ICAM-1. However, CD11a-deficient Tregs are functional in vivo but to a lesser extent then their WT counterparts. The data presented here clearly show that the β2-integrin family mediates more than adhesion related functions on T cells.
Disruption of the β2-Integrin CD11d (αDβ2) Gene Fails to Protect Against Experimental Autoimmune Encephalomyelitis

by

Jillian E. Adams, Matthew S. Webb, Xianchen Hu, Don Staunton, and Scott R. Barnum

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ABSTRACT

The fourth member of the $\beta_2$-integrin family of adhesion molecules, CD11d ($\alpha_\delta \beta_2$), is expressed on a wide variety of immune cells, however its function in autoimmune diseases, including EAE remains unknown. We induced EAE in wild type and CD11d$^{-/-}$ C57BL/6 mice using myelin oligodendrocyte glycoprotein (MOG$_{35-55}$) peptide. The clinical course and histopathology of EAE was identical in both groups of mice throughout the disease course. There were no significant differences in the infiltration of leukocyte subsets into the central nervous system or in the production of cytokines from T cells isolated from the spleen or spinal cord from both groups of mice. Our data demonstrate that CD11d is not required for the development of EAE and, to date, is the only $\beta_2$-integrin molecule whose deletion does not result in attenuated disease.

1. INTRODUCTION

A critical requirement for the development of multiple sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE) is the trafficking of immune effector cells into the central nervous system (CNS). These cells include macrophages and antigen-specific T cells which can participate in lesion formation and, in turn, demyelination (Sospedra and Martin, 2005). There are several families of cellular adhesion molecules known to function in the trafficking of effector cells to sites of inflammation, including the CNS (Archelos et al., 1999; Butcher et al., 1999; Campbell et al., 1998; Engelhardt, 2006; von Andrian and Mackay, 2000). In MS, the most notable of these are the $\alpha_4$ integrins, specifically $\alpha_4\beta_7$ and $\alpha_4\beta_1$, since treatment with antibody to $\alpha_4$ reduces the development of new lesions, resulting in fewer clinical relapses (Miller et al.,
2003). However inhibition of \( \alpha_4 \) integrins, by a variety of approaches, does not completely prevent disease, due most likely to the participation of other cellular adhesion molecules in the disease process (Engelhardt et al., 1998; Kent et al., 1995; Myers et al., 2005; Yednock et al., 1992).

In addition to the \( \alpha_4 \) integrins, members of the \( \beta_2 \)-integrin family, in particular CD11a and CD11b have been shown to contribute to EAE, however the results have often been contradictory. For example, treatment with anti-CD11a antibody in EAE produced results ranging from disease inhibition to exacerbation (Cannella et al., 1993; Gordon et al., 1995; Welsh et al., 1993; Willenborg et al., 1996). Similarly, several studies have shown variable levels of protection in antibody-mediated inhibition of CD11b. Those studies demonstrating attenuated disease attributed protection largely to prevention of myelin phagocytosis (Brocke et al., 1999; Bruck, 1997; Bruck and Friede, 1990; Gordon et al., 1995; Huitinga et al., 1993; Smith, 1999). Studies using CD11b-deficient mice, have shown that CD11b expression on T cells is critical for disease development, independent of cell trafficking into the CNS or phagocytosis (Bullard et al., 2005). More recent studies using CD11c-deficient mice have also shown a protective phenotype in EAE compared to control mice (Bullard et al., submitted). In contrast to these three \( \beta_2 \)-integrins family members, nothing is known regarding the role of CD11d in EAE.

Previous studies have demonstrated multiple roles for CD11d in immune responses including facilitation of macrophage activation and infiltration into inflamed tissues (Bao et al., 2004; Mabon et al., 2000; Saville et al., 2004), development of IgG complex induced lung injury (Shanley et al., 1998) and T cell proliferation (Wu et al., 2004). In a spinal cord injury model (SCI), treatment with antibody to CD11d markedly reduced
neutrophil and macrophage infiltration leading to improved recovery and reduced secondary damage (Gris et al., 2005; Gris et al., 2004; Mabon et al., 2000). These results, combined with those demonstrating reduced cellular infiltration in CD11b-deficient mice during EAE, suggested to us that loss of CD11d-mediated trafficking mechanisms would prove beneficial in EAE. We show here that deletion of CD11d, in contrast to other members of the β2-integrin family, has no effect on the development and progression of EAE. These data indicate that β2-integrin functions are not redundant in EAE and suggest differential functions in acute versus chronic inflammation.

2. MATERIALS AND METHODS

Mice

CD11d<sup>−/−</sup> mice were generated through the replacement of exons 1 and 2 of the CD11d gene with a neomycin cassette as previously described (Wu et al., 2004). All CD11-deficient mice were backcrossed for at least six generations onto the C57BL/6 background. Inbred C57BL/6 mice were used as controls for all experiments. All studies were performed with approval from the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

EAE Induction

Control and CD11d<sup>−/−</sup> mice were immunized with MOG35–55 peptide as previously described (Szalai et al., 2002). MOG peptide was synthesized by standard 9-fluorenylmethoxycarbonyl chemistry and was >95% pure as determined by reversed-phase HPLC (Biosynthesis International). Briefly, on day 0 mice were injected subcutaneously with MOG35-55 emulsified in complete Freud's adjuvant. Pertussis toxin was injected intraperitoneally on day 0 and 2. Onset and progression of EAE symptoms were
monitored daily using a standard clinical scale ranging from 0 to 6 as follows: 0, asymptomatic; 1, loss of tail tone; 2, flaccid tail; 3, incomplete paralysis of one or two hind limbs; 4, complete hind limb paralysis; 5, moribund; 6, dead. Mice scoring at least a 2 for more than 2 consecutive days were judged to have EAE. For each animal, a cumulative disease index (CDI) was calculated from the sum of the daily clinical scores observed between days 7 and 29.

Isolation of Spinal Cord Leukocytes

Spinal cords were removed from control and CD11d−/− mice with active EAE (day 10 and 21) following saline perfusion and were ground through a cell strainer, washed in PBS, resuspended in 40% Percoll, and layered on 70% Percoll. After centrifugation at 2000 rpm (RT, 25 min), cells at the interface were removed, washed in PBS, and stained as described below.

Flow Cytometry

Cells obtained from spinal cords, draining lymph nodes, or spleens were incubated with anti-CD16/32 (24G2, FcR block) to prevent nonspecific staining. Spinal cord leukocytes were stained with anti-CD45-FITC (30F11), anti-CD4-Sav-PerCP (GK1.5), anti-CD8-APC (53-6.7), anti-TNF-α-PE (MP6-XT22), and anti-IFN-γ-FITC (XMG1.2; all from eBioscience). To determine if $\beta_2$-integrin expression changed during EAE on T cells or macrophages from CD11d−/− mice relative to control mice, cells were isolated from spleens or lymph nodes at day 21 post-immunization, stained with anti-CD16/32 as described above and then stained with anti-CD3-FITC (145-2C11), or F4/80-FITC (BM8) and anti-CD11a-PE (M17/4), CD11b Biotin-Sav-PercP (M1-70) or anti-CD11c-APC (HL3). Stained cells were analyzed using a FACSCalibur (BD Biosciences) and data was analyzed using CellQuest software (BD Biosciences).
Ag-specific T cell proliferation assays were performed as described previously (Szalai et al., 2002). Single-cell suspensions from spleens obtained 21 days after EAE induction were cultured in 96-well plates at \(5 \times 10^5\) cells/well with increasing concentrations of MOG35–55 peptide or 3ug/ml ConA in triplicate. After 48 h, cultures were pulsed with \([^3H]\) thymidine for an additional 18 h, and incorporation of thymidine was measured. The in-vitro cytokine assays were performed essentially as described for the proliferation assay. Duplicate cultures were stimulated with MOG peptide (5 µg/ml). Culture supernatants were collected at 48 h for use in cytokine ELISAs. ELISA kits for TNF-\(\alpha\), IFN-\(\gamma\), IL-4, IL-2, IL-17 and TGF-\(\beta\)1 were purchased from R&D Systems. Each assay was performed according to the manufacturer’s instructions. Cytokine production by cultures of CD11d deficient cells is reported as a percentage of the wild-type cell production. The data are pooled from two separate experiments.

**Statistical Analysis**

Statistical significance between control and CD11d\(^-\) mice for active EAE experiments was calculated using the Mann-Whitney \(U\) test. Statistical significance in the proliferation, compensation analysis and cytokine production assays was tested using the student’s \(t\) test. All statistical analysis was done using Prism software v.4 (GraphPad).

### 3. RESULTS

**CD11d-Deficiency Does not Alter the Disease Course or Cellular Infiltration of Active EAE**

To assess the role of CD11d\(^-\) in EAE, we performed active EAE using MOG\(_{35-55}\) peptide. We induced EAE in control and CD11d\(^-\) mice and followed the course of disease for 29 days. The CD11d\(^-\) mice presented with a phenotype remarkably similar to the wild-type
mice as shown in Figure 1. The deletion of CD11d<sup>−/−</sup> does not alter the onset (15 days vs. 14 days; Table 1), incidence (100% for both groups) or the severity of disease as determined by cumulative disease index (CDI) for each group (46.5 vs. 45.6; Table 1).

We next determined if the extent of cellular infiltrate was similar between the two groups of mice. For these studies, we induced active EAE and isolated spinal cords 10 and 21 days later. Leukocytes were isolated from spinal cords as described in Materials and Methods and analyzed for total leukocyte infiltration (CD45<sup>+</sup> cells) and for T cell subsets (CD4<sup>+</sup> and CD8<sup>+</sup>). We observed essentially identical infiltration of CD45<sup>+</sup> cells in both groups of mice at day 10 (Figure 2A) and at later time points (data not shown). Neutrophil (Gr-1<sup>+</sup> cells) and macrophage (CD11b<sup>+</sup>) cells infiltration was also not significantly different (p>0.05, student’s t test) between wild type and CD11d<sup>−/−</sup> mice (data not shown). To determine if CD11d<sup>−/−</sup> T cells infiltrated the CNS to a similar extent as T cells from wild type mice, we isolated cells from spinal cords of control and CD11d<sup>−/−</sup> mice with active EAE and analyzed for the presence of CD4<sup>+</sup> and CD8<sup>+</sup> T cells. At day 10, the composition of the T cell infiltrate in CD11d<sup>−/−</sup> mice was not significantly different from that of wild type mice (Figure 2B, p>0.05, student’s t test). On Day 21 post-immunization, the spinal cords of control and CD11d<sup>−/−</sup> mice contained similar numbers of CD4<sup>+</sup> T cells (3.3% and 5.5% respectively), while CD11d<sup>−/−</sup> mice had ~2.5-fold more CD8<sup>+</sup> T cells than control mice (4.4% and 1.7%, respectively; Figure 2B). The difference in the numbers of CD4<sup>+</sup> T cells between the two groups was not statistically significant (p>0.05, student’s t test). To further characterize the cellular infiltrate in CD11d<sup>−/−</sup> versus wild type mice, we examined for the production of intracellular TNF-α and IFN-γ in the CD4<sup>+</sup> T cell population at days 10 and 21. Wild type and CD11d<sup>−/−</sup> CD4<sup>+</sup> T cells produced
comparable amounts of TNF-α at day 10 of active EAE (Figure 3). CD4+ T cells from CD11d−/− mice produce modestly elevated levels of IFN-γ early in disease compared to wild type mice, however, by day 21, both wild type and CD11d−/− T cells produce equivalent amounts of IFN-γ and very little TNF-α (Figure 3). None of the differences in cytokines produced by CD4+ T cells between the two groups of mice was significantly different (p>0.05, student’s t test).

