

CHARACTERIZATION OF B REGULATORY CELL-DERIVED-
INTERLEUKIN-35 CONTAINING EXOSOMES AND THEIR
IMMUNOSUPPRESSIVE EFFECT

by

Sahar Ali Alhakeem

A Dissertation Submitted in
Partial Fulfillment of the
Requirements for the Degree of

Doctor of Philosophy
in Health Sciences

at

The University of Wisconsin-Milwaukee

August 2022

ABSTRACT

CHARACTERIZATION OF B REGULATORY CELL-DERIVED-INTERLEUKIN-35 CONTAINING EXOSOMES AND THEIR IMMUNOSUPPRESSIVE EFFECT

by

Sahar Ali Alhakeem

University of Wisconsin-Milwaukee, 2022

Under the Supervision of Professor Charles E. Egwuagu, MPH, Ph.D and Professor Janis Eells,
Ph.D

Background: IL-35 has emerged as a potent immunosuppressive cytokine that plays a role in regulating autoimmune and inflammatory diseases. IL-35 production was thought to be restricted to T regulatory cells (Tregs) until the discovery of IL-35 producing B regulatory cells (i35-Bregs). The difficulty of *ex-vivo* production of bioactive heterodimeric IL-35 has impeded the use of IL-35 for immunotherapy. On the other hand, i35-Bregs provide advantages over therapeutic administration of IL-35 because they proliferate *in vivo* and can sustain delivery of IL-35 in target tissues. Therefore, understanding cues required for the development of i35-Bregs will facilitate optimization of their expansion *ex-vivo* for therapeutic purposes. Although the role of B-cell receptor (BCR) in the development of regulatory B cells including B10 and LAG-3 expressing natural plasma cells is well established, little is known about the role of BCR in the development of i35-Bregs. In this study, we examined different stimuli including BCR stimulation to induce i35-Breg expansion. We also characterized IL-35 expression by i35-Bregs to better understand the influence of different signals on the expression of IL35 subunits. There are many unsolved questions regarding IL-35 biology and i35-Breg mechanisms of action. Unlike IL-12 and IL-23, whose subunits are linked by disulfide bond, IL-35 subunits (EBI3 and IL-12p35) were presumed to be secreted independently and bind to each other by unknown

mechanisms. Recently, it was reported that Tregs express cell-surface IL-35 and release IL-35 bound to CD81 in exosomes. This observation raised the question of whether IL-35 subunits are also expressed on the surface of i35-Bregs in association with CD81, and if IL-35 is secreted in exosomes, that contribute to i35-Bregs' mechanism of action. Exosomes are small extracellular vesicles (50-150 nm in diameter) that readily cross the blood brain barrier (BBB) or brain-retina-barrier (BRB) and are therefore desirable vehicles to deliver biologics into CNS tissues. In this study, we investigated; (i) the expression of IL35 on the surface of i35-Bregs, (ii) the association of EBI3 and IL-12p35 subunits with CD81, (iii) the secretion of IL-35 containing exosomes (i35-exosomes) by i35-Bregs, (iv) therapeutic potential of i35-exosomes in CNS autoimmune diseases.

Methods: To determine the most optimal method for inducing i35-Breg expansion, we stimulated B cells with LPS, LPS + anti-CD40, anti-CD40, or anti-IgM (BCR) + anti-CD40, and examined i35-Breg induction by flow cytometry. We characterized the expression of IL-35 and the association of IL-35 subunits with CD81 by immunofluorescence staining and proximity ligation assay (PLA). Exosomes were isolated from the supernatant of i35-Breg culture by using ExoQuick ULTRA kit and their sizes were characterized by Nanoparticle Tracking Analysis and Tunable Resistive Pulse Sensing. To determine whether the exosomes contain IL-35, we used Western blotting to detect IL-35 subunits (IL-12p35 and EBI3). Finally, we assessed the effect of heterodimeric IL-35 and i35-exosome treatment on encephalitogenic T cells and regulatory lymphocytes by flow cytometry.

Results: Stimulating of B cells from naïve mice with a low concentration of anti-IgM (BCR) in combination with anti-CD40 induced the expansion of i35-Bregs up to ~44% of total B cells,

underscoring the role of BCR signaling strength in the induction of i35-Bregs. The i35-Bregs showed a polarized expression of IL-35 and colocalization of IL-35 subunits with CD81 on their plasma membrane. Supernatant of B cells stimulated with anti-IgM+anti-CD40 contained extracellular vesicles (EVs) of 50-150 nm in size that express the exosomes markers CD81, CD9, and CD63. Importantly, the exosomes contained IL-35 (i35-exosomes). While i35-Bregs/i35-exosomes generated in response to activation by anti-IgM+ anti-CD40 contain more IL-12p35, i35-Bregs/i35-exosomes generated in response to stimulation by LPS are enriched with EBI3 subunit, suggesting differential expression of IL-35 subunits depending on the stimuli. Importantly, IL-35 or i35-exosomes inhibited encephalitogenic T cells while inducing the expansion of regulatory lymphocytes.

Conclusions:

Data presented in this study show that stimulating B cells from naïve mice with a combination of anti-IgM+anti-CD40 is optimal for the expansion of i35-Bregs compared to other stimuli, establishing role of BCR signaling in i35-Breg development. Furthermore, IL-35 was found to be a membrane-bound cytokine in i35-Breg cells suggesting that cell-cell contact might underlie immunomodulatory functions mediated by i35-Bregs. i35-exosomes-mediated suppression of encephalitogenic T cells further indicates that i35-Bregs and the exosomes they secrete can be exploited for the treatment of CNS autoimmune diseases.

© Copyright by Sahar Ali Alhakeem
All Rights Reserved

TABLE OF CONTENTS

CHAPTER I: BACKGROUND AND LITERATURE REVIEW.....	1
Immune Homeostasis	1
Multiple Sclerosis.....	1
Regulatory B Cells	2
Types	2
Origin and Identification	5
Signals Involved in Induction and Expansion of Regulatory B Cells	6
Toll-Like Receptors	6
CD40.....	7
B Cell Receptor	8
IL-35 and the Discovery of i35-Bregs	12
IL-35 and i35-Bregs as Therapeutic Strategies for CNS Autoimmune Diseases.....	13
Extracellular Vesicles and Exosomes.....	15
Heterogeneity and Biogenesis	15
Tetraspanins and B cell Exosomes	19
IL-35 Containing Exosomes.....	20
Hypothesis and Specific Aims.....	22
References.....	23

CHAPTER II: B CELL RECEPTOR SIGNALING INDUCES THE EXPANSION OF IL-35-SURFACE EXPRESSING B REGULATORY CELLS.....	37
Abstract	37
Introduction	38
Materials and Methods	40
Mice	40
Generation of regulatory B cells.....	40
Flow cytometry for detection of regulatory B cells.....	41
Immunofluorescence staining and confocal imaging	45
Proximity ligation assay	46
Statistical analysis.....	47
Results.....	48
Activation of CD19 ⁺ B cells by BCR and CD40 signals induces expansion of i35-Bregs...48	
Activation of CD19 ⁺ B cells by BCR and CD40 signals does not induce the expansion of IL-10 Bregs.....	51
BCR/CD40 induced i35-Bregs express heterodimeric IL-35.....	54
IL-35 subunits are colocalized with CD81 on the surface of i35-Bregs	57
.....	61
Polarized distribution of IL-35 in BCR/CD40 stimulated B cells.....	62
Discussion	65

References.....	70
 CHAPTER III: IL-35 PRODUCING B REGULATORY CELLS SECRETE IL-35	
CONTAINING EXOSOMES WITH DIFFERENTIAL COMPOSITION BASED ON THE	
STIMULI.....	76
Abstract	76
Introduction	77
Materials and Methods	79
Mice	79
Generation of i35-Bregs	79
Isolation of i35-Breg exosomes.....	80
Exosomes size analysis-Nanoparticle Tracking Analysis (NTA) and Tunable Resistive	
Pulse Sensing (TRPS).....	81
ELISA.....	81
Western blot assay	81
Results.....	82
Characterization of B cell-derived extracellular vesicles (EVs)	82
Exosomes of B cells stimulated with anti-IgM/anti-CD40 contain IL-35.....	88
Differential expression of IL-35 subunits based on the stimulation used to generate i35-	
Bregs/i35-exosomes.....	93
Discussion	98
References.....	104

CHAPTER IV: IL-35 CONTAINING EXOSOMES SUPPRESS ENCEPHALITOGENIC T CELLS AND INDUCE REGULATORY LYMPHOCYTES.....	110
Abstract	110
Introduction	111
Material and Methods	114
Mice	114
Generation of i35-Bregs for i35-exosome collection	114
Exosomes isolation	115
EAE induction	115
EAE <i>ex-vivo</i> experiments	116
Flow cytometry	117
<i>In vivo</i> treatment of EAE mice with i35-exosomes and clinical scoring.....	120
Flow cytometry analyses of splenocytes and lymphoid cells collected from EAE mice	120
Isolation of brain and spinal cord infiltrating lymphocytes using percoll gradients	121
Statistical Analysis	122
Results.....	123
IL-35 suppresses encephalitogenic T cells and expands regulatory T cells	123
IL-35 induces IL-10 regulatory B cells and i35-Bregs in EAE.....	128
i35-exosomes suppress encephalitogenic T cells	131
i35-exosomes induce IL-35 and IL-10 producing B cells in EAE	134

Treatment with i35-exosomes has no significant effect on ongoing EAE	137
Mice treated with i35-exosomes showed no significant difference in frequency of inflammatory T cells compared to control mice.....	140
Discussion	143
References.....	149
CHAPTER V: SUMMARY AND CONCLUSIONS	154
References.....	163
APPENDIX	167
CURRICULUM VITAE	168

LIST OF FIGURES

Figure 1. Signals involved in induction of regulatory B cells	11
Figure 2. Biogenesis of extracellular vesicles	18
Figure 3. Sequential gating strategy to identify i35-Bregs and IL-10 producing Bregs	44
Figure 4. Combination of BCR and CD40 signals induces i35-Breg expansion	50
Figure 5. Combination of BCR and CD40 signals does not induce the expansion of IL-10 Bregs	53
Figure 6. BCR/CD40 induced i35-Bregs express heterodimeric IL-35.....	56
Figure 7. IL-35 subunits are colocalized with CD81 on the surface of i35-Bregs.....	59
Figure 8. IL-35 subunits are associated with CD81.....	61
Figure 9. Polarized distribution of IL-35 in BCR/CD40 stimulated B cells.....	64
Figure 10. EVs isolated from cell supernatant of i35-Bregs are within the size range of exosomes	85
Figure 11. EVs isolated from cell supernatant of i35-Bregs express exosome markers.....	87
Figure 12. Exosomes of B cells stimulated with anti-IgM/anti-CD40 contain IL-35.....	90
Figure 13. Exosomes of B cells stimulated with anti-IgM/anti-CD40 contain heterodimeric IL-35.....	92
Figure 14. Differential expression of IL-35 subunits based on the stimulation used to generate i-35Bregs	95
Figure 15. Differential expression of IL-35 subunits based on the stimulation used to generate i35-exosomes	97
Figure 16. Sequential gating strategy to identify inflammatory T cells and regulatory lymphocytes.....	119

Figure 17. IL-35 suppresses encephalitogenic T cells.....	125
Figure 18. IL-35 expands regulatory T cells in EAE.....	127
Figure 19. IL-35 induces IL-10 regulatory B cells and i35-Bregs in EAE.....	130
Figure 20. i35-exosomes suppress encephalitogenic T cells	133
Figure 21. i35-exosomes induce IL-35 and IL-10 producing B cells in EAE	136
Figure 22. Treatment with i35-exosomes has no significant effect on EAE disease	139
Figure 23. Mice treated with i35-exosomes showed no significant difference in frequency of inflammatory T cells compared to control mice	142
Figure 24. Proposed model of i35-Breg regulatory mechanisms.....	162
Figure 25. Assessment of Exosomal RNA by Agilent Analyzer	167

LIST OF ABBREVIATIONS

Bregs: B regulatory cells

i35-Bregs: IL-35 producing B regulatory cells

i27-Bregs: IL-27 producing B regulatory cells

B10: IL-10 producing B regulatory cells

Treg: T regulatory cells

i35-exosomes: IL-35 containing exosomes

APCs: Antigen presenting cells

DCs: Dendritic cells

LAG3: Lymphocyte Activating 3

PD-L1: Programmed death-ligand 1

PD-1: Programmed death-1

IL-35: Interleukin-35

IL-10: Interleukin-10

IL-27: Interleukin-27

IL-17: interleukin-17

IFN- γ , Interferon gamma

TGF-B: Transforming growth factor- β

MS: Multiple Sclerosis

CNS: Central nervous system

BBB: Blood brain barrier

MOG 35-55: Myelin oligodendrocyte glycoprotein 35-55

EAE: Experimental autoimmune encephalomyelitis

EAU: Experimental Autoimmune Uveitis

EBI3: Epstein-Barr Virus Induced 3

IL-12p35: Interleukin-12 subunit alpha

T_{FH}: T follicular helper

CpG: Unmethylated cytosine-phosphate-guanine

LPS: Bacterial lipopolysaccharides

TLR: toll-like receptor

FoxP3: Forkhead box P3

Th1: T helper type 1

Th17: T helper type 17

WT: Wild-type

CD: Cluster of differentiation

CD40L: CD40 ligand

BCR: B cell receptor

ELISA: enzyme-linked immunosorbent assay

PLA: Proximity Ligation Assay

PBS: phosphate buffered saline

PMA: Phorbol myristate acetate

I: Ionomycin

TNF- α : Tumor necrosis factor-alpha

IL: Interleukin

Ag: Antigen

IgM: Immunoglobulin M

Ig: Immunoglobulin

IRF4: Interferon Regulatory Factor 4

IRF8: Interferon Regulatory Factor 8

MHC: Major histocompatibility complex

MVBs: Multivesicular bodies

rER: Rough Endoplasmic Reticulum

EVs: Extracellular vesicles

DAPI: 4',6-diamidino-2-phenylindole

ACKNOWLEDGMENTS

I would like to express my sincerest gratitude to my mentors and advisors, Dr. Charles Egwuagu and Dr. Janis Eells, for their tremendous help and support guiding me through the learning process of this dissertation. Dr. Egwuagu provided me the invaluable opportunity of conducting my dissertation research in his laboratory at the National Eye Institute/NIH. I enjoyed discussing ideas with him, which broadened and sharpened my abilities as a research scientist. Dr. Eells's support and motivation empowered me throughout my dissertation research as she encouraged me to believe in myself. I also would like to thank Dr. Jeri-Anne Lyons for all she taught me during my time in her laboratory. I give my appreciation to all the members of the Egwuagu lab, with a special thank you to Dr. Cheng-Rong Yu for his support and Dr. Manoj Yadav for his help with confocal experiments. Thank you to Dr. Mary Mattapallil from Dr. Rachael Caspi's lab for her assistance with my EAE experiments. Additionally, I thank the rest of my dissertation committee: Dr. Dean Nardelli, Dr. Elizabeth Liedhegner, and Dr. Raymond Fleming for their time and feedback. Last, but not least, much appreciation to the NIH OITE, specifically Dr. Sharon Milgram, Dr. Phil Ryan, and Dr. Gail Seabold, for their help and support in crossing the finish line.

CHAPTER I: BACKGROUND AND LITERATURE REVIEW

Immune Homeostasis

Immune homeostasis is achieved by a balance between effector (pro-inflammatory) cells and regulatory (anti-inflammatory) cells. In case of infection, the number of effector cells that produce pro-inflammatory cytokines such as, T helper type 1 cells (Th1) that produce IFN- γ and T helper type 17 cells (Th17) that produce IL-17, increases to clear pathogens. The resolution of this inflammation is controlled by regulatory cells including T regulatory cells (Tregs) and B regulatory cells (Bregs). Both Tregs and Bregs suppress inflammation via the production of anti-inflammatory cytokines such as IL-10 (Fillatreau et al., 2002; Rubtsov et al., 2008), TGF- β (Kehrl et al., 1986; Surh & Sprent, 2012), IL-35 (Collison et al., 2007; Wang et al., 2014), and IL-27 (Choi et al., 2021; Do et al., 2016) or via inhibitory receptors such as lymphocyte activation gene-3 (LAG3) (Huang et al., 2004; Lino et al., 2018) and programmed death-ligand 1 (PD-L1) (Francisco et al., 2009; Khan et al., 2015). In addition, regulatory cells maintain tolerance to self-antigens. Failure of tolerance to self-antigen leads to the development of autoimmune diseases such as Multiple Sclerosis (MS).

Multiple Sclerosis

Multiple Sclerosis (MS) is an autoimmune inflammatory demyelinating disease of the central nervous system (CNS). In the United States alone, there are more than 400,000 people affected by MS (Dilokthornsakul et al., 2016). It is the most common cause of neurological disability among young people, and currently, there is no cure (Browne et al., 2014). Early symptoms of MS include dizziness, fatigue, numbness of limbs and blurred vision. At the late stage of the disease, patients suffer from loss of motor functions and vision (Cottrell et al., 1999).

MS is characterized by the activation of autoreactive immune cells, mainly Th1 and Th17 CD4⁺ T cells, that migrate across the blood-brain barrier (BBB) and attack the myelin sheath surrounding nerve cells. Inflammatory cytokines (IFN- γ , IL-17, and TNF- α) produced by lymphocytes and myeloid cells play an important pathological role in breaking the BBB, axonal demyelination and degeneration, and prolonging the immune response. Therefore, a major therapeutic goal is to suppress inflammation by suppressing the expansion of pathogenic cells that mediate the disease.

Most of the current understanding of MS pathogenesis and therapies is the result of experiments performed in the experimental autoimmune encephalomyelitis (EAE) model of MS. Upon immunization with CNS-specific proteins such as myelin oligodendrocyte glycoprotein 35-55 (MOG 35-55), the immune response starts in the draining lymph nodes where T cells are activated and differentiated. Subsequently, T cell will migrate, invade the CNS, and secrete pro-inflammatory cytokines such as IFN- γ and IL-17 resulting in the loss of the myelin sheath. Around day 8-10 post immunization, animals present with clinical and pathological features similar to MS in humans (Berard et al., 2010; Constantinescu et al., 2011). Experiments performed in the EAE model helped to identify protective mechanisms in autoimmune diseases including the identification of regulatory B cells and their therapeutic potentials in CNS inflammatory diseases.

Regulatory B Cells

Types

In MS, B cells play both pathogenic and protective roles. Their protective role is

suggested by reports that B-cell deficient mice do not recover from monophasic EAE (Fillatreau et al., 2002; Wolf et al., 1996). The earliest identified regulatory mechanism of B cells is through the production of IL-10, as mice with specific deletion of IL-10 in B cells failed to recover from EAE and showed enhanced Th1 response compared to wild-type (WT) mice (Fillatreau et al., 2002). IL-10 producing B cells from mice that had recovered from EAE conferred protection against EAE to IL-10 deficient mice (Fillatreau et al., 2002). In addition, IL-10 producing B cells were able to inhibit EAE when transferred at the onset of disease (Korniotis et al., 2016). Collectively, these studies support the protective role of B cells in EAE through the production of IL-10. The role of B cells as a regulatory player via the production of IL-10 was further supported in other models including colitis (Mizoguchi et al., 2002) and arthritis (Mauri et al., 2003). However, further studies have identified regulatory B cells that suppress inflammation through IL-10-independent mechanisms (Choi et al., 2021; Kehrl et al., 1986; Khan et al., 2015; Lino et al., 2018; Wang et al., 2014).

For example, regulatory B cells have been found to express TGF- β receptor and to produce TGF- β , which can work in an autocrine (act on producer cells) and paracrine (act on nearby cells) manner (Kehrl et al., 1986). In terms of an autocrine effect, TGF- β inhibits B cell proliferation and Ig production (Kehrl et al., 1986). In terms of a paracrine effect, it was found that TGF- β producing Bregs promote transplantation tolerance, in part, by expanding Tregs (Lee et al., 2014).

The most recently discovered types of regulatory B cells are IL-27 producing Bregs (i-27 Bregs) and IL-35 producing Bregs (i-35 Bregs). Our group generated i27-Bregs from a B-1a cell

lineage in the peritoneal cavity and human umbilical cord blood (Choi et al., 2021). Adoptive transfer of i27-Bregs ameliorated EAE and Experimental Autoimmune Uveitis (EAU), an animal model of uveitis that shares immunological and pathological features with EAE. Mice treated with i27-Bregs showed a decrease in inflammatory cells producing IL-17 and IFN γ , an increase in Bregs producing IL-27 and IL-35, an increase in Tregs, and an upregulation of the inhibitory molecules LAG-3 and programmed death receptor-1 (PD-1) by T cells (Choi et al., 2021). IL-27 structurally share the EBI3 subunit with IL-35. As i35-Bregs are the focus of this dissertation, we dedicated a separate section to discuss the emerging role of i35-Bregs in immune tolerance and their therapeutic potentials in autoimmune diseases.

Beside producing suppressive cytokines as we have discussed above, Bregs can regulate the immune response by expressing inhibitory molecules. Follicular helper T cells (T_{FH} cells) are involved in the humoral response by interacting with B cells to drive the formation of the germinal center. T_{FH} cells highly express the inhibitory receptor PD-1 which upon binding to its ligands PD-L1/2 inhibits T cell expansion and functions (Haynes et al., 2007). It was found that Bregs control the humoral and T cell responses through PD-L1/PD-1 interaction (Khan et al., 2015). Specifically, Khan et al., identified a subset of Bregs with a high expression of PD-L1 (PD-L1^{hi}) and a lower expression of IL-10, as compared to low to intermediate PD-L1 expressing (PD-L1^{lo/int}) B cells. These cells were able to attenuate the expansion of T_{FH} and antibody production independent of IL-10 (Khan et al., 2015). Another inhibitory molecule found to be expressed by Bregs is LAG3 (Lino et al., 2018). Lino et al., identified a subset of natural regulatory plasma cells that are LAG-3⁺ (Lino et al., 2018). The LAG3⁺CD138⁺ Bregs do present in naïve mice, however, they rapidly respond to infection by producing IL-10 within

hours. These cells also express PD-L1 and PD-L2. The increase of these cells correlated with suppression of memory T cell formation and impaired immunity against *Salmonella* infection (Lino et al., 2018).

In summary, the field of regulatory B cells is evolving as more subsets of Bregs are discovered emphasizing their previously unappreciated role in regulating immune tolerance. They suppress inflammation via a variety of mechanisms including the production of anti-inflammatory cytokines or the expression of inhibitory receptors. The therapeutic potential of Bregs in the treatment of autoimmune diseases necessitates the need for a better understanding of Breg phenotype and development.

Origin and Identification

While Tregs and Bregs have a lot in common, the origin and phenotype of Bregs remain ambiguous. Regulatory T cells originate from a specific Treg lineage in the thymus or are induced peripherally by TGF- β in the presence of a low dose of antigen (Chen et al., 2003; Kretschmer et al., 2005). In addition, Tregs can be identified by their master transcription factor Foxp3 (Fontenot et al., 2005). In contrast, it is unknown whether Bregs are generated from a distinct B regulatory cell lineage or any B cell could acquire a regulatory function as a response to environmental cues. Bregs are heterogenous in term of the phenotype and have been identified by their function such as the secretion of IL-10, IL-35, and or IL-27(Choi et al., 2021; Fillatreau et al., 2002; Wang et al., 2014). A recent report showed that IL-10 Bregs in human arise from different B cells subsets (Glass et al., 2022). Glass et al, sorted CD38⁺CD24⁺ transitional, CD45RB⁻CD27⁻CD38⁻CD24⁻ naive, CD45RB⁺CD27⁻ memory, CD45RB⁺CD27⁺ memory,

CD95⁺ memory, and CD19^{hi}CD11c⁺ effector cells and stimulated them with CpG (TLR9 ligand) and anti-CD40 in the presence of IL-2, IL-21, and IL-35. They found that IL-10 Bregs were generated from every subset but at different proportions (Glass et al., 2022). While a Breg master transcription factor has yet to be revealed, some transcription factors were found to play an important role in the development of regulatory B cells such as IRF4 and IRF8 (Choi et al., 2021). Mice with targeted deletion of IRF4 in B cells showed a decrease in i35-Bregs (Choi et al., 2021). On the other hand, mice with target deletion of IRF8 in B cells showed a decrease in i27-Bregs (Choi et al., 2021). This study suggests that Breg subsets are regulated by different transcriptional mechanisms. Until a Breg master transcription factor or a Breg specific marker is revealed, Bregs remain identified by their function.

Signals Involved in Induction and Expansion of Regulatory B Cells

One of the unanswered questions regarding B regulatory cells is how Bregs are induced *in vivo*? Current understanding implicates environmental cues in the induction of Bregs. Inflammatory cues such as cytokines, innate stimuli through toll-like receptors (TLR2/4/9), or antigen stimulation through B-cell receptor (BCR)/and the co-stimulatory receptor CD40, were found to be involved in the induction of Bregs (**Figure 1**). Stimulating B cells through these receptors is also used as a mechanism to generate and expand Bregs *in vitro* (**Figure 1**). In this section, we will discuss studies that support the role of TLRs, CD40, and BCR signaling in the induction of Bregs.

Toll-Like Receptors

Stimulating B cells through TLR4, by bacterial lipopolysaccharides (LPS), is one of the mechanisms to expand IL-10 producing Bregs (B10 cells) (Yanaba et al., 2009). Similarly, LPS was found to induce i35-Bregs *in vitro* and *in vivo* (Dambuza et al., 2017; Wang et al., 2014). In addition, stimulating B-1a cells with LPS induced i27-Bregs (Choi et al., 2021). Another innate receptor that was found to be involved in the induction of Bregs is TLR9 with bacterial unmethylated cytosine-phosphate-guanine (CPG) as an agonist. Stimulation of human B cells with CPG induced IL-10 producing Bregs that induced the expansion of Tregs *in vitro* (Mielle et al., 2018). Moreover, mice that lack MyD88, the downstream transcription factor of TLRs, in B cells failed to recover from EAE (Lampropoulou et al., 2008). These studies emphasize the importance of TLRs receptors in the induction of Bregs.

CD40

Co-stimulatory molecules such as CD40, which is involved in Breg maturation and response to antigen, were found to play an important role in Breg induction *in vivo* and *in vitro*. A study reported that mice with amplified CD40 signal showed an expansion of IL-10 producing Bregs (Poe et al., 2011). Moreover, EAE mice that are deficient in B cells and received B cells that lack CD40 (CD40^{-/-} B cells) or IL10^{-/-} B cells failed to recover from EAE, as compared to mice that received WT B cells (Fillatreau et al., 2002). Additionally, B cells from EAE mice that lack CD40 failed to produce IL-10 in response to MOG 35-55 re-stimulation (Fillatreau et al., 2002). The importance of CD40 signaling in the induction of human IL-10 producing Bregs was also reported (Komlósi et al., 2018). Another study showed that stimulating human B cells with CPG (TLR 9 agonist) and anti-CD40 increased the frequency of IL-10 producing Bregs, as compared to B cells stimulated with CPG alone (Bankó et al., 2017). Collectively, these studies

support the role of CD40 in the induction of Bregs and indicate that B cell:T cell interaction through CD40:CD40L is essential for the functional maturation of Bregs.

B Cell Receptor

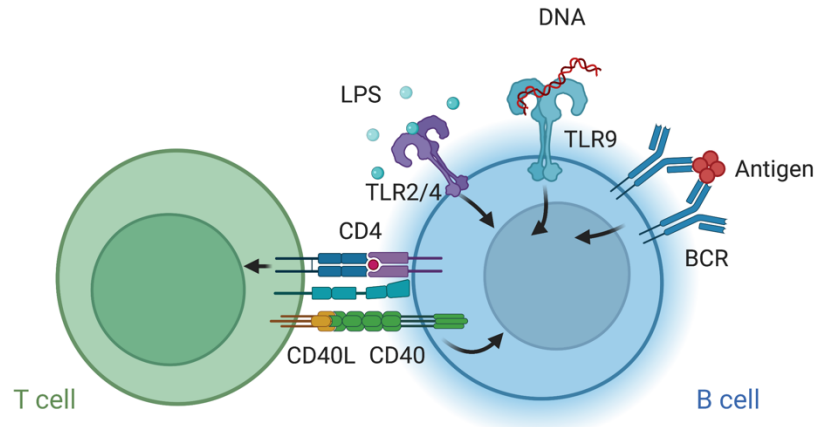
B cell receptor (BCR) signaling plays an important role in the development of B10 cells, and studies suggest that they exert their function in an antigen specific manner (Fillatreau et al., 2002; Yanaba et al., 2009; Yoshizaki et al., 2012). B cells from primed mice with autoimmune diseases produce IL-10 when stimulated by the BCR and CD40 (Fillatreau et al., 2002; Mauri et al., 2003). For example, B cells from EAE mice produce a high amount of IL-10 when stimulated with anti- κ + anti-CD40 (Fillatreau et al., 2002). However, stimulating B cells from naïve mice with anti-IgM+ anti-CD40 doesn't induce the expansion of IL-10 Bregs as compared to LPS (Yanaba et al., 2009). In addition, adding anti-CD40 or anti-IgM to LPS stimulation downregulates LPS induced IL-10 production in B cells from naïve mice (Yanaba et al., 2009). The reason for the different response to anti-IgM and anti-CD40 stimulation in B cells from primed mice vs naïve mice is not clear. It is possible that additional inflammatory cues *in vivo* lead to the functional maturation of IL-10 Bregs and enhance their regulatory response to BCR and CD40 stimulation.

Other studies support the role of BCR in the development of LAG-3⁺CD138^{hi} Bregs (Lino et al., 2018). Mice deficient in CD19 showed a reduced number of LAG-3⁺CD138^{hi} Bregs *in vivo*, and mice deficient in a negative regulator of BCR (CD72) showed an increased number of LAG-3⁺CD138^{hi} Bregs (Lino et al., 2018). In addition, BCR signaling were found to induce i27-Bregs. Stimulating mouse B-1a cells collected from the peritoneal cavity with anti-IgM+anti-

CD40 expanded i27-Bregs up to 37%, compared to 6% in unstimulated cells (Choi et al., 2021). Similarly, stimulating human B-1a cells collected from cord blood with anti-IgM+anti-CD40 expanded i27-Bregs up to 73% compared to 18% in unstimulated cells (Choi et al., 2021). Collectively, these studies showed that BCR signaling is involved in the induction and expansion of B10 cells, LAG-3⁺CD138^{hi} Bregs, and i27-Bregs.

While the role of BCR in the development of regulatory B cells, such as B10 and LAG-3 expressing natural plasma cells, is well established, as discussed, little is known about the role of BCR in IL-35 production or the development of i35-Bregs. Understanding the cues required for the development of i35-Bregs will facilitate the optimization of their expansion *ex-vivo* for therapeutic purposes. In the next section, we will elaborate on i35-Bregs and their regulatory functions, as well as their potential use as a therapeutic to autoimmune diseases.

In vivo



In vitro

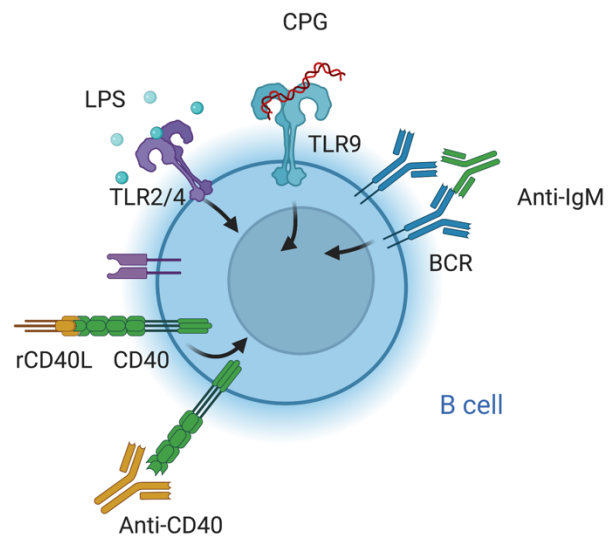


Figure 1. Signals involved in induction of regulatory B cells

The current understanding of regulatory B cell development implicates inflammatory cues in the induction of Bregs, including involvement of TLRs, CD40, and BCR. *In vivo*, TLR2/4 are activated by sensing LPS released from the wall of gram-negative bacteria, TLR9 is activated by sensing DNA, CD40 is activated by binding to CD40 ligand (CD40L) on the surface of T cells providing a co-stimulatory signal, and the BCR is activated by antigen. These signals can be mimicked *in vitro* to induce and expand Bregs: TLR2/4 are activated by LPS, TLR9 is activated by CPG, CD40 is activated by recombinant CD40L (rCD40L) or anti-CD40, and BCR is activated by anti-IgM. This image was created with BioRender.com

IL-35 and the Discovery of i35-Bregs

IL-35 is a member of the IL-12 cytokine family that includes heterodimeric cytokines with extensive subunit diversity. They are composed of an α -subunit with a helical structure similar to IL-6 and a β -subunit structurally related to soluble IL-6 α -receptor (IL-6R α). The number of identified cytokines in this family is increasing based on the mix-and-match of the following subunits: IL-12p35, IL12p40, IL23p19, IL27p28, and EBI3. There are five cytokines that have been identified to belong to this family, IL12 (IL-12p35/IL12p40), IL23 (IL23p19/IL12p40), IL27 (p28/EBI3), IL35 (IL-12p35/EBI3), and the bioengineered cytokine Fusokine (IL27p28/IL12p40) (Sun et al., 2015). As mentioned above, IL-35 shares the IL-12p35 subunit with IL12, and the EBI3 subunit with IL-27. IL-12 and IL-23 are considered pro-inflammatory cytokines and IL-35 and IL-27 are considered anti-inflammatory cytokines in autoimmune diseases. The two subunits of IL-12 and IL-23 are linked by a strong disulfide bond (Gubler et al., 1991; Oppmann et al., 2000). On the other hand, the two subunits of IL-27 and IL-35 are not covalently bound, rather, they are produced independently and little is known about factors that determine their heterodimerization (Devergne et al., 1997; Pflanz et al., 2002). When EBI3 is expressed alone, it accumulates in the endoplasmic reticulum suggesting that association with another subunit is needed for its secretion (Devergne et al., 1997). The expression of both EBI3 and IL-12p35 facilitates their secretion (Devergne et al., 1997). One of the limitations of using IL-35 as a therapeutic is the difficulty in producing heterodimeric IL-35. Understanding the mechanism (s) underlying the formation of heterodimeric IL-35 will advance therapeutic potential of IL-35.