**Splenic CD11d−/− T Cells and Macrophages Express Comparable Levels of β2-Integrins Compared to Wild Type Cells**

To determine if the expression of the remaining β2-integrin family members may have changed such that they might compensate for the loss of CD11d, we isolated T cells and macrophages from spleen and lymph nodes from CD11d−/− and wild type mice at day 21 post-immunization. We observed no statistically significant differences in the expression of CD11a-c on T cells from spleen or lymph nodes between CD11d−/− and wild type mice (Table 2). In contrast, CD11b and CD11c expression on CD11d−/− macrophages was significantly lower than that seen on control macrophages. There were no other significant changes in expression of the β2-integrins on macrophages isolated from CD11d−/− and wild type mice (table 2).

**Splenic CD11d−/− T Cells Show no Proliferative Deficits or Differences in Cytokine Production Compared to Wild Type T Cells**

We next looked for differences in the proliferative capacity and cytokine profile of T cells isolated from the spleens of CD11d−/− and wild type mice. An *in-vitro* proliferation assay
was performed as described previously (Bullard et al., 2005). Proliferation of MOG-sensitized T cells from wild type and CD11d<sup>-/-</sup> mice did not vary significantly (p>0.05, student’s t test) when restimulated with various concentrations of MOG peptide (Figure 4). We also examined for cytokine production in MOG-sensitized splenic T cells from wild type and CD11d<sup>-/-</sup> mice. No significant differences were observed in the levels of IFN-γ, TNF-α, IL-2, IL-17, IL-4, or TGF-β1 between the two groups of mice (Figure 5).

4. DISCUSSION

Members of the β<sub>2</sub>-integrin family of adhesion molecules are primarily known for their roles in leukocyte trafficking and, in the case of CD11b and CD11c, phagocytosis (reviewed in (Mayadas and Cullere, 2005; Underhill and Ozinsky, 2002; von Andrian and Mackay, 2000)). Although the results have often been contradictory, inhibition of β<sub>2</sub>-integrin functions via treatment with antibody generally results in a better disease outcome regardless of the model system employed. This is also true in the CNS, where anti-CD11a and anti-CD11b treatment reduced clinical signs of disease, cellular infiltration and phagocytosis of myelin (reviewed in (Barnum, 2001; Engelhardt, 2006)). Surprisingly the results reported in this study indicate that CD11d, unlike the other members of the β<sub>2</sub>-integrin family, is not required for development or progression of EAE. In contrast, in a SCI model system employing spinal cord transection, inhibition of CD11d through the use of anti-CD11d monoclonal antibodies, partially prevented the infiltration of neutrophils and macrophages (Mabon et al., 2000). The long-term effectiveness of antibody treatment was not assessed with respect to cellular infiltration, neuronal survival or functional outcome, and the protective effect was attributed largely to disruption of neutrophil
interaction with VCAM-1 on the vascular endothelium (Mabon et al., 2000). More recent SCI studies, in which the spinal cord was injured by compression (as opposed to transection), demonstrated not only reduced cellular infiltration but also reduced secondary injury and improved functional outcome for up to 12 weeks post treatment (Gris et al., 2005; Gris et al., 2004). Importantly, anti-CD11d treated mice had reduced myelin loss and more rapid remyelination compared to control mice (Gris et al., 2004).

Based on the treatment regimen in these studies (3 injections in 48 hrs) and the long-term improved outcome, it is clear that transient inhibition of leukocyte infiltration with anti-CD11d, during the acute phase response, provides significant neuroprotection.

The effect of blocking or deleting CD11d with respect to T cell infiltration has not been previously examined. In the present study we observed that both CD4$^+$ and CD8$^+$ T cells readily infiltrated the spinal cords of CD11d$^{-/-}$ mice during EAE (Figure 2B). In fact, our data indicate that greater frequencies of both T cell subsets migrate into the spinal cords of CD11d$^{-/-}$ mice during EAE compared to control mice. This data combined with the observation that CD45$^+$ cells (as representative of the total leukocyte infiltrate) also traffic readily into the spinal cords of CD11d$^{-/-}$ mice, indicates that CD11d is not required for CNS infiltration in EAE. Although it is possible that other $\beta_2$-integrins or members of other integrin families compensate for the loss of CD11d under these circumstances, we did not observe this by flow cytometry (Table 2) and, deletion of any of the remaining $\beta_2$-integrin $\alpha$-chains results in significant attenuation of EAE ((Bullard et al., 2005), J. Hu, D. Bullard and S.R. Barnum, unpublished observations).

Aside from cellular trafficking, it is possible that deletion of CD11d could affect T cell proliferation and effector functions in a way that would alter the course of EAE.
relative to control mice. However, we observed no difference in antigen-specific T cell proliferation in CD11d<sup>−/−</sup> and control mice (Figure 3), indicating that there is no intrinsic proliferative defect in the absence of CD11d. Wu and colleagues demonstrated that CD11d<sup>−/−</sup> T cells proliferate comparably to wild type T cells when the T cell receptor is bypassed on treatment with PMA and ionomycin (Wu et al., 2004). However, in the same study, it was shown that CD11d<sup>−/−</sup> T cells proliferate poorly on treatment with PHA, ConA and several superantigens including SEA, SEB and SEE (Wu et al., 2004). The reason(s) for these differences in proliferative capacity remain unclear. Similar to the proliferation assays using T cells from CD11d<sup>−/−</sup> mice, there were no differences in production of IFN-γ and TNF-α, in T cells isolated from the spinal cords, compared to control mice. In contrast, splenic T cells from CD11d<sup>−/−</sup> mice produced two-fold greater levels of IFN-γ, but lower levels of TNF-α compared to control mice. The reasons for differential production of cytokines in these two tissue sites is unclear but suggests that evaluation of T cell cytokine production in the spleen, is not necessarily representative of cytokine production at sites of demyelination.

Adhesion molecules and their respective ligands represent an attractive therapeutic target for demyelinating disease. Inhibition of effector lymphocyte and phagocytic cell trafficking into the CNS by inhibiting adhesion molecule interactions provides significant protection from clinical disease as reported in animal models and clinical trials (Bullard et al., 2005; Cannella et al., 1993; Engelhardt, 2006; Gordon et al., 1995; Miller et al., 2003; Nataf et al., 2000; Welsh et al., 1993; Willenborg et al., 1996). Although this strategy has proven effective for many adhesion molecules, it is not uniformly effective. For example, deletion of E and P selectins and PSGL-1 has no effect on the outcome of EAE (Brocke et
al., 1999; Engelhardt, 1997; Engelhardt et al., 2005; Osmers et al., 2005). The failure to protect against demyelinating disease through targeted inhibition of these molecules is likely due to redundancy of function for some family members through the utilization of unexpected receptor/ligand pairs or signaling pathways (Fagerholm et al., 2004; Hynes, 2002; Richter et al., 1990). Which of these possibilities account for the results we report here for CD11d remains to be fully explored. Our results support growing evidence that $\beta_2$-integrins participate in unique rather than highly overlapping functions and indicate that successful therapeutic targeting even of closely related family members might be disease-specific.

ACKNOWLEDGEMENTS

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REFERENCES


Figure 1. The clinical course of MOG-induced EAE in wild-type mice and CD11d−/− mice is identical. Active EAE was induced and symptoms were scored as described in the materials and methods. Results shown are the daily mean clinical scores for wild type (n=11) and CD11d−/−.
Figure 2. Leukocyte subsets in spinal cord of control and CD11d\textsuperscript{-/-} deficient mice with EAE are identical. Leukocytes isolated from spinal cords of control and CD11d\textsuperscript{-/-} mice as described in Materials and Methods were immunostained for CD4, CD8 and CD45. A) The level of CD45\textsuperscript{+} cells infiltrating the spinal cords of wild type (n=5) and CD11d\textsuperscript{-/-} (n=6) mice 10 days post-immunization are identical. B) The percentage of CD4\textsuperscript{+} T cells in the spinal cords of wild type and CD11d\textsuperscript{-/-} mice at days 10 (n=5 and 6, respectively) and 21 (n=4 and 5, respectively) post-immunization were nearly identical (upper left quadrant in each panel). The percentage of CD8\textsuperscript{+} T cells is modestly elevated in CD11d\textsuperscript{-/-} mice at day 21 post-immunization compared to wild type mice (lower right quadrant). The results shown are from cells pooled within each group of mice and a representative experiment is shown.
Figure 3. Intracellular levels of TNF-α and IFN-γ are identical in spinal cord-derived CD4+ T cells from wild type and CD11d−/− mice with active EAE. CD4+ T cells were isolated from the spinal cords of wild type and CD11d−/− mice at 10 (n=5 and 6, respectively) and 21 days (n=4 and 5, respectively) post-immunization and stained for intracellular TNF-α and IFN-γ as described in Materials and Methods. IFN-γ levels were modestly elevated at day 10 in CD11d−/− mice compared to wild type. Cytokine levels were similar for both groups at day 21. The results shown are from cells pooled within each group of mice.
FIGURE 4. CD11d^- T cells proliferate comparably to wild-type T cells. Encephalitogenic T cells enriched by nylon wool adherence from the spleens of wild type (n=4) or CD11d^- (n=4) mice with active EAE were co-cultured with irradiated splenic APCs plus MOG peptide. Cells were pulsed with [3H]thymidine and harvested at 18 h for determination of radioisotope incorporation. The results shown are from two experiments and are expressed as the mean ± SEM of fold induction of T cell proliferation relative to background proliferation.
FIGURE 5. The cytokine repertoire of CD11d<sup>−/−</sup> T cells is not significantly altered compared to wild-type T cells during EAE. Encephalitogenic T cells enriched by nylon wool adherence from the spleens of wild-type (n=6) or CD11d<sup>−/−</sup> (n=6) mice with active EAE were co-cultured with irradiated splenic APCs and restimulated with MOG peptide. Supernatants were collected 48h after stimulation and assayed by ELISA to quantitate production of each cytokine. The production of each cytokine for both CD11d<sup>−/−</sup> and wild type mice is presented as the mean ± S.D. in pg/ml. The data are the mean of two experiments.
**Table 1.** EAE Symptoms in Wild Type and CD11d-Deficient Mice

<table>
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<th>CD1&lt;sup&gt;A&lt;/sup&gt;</th>
<th>Disease Onset&lt;sup&gt;B&lt;/sup&gt;</th>
<th>Disease Incidence&lt;sup&gt;C&lt;/sup&gt;</th>
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<tr>
<td>CD11d&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>45.6</td>
<td>14d</td>
<td>100</td>
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<td>n=11</td>
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<sup>A</sup>Cumulative Disease Index is the mean of the sum of daily clinical scores observed between days 7 and 29.