IL-35 has emerged as a potent immunosuppressive cytokine that plays a role in regulating autoimmune and inflammatory diseases (Collison et al., 2007; Devergne et al., 1997; Shen et al., 2014; Wang et al., 2014). IL-35 was first identified to be produced by FOXP3⁺ Tregs (Collison et al., 2007). Tregs that lack EBI3 or IL-12p35 showed an impaired regulatory function and failed to regulate inflammatory bowel disease (Collison et al., 2007). Additionally, IL-35 induced the expansion of FOXP3⁺ IL-35 producing Treg (iT_R35 cells). Treatment with iT_R35 was able to prevent autoimmunity in Foxp3^{-/-} mice (Collison et al., 2010). These studies support the role of IL-35 as an immunosuppressive cytokine in autoimmune diseases.

IL-35 production was thought to be restricted to T regulatory cells until the discovery of IL-35 producing B regulatory cells (i35-Bregs) (Wang et al., 2014). Wang *et al.* found that recombinant IL-35 induced the expansion of a population of Bregs that produce IL-35 *in vitro* and *in vivo* (Wang et al., 2014). *In vitro*, stimulating B cells with LPS alone gave rise to a small population of IL-35 producing Bregs. The addition of rIL-35 increased the frequency of i35-Bregs up to 35%. It is of note that 17% of these cells were also IL-10⁺. This discovery added to IL-35 functions as an inducer of i35-Bregs, IL-10 Bregs, and Bregs with a dual function as producers of IL-35 and IL-10.

IL-35 and i35-Bregs as Therapeutic Strategies for CNS Autoimmune Diseases

IL-35 play a protective role in CNS autoimmune diseases. Shen *et al.*, found that mice with B-cell-restricted deficiency in IL-12p35 or EBI3 developed exacerbated EAE, while deficiency of p40 or p28 in B cells had no effect on the disease course (Shen et al., 2014). The mice with B cells deficient in IL-12p35 showed increased production of IL-17 and IFN- γ by

splenocytes, and increased in the infiltration of CD4⁺ T cells and phagocytes into the CNS, as compared to mice with WT B cells (Shen et al., 2014). While IL-35 exerts its function by activating STAT1 and STAT4 in T cells (Collison et al., 2012), it exerts its function through the binding to IL-12R β 2/IL-27Ra receptor and activating STAT1 and STAT3 signaling in B cells (Wang et al., 2014). Xie et al., found that mice that lack IL-12R β 2 developed severe EAE with increased T cell proliferation and infiltration into the CNS (Xie et al., 2016). Collectively, these data support the protective role of IL-35 secreted by B cells in EAE.

Our group found that treatment with recombinant IL-35 suppress EAU as shown by fundoscopy and histological analysis (Wang et al., 2014). This suppression was associated with a decrease in the frequency of Th17 and Th1 cells and an expansion of Tregs, IL-10 producing Bregs, and i35Bregs (Wang et al., 2014). Similarly, treatment of EAU mice with IL-35-induced Bregs ameliorated uveitis by inhibiting Th17 and Th1 and expanding Tregs and Bregs (Wang et al., 2014). Furthermore, mice that lack IL-12p35, EBI3, or IL-12R β 2 develop severe uveitis with a decrease in Breg levels (Wang et al., 2014). This study emphasizes the role of IL-35 in the expansion of Bregs and i35-Bregs and suggest its therapeutic use for CNS autoimmune diseases.

As IL-12p35 and EBI3 can be produced as monomers, our group questioned whether IL-35 single subunits have intrinsic regulatory effect. Indeed, we found that IL-12p35 inhibited the proliferation of Th17 cells and induced the expansion of Tregs and Bregs resulting in suppression of uveitis in EAU model (Dambuza et al., 2017). Similar results were found in the EAE model. IL-12p35 treatment ameliorated EAE by reducing cell infiltration into the CNS, inhibiting Th1 and Th17 cells, and expanding regulatory cells (Choi et al., 2017). EBI3 also showed an inhibitory effect on T cell proliferation (Dambuza et al., 2017). However, both

subunits showed less immunosuppressive effects compared to IL-35. These studies showed that EBI3 and IL-12p35 have intrinsic functions as monomers that partially resemble IL-35 effects.

Difficulty of *ex-vivo* production of bioactive heterodimeric IL-35 has impeded the use of IL-35 for immunotherapy. On the other hand, i35-Breg provides advantage over therapeutic administration of IL-35 because it proliferates *in vivo* and can sustain delivery of IL-35 in target tissues. However, there are some obstacles to using i35-Breg therapy for CNS autoimmune diseases. To use i35-Breg in patients, there is a need to produce a high amount of autologous i-35 Bregs to avoid an allogeneic immune response to the transferred cells. Another big challenge in treating CNS diseases is the need for a therapy that can passively cross the BBB without compromising it. Therefore, alternative approaches are needed to take advantage of the anti-inflammatory effect of IL-35.

Extracellular Vesicles and Exosomes

Heterogeneity and Biogenesis

Extracellular vesicles (EVs) are small vesicles that were first described in 1971 as being secreted by erythrocytes and platelets as a mechanism of eliminating extra proteins or as part of the coagulation cascade, respectively (Crawford, 1971; Schrier et al., 1971). The progress in EVs research over the last decades revealed that EVs are a group of small membranous vesicles that are secreted by all cells as a mechanism of intercellular communication. EVs mostly resemble their parent cells in term of the content and play an important role in many physiological processes. They contain proteins, lipids, metabolites, DNA, and RNAs that can be transferred to

recipient cells and modulate their phenotypes and/or functions (Kalluri & LeBleu, 2020). For example, Aiello *et al.*, found that Treg extracellular vesicles (Treg-EVs) contain miRNAs that inhibit T cell proliferation and that *in vivo* treatment with Treg-EV prolonged kidney allograft survival in rats (Aiello et al., 2017).

The size of EVs ranges between 50-1000nm and they are heterogenous in nature. They can be divided based on size, biogenesis, and composition into two subgroups: exosomes with size range (50-150 nm), and microvesicles with size range (50-1000 nm) (Zhang et al., 2018). Microvesicles are generated by outward budding of the plasma membrane (Del Conde et al., 2005) (**Figure 2**). In contrast, exosomes are mainly generated from the endosome (Fritzsche et al., 2002; Kalluri & LeBleu, 2020; Vidal et al., 1997). Plasma membrane inward invagination leads to the encapsulation of extracellular molecules with cell surface molecules to form early endosomes (**Figure 2**). Sorting and exchange of the cargo occurs between endosomes, endoplasmic reticulum, and Golgi apparatus. Further maturation of early endosome leads to the formation of endosomal multivesicular bodies (MVBs) that contain vesicles (**Figure 2**). MVBs are either recycled by lysosomes or transported to the plasma membrane and exosomes are released through exocytosis (Kahlert & Kalluri, 2013; Kalluri & LeBleu, 2020; van Niel et al., 2018) (**Figure 2**). While exosomes are mainly originated from endosomes, some immune cells produce exosomes from the plasma membrane suggesting an alternative mechanism of exosome production (Booth et al., 2006; Fordjour et al., 2019; Gurung et al., 2021).

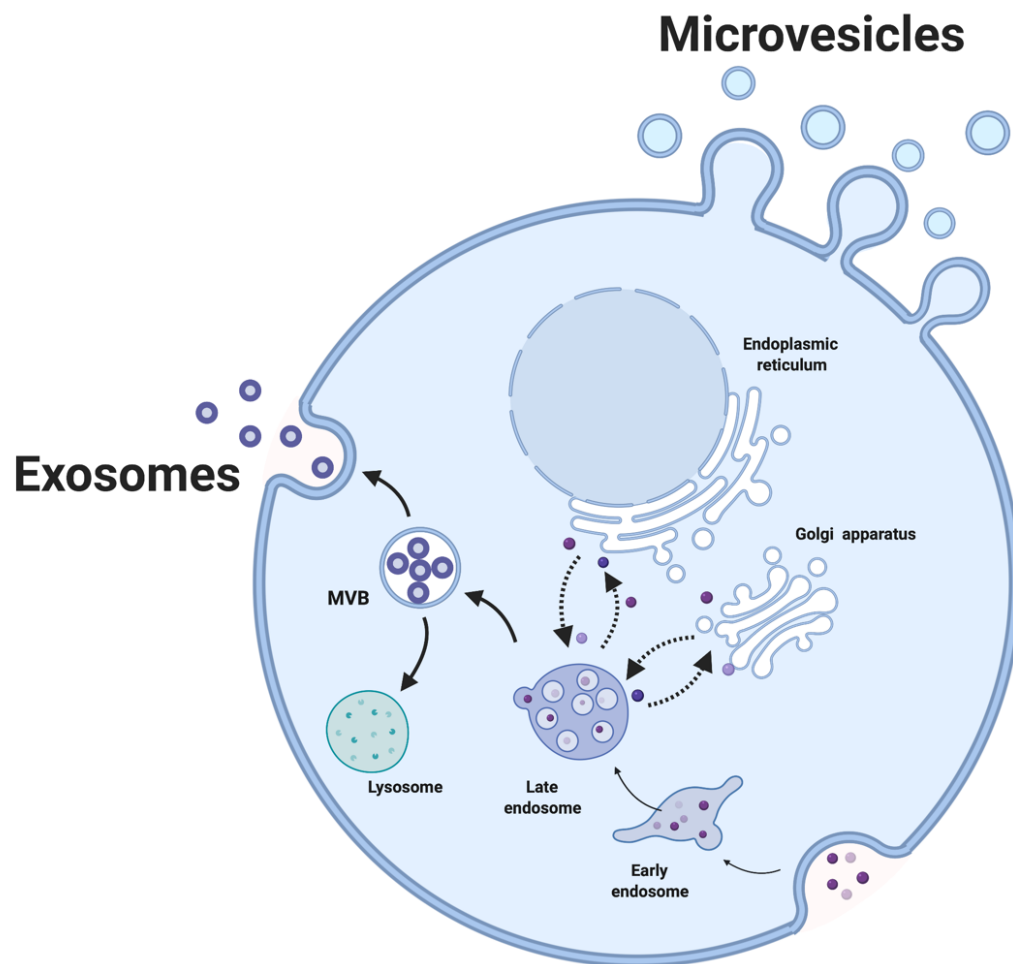


Figure 2. Biogenesis of extracellular vesicles

Extracellular vesicles can be divided into microvesicles and exosomes based on their biogenesis pathway. Microvesicles are generated by outward budding of the plasma membrane. Exosomes are originated from the endosome. Plasma membrane inward invagination leads to the encapsulation of extracellular molecules with plasma membrane molecules to form early endosomes. Sorting and exchange of the cargo occurs between late endosomes, endoplasmic reticulum, and the Golgi apparatus. Further maturation of early endosome leads to the formation of endosomal multivesicular bodies (MVBs) that contain vesicles. MVBs are either directed to be recycled by lysosomes or transported to the plasma membrane for exosomes to be released through exocytosis. This image was created with BioRender.com

Tetraspanins and B cell Exosomes

Tetraspanins are cell surface proteins composed of intracellular, extracellular, and transmembrane domains. They are involved in the regulation of different cellular functions, signaling, and protein trafficking as they interact with a variety of signaling proteins (Yeung et al., 2018). One of the main mechanisms by which tetraspanins act is by interacting with signaling proteins and modulating their signaling activity (Hemler, 2005). For example, in B cells, CD81 plays a role in BCR signaling by forming a complex with CD19 and regulating its trafficking from the Rough Endoplasmic Reticulum (rER) to the plasma membrane where it forms the CD81/CD19/CD21 B cell co-receptor (Shoham et al., 2003). Upon B cell activation through the BCR, CD81 undergoes conformational changes leading to the release of CD19 to freely diffuse along the membrane and interact with the BCR, leading to amplified signaling through the BCR and activation of the B cells (Susa et al., 2020).

Exosomes are enriched in tetraspanins and, therefore, the tetraspanins CD81, CD9, and CD63 are used as exosome markers to differentiate them from microvesicles (Perez-Hernandez et al., 2013). Tetraspanins in exosomes are not solely markers, rather, they are involved in cargo sorting. For example, CD81 and CD9 are involved in the trafficking of MHC-II to B cells exosomes and dendritic cell exosomes, respectively (Buschow et al., 2009; Saunderson et al., 2008; Schick & Levy, 1993; Wubbolts et al., 2003). Exosome secretion by B cells is enhanced following activation through BCR, CD40, MHCII, and IL-4 receptor (Muntasell et al., 2007; Saunderson et al., 2008). Primary B cells activated with anti-CD40/IL-4 secrete exosomes that are representative of B cells (Saunderson et al., 2008). They express MHC-I, MHC-II, CD45RA, Ig, and the tetraspanins CD9 and CD81 (Saunderson et al., 2008). Little is known about the

biological function of B cell exosomes. However, the polarized secretion of exosomes by B cells upon interacting with T cells or Antigen Presenting Cells (APCs) indicates their possible involvement in antigen processing and presentation (Calvo & Izquierdo, 2020). Given that all cells produce exosomes and that exosomes mostly resemble their parent cells, we hypothesized that i35-Bregs secrete exosomes with immunoregulatory effects. Exosomes as nanovesicles that pass the BBB could provide a new therapeutic strategy that takes advantages of i35-Bregs.

IL-35 Containing Exosomes

To understand how the two subunits of IL-35 come together, given that they are not covalently linked, and to develop other approaches to use IL-35 as a therapeutic, we asked whether IL-35 subunits are assembled and secreted in exosomes. Recently, we found that i35-Bregs produce exosomes that contain IL-35 (i35-exosomes). Mice treated with i35-exosomes showed mild EAU with a decrease in retinal pathology, preserved retinal function, and a decrease in Th17 cell levels in the retina, spleen, and lymph nodes. These findings were associated with the expansion of IL-10 and IL-35 producing Tregs (Kang et al., 2020).

Concurrently, Sullivan *et al.*, found that IL-35 is expressed on the surface of FOXP3⁺ Tregs. In addition, IL-35 was expressed on the surface of FOXP3⁻ T cells that do not produce EBI3, suggesting that these cells acquired IL-35 expression by taking up exosomes (J. A. Sullivan et al., 2020). In fact, Sullivan *et al.*, found that i35-exosomes secreted by IL-35 producing Tregs were taken up by conventional T cells, leading them to acquire regulatory functions (J. A. Sullivan et al., 2020). This mechanism is termed “infectious tolerance”. It seems that infectious tolerance is a unique property of IL-35 since it was not documented for other

immunosuppressive cytokines such as IL-10 and TGF- β (Jeremy A. Sullivan et al., 2020). They also found that IL-35 subunits secreted by Tregs were bound to CD81 on the surface of exosomes and were not in a water-soluble form (J. A. Sullivan et al., 2020). However, it is unknown whether this is the case with i35-Bregs.

In this dissertation, we examined the expression of IL35 on the surface of i35-Bregs and whether IL-35 subunits are in association with CD81. The expression of IL-35 on the surface of i35-Bregs will reveal cell-cell contact as a mechanism by which i35-Bregs exert their function. The production of IL-35 in biological carriers, that pass the BBB and propagate immunosuppression, makes i35-exosomes an attractive therapeutic strategy for CNS inflammatory diseases. Given the pathophysiological similarities between EAU and EAE, we examined whether i35-exosomes suppress EAE.

Hypothesis and Specific Aims

IL-35 and i35-Bregs are promising therapeutic strategies for autoimmune diseases. However, little is known about the mechanisms of IL-35 heterodimerization and i35-Breg induction which limits their therapeutic use. The overall objective of this dissertation is to advance our understanding of IL-35 biology and facilitate the development of i35-Breg-derived exosomes as a potential novel cell-free treatment for CNS autoimmune diseases. The hypothesis of this dissertation is: *IL-35 is expressed on the surface of i35-Bregs in association with CD81 and secreted in exosomes that can be used to treat CNS autoimmune diseases.* This hypothesis was tested by accomplishing the following specific aims.

1. Optimize the generation of i35-Bregs
2. Characterize IL-35 expression in i35-Bregs
 - A. Examine the expression of IL-35 on the surface of i35-Bregs
 - B. Examine the association of IL35 subunits with CD81
3. Isolate and characterize i35-exosomes
 - A. Isolate exosomes produced by i35-Bregs
 - B. Determine their size and the expression of exosome markers
 - C. Examine the expression of IL-35 in isolated exosomes
4. Determine the effect of i35-exosome treatment in EAE
 - A. Examine the effect of IL-35 on inflammatory and regulatory cells from EAE mice *in vitro*
 - B. Examine the effect of i35-exosomes on inflammatory and regulatory cells from EAE mice *in vitro*

C. Examine the effect of i35-exosomes treatment on clinical EAE

References

- Aiello, S., Rocchetta, F., Longaretti, L., Faravelli, S., Todeschini, M., Cassis, L., Pezzuto, F., Tomasoni, S., Azzollini, N., Mister, M., Mele, C., Conti, S., Breno, M., Remuzzi, G., Noris, M., & Benigni, A. (2017). Extracellular vesicles derived from T regulatory cells suppress T cell proliferation and prolong allograft survival. *Sci Rep*, 7(1), 11518. <https://doi.org/10.1038/s41598-017-08617-3>
- Bankó, Z., Pozsgay, J., Szili, D., Tóth, M., Gáti, T., Nagy, G., Rojkovich, B., & Sármay, G. (2017). Induction and Differentiation of IL-10–Producing Regulatory B Cells from Healthy Blood Donors and Rheumatoid Arthritis Patients. *The Journal of Immunology*, 198(4), 1512-1520. <https://doi.org/10.4049/jimmunol.1600218>
- Berard, J. L., Wolak, K., Fournier, S., & David, S. (2010). Characterization of relapsing-remitting and chronic forms of experimental autoimmune encephalomyelitis in C57BL/6 mice. *Glia*, 58(4), 434-445. <https://doi.org/10.1002/glia.20935>
- Booth, A. M., Fang, Y., Fallon, J. K., Yang, J. M., Hildreth, J. E., & Gould, S. J. (2006). Exosomes and HIV Gag bud from endosome-like domains of the T cell plasma membrane. *J Cell Biol*, 172(6), 923-935. <https://doi.org/10.1083/jcb.200508014>
- Browne, P., Chandraratna, D., Angood, C., Tremlett, H., Baker, C., Taylor, B. V., & Thompson, A. J. (2014). Atlas of Multiple Sclerosis 2013: A growing global problem with widespread inequity. *Neurology*, 83(11), 1022-1024. <https://doi.org/10.1212/wnl.0000000000000768>

- Buschow, S. I., Nolte-'t Hoen, E. N., van Niel, G., Pols, M. S., ten Broeke, T., Lauwen, M., Ossendorp, F., Melief, C. J., Raposo, G., Wubbolts, R., Wauben, M. H., & Stoorvogel, W. (2009). MHC II in dendritic cells is targeted to lysosomes or T cell-induced exosomes via distinct multivesicular body pathways. *Traffic*, 10(10), 1528-1542.
<https://doi.org/10.1111/j.1600-0854.2009.00963.x>
- Calvo, V., & Izquierdo, M. (2020). Inducible Polarized Secretion of Exosomes in T and B Lymphocytes. *Int J Mol Sci*, 21(7). <https://doi.org/10.3390/ijms21072631>
- Chen, W., Jin, W., Hardegen, N., Lei, K. J., Li, L., Marinos, N., McGrady, G., & Wahl, S. M. (2003). Conversion of peripheral CD4⁺CD25⁻ naive T cells to CD4⁺CD25⁺ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med*, 198(12), 1875-1886. <https://doi.org/10.1084/jem.20030152>
- Choi, J. K., Dambuza, I. M., He, C., Yu, C. R., Uche, A. N., Mattapallil, M. J., Caspi, R. R., & Egwuagu, C. E. (2017). IL-12p35 Inhibits Neuroinflammation and Ameliorates Autoimmune Encephalomyelitis. *Front Immunol*, 8, 1258.
<https://doi.org/10.3389/fimmu.2017.01258>
- Choi, J. K., Yu, C. R., Bing, S. J., Jittayasothorn, Y., Mattapallil, M. J., Kang, M., Park, S. B., Lee, H. S., Dong, L., Shi, G., Caspi, R. R., & Egwuagu, C. E. (2021). IL-27-producing B-1a cells suppress neuroinflammation and CNS autoimmune diseases. *Proc Natl Acad Sci U S A*, 118(47). <https://doi.org/10.1073/pnas.2109548118>
- Collison, L. W., Chaturvedi, V., Henderson, A. L., Giacomini, P. R., Guy, C., Bankoti, J., Finkelstein, D., Forbes, K., Workman, C. J., Brown, S. A., Rehg, J. E., Jones, M. L., Ni, H. T., Artis, D., Turk, M. J., & Vignali, D. A. (2010). IL-35-mediated induction of a

- potent regulatory T cell population. *Nat Immunol*, 11(12), 1093-1101.
<https://doi.org/10.1038/ni.1952>
- Collison, L. W., Delgoffe, G. M., Guy, C. S., Vignali, K. M., Chaturvedi, V., Fairweather, D., Satoskar, A. R., Garcia, K. C., Hunter, C. A., Drake, C. G., Murray, P. J., & Vignali, D. A. (2012). The composition and signaling of the IL-35 receptor are unconventional. *Nat Immunol*, 13(3), 290-299. <https://doi.org/10.1038/ni.2227>
- Collison, L. W., Workman, C. J., Kuo, T. T., Boyd, K., Wang, Y., Vignali, K. M., Cross, R., Sehy, D., Blumberg, R. S., & Vignali, D. A. (2007). The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature*, 450(7169), 566-569.
<https://doi.org/10.1038/nature06306>
- Constantinescu, C. S., Farooqi, N., O'Brien, K., & Gran, B. (2011). Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS). *Br J Pharmacol*, 164(4), 1079-1106. <https://doi.org/10.1111/j.1476-5381.2011.01302.x>
- Cottrell, D. A., Kremenichutzky, M., Rice, G. P., Koopman, W. J., Hader, W., Baskerville, J., & Ebers, G. C. (1999). The natural history of multiple sclerosis: a geographically based study. 5. The clinical features and natural history of primary progressive multiple sclerosis. *Brain*, 122 (Pt 4), 625-639. <https://doi.org/10.1093/brain/122.4.625>
- Crawford, N. (1971). The presence of contractile proteins in platelet microparticles isolated from human and animal platelet-free plasma. *Br J Haematol*, 21(1), 53-69.
<https://doi.org/10.1111/j.1365-2141.1971.tb03416.x>
- Dambuza, I. M., He, C., Choi, J. K., Yu, C. R., Wang, R., Mattapallil, M. J., Wingfield, P. T., Caspi, R. R., & Egwuagu, C. E. (2017). IL-12p35 induces expansion of IL-10 and IL-35-

- expressing regulatory B cells and ameliorates autoimmune disease. *Nat Commun*, 8(1), 719. <https://doi.org/10.1038/s41467-017-00838-4>
- Del Conde, I., Shrimpton, C. N., Thiagarajan, P., & López, J. A. (2005). Tissue-factor-bearing microvesicles arise from lipid rafts and fuse with activated platelets to initiate coagulation. *Blood*, 106(5), 1604-1611. <https://doi.org/10.1182/blood-2004-03-1095>
- Devergne, O., Birkenbach, M., & Kieff, E. (1997). Epstein–Barr virus-induced gene 3 and the p35 subunit of interleukin 12 form a novel heterodimeric hematopoietin. *Proceedings of the National Academy of Sciences*, 94(22), 12041-12046. <https://doi.org/10.1073/pnas.94.22.12041>
- Dilokthornsakul, P., Valuck, R. J., Nair, K. V., Corboy, J. R., Allen, R. R., & Campbell, J. D. (2016). Multiple sclerosis prevalence in the United States commercially insured population. *Neurology*, 86(11), 1014-1021. <https://doi.org/10.1212/wnl.0000000000002469>
- Do, J. s., Visperas, A., Sanogo, Y. O., Bechtel, J. J., Dvorina, N., Kim, S., Jang, E., Stohlman, S. A., Shen, B., Fairchild, R. L., Baldwin Iii, W. M., Vignali, D. A. A., & Min, B. (2016). An IL-27/Lag3 axis enhances Foxp3⁺ regulatory T cell–suppressive function and therapeutic efficacy. *Mucosal Immunology*, 9(1), 137-145. <https://doi.org/10.1038/mi.2015.45>
- Fillatreau, S., Sweenie, C. H., McGeachy, M. J., Gray, D., & Anderton, S. M. (2002). B cells regulate autoimmunity by provision of IL-10. *Nat Immunol*, 3(10), 944-950. <https://doi.org/10.1038/ni833>
- Fontenot, J. D., Rasmussen, J. P., Williams, L. M., Dooley, J. L., Farr, A. G., & Rudensky, A. Y. (2005). Regulatory T Cell Lineage Specification by the Forkhead Transcription Factor

Foxp3. *Immunity*, 22(3), 329-341.

<https://doi.org/https://doi.org/10.1016/j.immuni.2005.01.016>

Fordjour, F. K., Daaboul, G. G., & Gould, S. J. (2019). A shared pathway of exosome biogenesis operates at plasma and endosome membranes. *bioRxiv*, 545228.

<https://doi.org/10.1101/545228>

Francisco, L. M., Salinas, V. H., Brown, K. E., Vanguri, V. K., Freeman, G. J., Kuchroo, V. K., & Sharpe, A. H. (2009). PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. *Journal of Experimental Medicine*, 206(13), 3015-3029.

<https://doi.org/10.1084/jem.20090847>

Fritzsche, B., Schwer, B., Kartenbeck, J., Pedal, A., Horejsi, V., & Ott, M. (2002). Release and intercellular transfer of cell surface CD81 via microparticles. *J Immunol*, 169(10), 5531-

5537. <https://doi.org/10.4049/jimmunol.169.10.5531>

Glass, M. C., Glass, D. R., Oliveria, J.-P., Mbiribindi, B., Esquivel, C. O., Krams, S. M., Bendall, S. C., & Martinez, O. M. (2022). Human IL-10-producing B cells have diverse states that are induced from multiple B cell subsets. *Cell Reports*, 39(3), 110728.

<https://doi.org/https://doi.org/10.1016/j.celrep.2022.110728>

Gubler, U., Chua, A. O., Schoenhaut, D. S., Dwyer, C. M., McComas, W., Motyka, R., Nabavi, N., Wolitzky, A. G., Quinn, P. M., Familletti, P. C., & et al. (1991). Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor. *Proc Natl Acad Sci U S A*, 88(10), 4143-4147.

<https://doi.org/10.1073/pnas.88.10.4143>

- Gurung, S., Perocheau, D., Touramanidou, L., & Baruteau, J. (2021). The exosome journey: from biogenesis to uptake and intracellular signalling. *Cell Communication and Signaling*, 19(1), 47. <https://doi.org/10.1186/s12964-021-00730-1>
- Haynes, N. M., Allen, C. D., Lesley, R., Ansel, K. M., Killeen, N., & Cyster, J. G. (2007). Role of CXCR5 and CCR7 in follicular Th cell positioning and appearance of a programmed cell death gene-1high germinal center-associated subpopulation. *J Immunol*, 179(8), 5099-5108. <https://doi.org/10.4049/jimmunol.179.8.5099>
- Hemler, M. E. (2005). Tetraspanin functions and associated microdomains. *Nature Reviews Molecular Cell Biology*, 6(10), 801-811. <https://doi.org/10.1038/nrm1736>
- Huang, C. T., Workman, C. J., Flies, D., Pan, X., Marson, A. L., Zhou, G., Hipkiss, E. L., Ravi, S., Kowalski, J., Levitsky, H. I., Powell, J. D., Pardoll, D. M., Drake, C. G., & Vignali, D. A. (2004). Role of LAG-3 in regulatory T cells. *Immunity*, 21(4), 503-513. <https://doi.org/10.1016/j.immuni.2004.08.010>
- Kahlert, C., & Kalluri, R. (2013). Exosomes in tumor microenvironment influence cancer progression and metastasis. *Journal of molecular medicine (Berlin, Germany)*, 91(4), 431-437. <https://doi.org/10.1007/s00109-013-1020-6>
- Kalluri, R., & LeBleu, V. S. (2020). The biology, function, and biomedical applications of exosomes. *Science*, 367(6478). <https://doi.org/10.1126/science.aau6977>
- Kang, M., Choi, J. K., Jittayasothorn, Y., & Egwuagu, C. E. (2020). Interleukin 35-Producing Exosomes Suppress Neuroinflammation and Autoimmune Uveitis. *Front Immunol*, 11, 1051. <https://doi.org/10.3389/fimmu.2020.01051>

- Kehrl, J. H., Roberts, A. B., Wakefield, L. M., Jakowlew, S., Sporn, M. B., & Fauci, A. S. (1986). Transforming growth factor beta is an important immunomodulatory protein for human B lymphocytes. *J Immunol*, 137(12), 3855-3860.
- Khan, A. R., Hams, E., Floudas, A., Sparwasser, T., Weaver, C. T., & Fallon, P. G. (2015). PD-L1hi B cells are critical regulators of humoral immunity. *Nat Commun*, 6, 5997. <https://doi.org/10.1038/ncomms6997>
- Komlósi, Z. I., Kovács, N., van de Veen, W., Kirsch, A. I., Fahrner, H. B., Wawrzyniak, M., Rebane, A., Stanic, B., Palomares, O., Rückert, B., Menz, G., Akdis, M., Losonczy, G., & Akdis, C. A. (2018). Human CD40 ligand-expressing type 3 innate lymphoid cells induce IL-10-producing immature transitional regulatory B cells. *J Allergy Clin Immunol*, 142(1), 178-194.e111. <https://doi.org/10.1016/j.jaci.2017.07.046>
- Korniotis, S., Gras, C., Letscher, H., Montandon, R., Mégret, J., Siegert, S., Ezine, S., Fallon, P. G., Luther, S. A., Fillatreau, S., & Zavala, F. (2016). Treatment of ongoing autoimmune encephalomyelitis with activated B-cell progenitors maturing into regulatory B cells. *Nat Commun*, 7, 12134. <https://doi.org/10.1038/ncomms12134>
- Kretschmer, K., Apostolou, I., Hawiger, D., Khazaie, K., Nussenzweig, M. C., & von Boehmer, H. (2005). Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol*, 6(12), 1219-1227. <https://doi.org/10.1038/ni1265>
- Lampropoulou, V., Hoehlig, K., Roch, T., Neves, P., Gómez, E. C., Sweenie, C. H., Hao, Y., Freitas, A. A., Steinhoff, U., Anderton, S. M., & Fillatreau, S. (2008). TLR-Activated B Cells Suppress T Cell-Mediated Autoimmunity. *The Journal of Immunology*, 180(7), 4763-4773. <https://doi.org/10.4049/jimmunol.180.7.4763>

- Lee, K. M., Stott, R. T., Zhao, G., SooHoo, J., Xiong, W., Lian, M. M., Fitzgerald, L., Shi, S., Akrawi, E., Lei, J., Deng, S., Yeh, H., Markmann, J. F., & Kim, J. I. (2014). TGF- β -producing regulatory B cells induce regulatory T cells and promote transplantation tolerance. *European journal of immunology*, 44(6), 1728-1736.
<https://doi.org/10.1002/eji.201344062>
- Lino, A. C., Dang, V. D., Lampropoulou, V., Welle, A., Joedicke, J., Pohar, J., Simon, Q., Thalmensi, J., Baures, A., Flühler, V., Sakwa, I., Stervbo, U., Ries, S., Jouneau, L., Boudinot, P., Tsubata, T., Adachi, T., Hutloff, A., Dörner, T., Zimmer-Strobl, U., de Vos, A. F., Dahlke, K., Loh, G., Korniotis, S., Goosmann, C., Weill, J. C., Reynaud, C. A., Kaufmann, S. H. E., Walter, J., & Fillatreau, S. (2018). LAG-3 Inhibitory Receptor Expression Identifies Immunosuppressive Natural Regulatory Plasma Cells. *Immunity*, 49(1), 120-133.e129. <https://doi.org/10.1016/j.immuni.2018.06.007>
- Mauri, C., Gray, D., Mushtaq, N., & Londei, M. (2003). Prevention of arthritis by interleukin 10-producing B cells. *J Exp Med*, 197(4), 489-501. <https://doi.org/10.1084/jem.20021293>
- Mielle, J., Audo, R., Hahne, M., Macia, L., Combe, B., Morel, J., & Daien, C. (2018). IL-10 Producing B Cells Ability to Induce Regulatory T Cells Is Maintained in Rheumatoid Arthritis [Original Research]. *Frontiers in Immunology*, 9.
<https://doi.org/10.3389/fimmu.2018.00961>
- Mizoguchi, A., Mizoguchi, E., Takedatsu, H., Blumberg, R. S., & Bhan, A. K. (2002). Chronic intestinal inflammatory condition generates IL-10-producing regulatory B cell subset characterized by CD1d upregulation. *Immunity*, 16(2), 219-230.
[https://doi.org/10.1016/s1074-7613\(02\)00274-1](https://doi.org/10.1016/s1074-7613(02)00274-1)