<sup>B</sup>Disease onset is defined as the first day of two consecutive days with a clinical score of two or more.

<sup>C</sup>Disease incidence is defined as the percent of mice that displayed any clinical signs of disease.

**Table 2.** Changes in Expression of β<sub>2</sub>-Integrins on T Cells and Macrophages in CD11d-Deficient Mice During EAE

<table>
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<th>CD11c</th>
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<td>T cells</td>
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<td>Spleen (CD11d&lt;sup&gt;−/−&lt;/sup&gt;)</td>
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<td>Lymph node (WT)</td>
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<td>Lymph node (CD11d&lt;sup&gt;−/−&lt;/sup&gt;)</td>
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<td>Macrophages</td>
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<td>Spleen (CD11d&lt;sup&gt;−/−&lt;/sup&gt;)</td>
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<td>Lymph nodes (CD11d&lt;sup&gt;−/−&lt;/sup&gt;)</td>
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<td>2.0 ± 0.9</td>
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<sup>A</sup>Data are presented as the mean ± S.D. from three mice.

<sup>B</sup>Significantly different compared to spleens from CD11d<sup>−/−</sup> mice, p=0.02.

<sup>C</sup>Significantly different compared to spleens from CD11d<sup>−/−</sup> mice, p=0.01.
γδ T Cells in Experimental Autoimmune Encephalomyelitis: Early Trafficking Events and Cytokine Requirements

by

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ABSTRACT

γδ T cells play an important role in the development of experimental autoimmune encephalomyelitis (EAE), the animal model of multiple sclerosis. We have previously shown that these cells traffic to the CNS during EAE with concurrent increased expression of β2-integrins and production of IFN-γ and TNF-α. To extend these studies, we transferred bioluminescent γδ T cells to wild type mice and followed their movement through the acute stages of disease. We found that γδ T cells rapidly migrated to the site of myelin oligodendrocyte glycoprotein (MOG) peptide injection and underwent massive expansion. Within six days after EAE induction, bioluminescent γδ T cells were found in the spinal cord and brain, peaking in number between days ten and twelve and then rapidly declining by day fifteen. Reconstitution of γδ T cell−/− mice with γδ T cells derived from β2-integrin-deficient mice (CD11a, -b or -c) demonstrated that γδ T cell trafficking to the CNS during EAE is independent of this family of adhesion molecules.

We also examined the role of γδ T cell-produced INF-γ and TNF-α in EAE and found that production of both cytokines by γδ T cells was required for full development of EAE. IFN-γ in particular was critical for disease since its absence resulted in significantly delayed and attenuated EAE. These results indicate that γδ T cells are critical in setting the stage for the development of EAE and suggest a therapeutic target in demyelinating disease.

INTRODUCTION

γδ T cells are one of several T cell subsets that contribute to the development of experimental autoimmune encephalomyelitis (EAE), a T cell-mediated autoimmune
disease of the central nervous system that mimics many aspects of the human disease multiple sclerosis (MS) (1-3). Cellular infiltration of the brain and spinal cord by several leukocyte subsets, including γδ T cells (4-8), is a characteristic feature of both EAE and MS (1, 3). Although it has been appreciated for some time that γδ T cells produce cytokines that contribute to the pro-inflammatory milieu (9-15) and express adhesion molecules that may be critical for initial priming, trafficking to, and infiltration of the CNS (15-17), the significance of their contribution to demyelinating disease remains controversial.

γδ T cells are considered innate immune T cells by virtue of their limited T cell receptor repertoire, tissue-specific homing patterns, and recognition of non-traditional T cell antigens (18-22). Activation of γδ T cells occurs on presentation of phosphoantigens, WC1 molecules or self-antigens by non-classical MHC molecules with cytokines and TLRs providing co-stimulation (23-31). Regardless of the priming event(s), trafficking mechanisms employed by γδ T cells in demyelinating disease remain ill-defined. Studies have implicated VLA-4 as a participant in γδ T cell adhesion to endothelium, epithelium or fibroblasts, and in transmigration (16, 17, 32, 33), but none have implicated VLA-4 in migration of γδ T cells to the CNS. In contrast, γδ T cells express all four members of the β2-integrin family of adhesion molecules and expression increases through the course of MOG-induced EAE (15). Importantly, deletion of three of the four β2-integrins (CD11a-c) results in significantly attenuated disease (34-38), implicating, but not directly proving, a role for these adhesion molecules in γδ T cell trafficking into the CNS during disease. Cytokine-mediated modulation of demyelinating disease by γδ T cells, either in humans or animal models, although the subject of numerous studies, also remains controversial.
Early studies examined the contribution of γδ T cell-produced cytokines and chemokines after antibody-mediated depletion of γδ T cells (10, 11) and implicated these cells in the production of TNF-α, IFN-γ, IL-1, IL-6, IL-12 and several others. Although informative, these studies could not directly attribute cytokine production to γδ T cells as they used spinal cord homogenate to analyze cytokine levels. In addition, γδ T cell depletion was not monitored over the disease course in these studies, raising the possibility of undefined levels of γδ T cell-mediated cytokine production as the disease progressed. Other studies have shown that γδ T cells producing IFN-γ and IL-4 are significantly elevated during EAE (12). Similar results were reported by Gao and colleagues (39), however in this study it was shown that in both the spleen and CNS of normal mice, there were substantial numbers of CD3+ and γδ T cells secreting both IFN-γ and IL-4. Since the CNS of normal mice is usually devoid of any lymphocyte subset, and particularly of γδ T cells, the meaning of these results is unclear. More recent studies have indicated that γδ T cells act in an antigen-independent fashion to modulate cytokine production (IL-12 and IFN-γ) and thus the early effector phase of the immune response in EAE (13, 14). Ponomarev and colleagues have suggested that the immunomodulatory effect of γδ T cells in EAE is independent of their ability to produce IFN-γ (14). In this report, we examine the trafficking of bioluminescent γδ T cells in the inductive and acute phases of EAE as well as the requirement for β2-integrins in trafficking. We observed that γδ T cells rapidly migrate to and expand at the site of MOG peptide injection. By day six after EAE induction, bioluminescent γδ T cells were found in the brain and spinal cord, however, by day fifteen the CNS was largely devoid of γδ T cells. Reconstitution of γδ T cell−/− mice with γδ T cells derived from β2-integrin-deficient mice demonstrated that γδ T cell
trafficking to the CNS is independent of this family of adhesion molecules. In addition, we examined the role of INF-γ and TNF-α production by γδ T cells with respect to the development of EAE. We found that production of both cytokines by γδ T cells was required for fulminant EAE and that disease was significantly delayed and attenuated when mice were reconstituted with IFN-γ−/− γδ T cells. These results indicate that γδ T cells are critical in setting the stage for the development of EAE and may offer an unforeseen therapeutic target in demyelinating disease.

MATERIALS AND METHODS

Mice

Tcrdtm1Mom mice, deficient in γδ T cells, were obtained from Jackson laboratories. TNF-α−/− and IFN-γ−/− mice were generous gifts from Drs. David Chaplin and Alan Zajac (Department of Microbiology, University of Alabama at Birmingham), respectively. CD11a−/−, CD11b−/−, and CD11c−/− mice have been previously described (40-42). For all studies, we used the β2-integrin deficient mice at an N16 backcross onto C57BL/6. The luciferase transgenic mouse line (T-lux), expressing firefly luciferase under the control of the human CD2 promoter, was generated in the C57BL/6 background as previously described (43). T-lux mice express luciferase activity in all CD3+ cells and bioluminescence generated by this enzyme is directly proportional to the number of cells expressing the gene, allowing real-time assessment of T cell proliferation and migration in vivo. For some experiments, LFA-1 deficient T-lux transgenic mice were generated by intercrossing the CD11a−/− and T-lux mice. Inbred T-lux transgenic or non-transgenic
C57BL/6 mice were used as controls for all experiments. All studies were performed with approval from the UAB IACUC.

**Bioluminescent Imaging**

Mice were subjected to bioluminescent imaging as previously described (43). Briefly, mice were anesthetized with isofluorane and placed in a light-tight chamber. The photographic (gray-scale) reference image was obtained at 10 minutes after D-luciferin injection; the bioluminescent image was collected immediately thereafter. Images were obtained with a CCD camera cooled to –120°C, using the IVIS Imaging System (Xenogen Corp., Alameda, CA) with the field of view set at 10 cm height. The photographic images were taken at a 0.2 second exposure, 8 f/stop, 2 binning (resolution), and an open filter. The bioluminescent images used exposures of 600 seconds, 1f/stop, 16 binning and open filter. The bioluminescent and gray-scale images were overlaid using Living Image software (Xenogen Corp.). Igor image analyses software (Wavemetrics, Lake Oswego, OR) was employed to obtain a pseudocolor image representing bioluminescence intensity (blue, least intense; red, most intense). The total counts were normalized to image acquisition. Ex vivo images were obtained by removal of brain and spinal cord and imaging at 3X magnification after treatment with the Luciferase Assay system (Promega).

**Flow Cytometry and Intracellular Cytokine Staining**

Cells obtained from draining lymph nodes, spleens, and spinal cords were incubated with anti-CD16/32 (FcR block, eBioscience) to prevent nonspecific staining. Cells were incubated for 30 minutes in the dark at 4°C with anti-γδ TCR FITC (GL3, BD Pharmingen). All antibodies were diluted in FACS buffer (1X PBS, 2% FCS, 0.1% NaN3). Immunofluorescence analyses were performed using a FACSCalibur and
CellQuest software (BD Biosciences).

Reconstitution

γδ T cells were isolated from spleen, lymph nodes, and thymus of wild type, T-lux, CD11a−/−, LFA-1−/−/T-lux, CD11b−/−, CD11c−/−, TNF-α−/−, and IFN-γ−/− mice using the magnetic bead isolation kits from Miltenyi Biotech. γδ T cells (5 x 10⁵, > 90% pure) were injected retro-orbitally into γδ T cell−/− mice. Active EAE was induced as described below two days after reconstitution.