- Muntasell, A., Berger, A. C., & Roche, P. A. (2007). T cell-induced secretion of MHC class II-peptide complexes on B cell exosomes. *Embo j*, 26(19), 4263-4272.
<https://doi.org/10.1038/sj.emboj.7601842>
- Oppmann, B., Lesley, R., Blom, B., Timans, J. C., Xu, Y., Hunte, B., Vega, F., Yu, N., Wang, J., Singh, K., Zonin, F., Vaisberg, E., Churakova, T., Liu, M.-r., Gorman, D., Wagner, J., Zurawski, S., Liu, Y.-J., Abrams, J. S., Moore, K. W., Rennick, D., de Waal-Malefyt, R., Hannum, C., Bazan, J. F., & Kastelein, R. A. (2000). Novel p19 Protein Engages IL-12p40 to Form a Cytokine, IL-23, with Biological Activities Similar as Well as Distinct from IL-12. *Immunity*, 13(5), 715-725. [https://doi.org/https://doi.org/10.1016/S1074-7613\(00\)00070-4](https://doi.org/https://doi.org/10.1016/S1074-7613(00)00070-4)
- Perez-Hernandez, D., Gutiérrez-Vázquez, C., Jorge, I., López-Martín, S., Ursa, A., Sánchez-Madrid, F., Vázquez, J., & Yáñez-Mó, M. (2013). The Intracellular Interactome of Tetraspanin-enriched Microdomains Reveals Their Function as Sorting Machineries toward Exosomes*. *Journal of Biological Chemistry*, 288(17), 11649-11661.
<https://doi.org/https://doi.org/10.1074/jbc.M112.445304>
- Pflanz, S., Timans, J. C., Cheung, J., Rosales, R., Kanzler, H., Gilbert, J., Hibbert, L., Churakova, T., Travis, M., Vaisberg, E., Blumenschein, W. M., Mattson, J. D., Wagner, J. L., To, W., Zurawski, S., McClanahan, T. K., Gorman, D. M., Bazan, J. F., de Waal Malefyt, R., Rennick, D., & Kastelein, R. A. (2002). IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4⁺ T cells. *Immunity*, 16(6), 779-790. [https://doi.org/10.1016/s1074-7613\(02\)00324-2](https://doi.org/10.1016/s1074-7613(02)00324-2)

- Poe, J. C., Smith, S. H., Haas, K. M., Yanaba, K., Tsubata, T., Matsushita, T., & Tedder, T. F. (2011). Amplified B lymphocyte CD40 signaling drives regulatory B10 cell expansion in mice. *PLoS One*, 6(7), e22464. <https://doi.org/10.1371/journal.pone.0022464>
- Rubtsov, Y. P., Rasmussen, J. P., Chi, E. Y., Fontenot, J., Castelli, L., Ye, X., Treuting, P., Siewe, L., Roers, A., Henderson, W. R., Jr., Muller, W., & Rudensky, A. Y. (2008). Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity*, 28(4), 546-558. <https://doi.org/10.1016/j.immuni.2008.02.017>
- Saunderson, S. C., Schuberth, P. C., Dunn, A. C., Miller, L., Hock, B. D., MacKay, P. A., Koch, N., Jack, R. W., & McLellan, A. D. (2008). Induction of exosome release in primary B cells stimulated via CD40 and the IL-4 receptor. *J Immunol*, 180(12), 8146-8152. <https://doi.org/10.4049/jimmunol.180.12.8146>
- Schick, M. R., & Levy, S. (1993). The TAPA-1 molecule is associated on the surface of B cells with HLA-DR molecules. *J Immunol*, 151(8), 4090-4097.
- Schrier, S. L., Godin, D., Gordon Gould, R., Swyryd, B., Junga, I., & Seeger, M. (1971). Characterization of microvesicles produced by shearing of human erythrocyte membranes. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 233(1), 26-36. [https://doi.org/https://doi.org/10.1016/0005-2736\(71\)90354-3](https://doi.org/https://doi.org/10.1016/0005-2736(71)90354-3)
- Shen, P., Roch, T., Lampropoulou, V., O'Connor, R. A., Stervbo, U., Hilgenberg, E., Ries, S., Dang, V. D., Jaimes, Y., Daridon, C., Li, R., Jouneau, L., Boudinot, P., Wilantri, S., Sakwa, I., Miyazaki, Y., Leech, M. D., McPherson, R. C., Wirtz, S., Neurath, M., Hoehlig, K., Meinl, E., Grützkau, A., Grün, J. R., Horn, K., Kühl, A. A., Dörner, T., Bar-Or, A., Kaufmann, S. H. E., Anderton, S. M., & Fillatreau, S. (2014). IL-35-producing B

- cells are critical regulators of immunity during autoimmune and infectious diseases. *Nature*, 507(7492), 366-370. <https://doi.org/10.1038/nature12979>
- Shoham, T., Rajapaksa, R., Boucheix, C., Rubinstein, E., Poe, J. C., Tedder, T. F., & Levy, S. (2003). The Tetraspanin CD81 Regulates the Expression of CD19 During B Cell Development in a Postendoplasmic Reticulum Compartment. *The Journal of Immunology*, 171(8), 4062-4072. <https://doi.org/10.4049/jimmunol.171.8.4062>
- Sullivan, J. A., AlAdra, D. P., Olson, B. M., McNeel, D. G., & Burlingham, W. J. (2020). Infectious Tolerance as Seen With 2020 Vision: The Role of IL-35 and Extracellular Vesicles [Review]. *Frontiers in Immunology*, 11(1867). <https://doi.org/10.3389/fimmu.2020.01867>
- Sullivan, J. A., Tomita, Y., Jankowska-Gan, E., Lema, D. A., Arvedson, M. P., Nair, A., Bracamonte-Baran, W., Zhou, Y., Meyer, K. K., Zhong, W., Sawant, D. V., Szymczak-Workman, A. L., Zhang, Q., Workman, C. J., Hong, S., Vignali, D. A. A., & Burlingham, W. J. (2020). Treg-Cell-Derived IL-35-Coated Extracellular Vesicles Promote Infectious Tolerance. *Cell Rep*, 30(4), 1039-1051.e1035. <https://doi.org/10.1016/j.celrep.2019.12.081>
- Sun, L., He, C., Nair, L., Yeung, J., & Egwuagu, C. E. (2015). Interleukin 12 (IL-12) family cytokines: Role in immune pathogenesis and treatment of CNS autoimmune disease. *Cytokine*, 75(2), 249-255. <https://doi.org/10.1016/j.cyto.2015.01.030>
- Surh, C. D., & Sprent, J. (2012). TGF- β puts the brakes on homeostatic proliferation. *Nat Immunol*, 13(7), 628-630. <https://doi.org/10.1038/ni.2345>

- Susa, K. J., Seegar, T. C., Blacklow, S. C., & Kruse, A. C. (2020). A dynamic interaction between CD19 and the tetraspanin CD81 controls B cell co-receptor trafficking. *Elife*, 9, e52337. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7228769/pdf/elife-52337.pdf>
- van Niel, G., D'Angelo, G., & Raposo, G. (2018). Shedding light on the cell biology of extracellular vesicles. *Nature Reviews Molecular Cell Biology*, 19(4), 213-228. <https://doi.org/10.1038/nrm.2017.125>
- Vidal, M., Mangeat, P., & Hoekstra, D. (1997). Aggregation reroutes molecules from a recycling to a vesicle-mediated secretion pathway during reticulocyte maturation. *J Cell Sci*, 110 (Pt 16), 1867-1877. <https://doi.org/10.1242/jcs.110.16.1867>
- Wang, R. X., Yu, C. R., Dambuza, I. M., Mahdi, R. M., Dolinska, M. B., Sergeev, Y. V., Wingfield, P. T., Kim, S. H., & Egwuagu, C. E. (2014). Interleukin-35 induces regulatory B cells that suppress autoimmune disease. *Nat Med*, 20(6), 633-641. <https://doi.org/10.1038/nm.3554>
- Wolf, S. D., Dittel, B. N., Hardardottir, F., & Janeway, C. A., Jr. (1996). Experimental autoimmune encephalomyelitis induction in genetically B cell-deficient mice. *J Exp Med*, 184(6), 2271-2278. <https://doi.org/10.1084/jem.184.6.2271>
- Wubbolts, R., Leckie, R. S., Veenhuizen, P. T. M., Schwarzmann, G., Möbius, W., Hoernschemeyer, J., Slot, J.-W., Geuze, H. J., & Stoorvogel, W. (2003). Proteomic and Biochemical Analyses of Human B Cell-derived Exosomes: POTENTIAL IMPLICATIONS FOR THEIR FUNCTION AND MULTIVESICULAR BODY FORMATION*. *Journal of Biological Chemistry*, 278(13), 10963-10972. <https://doi.org/https://doi.org/10.1074/jbc.M207550200>

- Xie, C., Ciric, B., Yu, S., Zhang, G. X., & Rostami, A. (2016). IL-12R β 2 has a protective role in relapsing-remitting experimental autoimmune encephalomyelitis. *J Neuroimmunol*, 291, 59-69. <https://doi.org/10.1016/j.jneuroim.2015.12.009>
- Yanaba, K., Bouaziz, J.-D., Matsushita, T., Tsubata, T., & Tedder, T. F. (2009). The Development and Function of Regulatory B Cells Expressing IL-10 (B10 Cells) Requires Antigen Receptor Diversity and TLR Signals. *The Journal of Immunology*, 182(12), 7459-7472. <https://doi.org/10.4049/jimmunol.0900270>
- Yeung, L., Hickey, M. J., & Wright, M. D. (2018). The Many and Varied Roles of Tetraspanins in Immune Cell Recruitment and Migration [Review]. *Frontiers in Immunology*, 9(1644). <https://doi.org/10.3389/fimmu.2018.01644>
- Yoshizaki, A., Miyagaki, T., DiLillo, D. J., Matsushita, T., Horikawa, M., Kountikov, E. I., Spolski, R., Poe, J. C., Leonard, W. J., & Tedder, T. F. (2012). Regulatory B cells control T-cell autoimmunity through IL-21-dependent cognate interactions. *Nature*, 491(7423), 264-268. <https://doi.org/10.1038/nature11501>
- Zhang, H., Freitas, D., Kim, H. S., Fabijanic, K., Li, Z., Chen, H., Mark, M. T., Molina, H., Martin, A. B., Bojmar, L., Fang, J., Rumpersaud, S., Hoshino, A., Matei, I., Kenific, C. M., Nakajima, M., Mutvei, A. P., Sansone, P., Buehring, W., Wang, H., Jimenez, J. P., Cohen-Gould, L., Paknejad, N., Brendel, M., Manova-Todorova, K., Magalhães, A., Ferreira, J. A., Osório, H., Silva, A. M., Massey, A., Cubillos-Ruiz, J. R., Galletti, G., Giannakakou, P., Cuervo, A. M., Blenis, J., Schwartz, R., Brady, M. S., Peinado, H., Bromberg, J., Matsui, H., Reis, C. A., & Lyden, D. (2018). Identification of distinct nanoparticles and subsets of extracellular vesicles by asymmetric flow field-flow

fractionation. *Nature Cell Biology*, 20(3), 332-343. <https://doi.org/10.1038/s41556-018-0040-4>

CHAPTER II: B CELL RECEPTOR SIGNALING INDUCES THE EXPANSION OF IL-35-SURFACE EXPRESSING B REGULATORY CELLS

Abstract

IL-35 and i35-Bregs are promising therapeutic strategies for autoimmune diseases. However, there are some challenges that need to be addressed to facilitate their therapeutic use. For example, there is difficulty in producing bioactive heterodimeric IL-35 *ex-vivo* given that IL-35 subunits (EBI3 and IL-2p35) are secreted independently and bind to each other by unknown mechanisms. While i35-Bregs can sustain delivery of IL-35 in target tissues, there is a need to expand them *ex-vivo* for therapeutic purposes. We have investigated different stimuli to induce the expansion of i35-Bregs. We found that i35-Bregs were induced by stimulation with LPS, LPS + anti-CD40, anti-CD40, or anti-IgM (BCR) + anti-CD40. Stimulating B cells with a low concentration of anti-IgM in a combination of anti-CD40 induced the maximum expansion of i35-Bregs up to ~44% of total B cells thus underscoring the role of BCR signal strength in the induction of i35-Bregs. Using Proximity Ligation Assay, we confirmed that anti-IgM+anti-CD40 induced i35-Bregs produce heterodimeric IL-35. Additionally, we found that IL35 subunits are expressed on the surface of i35-Bregs in colocalization with the tetraspanin CD81. Using Proximity Ligation Assay, we confirmed that EBI3 and IL-12p35 are in association with CD81. Collectively, our study suggested a role of BCR signaling in i35-Breg development and found that stimulating B cells from naïve mice with a combination of anti-IgM+anti-CD40 is optimal for the expansion of i35-Bregs compared to other stimuli. We showed that IL-35 is expressed on the surface of i35-Bregs suggesting cell-cell contact as an immunomodulatory mechanism of i35-

Bregs. The association of EBI3 and IL-12p35 with CD81 provides insight into IL-35 heterodimerization process.

Introduction

IL-35 is a member of the IL12 family of heterodimeric cytokines that includes IL12 (IL-12p35/IL12p40), IL23 (IL23p19/IL12p40), IL27 (p28/EBI3), and IL35 (IL-12p35/EBI3) (Sun et al., 2015). IL-35 is a potent immunosuppressive cytokine that plays a role in regulating autoimmune diseases. It was thought that IL-35 production is restricted to T regulatory cells until IL-35 producing B regulatory cells were discovered (Collison et al., 2007; Wang et al., 2014). Our group found that recombinant IL-35 induced the expansion of a population of Bregs that produce IL-35 (i35-Bregs) *in vitro* and *in vivo* resulting in the amelioration of Experimental Autoimmune Uveitis (EAU), an animal model of uveitis (Wang et al., 2014). Studies by our group and others supported the protective role of regulatory B cells in autoimmune diseases and their potential use as a therapeutic strategy for inflammatory diseases (Shen et al., 2014; Wang et al., 2014). However, little is known about signals required for the expansion of regulatory B cells.

Accumulating evidence implicates environmental cues in the induction of Bregs including stimulation through the toll-like receptors (TLRs) or through the B-cell receptor (BCR)/ and the co-stimulatory receptor CD40. These receptors are involved in B cell activation *in vivo* and *in vitro*. Stimulating B cells through TLR2/4 by LPS is one of the mechanisms to expand IL-10 producing Bregs (B10 cells) (Yanaba et al., 2009), i35-Bregs (Dambuza et al., 2017; Wang et al., 2014), and IL-27 producing Bregs (i27-Bregs) (Choi et al., 2021).

Additionally, BCR signaling was found to be involved in the development of B10 cells (Fillatreau et al., 2002; Yanaba et al., 2009; Yoshizaki et al., 2012) and LAG-3⁺CD138^{hi} Bregs (Lino et al., 2018). Mice deficient in CD19, a co-receptor of BCR, showed a reduced number of LAG-3⁺CD138^{hi} Bregs *in vivo*. On the other hand, mice deficient in CD72, a negative regulator of BCR, showed increased number of LAG-3⁺CD138^{hi} Bregs (Lino et al., 2018). Collectively, these studies support the role of BCR in the development of B10 and LAG3⁺ regulatory B cells. However, little is known about the role of BCR in IL-35 production or the development of i35-Bregs. Understanding signals involved in the induction of i35-Bregs will facilitate their expansion *ex-vivo* for therapeutic purposes.