Active EAE

For active EAE, control, γδ T cell deficient, and reconstituted mice were immunized with MOG peptide35-55 (Biosynthesis, Lewisville, TX) as described (34). Onset and progression of EAE were monitored daily using a standard clinical scale ranging from 0 to 6 as follows: 0, asymptomatic; 1, loss of tail tone; 2, flaccid tail; 3, incomplete paralysis of one or two hind limbs; 4, complete hind limb paralysis; 5, moribund; and 6, death. Only mice with a score of at least a 2 (flaccid tail) for more than 2 consecutive days were judged to have EAE. For each animal a cumulative disease index was calculated from the sum of the daily clinical scores observed between day 0 and day 30.

Statistics

Statistical significance for the bioluminescent images was measured using Anova with Tukey’s post test. Clinical disease courses were analyzed using Wilcoxon Signed Rank test and disease onset was analyzed using the Student’s t-test.

RESULTS

Trafficking of γδ T Cells Prior to Disease Onset and in Acute EAE
We induced EAE in γδ T cell+ mice reconstituted with T-lux γδ T cells and performed in vivo bioluminescent imaging to visualize trafficking of γδ T cells before onset of disease symptoms. γδ T cells were found predominantly in the gut but could also be seen in various lymph nodes, primarily the cervical lymph nodes almost immediately after transfer as seen in ventral imaging of the mice (Figure 1A, upper panel). By day three of active EAE, the γδ T cells began accumulating in the cervical lymph nodes and the spleen and underwent expansion. Dorsal imaging of the mice revealed that γδ T cells rapidly traffic to the site of MOG injection (Figure 1A, lower panel). By day three after induction γδ T cells accumulated at both injection sites and underwent continuous expansion through day nine post disease induction. Quantification of the images shows a significant increase in signal on the dorsal view of the mice when compared to the ventral view on days eight and nine of EAE suggesting little to no expansion of γδ T cells in the gut or other peripheral lymphoid tissues (Figure 1B). Quantification of bioluminescent signal solely from the injection sites indicates that the increased dorsal bioluminescent signal was due largely to expansion of the γδ T cells in this location (Figure 1C). To further characterize the trafficking patterns of γδ T cells during EAE, we performed ex vivo imaging of spinal cord and brains from γδ T cell+ mice reconstituted with T-lux γδ T cells at several time points after disease induction. γδ T cells were seen initially in the sacral region of the spinal cord at day 6 and, as disease progressed, the cells were found through out the spinal cord (Figure 2A). Similarly brain infiltration was observed starting at day six post EAE induction and, by day nine, γδ T cells fully infiltrated the brain (Figure 2B). γδ T cell infiltration into the brain peaks at day 12, since by day 15, γδ T cells could not be detected by bioluminescent imaging (Figure 2B and data not shown).
These results are supported by flow cytometric data in which spinal cords were isolated at multiple time points post EAE induction and assessed for γδ T cell infiltration (Figure 2C). We observed a peak in γδ T cell infiltration between days 10 and 12 that matches the bioluminescent imaging data followed by a rapid decline.

**Trafficking of LFA-1−/− γδ T Cells in EAE**

To determine if LFA-1 was critical to the trafficking of γδ T cells during EAE, *in vivo* bioluminescent images of actively induced γδ T cell deficient mice reconstituted with either wild type T-lux or LFA-1−/−/T-lux γδ T cells were taken at various time points post-EAE induction (Figure 3A). Ventral images showed no difference in localization of wild type and LFA-1−/− γδ T cells in the gut and the cervical lymph nodes of mice with active EAE (data not shown). Dorsal imaging demonstrated that wild type and LFA-1−/− γδ T cells had localized and expanded at the site of immunization by day seven of active EAE (Figure 3A, upper panel), similar to the results shown in figure 1. By day nine of EAE and continuing through day eleven, wild type γδ T cells expanded throughout the dorsal region of the mouse and began to localize in the brachial lymph nodes and the brain. In contrast, at the same time points LFA-1−/− γδ T cells left the site of immunization and did not continue expanding (Figure 3A, lower panel). Quantitation of bioluminescent signal from the images verified that wild type and LFA-1−/− γδ T cell localization and expansion was similar on day nine but thereafter LFA-1−/− γδ T cell bioluminescent signaled decreased while the wild type signal remained high until day 13 of EAE (Figure 3B). These data suggest that γδ T cells require LFA-1 for retention at a priming site and that LFA-1 is required, directly or indirectly, for γδ T cell co-stimulation.
**γδ T Cells do not Require β2-Integrins for the Development of EAE**

It has been previously shown that γδ T cell\(^{-/-}\) mice develop significantly delayed and attenuated disease compared to wild type mice (Spahn et al., 1999) and we confirmed these original observations. In our hands EAE in γδ T cell\(^{-/-}\) mice was significantly delayed (p=0.04, Wilcoxon sign rank test) and attenuated (p<0.0001, Wilcoxon sign rank test) (Figure 4A, Table 1). Furthermore, when γδ T cell\(^{-/-}\) mice are reconstituted with wild type γδ T cells EAE mirrored that of wild type mice. Reconstituted γδ T cell\(^{-/-}\) mice developed EAE with a clinical severity closely approximating wild type disease (CDI 54.8 vs. 59.8, respectively) (Figure 4A, Table 1). To determine if expression of β2-integrins on γδ T cells is critical to the development of clinical disease we reconstituted γδ T cell\(^{-/-}\) mice with CD11a\(^{-/-}\), CD11b\(^{-/-}\), or CD11c\(^{-/-}\) γδ T cells, induced EAE and monitored mice for disease symptoms. We found that reconstitution with γδ T cells deficient in the various β2-integrin α-chains resulted in disease severity comparable to reconstitution with wild type γδ T cells. A representative example of the EAE disease course after reconstitution with CD11a\(^{-/-}\) γδ T cells is shown in Figure 4B. Similar results were obtained on reconstitution with CD11b\(^{-/-}\) and CD11c\(^{-/-}\) γδ T cells (data not shown). Overall EAE parameters for reconstitution with CD11a-CD11c\(^{-/-}\) γδ T cells are shown in Table 2. These data indicate that β2-integrins on γδ T cell are not critical to the development of EAE, at least in the experimental setting we employed. These results contrast sharply with the role of β2-integrins on αβ T cells in EAE (34-36, 38).

**INF-γ and TNF-α Produced by γδ T Cells are Critical for full Development of EAE**

Several studies have shown that γδ T cells produce IFN-γ and TNF-α in the spinal
cord during EAE (12-15, 39). To determine if this production is critical to the
development of EAE we reconstituted γδ T cell−/− mice with IFN-γ−/− or TNF-α−/− γδ T
cells. Reconstitution with IFN-γ−/− γδ T cells resulted in a significantly reduced
(p<0.0001, Wilcoxon sign rank test) and delayed (p=0.04, unpaired T-test) clinical
disease course compared to reconstitution with wild type γδ T cells (Figure 5A). The
cumulative disease index and incidence rate was markedly lower in the IFN-γ−/−
reconstituted mice than in mice reconstituted with wild type γδ T cells (Table 3).
Reconstitution with TNF-α−/− γδ T cells also failed to induce fulminant EAE (Figure 5B,
p<0.0004, Wilcoxon sign rank test), with a similar reduction in cumulative disease
index and incidence rate compared to mice reconstituted with wild type γδ T cells
(Table 3). These data indicate that IFN-γ and TNF-α production by γδ T cells is
critical for the development of severe EAE, particularly in the chronic phase of
disease.

DISCUSSION

The results we report here indicate that γδ T cells play a critical role early in the
development of EAE. We visualized γδ T cell trafficking in vivo during active EAE
using bioluminescence and observed that γδ T cells rapidly accumulated at the site of
MOG peptide immunization after disease induction and underwent massive
expansion between days three and six. Based on imaging, γδ T cells did not migrate
to and expand in the spleen and other secondary lymphoid organs to any significant
degree, with the exception of the cervical lymph nodes at day three and the brachial
lymph nodes at day nine. These data demonstrate that γδ T cells, like CD4+ T cells
(44-47), drain to cervical nodes and raise the possibility that brachial lymph nodes
may also serve as a draining lymph node for the CNS, at least for γδ T cells. Expansion of γδ T cells continued at the site of MOG injection up to day eleven, however, by day thirteen there was marked decline in expansion. Interestingly, we observed similar but delayed kinetics of γδ T cell trafficking to the CNS. γδ T cells entered the sacral region of the spinal cord and the cerebellum of the brain by day six, trafficked throughout the parenchyma of the spinal cord and brain peaking between days ten and twelve and then rapidly declined by day fifteen in all tissues. These kinetics are consistent with γδ T cells playing an important role in demyelinating disease well before clinical signs of disease and when few CD4 and CD8 T cells have reached the CNS (15), most likely in priming events important for acute disease development.

The dramatic expansion of γδ T cells at the site of MOG peptide injection raises questions regarding priming events for these cells in demyelinating disease. Proteins from mycobacterium tuberculosis (MT), a component of the adjuvant used to induce EAE, are potent antigens for γδ T cells (48). More recently it has been shown that the expansion of γδ T cells seen after the induction of experimental autoimmune uveitis can be attributed to the MT in the adjuvant and the pertussis toxin injected at the time of immunization (31). Taken together, these observations suggest that the MT and pertussis toxin used to induce EAE may be in large part responsible for the localization, activation, and proliferation of the γδ T cells, at the site of immunization. However, the rapid entry of γδ T cells into the CNS suggests that some of the γδ T cells expanding at the immunization site are antigen-specific and directed toward the MOG35-55 peptide. Antigen-specific CD4+ T cells have also
been shown to migrate to MOG peptide injection sites (49). However in this model system MOG-specific T cells were adoptively transferred into mice with an ongoing *Mycobacterium bovis* infection making it difficult to determine if CD4+ T cells normally traffic to the MOG emulsion. In our hands, naïve αβ T cells did not traffic to the injection site (data not shown) suggesting that the trafficking observed by Sewell and colleagues is activation-dependent or, given the nature of their model system, that γδ T cells are unique among T cells subsets in trafficking to the MOG injection site.

The β2-integrin family of adhesion molecules plays an important role in the development of EAE (34-36, 38). Deletion of CD11a, CD11b, or CD11c results in significantly delayed and attenuated disease due, in part, to reduced trafficking of T cells to the CNS. It has recently been shown that β2-integrins are differentially expressed on αβ and γδ T cells throughout EAE (15), but it is unclear how deletion of any one of the β2-integrins on γδ T cells contributes to disease development and progression. To answer this question we performed reconstitution experiments in which γδ T cells, deficient in CD11a, CD11b, or CD11c, were transferred to γδ T cell−/− mice. To our surprise all of the β2-integrin mutant γδ T cells were able to restore disease severity comparable to that seen in reconstitutions using wild type γδ T cells. These data indicate that the disease phenotypes seen in β2-integrin-deficient mice during EAE are independent of γδ T cell mediated expression of β2-integrins. Previous studies have demonstrated that anti-LFA-1 antibodies prevented the adhesion of γδ T cells to endothelial monolayers in vitro (16, 17). Such studies do not however sufficiently replicate in vivo events during EAE and specific targeting of γδ T
cells with anti-LFA-1 antibodies during disease would be technically challenging. Our bioluminescent data clearly demonstrated that LFA-1−/− γδ T cells trafficked to the sites of MOG injection and expanded in a fashion comparable to that of wild type γδ T cells. Despite these initial similarities, LFA-1−/− γδ T cells were either not retained at the MOG injections sites or had altered proliferation kinetics compared to wild type γδ T cells based on the rapid clearance (compared to wild type γδ T cells) from injections site. Although these data are consistent with activation deficits inLFA-1−/− γδ T cells, this seems unlikely since the clinical course of disease was not changed relative to wild type γδ T cells. We cannot rule out the possibility that loss of a given β2-intergrin on γδ T cells is compensated for by other family members and/or that other adhesion molecules are utilized by γδ T cells for migration to priming sites and to the CNS. Our results suggest that a likely candidate for γδ T cell trafficking to the CNS may be VLA-4, based on studies in several inflammatory model systems (16, 17, 33, 50-52).

IFN-γ is produced in the CNS during EAE by infiltrating T cells and thought to help drive early stages of the disease (53, 54). From a therapeutic point of view, IFN-γ is considered central to pathogenic and inflammatory mechanisms based on the results of clinical trials in which IFN-γ exacerbated disease (55, 56). However, IFN-γ may also help to regulate inflammation since IFN-γ−/− mice develop exacerbated, sometimes fatal EAE, during the chronic phase of disease (57-60). Previous studies have suggested that IFN-γ production by γδ T cells is not critical to disease development and progression; γδ T cells were required solely for induction of IFN-γ by CD4 and CD8 T cells (14). Evidence supporting this potential mechanism was
derived from quantitation of mRNA levels for IFN-γ in bone marrow chimeric mice 
reconstituted with γδ T cells derived from wild type and IFN-γ−/− mice. Interpretation 
of these studies is complicated by the fact that IFN-γ protein levels were not 
assessed, nor was the disease course monitored to allow correlation to IFN-γ 
production. In contrast, we found that the production of IFN-γ by γδ T cells is critical 
to EAE development. Reconstitution studies using congenic C57BL/6 mice (as 
opposed to mixed B6.129 x B10.PL backgrounds) clearly demonstrated that 
IFN-γ production by γδ T cells contributes to disease onset and development. We 
cannot determine from our studies if γδ T cell-mediated IFN-γ production modulated 
IFN-γ production by αβ T cells, but previous studies from our laboratory and others 
have demonstrated that before and during the acute phase of EAE, the majority of γδ 
T cells (70% or more) produce IFN-γ, while αβ T cells produce significantly less (14, 
15). These data suggest that IFN-γ production by γδ T cells may be central to initial 
inflammatory events during EAE.

TNF-α has also been shown to be an important cytokine in MS and EAE 
pathogenic mechanisms. TNF-α−/− mice are protected from EAE and transgenic mice 
expressing TNF-α in the CNS develop spontaneous neurodegenerative disease 
characterized by cellular inflammation and neuronal damage (61-64). These studies 
did not establish cellular subset production of TNF-α or correlate the corresponding 
functional role of TNF-α to that cell type in EAE. Likewise, in studies where γδ T cells 
were depleted by antibody treatment, TNF-α levels were markedly reduced, but 
which cells were producing TNF-α and how production by those cells modulated the 
disease course was not addressed (11). To overcome the technical limitations of γδ T
cell depletion, we performed reconstitution studies in which γδ T cell−/− mice were
given TNF-α−/− γδ T cells and observed that TNF-α production by γδ T cells modulated
the chronic phase of EAE. The disease phenotype in these reconstitutions was more
severe than that observed for IFN-γ−/− γδ T cell reconstitutions suggesting that TNF-α
production by γδ T cells is not as crucial as IFN-γ to disease development and
progression. Nonetheless, γδ T cells in the spinal cord during EAE are a significant
source of TNF-α production, perhaps even the most significant source of this
cytokine on a per cell basis (15). These results raise the possibility that TNF-α
additively or synergistically acts in concert with IFN-γ to modulate early disease
development. γδ T cells are innate-like lymphocytes and it has been proposed that
they may provide a link between the innate and adaptive immune response. It has
recently been shown that activated murine γδ T cells can present MOG35-55 peptide to
naïve CD4 T cells (31). γδ T cells also interact with dendritic cells driving their
maturation through TNF-α and increasing IL-12 production by dendritic cells
through IFN-γ-mediated mechanisms (13, 65-67). Antibody depletion of γδ T cells in
EAE results in delayed cytokine and chemokine production in the CNS (10, 11),
possibly by hindering the generation of the adaptive immune response normally
augmented by γδ T cells. Our data suggest that local production of IFN-γ and/or
TNF-α is important for dendritic cell maturation or programming which aides in
initiating an immune response to myelin antigens. Concurrently, γδ T cells are also
entering the CNS, and in situ production of IFN-γ and TNF-α may activate APCs in the
CNS and prime the microenvironment for CD4+ and CD8+ T cell recruitment and
activation. In this scenario, γδ T cells play a critical role in disease initiation that has
largely been overlooked both mechanistically and therapeutically. Deletion of γδ T cells in demyelinating disease may be advantageous in that the αβ T cell compartment would remain unaffected, thereby reducing the risk of therapeutically-induced immunodeficiencies.

ACKNOWLEDGEMENTS

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REFERENCES


50. Avdalovic, M., D. Fong, and B. Formby. 1993. Adhesion and costimulation of proliferative responses of human gamma delta T cells by interaction of VLA-4 and


61. Probert, L., K. Akassoglou, G. Kassiotis, M. Pasparakis, L. Alexopoulou, and G.


Figure 1. Bioluminescent imaging of early trafficking events of γδ T cells during active EAE. γδ T cell deficient mice were reconstituted with T-lux γδ T cells as described in materials and methods. EAE was induced and imaging was performed as described in materials and methods daily from day 0 to 9. (A) Each panel shows a representative ventral or dorsal image from one mouse at the indicated time points. Pseudo-color scale is shown to emphasize individual organ structures and areas in which γδ T cells have accumulated. Arrows indicate cervical and inguinal lymph nodes on ventral images. Arrows on dorsal images indicate spleen and the two sites of MOG peptide immunization. Quantification of bioluminescent signal from the whole body in ventral (n = 5, filled circles) and dorsal (n = 5, open circles) images (B) and the sites of injection in the dorsal images (C) are shown in counts/sec. The dorsal images had significantly (p <0.05) more bioluminescent signal on days 8 and 9 of EAE as measured by Anova.
FIGURE 2. Bioluminescent imaging of γδ T cells in the brain and spinal cord during EAE. Mice deficient in γδ T cells were reconstituted with T-lux γδ T cells as described in materials and methods. Active EAE was induced in reconstituted mice and ex vivo bioluminescent images of the spinal cord (A, n = 3) and brain (B, n = 3) at indicated days were taken. The lower panel in B is the same brain sectioned at midline to show bioluminescent γδ T cells in the parenchyma and cerebellum. Representative images with a pseudo-color scale are shown to emphasize areas in which γδ T cells have accumulated. (C) Representative histograms show γδ (bold line) T cells in the spinal cord of EAE-induced mice 6, 10, 12, 15 days post immunization. Percentages of positive cells are indicated in each histogram. Cells were pooled from at least 4 mice per experiment and data shown is representative of at least 3 independent experiments. Day 6 (n=5) are from one experiment. The gray area represents control staining.
FIGURE 3. Bioluminescent imaging of wild type and LFA-1⁻/⁻ γδ T cells during EAE. γδ T cell deficient mice were reconstituted with T-lux and LFA-1⁻/⁻/T-lux γδ T cells as described in materials and methods. EAE was induced and imaging was performed as described in materials and methods. Representative dorsal (A) images from individual mice are shown from the indicated time points. A Pseudo-color scale is shown to highlight organ structures and areas in which the γδ T cells have accumulated. Arrows indicate sites of immunization and brachial lymph nodes. Bioluminescence from the whole body of dorsal images (B) was quantitated (WT filled circles, n = 4 and LFA-1⁻/⁻ open circles, n = 4).
Figure 4. The clinical course of active EAE in γδ T cell−/− mice reconstituted with wild type or LFA-1−/− γδ T cells is comparable to that of control mice. (A) γδ T cell deficient mice were reconstituted with wild type γδ T cells and active EAE was induced in reconstituted (open circles, n = 19), γδ T cell−/− (open squares, n = 22), and control mice (filled circles, n = 20). Clinical disease was monitored for 30 days as described in materials and methods. Mice deficient in γδ T cells had a significantly reduced clinical disease course compared to control mice (p<0.0001, Wilcoxon signed rank test), while there was no significant difference in EAE between control and γδ T cell−/− mice reconstituted with wild type γδ T cells. (B) γδ T cell−/− mice were reconstituted with wild type γδ T cells or LFA-1−/− γδ T cells and EAE was induced. The course of disease in wild type (filled circles, n=4) and LFA-1−/− γδ T cell (open circles, n=4) reconstituted mice was not significantly different. Where not visible errors bars are contained within the symbol.
**Figure 5.** IFN-γ and TNF-α produced by γδ T cells are critical to clinical disease severity in active EAE. (A) γδ T cell−/− mice were reconstituted with wild type (n = 7, filled circles), or IFN-γ−/− γδ T cells (n=9, open circles), active EAE was induced, and signs of disease monitored for 30 days as described in materials and methods. Disease onset was significantly delayed (p=0.046; unpaired t-test) and overall severity was reduced (p=0.0001; Wilcoxon sign rank test) on reconstitution with IFN-γ−/− γδ T cells (B) Same as A except γδ T cell−/− mice were reconstituted with wild type (filled circles, n=5) or TNF-α−/− γδ T cells (open circles, n=5) and followed by active EAE induction. Disease severity was significantly lower in γδ T cell−/− mice reconstituted with TNF-α−/− γδ T cells (p=0.0004. Wilcoxon sign rank test). Where not visible errors bars are contained within the symbol.
### TABLE 1
EAE in γδ T Cell Deficient Mice Reconstituted With Wild Type γδ T Cells

<table>
<thead>
<tr>
<th></th>
<th>Onset(^a)</th>
<th>CDI(^b)</th>
<th>Incidence(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (n=20)</td>
<td>12.5d</td>
<td>59.8</td>
<td>100%</td>
</tr>
<tr>
<td>γδ TCR(^{-/-}) (n=22)</td>
<td>13.4d</td>
<td>36.8</td>
<td>91%</td>
</tr>
<tr>
<td>γδ TCR(^{-/-}) + wild type γδ T cells (n=25)</td>
<td>13.1d</td>
<td>54.8</td>
<td>100%</td>
</tr>
</tbody>
</table>

\(^a\) Disease onset is the first of 2 consecutive days with a clinical score of 2 or more.