Although the role of IL-35 and i35-Bregs in suppressing inflammation is well established, there are unsolved questions regarding IL-35 biology and i35-Breg mechanisms of action. IL-35 subunits (EBI3 and IL-2p35) are not linked by a disulfide bond, rather, they are secreted independently and bind to each other by unknown mechanisms (Devergne et al., 1997). The ambiguity of IL-35 heterodimerization mechanism has impeded its use for immunotherapy. Recently, Sullivan *et al.*, reported that IL-35 is expressed on the cell-surface of T regulatory cells (Sullivan et al., 2020). Given that EBI3 and IL-12p35 lack a membrane anchoring motif, further examination showed that EBI3 and IL-12p35 subunits are released bound to the tetraspanin CD81 by Tregs (Sullivan et al., 2020). One of the main functions of tetraspanins is to regulate protein trafficking. Sullivan *et al.* findings suggest that CD81 might be involved in EBI3 and IL-12p35 heterodimerization. However, it is unclear whether IL-35 expression on the cell membrane is a characteristic of IL-35 producing Tregs or it is a unique feature of IL-35 that presents in other IL-35 producing cells. These observations led us to

hypothesize that IL-35 subunits are produced in association with CD81 and expressed on the surface of i35-Bregs.

In this study, we show that stimulating B cells with anti-IgM+anti-CD40 induces i35-Bregs in a higher percentage compared to LPS, LPS+ anti-CD40, or anti-CD40 stimulation. Interestingly, a low concentration of anti-IgM (1 μ g) in combination with anti-CD40 resulted in a higher frequency i35-Bregs than the use of 5 μ g of anti-IgM+anti-CD40. By using Proximity Ligation Assay (PLA), we further support the induction of i35-Bregs that produce heterodimeric IL-35 by anti-IgM+anti-CD40 stimulation. Moreover, we show that i35-Bregs express IL-35 on the surface in colocalization with CD81. We further confirmed that IL-12p35 and EBI3 are in association with CD81. Collectively, our findings support the role of BCR signaling in the induction of i35-Bregs and show that IL-35, unlike other cytokines, is expressed on the cell surface possibly mediating suppression by cell-cell contact mechanisms.

Materials and Methods

Mice

Wild-type C57BL/6J were purchased from Jackson Laboratory. Mice were bred, maintained, and treated in accordance with NIH Animal Care and Use Committee guidelines.

Generation of regulatory B cells

C57BL/6 WT mice were euthanized by CO₂ asphyxiation and the spleens were collected. The spleens were placed on cell strainers (Corning Falcon) and homogenized by gently rubbing the spleens with the plunger of a disposable 1 mL syringe. The cell strainers were washed with

RPMI and the effluent single cell suspensions were collected and washed twice with RPMI by centrifugation at 300 ×g for 10 minutes. Red blood cells were lysed by adding 1 mL of ACK buffer to pelleted splenocytes and incubating for 5 minutes at room temperature. The cells were then washed twice with RPMI to remove ACK buffer by centrifugation at 300 ×g for 10 minutes. The pelleted cells were resuspended in RPMI and passed through a 40-µm pore-size cell strainer to remove tissue fragments. The cells were counted by using Vi-Cell XR (Beckman Coulter, Indianapolis, IN). B cells were sorted using CD19 MicroBeads (Miltenyi Biotec) as per manufacturer's instructions. Briefly, 10 µL of CD19 magnetic beads were added per 10⁷ total cells, mixed, and incubated for 15 minutes at 4 °C. Cells were then washed and passed through LS MACS column placed in the magnetic MACs separator. MACs column was removed from the separator, flushed out with MACs buffer, and the effluent containing B cells was collected. B cells were counted by using Vi-Cell XR (Beckman Coulter, Indianapolis, IN) and resuspended to make 2 x 10⁶ cell/ mL in complete RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (Hyclone), 1% penicillin/streptomycin (Invitrogen), 1% L-glutamine (Sigma-Aldrich), 0.2% β-mercaptoethanol (Gibco), and HEPES. Cells were stimulated with 5 µg/mL LPS (Sigma), 10 µg/mL anti-CD40 (BioXcell), 5 µg/mL LPS+10 µg/mL anti-CD40, 1 µg/mL anti-IgM (Jackson ImmunoResearch)+10 µg/mL anti-CD40, or 5 µg/mL anti-IgM+10 µg/mL anti-CD40. Unstimulated cells were used as a control. The cells were incubated at 37°C in 5 % CO₂ for 72 hours and then proceeded with flow cytometry staining for the identification of regulatory B cells.

Flow cytometry for detection of regulatory B cells

After 72h of cell stimulation as described in the previous section, the cells were stimulated with PMA (20 ng/mL)/ionomycin (1 μ M) and GolgiStop (BD Bioscience) for 4 hours. B cells were washed twice with PBS and stained with Fixable Viability Dye eFluor™ 450 (eBioscience) (diluted as 1:1000 in PBS) for 5 minutes. The cells were washed with complete RPMI and stained with anti-B220 (FITC) and anti-CD-19 (BV510) for 30 minutes at 4 °C. Intracellular cytokine staining was performed using the BD Biosciences Cytofix/Cytoperm kit per manufacturer's instructions. Briefly, the cells were permeabilized by incubating with Cytofix/Cytoperm for 20 minutes at 4 °C. The cells were then washed and stained with anti-EBI3 (PerCp), anti-IL-12p35 (APC), and IL-10 (PE) overnight at 4 °C. Cells stained as Fluorescence Minus One (FMO) were used as controls. The data were collected by using flow cytometry CytoFlex, and the data analysis was performed by using FlowJo software. i35-Breg were identified as B220⁺CD19⁺EBI-3⁺IL-12p35⁺ and IL-10 producing Bregs were identified as B220⁺CD19⁺IL-10⁺ using the gating strategy in (**Figure 3**).

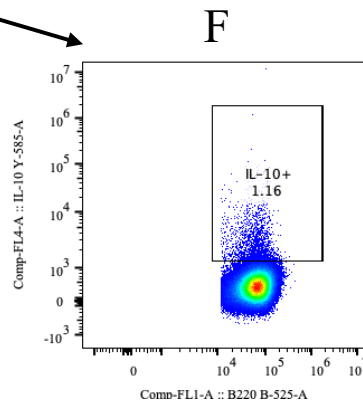
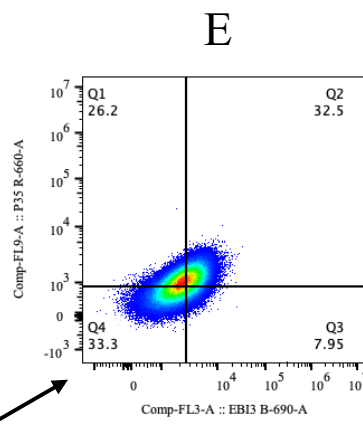
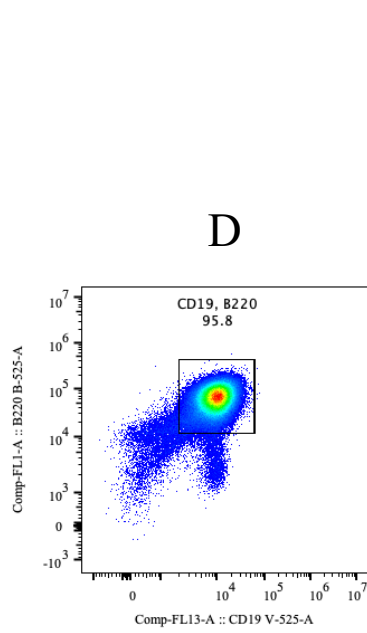
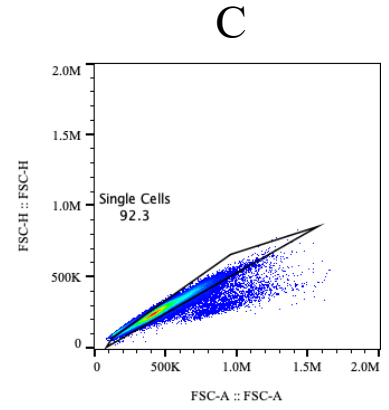
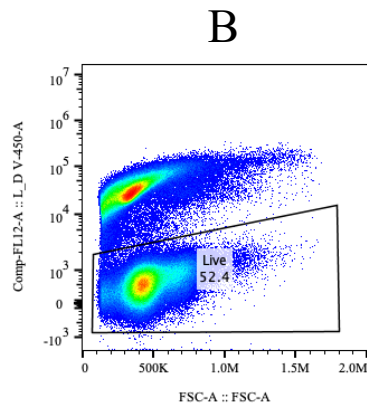
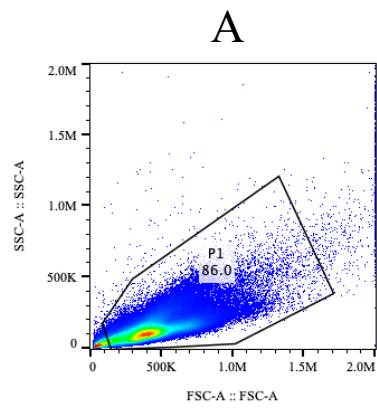


Figure 3. Sequential gating strategy to identify i35-Bregs and IL-10 producing Bregs

A) Cells were first gated based on the forward (FSC) vs side scatter (SSC). **B)** Viable cells were selected based on FSC vs Fixable Viability Dye eFluor™ 450. **C)** Single cells were gate based on FSC-A vs FSC-H. **D)** Cells were gated based on CD19 vs B220 and B cells were identified as CD19⁺B220⁺. **E)** B cells were gated based on IL-12p35 and EBI3 to identify i35-Bregs as IL-12p35⁺EBI3⁺. **F)** B cells were gated based on B220 vs IL-10 to identify IL-10 producing B cells as B220⁺ IL-10⁺.

Immunofluorescence staining and confocal imaging

We used Immunofluorescence staining to examine the expression of IL-35 subunits and CD81 on the surface of i35-Bregs by confocal microscopy. Poly-L-Lysin coated slides were prepared by adding 100 μ l of 0.01% (w/v) Poly-L-Lysin solution (Sigma-Aldrich) to the slide for 5 minutes at room temperature. Excess Poly-L-Lysin was removed, and slides were washed twice with dH₂O and left to air dry. i35Bregs were generated by anti-IgM/anti-CD40 stimulation for 72h as previously described. The cells were collected, washed twice with PBS at 300xg for 5 minutes, and fixed using BD Biosciences Cytofix/Cytoperm for 20 minutes at 4 °C. The cells were then washed with PBS and resuspended in PBS to achieve 2 x10⁶ cells/mL. 100 μ l of the cell suspension were placed to adhere on Poly-L-Lysin coated slides for 20-30 minutes at room temperature. After 30 minutes, excess cells were removed, the slide were allowed to dry, and stored in -20 °C until the staining. For antigen retrieval, Tris-EDTA buffer was heated until boiling and the slides were placed in the buffer until it reaches room temperature then washed twice for 5 minutes with PBS. For immunofluorescence staining, the cells were blocked with ReadyProbes™ Mouse-on-Mouse IgG Blocking Solution (Invitrogen) for 30 minutes, then, blocked with (10% goat serum, 1% BSA, 3% skim milk, 0.02% sodium azide and 0.1% Triton) for another 30 minutes at room temperature. The cells were stained with primary antibodies, rabbit anti-CD81 (D5O2Q, Cellsignal), Rat anti-IL-12p35 (Clone: C18.2, eBioscience™), and mouse anti-EBI3 (sc-166158, SantaCruz) for 24h at 4°C. Primary antibodies were washed twice for 5 minutes with washing buffer (PBS with 0.05% tween 20). The cells were then stained with secondary antibodies, Alexa Fluor Plus 647 Goat anti-Mouse IgG (A32728 Invitrogen), Alexa Fluor 488 Goat anti-rat IgG (A11006 Invitrogen), and Alexa Fluor 568 Goat anti-rabbit IgG

(A11036 Invitrogen) for 1 hour at room temperature. Slides were washed 3 times for 5 minutes with the washing buffer. The slides were stained with DAPI (1:1000 dilution in washing buffer) (Cat#62248, Thermo Scientific) for 5 minutes at room temperature. The slides were washed one time with washing buffer, mounted using VECTASHIELD Vibrance anti-fade Mounting Media (Vector Laboratories), and covered with glass coverslip. The images were acquired by Zeiss LSM 880 confocal microscope. The images were processed using Imaris 9.7.0 to determine the colocalization of IL-12p35, EBI3, and CD81.

Proximity ligation assay

The Duolink Proximity Ligation Assay kit (Sigma Aldrich) was used to demonstrate the association between IL-12p35 and EBI3 subunits that form the heterodimeric IL-35, EBI3 and CD81, and IL-12p35 and CD81. i35 Bregs were generated by anti-IgM/anti-CD40 stimulation and slides were prepared as mentioned previously. After antigen retrieval, the cells were stained per manufacturer's instructions. Briefly, the cells were blocked with Duolink blocking solution in a heated humidity chamber for 60 minutes at 37 °C. The slides were then incubated with primary antibodies mouse anti-EBI3 (sc-166158, SantaCruz) + rabbit anti-IL-12p35 (ab131039, Abcam), rabbit anti-CD81 (D5O2Q, Cellsignal) + mouse anti-EBI3 (sc-166158, SantaCruz), or rabbit anti-CD81 (D5O2Q, Cellsignal) + mouse anti-IL-12p35 for 24 hours at 4°C. Then, the cells were washed twice with washing buffer and incubated with secondary antibodies conjugated with oligonucleotides (PLA probes) in a pre-heated humidity chamber for 1 hour at 37 °C. The slides were then washed twice for 5 minutes with washing buffer and incubated with connector oligos and ligase in a pre-heated humidity chamber for 30 minutes at 37 °C. When the two molecules of interest are in close proximity (within 40 nm), the connector oligo join the PLA

probes and become ligated. Then, the formed circular DNA template was amplified by DNA polymerase in a pre-heated humidity chamber for 100 minutes at 37 °C. Complementary detection oligos conjugated to fluorochromes hybridized to repeating sequences in the amplicons. The slides were stained with DAPI (Cat#62248, Thermo Scientific) and mounted using VECTASHIELD Vibrance anti-fade Mounting Media (vector Laboratories). Associated IL-12p35:EBI3, IL-12p35:CD81, or EBI3:CD81 were detected as fluorescent spots by Zeiss LSM 880 confocal microscopy and images were processed using Imaris 9.7.0.

Statistical analysis

Statistical analysis was performed by using Prism 9 software. Student's *t*-test or one-way ANOVA followed by Post-hoc Tukey test were performed to determine differences between groups. P value of <0.05 was considered statistically significant.

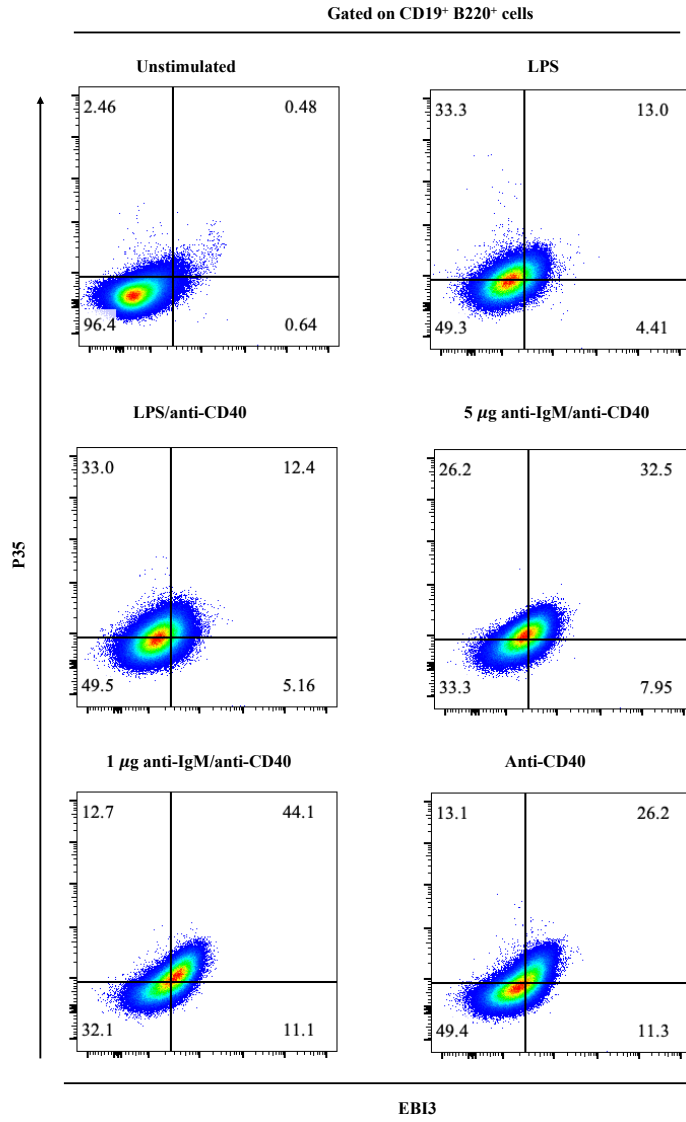
Results

Activation of CD19⁺ B cells by BCR and CD40 signals induces expansion of i35-

Bregs

Regulatory B cells are induced by inflammatory cues such as activation through TLR2/4, BCR, and CD40. However, little is known about signals involved in the development of i35-Bregs. To better understand cues required for the induction of i35-Bregs, we examined different stimulation conditions to induce the expansion of i35-Bregs. The stimulation conditions involved activating CD19⁺ B cells isolated from mouse spleen through TLR2/4 by using LPS (innate signal), BCR by using anti-IgM (mimicking antigen), and or CD40 by using anti-CD40 (co-stimulatory signal) for 72h. Our data show that compared to unstimulated cells, LPS or LPS/CD40 expanded the cells up to 13% (**Figure 4**). Stimulating the cells with anti-CD40 alone was sufficient to induce the expansion of i35-Bregs up to ~ 26% (**Figure 4**). Among the different stimulation conditions, the combination of BCR/CD40 stimulation (anti-IgM/anti-CD40) induced the highest percentages of i35-Bregs (~32-44%) (**Figure 4**). Interestingly, B cells stimulated with a lower concentration of anti-IgM (1 µg) in combination with anti-CD40 induced a higher percentage of i35-Bregs ~44% compared to cells stimulated with anti-IgM (5 µg)/anti-CD40 ~32 % (**Figure 4**). Collectively, our data show that anti-IgM/anti-CD40 is more robust in inducing i35-Bregs than LPS or LPS/anti-CD40 and suggest that a weak BCR signal promotes i35-Bregs induction.