\(^b\) Cumulative disease index is the mean sum of daily clinical scores from day 0 to 30.

\(^c\) Incidence is defined as the percent of mice that displayed any signs of clinical disease.

### TABLE 2
EAE in γδ T Cell Deficient Mice Reconstituted With β2-Integrin Ceficient γδ T Cells

<table>
<thead>
<tr>
<th></th>
<th>Onset(^a)</th>
<th>CDI(^b)</th>
<th>Incidence(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>γδ TCR(^{-/-}) + wild type γδ T cells (n=4)</td>
<td>12.5d</td>
<td>55.6</td>
<td>100%</td>
</tr>
<tr>
<td>γδ TCR(^{-/-}) + CD11a(^{-/-}) (n=4)</td>
<td>11.7d</td>
<td>59.3</td>
<td>100%</td>
</tr>
<tr>
<td>γδ TCR(^{-/-}) + wild type γδ T cells (n=6)</td>
<td>13.8d</td>
<td>47</td>
<td>100%</td>
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<tr>
<td>γδ TCR(^{-/-}) + CD11b(^{-/-}) (n=9)</td>
<td>11.5d</td>
<td>48</td>
<td>100%</td>
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<tr>
<td>γδ TCR(^{-/-}) + wild type γδ T cells (n=8)</td>
<td>14.4</td>
<td>47.8</td>
<td>100%</td>
</tr>
<tr>
<td>γδ TCR(^{-/-}) + CD11c(^{-/-}) (n=5)</td>
<td>13.6d</td>
<td>49.5</td>
<td>100%</td>
</tr>
</tbody>
</table>

\(^a\) Disease onset is the first of 2 consecutive days with a clinical score of 2 or more.

\(^b\) Cumulative disease index is the mean sum of daily clinical scores from day 0 to 30.

\(^c\) Incidence is defined as the percent of mice that displayed any signs of clinical disease.
<table>
<thead>
<tr>
<th></th>
<th>Onset</th>
<th>CDI</th>
<th>Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \gamma \delta ) TCR(^{-/-}) + wild type ( \gamma \delta ) T cells (n=5)</td>
<td>14.6d</td>
<td>52.6</td>
<td>100%</td>
</tr>
<tr>
<td>( \gamma \delta ) TCR(^{-/-}) + TNF-( \alpha )^{-/-} (n=5)</td>
<td>14.3d</td>
<td>36.4</td>
<td>80%</td>
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<tr>
<td>( \gamma \delta ) TCR(^{-/-}) + wild type ( \gamma \delta ) T cells (n=7)</td>
<td>13d</td>
<td>62.2</td>
<td>100%</td>
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<tr>
<td>( \gamma \delta ) TCR(^{-/-}) + IFN-( \gamma )^{-/-} (n=9)</td>
<td>14.7d</td>
<td>31.45</td>
<td>78%</td>
</tr>
</tbody>
</table>

\(^{a}\) Disease onset is the first of 2 consecutive days with a clinical score of 2 or more.

\(^{b}\) Cumulative disease index is the mean sum of daily clinical scores from day 0 to 30.

\(^{c}\) Incidence is defined as the percent of mice that displayed any signs of clinical disease.
LFA-1 is Critical for Regulatory T Cell Homeostasis and Function

by

Jillian E. Wohler, Dan C. Bullard, Trent Schoeb, Scott R. Barnum

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Format adapted for dissertation
ABSTRACT

The suppressive mechanisms used by T regulatory cells (Tregs) to control peripheral tolerance remain an active area of research. Cellular adhesion molecules involved in cell-to-cell mediated suppression by Tregs are not well characterized. We found that the majority of Tregs (CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>) expressed LFA-1 and most strikingly, the frequency of Tregs in LFA-1<sup>−</sup> mice was significantly lower (~50%) in the spleen, lymph nodes, and Peyer’s Patches compared to wild type controls. The reduction in Treg populations may in part be due to a reduced capacity of LFA-1<sup>−</sup> CD4<sup>+</sup>CD25<sup>−</sup> cells to be induced to become Tregs in the lymph nodes. Importantly, we found that LFA-1<sup>−</sup> Tregs fail to suppress T cell responses in vitro and in vivo. Treg mediated-suppression does not depend on LFA-1 interactions with ICAM-1 on the surface of responder cells. Our data demonstrate that LFA-1 plays a critical role in regulatory T cell homeostasis and function.

INTRODUCTION

Regulatory T cells (Tregs) are critical for maintaining peripheral tolerance, through their ability to suppress T cells at all stages of an immune response (1-6). Many immunosuppressive mechanisms have been attributed to Tregs including inhibition through cell-to-cell contact, release of soluble factors, competition for growth factors, and others (7-9). Migration of Tregs to sites of inflammation is critical to their function and they express higher levels of CD103, L-selectin, P-selectin, and ICAM-1 than effector T cells (10). Some adhesion molecules, such as CD103 and L-selectin, define Treg subsets based on immunosuppressive function, while others including ICAM-1 and P-selectin, do not appear to be critical to the suppressive mechanisms of Treg cells (11). Although little
is known about cellular adhesion molecules utilized by Tregs for contact-mediated effector T cell suppression, β2-integrins have been implicated in this function. CD18-deficient mice, the common β-chain for each family member, have reduced Treg populations and CD18−/− Tregs fail to suppress T cell responses in vivo (12). However, CD18−/− mice do not express any of the β2-integrins (LFA-1, Mac-1, p150,95, and αD) and thus which family member or members are important for Treg immunosuppression remains unclear. It has recently been shown that LFA-1 is critical to the development and progression of EAE (13, 14), however transfer of wild type encephalitogenic T cells to LFA-1−/− mice, unexpectedly resulted in exacerbated disease (14). These results suggested the possibility of defective Tregs in LFA-1−/− recipients and that LFA-1 expression on Tregs is important for suppression of effector T cells. We now report that LFA-1−/− mice have significantly reduced levels of Tregs (CD4+CD25+Foxp3+) throughout their secondary lymphoid system, but elevated numbers in the thymus. In addition, in vivo conversion efficiency of LFA-1−/− CD4+CD25− T cells to Tregs depends on the presence or absence of LFA-1 in the host. Importantly, LFA-1−/− Tregs are nonfunctional in in vitro suppression assays and poorly immunosuppressive in experimental colitis. These data demonstrate that LFA-1 plays a major role in Treg function and may be a central receptor in cell-to-cell contact mechanisms utilized by Tregs to suppress effector cells.

MATERIALS AND METHODS

Mice

CD45.1 mice were obtained from Jackson labs (Bar Harbor, ME). CD45.2 and RAG1−/− mice were provided by the genomic core facilities at the University of Alabama,
Birmingham. CD11a\(^{-/-}\), CD11b\(^{-/-}\), CD11c\(^{-/-}\), and ICAM\(^{null}\) mice were generated as previously described and are on the C57BL/6 background (backcrossed greater than eight generations) (15-18). C57BL/6 mice were used as controls for all experiments. All studies were performed with approval from the UAB IACUC.

**Flow Cytometry**

Cells obtained from lymph nodes (inguinal, mesenteric and axillary), spleens, blood, thymus, and Peyer’s patches were stained with anti-CD4-FITC and anti-CD25-PE (eBioscience). Cells were permeabilized using the eBioscience regulatory T cell staining kit and then stained with anti-Foxp3-APC. Stained cells were analyzed using a FACSCalibur and data was analyzed using CellQuest software (BD Biosciences).

**Treg Isolation**

CD4\(^{+}\)CD25\(^{+}\) cells were isolated or depleted from thymus, spleens, lymph nodes and Peyer’s patches of wild type, CD11a\(^{-/-}\), CD11b\(^{-/-}\), CD11c\(^{-/-}\), and ICAM\(^{null}\) mice using the Miltenyi regulatory T cell isolation kit, as per the manufacturer’s instructions. Isolated CD4\(^{+}\)CD25\(^{+}\) populations contained greater than 95% Foxp3 expressing cells.

**Treg Suppression Assay**

CD4\(^{+}\)CD25\(^{-}\) responder T cells (5 x 10\(^4\)) from control mice were stimulated in round bottom 96-well plates using anti-CD3 (145-2C11, eBiosciences, 0.75 \(\mu\)g/ml) and CD28 (37.51 BD Biosciences, 4 \(\mu\)g/ml) antibodies along with CD4\(^{+}\)CD25\(^{+}\) Tregs from wild type mice (1 x 10\(^5\)) or LFA-1\(^{-/-}\) mice (1 x 10\(^5\)) as previously described (19). Cells were pulsed after 48 hr with \(^{3}\)H-thymidine (1 \(\mu\)Ci) for an additional 18 hrs, harvested and thymidine incorporation determined.

**Peripheral Treg Generation**
CD4^+CD25^- cells were isolated from wild type and LFA-1^- CD45.2 mice and then retro-orbitally injected (2x10^6) into irradiated (500R) CD45.1 recipients. In some experiments CD45.1 CD4^-CD25^- cells were isolated from wild type mice and retro-orbitally injected (2x10^6) into CD45.2 wild type or LFA-1^- mice. Six weeks later spleens and lymph nodes were harvested and the percentage of CD45.2 or CD45.1 Treg cells were measured using FACS analysis.

Colitis

CD45RB^{hi} and CD45RB^{lo} cells were sorted from CD4^+ cells isolated from spleens and lymph nodes of wild type or LFA-1^- mice with MACS beads. CD4^-CD45RB^{hi} cells (5x10^5) were injected (i.p.) into RAG1^- recipients colonized with *H. hepaticus*. In some experiments wild type or LFA-1^- CD4^-CD45RB^{lo} cells (5x10^5) were co-injected with wild type CD4^-CD45RB^{hi} cells. Six weeks post-injection colons were prepared for histological analysis.

RESULTS AND DISCUSSION

*Altered Treg populations in secondary lymphoid tissues and the thymus*

In recent studies, we found that transfer of wild type encephalitogenic T cells to LFA-1^- recipients resulted in significantly exacerbated EAE compared to wild type-to-wild type transfers (14). T cells in these transfer experiments expanded dramatically and were readily detected in cervical and axillary lymph nodes, spinal cord and brains. These data raised the possibility that LFA-1^- mice have an immune regulatory defect in their Treg compartment. To address this question, we first examined blood, thymus, and secondary lymphoid organs including lymph nodes, spleen and Peyer’s patches for the frequency of
Tregs in wild type and LFA-1<sup>−/−</sup> mice. LFA-1<sup>−/−</sup> mice had significantly lower Treg populations in the spleen, lymph nodes, and Peyer’s Patches, but surprisingly had increased numbers of Tregs in the thymus (Fig 1A). The increased thymic Treg population in LFA-1<sup>−/−</sup> mice is unique in the β<sub>2</sub>-integrin adhesion molecule family as no difference was observed in the number of thymic Tregs in CD11b<sup>−/−</sup> or CD11c<sup>−/−</sup> mice compared to wild type mice (Fig 1B). We did not find any differences in the double or single positive CD4/CD8 profile of Tregs in the thymus of LFA-1<sup>−/−</sup> mice compared to wild type mice, suggesting that the absence of LFA-1 alone does not delay Treg development in the thymus (data not shown). We also did not observe sexual dimorphism or age-related effects that might account for the higher Treg population in the thymus. The elevated thymic Treg population in LFA-1<sup>−/−</sup> mice may be due, in part, to a compensatory mechanism to repopulate the periphery where Treg numbers are reduced or an incompletely penetrant LFA-1-dependent emigration defect. Interestingly, our results contrast with those of Marski and colleagues who reported reduced numbers of Tregs in the thymus of CD18<sup>−/−</sup> mice (12). The reasons for this are unclear given our observations in CD11b- and CD11c-deficient mice (Fig. 1B), however the absence of all β<sub>2</sub>-integrins in CD18<sup>−/−</sup> mice may lead to altered Treg thymic development or perhaps accelerated Treg thymic emigration.

In vivo allogenic conversion efficiency of LFA-1<sup>−/−</sup> CD4<sup>+</sup>CD25<sup>−</sup> T cells into Tregs depends on the presence or absence of LFA-1 in the recipient

The reduced Treg populations in LFA-1<sup>−/−</sup> mice could be attributed in part to a reduced capacity of naive Tregs in the periphery to differentiate to Foxp3<sup>+</sup> cells (20, 21). To assess
this possibility, we performed allogenic transfers to examine the ability of LFA-1−/− CD4+CD25− T cells to induce Foxp3 expression. In control experiments, we found that 17% of wild type CD4+CD25+CD45.2+ cells converted to CD4+CD25+Foxp3+ Tregs in the spleen and lymph nodes of the wild type CD45.1 recipient mice within six weeks after transfer (Fig 2A). A similar level of peripheral conversion to Tregs (15%) was observed in the spleen using LFA-1−/− CD45.2 effector T cells. In contrast, a significantly lower proportion of LFA-1−/− CD4+CD25+CD45.2+ cells (11% of total CD45.2+ cells, p=0.001) were able to convert into CD4+CD25+Foxp3+ Tregs in lymph nodes after transfer to wild type CD45.1 recipients (Fig 2A). These data indicate that LFA-1−/− T cells are able to turn on Foxp3 expression in the periphery, but that the efficiency of conversion maybe organ-specific and this may account, in part, for the reduced Treg populations in lymph nodes of LFA-1−/− mice. To ensure that the data represented a reduction in the induction of Foxp3 rather than a defect in trafficking of LFA-1−/− T cells, we calculated cell numbers recovered from the lymph nodes and found no differences (Fig 2B). We next asked if LFA-1 expression in the recipient mouse was required for conversion of CD4+CD25− T cells to Tregs. We found comparable conversion of wild type CD4+CD25+CD45.1+ cells to CD4+CD25+Foxp3+ Tregs in the spleen and lymph nodes of the wild type CD45.2 recipients and in the spleen of LFA-1−/− CD45.2 recipients (Fig 2B). Unexpectedly, we observed a two-fold higher conversion of wild type CD4+CD25+CD45.1+ cells to Tregs in the lymph nodes of LFA-1−/− CD45.2 recipients (9.1% vs. 5%, p=0.006), a finding that may suggest a compensatory mechanism to increase Treg numbers in the LFA-1-deficient background. Again these results do not seem to be driven by trafficking defects, given the cell numbers recovered from the lymph nodes were equivalent (Fig 2D).
**LFA-1**−/− Tregs are unable to suppress wild type CD4+ proliferation in vitro

Onishi and colleagues (22) demonstrated that LFA-1−/− Tregs could not suppress T cell proliferation on dendritic cells, but did not determine if LFA-1−/− Tregs could suppress effector T cell proliferation. As expected, wild type Tregs readily suppressed effector T cell proliferation in response to antigen-independent stimulation using anti-CD3 and anti-CD28 antibodies. In contrast, LFA-1−/− CD4+CD25+ cells completely failed to suppress wild type effector T cell proliferation (Fig 3A). A predominant ligand for LFA-1 is ICAM-1 and interactions between LFA-1 and ICAM-1 are critical to the formation of the immune synapse and in providing co-stimulation during T cell activation (23). To determine if ICAM-1 is critical for Treg-mediated suppression, we stimulated ICAM-1null effector T cells, in vitro, in the presence of wild type Tregs and analyzed proliferation levels. To our surprise, wild type Tregs were capable of suppressing ICAM-1null effector T cells (Fig 3B), suggesting that LFA-1 interaction with ICAM-1 is not critical to the function of Tregs.

**LFA-1**−/− Tregs poorly suppress inflammation in experimental colitis

To test the ability of LFA-1−/− Tregs to suppress effector T cell responses in vivo, we examined the ability of LFA-1−/− Tregs to prevent the development of experimental colitis. Transferring wild type CD4+CD45RBhi cells into RAG-1−/− mice induced colitis. Six weeks after transfer, severe colitis, characterized by inflammatory cell infiltration, epithelial hyperplasia, and goblet cell loss, had developed in the recipient mice (Fig 4). Colitis was significantly prevented by co-transfer of wild type CD4+CD45RBlow cells with
wild type CD4⁺CD45RB⁹ cells and by co-transfer of LFA-1⁺CD4⁺CD45R¹⁰ cells with wild type CD4⁺CD45RB⁹ cells (Fig. 4). LFA-1⁻CD4⁺CD45R¹⁰ cells did not suppress development of colitis to the extent observed with wild type Tregs, however, the difference was not statistically significant (p=0.051, students t-test). Previous studies using CD18⁻/⁻ mice and a similar colitis model, demonstrated that CD18⁻/⁻ Tregs (which do not express any β₂-integrins) could not suppress disease development (12). The loss of Treg suppressive function in CD18⁻/⁻ mice was attributed to the absence of LFA-1, however studies with LFA-1⁻/⁻ mice were not performed to substantiate this claim. The fact that we did not see similar levels of suppression with LFA-1⁻/⁻ Tregs, suggests that an additional β₂-integrin or a combination of several β₂-integrins may be required for in vivo Treg immunosuppression.

Mechanisms of Treg function have been broadly categorized to include suppression by inhibitory cytokines (IL-10 and TGF-β), cytolysis in a perforin and granzyme-dependent fashion, metabolic disruption either by IL-2 sequestration or generation of adenosine and, targeting dendritic cell function and/or maturation (reviewed in (7-9)). LFA-1 could potentially contribute to any one of these functions either through adhesive mechanisms (cytolysis and dendritic cell maturation) or through appropriate co-stimulation that may modulate inhibitory cytokine production or the adenosine suppressive pathway. We examined the LFA-1⁻/⁻ Tregs for changes in the expression of CD39 and CD73 on, seeking a link between LFA-1 and the adenosine suppression pathway, but found no difference compared to wild type Tregs (data not shown). An important observation in our study was that LFA-1 alone does not appear to be the only β₂-integrin playing a role in Treg-
mediated immunosuppression. Utilization of multiple $\beta_2$-integrins by Tregs to modulate their function could operate at several levels, including 1) increasing the ligand/receptor pairings between Tregs and thymic epithelium, potentially contributing to various stages in Treg development, 2) controlling their emigration from the thymus into the periphery and, 3) allowing differential co-stimulation during Treg-mediated regulation of immune responses, perhaps contributing to the fine-tuning of the overall response. Our findings point to unique roles for LFA-1 in Treg biology and indicate a broader role in immune responses for $\beta_2$-integrins in general.

ACKNOWLEDGEMENTS

The authors thank Drs. Kari Dugger and Robin Lorenz for helpful discussions and critical reading of the manuscript.

DISCLOSURES

The authors have no financial conflicts to disclose.

REFERENCES


**Figure 1.** Frequencies of regulatory T cells in LFA-1⁻/⁻ mice. A, Cells isolated from control or LFA-1⁻/⁻ blood, spleen, lymph node, Peyer’s patch, and thymus using a Miltenyo regulatory T cell kit were stained with CD4, CD25, and Foxp3 antibodies, as described in materials and methods. Data shown is gated on CD4⁺ cells. Statistical analysis was performed for each wild type and LFA-1⁻/⁻ tissue using the student’s t-test (**, p≤0.01 and ***, p≤0.001). B, The thymus of LFA-1⁻/⁻ mice has elevated frequency of Tregs compared to other β₂-integrin-deficient mice. Cells were isolated from thymus of control, CD11a⁻/⁻, CD11b⁻/⁻ and CD11c mice and immunostained as described in A. Data shown are gated on CD4⁺ cells. Statistical analysis was performed using Anova (p≤0.001).
Figure 2. LFA-1 deficiency prevents peripheral Treg generation from CD4^{+}CD25^{-} T cells. A, CD4^{+}CD25^{-} cells were isolated from CD45.2 wild type or LFA-1^{-/-} mice and injected into wild type CD45.1 recipients as described in materials or methods. C, CD4^{+}CD25^{-} cells were isolated from wild type CD45.1 mice and injected into wild type or LFA-1^{-/-} CD45.2 recipients. Six weeks post-injection the generation of peripheral Tregs was measured in the spleen and lymph node by FACS analysis. Cells were stained for CD4, CD25, CD45.1 or CD45.2, and Foxp3. Data shown is gated on CD4 and CD45.2 (A) or CD45.1 (C) cells. Cell numbers recovered from lymph nodes are shown in B and D which correspond to experiments described in A and C respectively. Statistical analysis was performed for each tissue measured using the student’s t-test (**, p≤0.01 and ***, p≤0.001).
Figure 3. LFA-1<sup>−/−</sup> Tregs fail to suppress CD4<sup>+</sup> effector cell proliferation. In vitro suppression assays were performed as described in materials and methods using wild type or ICAM<sup>null</sup> CD4<sup>+</sup>CD25<sup>−</sup> responder cells and either control CD4<sup>+</sup>CD25<sup>+</sup> or LFA-1<sup>−/−</sup> CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells. Cultures were stimulated with soluble anti-CD3 and anti-CD28 antibodies for 72 hours and were pulsed with <sup>3</sup>H-thymidine for the last 18 hours. A, The results are shown as fold-increase over baseline controls for WT CD4<sup>+</sup>CD25<sup>−</sup> cells alone (n=6), control CD4<sup>+</sup>CD25<sup>−</sup> cells with wild type CD4<sup>+</sup>CD25<sup>+</sup> cells (n=7), and for wild type CD4<sup>+</sup>CD25<sup>−</sup> cells with LFA-1<sup>−/−</sup> CD4<sup>+</sup>CD25<sup>+</sup> cells (n=5). B, wild type Treg cells are able to suppress proliferation of ICAM<sup>null</sup> CD4<sup>+</sup> T cells. The results shown are fold-increase over baseline controls for wild type CD4<sup>+</sup>CD25<sup>−</sup> cells alone (n=6), wild type CD4<sup>+</sup>CD25<sup>−</sup> cells with wild type CD4<sup>+</sup>CD25<sup>+</sup> cells (n=7), ICAM<sup>null</sup> CD4<sup>+</sup>CD25<sup>−</sup> cells alone (n=5), and for ICAM<sup>null</sup> CD4<sup>+</sup>CD25<sup>−</sup> cells with wild type CD4<sup>+</sup>CD25<sup>+</sup> cells (n=5). Statistical analysis was performed using Anova (**, p≤0.01 and ***, p≤0.001).
Figure 4. LFA-1<sup>−/−</sup> Tregs suppress colitis development comparably to wild type Tregs. RAG-1<sup>−/−</sup> mice colonized with *H. hepaticus* were injected with CD4<sup>+</sup>CD45RB<sup>hi</sup> cells (n=4) alone or with wild type (n=5) or LFA-1<sup>−/−</sup> (n=5) CD4<sup>+</sup>CD45RB<sup>lo</sup> cells as described in materials and methods. Six weeks post-injection colitis development was measured by histopathological analysis of colons. Colons were scored to assess the inflammation and tissue disruption throughout the tissue. LFA-1<sup>−/−</sup> CD4<sup>+</sup>CD45RB<sup>lo</sup> cells did not suppress colitis development significantly different than wild type CD4<sup>+</sup>CD45RB<sup>lo</sup> cells (p = 0.051, student’s t-test).
CONCLUSIONS