A



B

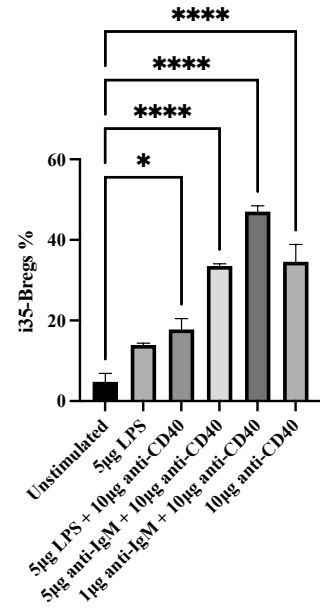


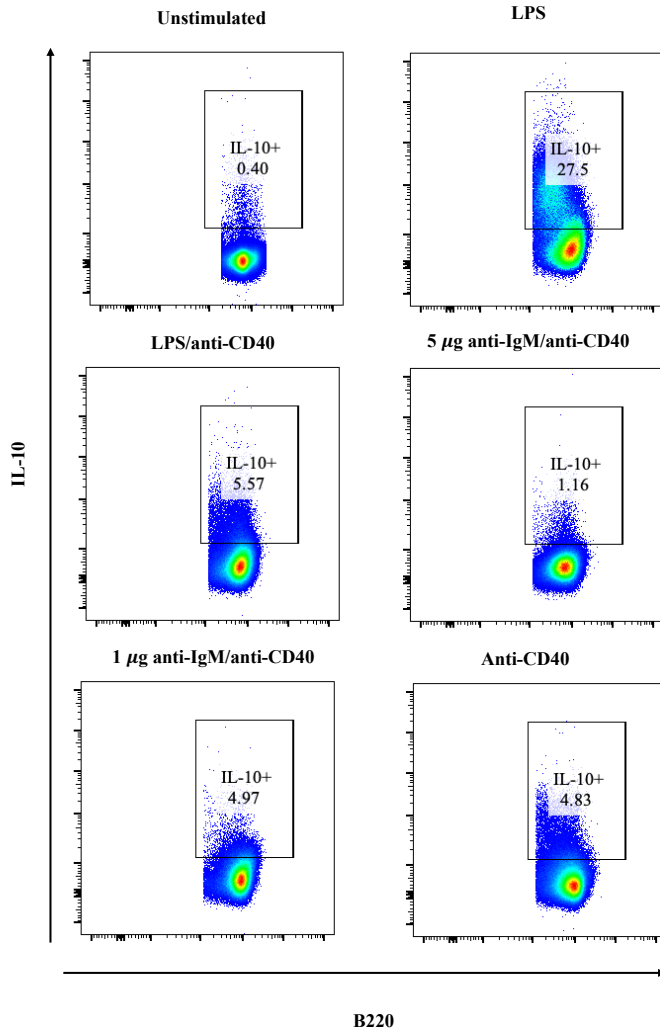
Figure 4. Combination of BCR and CD40 signals induces i35-Breg expansion

Isolated CD19⁺ B cells from the spleen of C57BL/6 mice were stimulated with 5 µg LPS, 5 µg LPS + 10 µg anti-CD40, 5 µg anti-IgM + 10 µg anti-CD40, 1 µg anti-IgM + 10 µg anti-CD40, or 10 µg anti-CD40 for 72 hours. Unstimulated B cells were used as a control. To detect intracellular IL-35 by flow cytometry, cells were then stimulated with PMA/I in the presence of GolgiStop for 4 hours. **A)** Representative flow cytometry plots of intracellular cytokine staining analysis. B cells were identified as CD19⁺B220⁺ and quadrants indicate percent of B cells IL-12p35⁻EBI3⁻, IL-12p35⁺EBI3⁻, IL-12p35⁻EBI3⁺, IL-12p35⁺EBI3⁻, or IL-12p35⁺EBI3⁺. **B)** Frequency of IL-12p35⁺EBI3⁺ B cells (i35-Bregs) from the upper right quadrants of **(A)**. *P* value was calculated using one-way ANOVA, **p*<0.05, *****p*<0.0001. Error bars indicate SEM. Data are from two independent experiments with two replicates/condition/experiment.

Activation of CD19⁺ B cells by BCR and CD40 signals does not induce the expansion of IL-10 Bregs

As IL-35 induces the expansion of both IL-10 producing Bregs and i35-Bregs (Wang et al., 2014), we examined whether the different stimuli induce the expansion of IL-10 Bregs at a similar proportion as i35-Bregs. CD19⁺ B cells were isolated from the spleen of C57BL/6 mice and stimulated with 5 µg LPS, 5 µg LPS + 10 µg anti-CD40, 5 µg anti-IgM + 10 µg anti-CD40, 1µg anti-IgM + 10 µg anti-CD40, or 10µg anti-CD40 for 72h. Our data show that LPS significantly induced the expansion of IL-10 Bregs compared to unstimulated B cells and gave the highest percentage of IL-10 B cells ~27% compared to other stimulation conditions (**Figure 5**). While anti-IgM/ anti-CD40 stimulation highly induced i35-Bregs (**Figure 4**), B cells stimulated with anti-IgM/ anti-CD40 showed a low frequency of IL-10 producing B cells (**Figure 5**). Additionally, the percentage of IL-10 Bregs is lower with a stronger BCR signal as mimicked by increasing the concentration of anti-IgM (**Figure 5**). While 5 µg anti-IgM + 10 µg anti-CD40 induced ~ 5% of IL-10 producing Bregs, 1µg anti-IgM + 10 µg anti-CD40 induced ~ 1% of IL-10 Bregs. Stimulating B cells with anti-CD40 alone also induced a low percentage of IL-10 producing Bregs ~ 4% (**Figure 5**). Similarly, adding a co-stimulatory signal (anti-CD40) to LPS stimulation downregulated IL-10 production by LPS. This effect suggests that CD40 signaling antagonizes IL-10 production by LPS in B cells from naïve mice (**Figure 5**). Collectively, our data show that LPS is a potent inducer of IL-10 Bregs and that anti-IgM/anti-CD40 stimulation specifically induces the expansion of i35-Bregs in B cells from naïve mice.

A



B

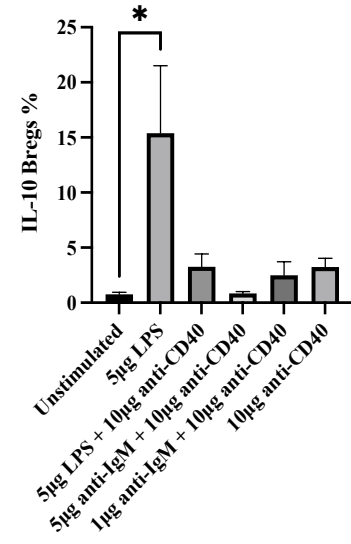


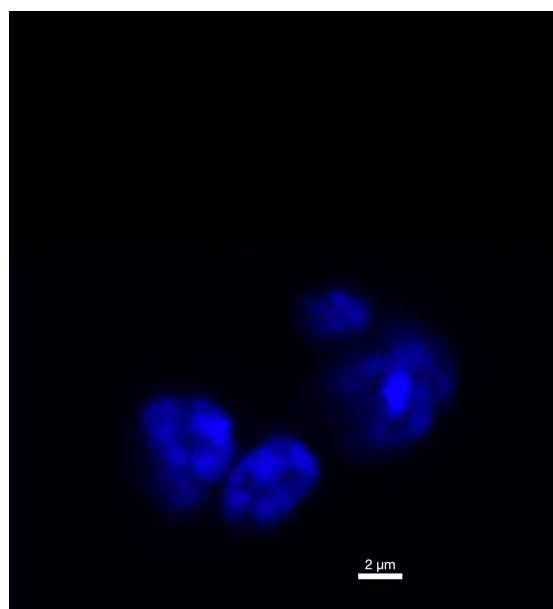
Figure 5. Combination of BCR and CD40 signals does not induce the expansion of IL-10 Bregs

Isolated CD19⁺ B cells from the spleen of C57BL/6 mice were stimulated with 5 µg LPS, 5 µg LPS+ 10 µg anti-CD40, 5 µg anti-IgM+10 µg anti-CD40, 1 µg anti-IgM+10 µg anti-CD40, or 10 µg anti-CD40 for 72h. Unstimulated B cells were used as a control. To detect intracellular IL-10 by flow cytometry, cells were then stimulated with PMA/I in the presence of GolgiStop for 4 hours. **A)** Representative flow cytometry plots of intracellular cytokine staining analysis. Gate indicates percent of B cells (identified as B220⁺) expressing IL-10. **B)** Frequency of IL10⁺ B cells from **(A)**. *P* value was calculated using one-way ANOVA, **p*<0.05. Error bars indicate SEM. Data are from 2 independent experiments of 2 replicates/condition/experiment.

BCR/CD40 induced i35-Bregs express heterodimeric IL-35

Unlike IL-12 and IL-23, IL-35 subunits are not covalently linked, rather, they are secreted independently and factors that promote their association are poorly understood. IL-35 producing cells are usually identified as a double positive of EBI3 and IL-12p35 by using two antibodies, anti-EBI3 and anti-IL-12p35. The lack of antibodies that recognize the heterodimeric IL-35 made it difficult to conclude whether a cell type does produce heterodimeric IL-35. By using flow cytometry, we have shown earlier that anti-IgM + anti-CD40 stimulation induces the expansion of i35-Bregs as indicated by cells that are positive for EBI3 and IL-12p35 (**Figure 4**). To confirm whether B cells stimulated with anti-IgM + anti-CD40 produce heterodimeric IL-35, we used Proximity Ligation Assay (PLA) that detects interacting molecules see (Materials & Methods). In this assay, we used anti-IL-12p35 and anti-EBI3 antibodies and the generated signal indicates that the two subunits are in a close proximity (within 40nm). Here, we show that anti-IgM + anti-CD40 induced i35-Bregs indeed express heterodimeric IL-35 as indicated by PLA signals compared to the negative control (**Figure 6**). These data further support that BCR/CD40 signals induce the expression of heterodimeric IL-35.

Negative



IL-35/DAPI

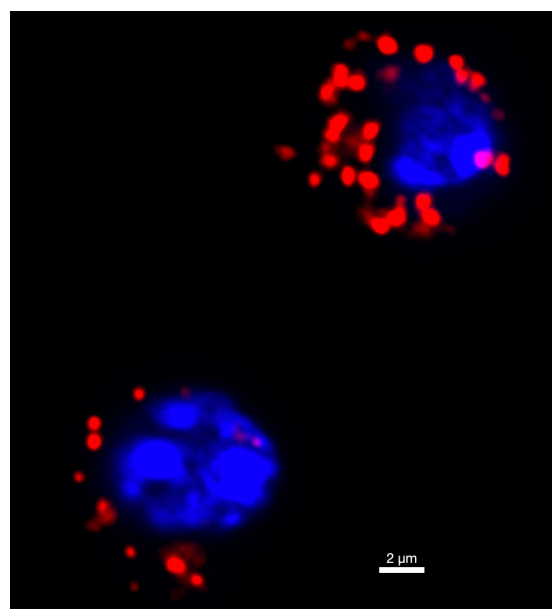


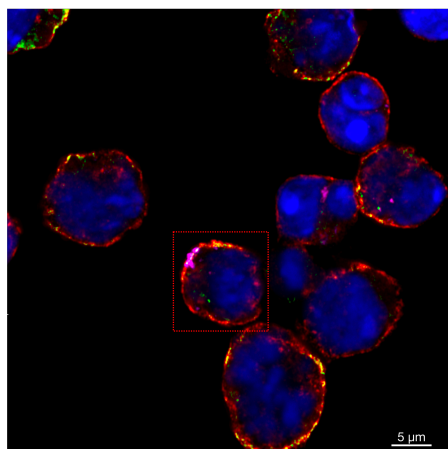
Figure 6. BCR/CD40 induced i35-Bregs express heterodimeric IL-35

i35-Bregs were generated by anti-IgM/anti-CD40 stimulation for 72h and examined for the expression of heterodimeric IL-35 by Proximity Ligation Assay (PLA). Cells were fixed, permeabilized, stained with or without primary anti-IL-12p35 and anti-EBI3, and secondary PLA probes following the PLA protocol as indicated in *Materials and Methods*. Cells were examined by confocal microscopy and all images were taken with 60x magnification. Left image of i35-Bregs stained without primary anti-IL-12p35 and anti-EBI3 as a negative control shows no signal except DAPI fluorescence of nuclei (blue). Right image of i35-Bregs stained with primary anti-IL-12p35 and anti-EBI3 shows heterodimeric IL-35 (red).

IL-35 subunits are colocalized with CD81 on the surface of i35-Bregs

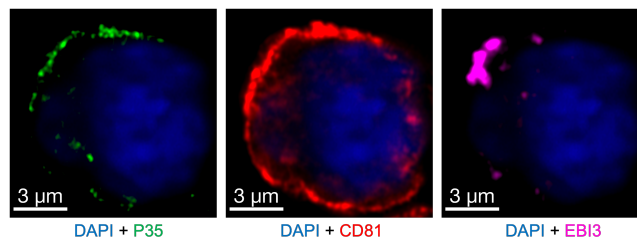
A recent report showed that EBI3 and IL-12p35 are expressed on the surface of IL-35 producing T cells (Sullivan et al., 2020). Given that EBI3 and IL-12p35 lack a transmembrane domain, further investigation showed that the two subunits are associated with the tetraspanin CD81 on the surface of IL-35 producing Tregs (Sullivan et al., 2020). To examine whether this is the case with i35-Bregs, we activated B cells with anti-IgM/anti-CD40 for 72h and then stained them for imaging by confocal microscopy. We show that similar to Tregs, IL-35 subunits are expressed on the surface of i35-Bregs (**Figure 7, B**). The two subunits are colocalized with CD81, suggesting the possible interaction of IL-12p35 and EBI3 with CD81 in i35-Bregs (**Figure 7, C**). Using PLA, we confirmed that IL-12p35 and EBI3 are associated with CD81(**Figure 8**). Collectively, our data show that IL-35 is expressed on the surface of anti-IgM/anti-CD40 induced i35-Bregs in association with CD81. These data provide additional evidence that IL-35 has a unique feature of being a cell membrane associated cytokine.

A



DAPI + P35 + CD81 + EBI3

B

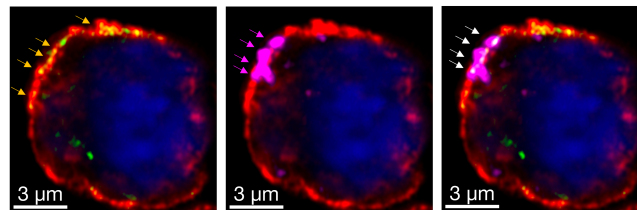


DAPI + P35

DAPI + CD81

DAPI + EBI3

C



DAPI + P35 + CD81

DAPI + EBI3 + CD81

DAPI + P35 + CD81 + EBI3

Figure 7. IL-35 subunits are colocalized with CD81 on the surface of i35-Bregs

i35-Bregs were generated by anti-IgM/anti-CD40 stimulation for 72 hours. Cells were then fixed, permeabilized, and stained for IL-12p35, EBI3, and CD81. **A)** Confocal images of i35-Bregs show DAPI fluorescence of nuclei (blue), IL-12p35 (green), EBI3 (magenta), and CD81 (red). A focus on a cells that show colocalization of IL-12p35, EBI3, and CD81 on the surface (white). **B)** confocal images of individual and merged channels show the expression of IL-12p35 (green), CD81 (red), or EBI3 (magenta) on the surface of i35-Bregs. **C)** Images of merged channels shows the colocalization of IL-12p35 and CD8 (yellow), the colocalization of EBI3 and CD81(magenta), and the colocalization of IL-35 (IL12p3:EBI3) and CD81 (white). All images were taken with 60x magnification.