The data presented here contribute to the body of evidence that adhesion molecules, specifically the β2-integrins, contribute to many facets of T cell biology. During autoimmune demyelinating disease, all of the β2-integrins except CD11d contribute to the ability of T cells to drive disease progression. CD11a−/−, CD11b−/−, and CD11c−/− mice all present with reduced disease severity in EAE [128-131]. Lymphocytes from CD11b−/− mice entered the CNS during active EAE in frequencies equivalent to those seen in WT mice, however infiltration in CD11c−/− mice was dramatically reduced [128, 129]. Encephalitogenic CD11b−/− T cells were not able to induce transferred EAE in WT mice, providing further evidence that trafficking defects were not solely contributing to the CD11b−/− disease phenotype. These T cells also proliferated poorly in response to MOG antigen and produced an altered cytokine profile possibly contributing to their inability to cause disease [129]. CD11c-deficient T cells were also unable to transfer fulminate EAE, however this defect may be due in part to reduced trafficking into the CNS. CD11c-deficient T cells did produce an altered cytokine profile in response to antigen re-stimulation, which may also contribute to the reduced severity of disease [128]. We report here that unique to the β2-integrin family, CD11d-deficiency had no affect on the progression of EAE. CD11d−/− T cells were able to migrate into the CNS, proliferate, and produce cytokines comparably to WT T cells [131]. Given this, we chose to focus on CD11a, CD11b, and CD11c for future studies examining T cell functions.
The contribution of various T cell subsets to the phenotypes seen in β2-integrin-deficient T cells remains unclear. The fold induction in expression of the β2-integrins during EAE is higher on γδ T cells when compared to αβ T cells [110]. Given this, we next explored the possibility that γδ T cells contributed to the observed defects in β2-integrin-deficient T cell function during EAE. In order to fully characterize possible mechanisms of β2-integrin involvement on γδ T cells, it was important to determine how they migrated early in disease. In doing this, we found that γδ T cells migrate to the immunization sites prior to disease onset and expand in this area. The adjuvant used to induce EAE contains proteins from mycobacterium tuberculosis, which is a potent stimulator of γδ T cells. This may account in part for the strong localization and expansion of γδ T cells in the area of immunization [24, 133]. Concurrent to this localization and expansion, γδ T cells were entering the brain and spinal cord suggesting that there is some degree of antigen specificity to the γδ T cell response. Previous studies have suggested that CD11a plays a role in transendothelial migration of γδ T cells [134, 135], thus we examined the migration patterns of CD11a-deficient γδ T cells. We report that these cells are able to localize at the injection sites similar to WT γδ T cells however, they are not retained in the area and fail to proliferate comparably to WT cells. This defect was not significant enough to prevent the development of clinical EAE given that γδ T cell−/− mice reconstituted with CD11a-deficient γδ T cells developed clinical disease with equivalent severity to mice reconstituted with WT γδ T cells. We next examined the role of CD11b and CD11c-deficient γδ T cells and found that these cells were also able to drive clinical disease comparably to WT γδ T cells. Although we cannot rule out compensation from other family members in the α-chain deficient mice, our data strongly
suggest that the β2-integrin family is not critical to the ability of γδ T cells to drive disease progression and that other T cell subsets are responsible for the observed reduction in EAE severity.

Previous studies from our lab had shown that a high frequency of γδ T cells in the spinal cords of mice with EAE produced IFN-γ and/or TNF-α [110], here we set out to determine if the production of these cytokines contributed to the ability of γδ T cells to contribute to disease pathogenesis. We found that reconstitution of γδ T cell−/− mice with IFN-γ or TNF-α-deficient γδ T cells was unable to restore clinical disease severity. This indicates that the production of both IFN-γ and TNF-α by γδ T cells is vital for the participation of these cells in the development of EAE. γδ T cells have the ability to present MOG peptide to naïve CD4+ T cells and thus, that the production of IFN-γ or TNF-α by γδ T cells may influence the immune response generated during the initiation of EAE [24]. IFN-γ has also been shown to modulate dendritic cell functions by increasing the production of IL-12 resulting in alterations in T cell differentiation [34]. It has also been shown that γδ T cells interact with dendritic cells, and the production of TNF-α may drive maturation of these cells [136-138]. Given this, the production of IFN-γ and TNF-α by γδ T cells at the site of immunization may be contributing to dendritic cell maturation, resulting in changes in the priming of CD4+ T cells in the lymph nodes. It is also likely that the production of these cytokines by γδ T cells in the CNS early in disease helps to prime the microenvironment and possibly to mature local dendritic cells aiding in initial inflammatory processes leading to the development of the clinical symptoms of EAE.

Finally, in an effort to explain earlier findings suggesting that CD11a−/− mice have regulatory defects, we examined the role that CD11a plays on regulatory T cells [130].
study by Marski and colleagues showed that CD18-deficient Tregs are unable to suppress T cells responses both in vitro and in vivo, and that there are reduced frequencies of Tregs in the secondary lymphoid tissues in these mice [132]. The effects seen in this study were attributed to CD11a, however no data were provided to substantiate this claim. The CD18−/− mice fail to express any of the β2-integrins and Tregs do express CD11a, CD11b, and CD11c any of which could potentially contribute to the function of these cells (Data not shown). Given this, we examined the frequency of Tregs in CD11a−/− mice and found that the secondary lymphoid compartments contained significantly lower frequencies of Tregs when compared to WT mice. In contrast to this, we found that the thymus of CD11a−/− mice had a significantly increased proportion of Tregs compared to WT thymus. We did not however observe any maturational defects in Tregs in the CD11a−/− thymus when examining the CD4/CD8 single and double positive ratios. The decreased frequency of Tregs in the CD11a−/− lymph nodes may be due in part to a reduced capacity of CD11a-deficient T cells to convert into inducible Tregs. This defect was not found in the spleen and seems to be organ specific in nature and not due to an inability of the CD11a-deficient T cells to migrate into the lymph nodes. We did not find any defect in WT T cells converting into inducible Tregs in the periphery of CD11a−/− mice, in fact these cells were able to convert at a higher rate in the lymph node of CD11a−/− mice than in WT mice. This indicates that the CD11a-deficient environment does not contribute to the reduced inducible Treg compartment. The dysregulation of Treg frequencies and peripheral Treg generation in CD11a−/− mice indicates that this member of the β2-integrin family plays an important role in Treg homeostasis.
The study conducted by Marski and colleagues also found that CD18-deficient Tregs fail to suppress T cell responses [132]. To determine whether CD11a contributes to this phenotype we tested the function of CD11a-deficient Tregs both in vitro and in vivo. We show here that CD11a−/− Tregs fail to suppress WT effector T cell responses in vitro. Our findings provide further confirmation of a recent study from Sakaguchi’s group in which CD11a-deficient Tregs fail to suppress T cell responses in the presence of dendritic cells in vitro [139]. This study focused on the inability of CD11a-deficient Tregs to compete with WT T cells for contact with dendritic cells, thus reducing their capacity to suppress T cell proliferation. We show here that in cultures with only CD11a-deficient Tregs and WT effector T cells that CD11a-deficiency interferes with suppression mechanisms that act directly on target T cells. Surprisingly, the immunosuppressive functions of CD11a are not mediated through interactions with its primary ligand ICAM-1 on target T cells. Illustrated by the ability WT Tregs to fully suppress ICAMNull effector T cells in vitro. The failure of CD11a-deficient Tregs to suppress T cell responses does not carry over to in vivo disease models. In contrast to studies done using CD18-deficient Tregs, which completely fail to suppress experimental colitis development [132], we found that CD11a-deficient Tregs, while not able to fully suppress disease development, did significantly reduce disease severity. This suggests that other β2-integrin family members may be important to Treg suppression in vivo. It is also possible that deletion of the entire family had an additive effect that may not be recapitulated by any of the individual α-chain deficient cells.

Given the findings presented here, it is evident that the β2-integrin family of adhesion molecules plays an important role on T cells beyond mediating trafficking
events, and that these functions vary widely between T cell subsets. Although it seems that the family is not critical to γδ T cell function during demyelinating disease, trafficking patterns were altered in CD11a-deficient γδ T cells and this may be important for other functions of these T cells. Due to the high expression of the β2-integrin family on γδ T cells, examining the role of this family of molecules on γδ T cell subsets in other disease models may provide more insight into γδ T cell biology. The in vivo suppression data with CD11a-deficient Tregs indicates further studies examining CD11b and CD11c on regulatory T cells are needed to assess the contribution of these β2-integrins to Treg biology. The β2-integrin family presents an attractive target for treatment of demyelinating disease given the protection seen in EAE, however, caution should be used due to the differing functions these molecules mediate on T cells, particularly on regulatory T cells.
REFERENCES


APPENDIX A

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: November 28, 2007
TO: Scott R. Barnum, Ph.D.
    BBRB-842 2170
    FAX: 934-4985

FROM: Judith A. Kapp, Ph.D., Chair
       Institutional Animal Care and Use Committee

SUBJECT: Title: The Role of C3 and C3 Receptors in EAE
         Sponsor: NIH
         Animal Project Number: 071106914

On November 28, 2007, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

<table>
<thead>
<tr>
<th>Species</th>
<th>Use Category</th>
<th>Number in Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>C</td>
<td>205</td>
</tr>
</tbody>
</table>

Animal use is scheduled for review one year from November 2007. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 071106914 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.