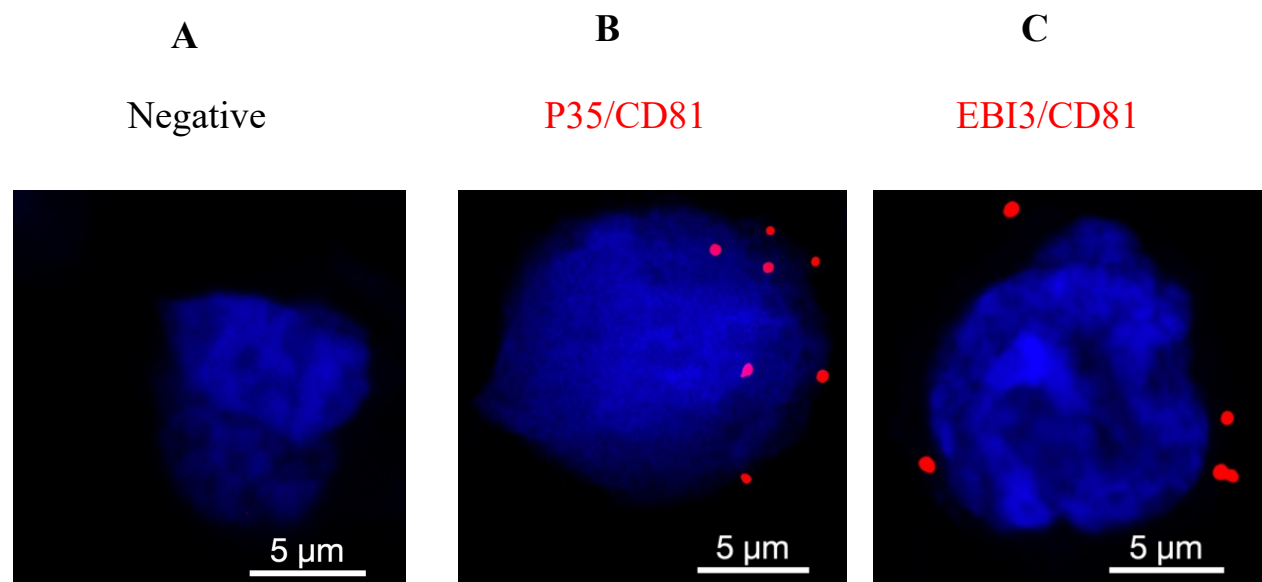


Figure 8. IL-35 subunits are associated with CD81

i35-Bregs were generated by anti-IgM/anti-CD40 stimulation for 72h and examined for the association of IL-35 subunits with CD81 by PLA. Cells were fixed, permeabilized, and stained with primary anti-IL-12p35 and anti-CD81, or anti-EBI3 and anti-CD81, or no primary antibodies as a negative control. Then, secondary PLA probes were added following the PLA protocol as indicated in *Materials and Methods*. Cells were examined by confocal microscopy and all images were taken with 60x magnification. **A)** Image of i35-Bregs stained without primary antibodies as a negative control shows no signal except DAPI fluorescence of nuclei (blue). **B)** Image of i35-Bregs stained with primary anti-IL-12p35 and anti-CD81 shows the association of IL-12p35:CD81 as (red). **C)** Image of i35-Bregs stained with primary anti-EBI3 and anti-CD81 shows the association of EBI3:CD81 as (red).

Polarized distribution of IL-35 in BCR/CD40 stimulated B cells

B cells stimulated with BCR undergo morphological changes leading to cell polarization and the formation of immune synapses (Thyagarajan et al., 2003; M. I. Yuseff et al., 2013). Additionally, some studies suggest that some cytokines are secreted at the immune synapses. During confocal imaging of i35-Bregs induced by anti-IgM+anti-CD40 for 72h, interestingly, we noticed that IL-35 is not uniformly distributed in B cells stimulated with BCR/CD40, rather, it was polarized to one direction (**Figure 9, A**). IL-35 colocalized with CD81 in a pattern that resembled a BCR cap or BCR microdomain, a pattern of BCR clustering induced by antigen or anti-BCR and formed during B cell immune synapses (**Figure 9, B**). While further investigation is needed to confirm IL-35 colocalization with the BCR, the polarized expression of IL-35 suggests that IL-35 are targeted to be secreted into the synapse area.

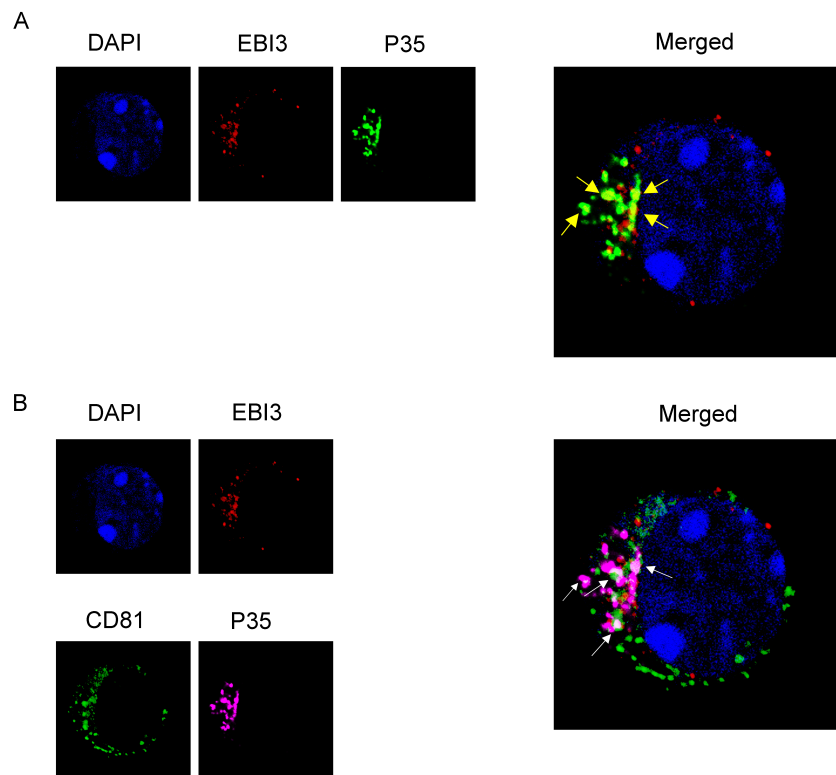


Figure 9. Polarized distribution of IL-35 in BCR/CD40 stimulated B cells

i35-Bregs were generated by anti-IgM/anti-CD40 stimulation for 72h and examined for the expression of EBI3, IL-12p35, and CD81. Cells were fixed, permeabilized, stained with anti-IL-12p35, anti-EBI3, anti-CD81, and DAPI as indicated in *Materials and Methods*. Cells were examined by confocal microscopy and all images were taken with 60x magnification.

A) Confocal images of i35-Bregs show DAPI fluorescence of nuclei (blue), polarized expression of EBI3 (red) and IL-12p35 (green). The merged image shows polarized expression of IL-35 as indicated by colocalization of EBI3 and IL-12p35 (yellow). **B)** Confocal images of i35-Bregs show DAPI fluorescence of nuclei (blue), EBI3 (red), CD81 (green), and IL-12p35 (magenta). The merged image shows polarized colocalization of IL-35 (EBI3:IL-12p35) and CD81 (white).

Discussion

In this study, we have studied different stimuli to induce the expansion of i35-Bregs. Consistent with a previous report from our group (Wang et al., 2014), we found that LPS stimulation slightly induced the expansion of i35-Bregs, up to 13% compared to unstimulated B cells (**Figure 4**). Stimulating B cells with anti-CD40 alone was sufficient to induce the expansion of i35-Bregs up to ~ 26% (**Figure 4**). There is cumulative evidence that LPS and CD40 signals induce the expansion of regulatory B cells. LPS stimulation was found to induce the expansion of B10 cells (Yanaba et al., 2009) and i27-Bregs (Choi et al., 2021). Additionally, B cells that lack CD40 do not produce IL-10 (Fillatreau et al., 2002). Our findings further support the involvement TLRs and CD40 signaling in Breg induction.

Among the different stimulation conditions, stimulating B cells from naïve mice with anti-IgM+anti-CD40 induced the highest percentage of i35-Bregs compared to LPS, LPS+ anti-CD40, or anti-CD40 (**Figure 4**). Interestingly, B cells stimulated with a lower concentration of anti-IgM (1 μ g), in combination with anti-CD40, showed a higher percentage of i35-Bregs (~44%), compared with cells stimulated with 5 μ g anti-IgM+anti-CD40 (~32%) (**Figure 4**). These findings suggest that a lower BCR signal is sufficient for the induction of i35-Bregs. This resembles the role of TCR signaling strength in the generation of Tregs. Different studies showed that low TCR signaling favors the generation of regulatory T cells (Gomez-Rodriguez et al., 2014; Kretschmer et al., 2005). Gomez-Rodriguez et al., showed that while 0.1 μ g of anti-CD3 induced $65.3 \pm 3.1\%$ of FoxP3⁺ cells under iTreg cell differentiation conditions, 5 μ g of anti-CD3 induced $29.7 \pm 2.9\%$ of FoxP3⁺ cells (Gomez-Rodriguez et al., 2014). It is possible that similar to T cells, the concentration of antigen influences the generation of regulatory B cells.

BCR signaling was reported to be involved in the development of other subsets of regulatory B cells such as B10 and LAG-3⁺CD138^{hi} Bregs (Fillatreau et al., 2002; Lino et al., 2018; Yanaba et al., 2009; Yoshizaki et al., 2012). Our findings extend the importance of BCR signaling in Breg induction by showing that i35-Bregs are maximally induced under BCR/CD40 stimulation. Furthermore, our study supports the role of BCR signaling strength in the generation of i35-Bregs.

Given that IL-35 also induces the expansion of IL-10 producing Bregs, we further examined whether anti-IgM+anti-CD40 stimulation will induce IL-10 Bregs. While anti-IgM+anti-CD40 induced the maximum expansion of i35-Bregs compared to other stimuli (**Figure 4**), it did not induce significant expansion of IL-10 producing Bregs (**Figure 5**). This finding is consistent with previous reports that showed that BCR activation of B cells from naïve mice downregulates the production of IL-10 *in vitro*, however, it induces IL-10 production by primed B cells or *in vivo* (Catalán et al., 2021; Jin et al., 2013; Yanaba et al., 2009; Yanaba et al., 2008). We show that, in contrast to i35-Bregs, IL-10 producing Bregs were maximally expanded by LPS stimulation (**Figure 5**). Similarly, Yanaba *et al.*, showed that LPS stimulation could induce IL-10 production within 5 hours and the frequency of IL-10 producing B cells increases at 48h post-stimulation (Yanaba et al., 2009). Our previous report showed that a percentage of Bregs that were induced by LPS stimulation in the presence of rIL-35 produce both IL-10 and IL-35 (Wang et al., 2014). Whether LPS-induced Bregs have suppressive advantages over anti-IgM+anti-CD40 induced Bregs due to the dual expression of two suppressive cytokines, IL-10 and IL-35, remains to be elucidated. Taken together, these findings suggest that anti-IgM+anti-CD40 stimulation specifically induces the expansion of i35-Bregs, and raises the question of how

BCR signaling induces IL-35 and bypasses IL-10 induction in B cells from naïve mice? To answer this question, we will need to understand the BCR downstream signaling molecules that control IL-35 production. Investigating BCR downstream signaling molecules that control the generation of i35-Bregs will open a new window of research and advance our understanding of regulatory B cell development.

IL-35 shares the IL-12p35 subunit with IL12, and the EBI3 subunit with IL-27. IL-35 subunits are not covalently linked, rather, they are secreted independently and factors that promote their association are poorly understood (Devergne et al., 1997). Cells that produce IL-35 usually identified by using two antibodies, anti-EBI3 and anti-IL-12p35 (Wang et al., 2014). The lack of antibodies that recognize the heterodimeric IL-35 made it difficult to conclude whether a cell type does produce heterodimeric IL-35. By using PLA assay that detect protein interactions, we confirmed that BCR/CD40 induced i35-Bregs produce heterodimeric IL-35 (**Figure 6**). When EBI3 is expressed alone, it accumulates in the endoplasmic reticulum suggesting that association with another subunit is needed for its secretion (Devergne et al., 1997). The expression of both EBI3 and IL-12p35 subunits facilitates their secretion (Devergne et al., 1997). Our finding further supports the successful generation of regulatory B cells that produce heterodimeric IL-35 by using anti-IgM/anti-CD40 stimulation.

In our experience, the concentration of heterodimeric IL-35 in cell lysates is always higher than the cytokine concentration in cell supernatants. We and others detected low levels of heterodimeric IL-35 in the supernatant of cells that over express IL-35 (Devergne et al., 1997; Egwuagu et al., 2015; Wang et al., 2014). The reasons behind the difficulty of detecting

heterodimeric IL-35 in the supernatant are not clear and were mainly attributed to the instability of EBI3:IL-12p35 bond. Whether there is another reason behind the low concentration of heterodimeric IL-35 in the supernatant of cells that overexpress IL-35 was not clear at that time. A recent report showed that EBI3 and IL-12p35 are expressed on the surface of IL-35 producing T cells (Sullivan et al., 2020). We examined whether IL-35 is expressed on the cell membrane of i35-Bregs generated by anti-IgM+anti-CD40 stimulation. We found that similar to Tregs, IL-35 is expressed on the surface of i35-Bregs (**Figure 7, C**). Our findings and those by others (Sullivan et al., 2020) corroborate the unique feature of IL-35 as being a membrane bound cytokine. In addition, the surface expression suggests that IL-35 expressing B cells could exert their regulatory effect by interacting with IL-35- receptors on other cells' surface.

CD81 was found to be associated with EBI3 and IL-12p35 in Tregs facilitating their expression on the cell membrane (Sullivan et al., 2020). In line with this finding, we found that IL-35 co-localize with CD81 on the surface of i35-Bregs (**Figure 7, C**). By using Proximity Ligation Assay, we confirmed that IL-12p35 and EBI3 are associated with CD81 (**Figure 8**). While these findings advance our understanding of IL-35 biology, there are questions that need to be answered. The tetraspanin CD81 is also interacting with CD19 in B cells (Bradbury et al., 1992). Upon B cell activation via the BCR, CD81 undergoes conformational changes leading to the release of CD19 to freely bind to the BCR complex (Susa et al., 2020). This requirement of the BCR signal for CD19 dissociation from CD81 raises the question of what signals lead to the release of IL35-subunits from CD81 and the dimerization of the two subunits to form IL-35. Answering this question will advance our understanding of IL-35 heterodimerization and accelerate its use as an immunotherapy.

Upon interacting with APCs to capture an antigen or with Th cells to present an antigen, B cells undergo morphological changes leading to a polarized phenotype that facilitates synapse formation. These changes include actin reorganization, polarization of microtubule organization center (MTOC), and recruitment of antigen processing and presentation machineries (M.-I. Yuseff et al., 2013). Our data show a polarized expression of IL-35 in B cells stimulated by BCR (**Figure 9**). The staining pattern of IL-35 resembles the staining pattern of BCR cap (a pattern that indicates BCR clustering at the immune synapses due to the involvement of the BCR in antigen uptake) (Patterson et al., 2006; Yan et al., 2005). This finding suggests that IL-35 could be secreted, in a polarized manner, at the immunological synapse. Some cytokines were found to be enriched in the immune synapse area and secreted in a polarized manner such as IL-12 in DC immune synapse (Borg et al., 2004; Pulecio et al., 2010), IL-2 and IFN γ in T cell synapses (Huse et al., 2006). The polarized secretion of IL-35 could help in a more targeted and robust effect upon i35-Breg contact with T cells or APCs. This finding provides additional evidence that i35-Bregs could exert their regulatory function in a cell-cell contact manner. While the effects of i35-Bregs on T cell functions are well studied, little is known about the effect of i35-Bregs on innate immune cells. Elucidating whether i35-Bregs can modulate APCs cells in a cell-cell contact manner could reveal an unappreciated function of i35-Bregs.

In conclusion, our findings support the role of BCR signaling in i35-Breg induction. Our study provides insights on the mechanism of IL-35 heterodimerization and i35-Breg mechanism of actions. We showed that IL-35 is expressed on the surface suggesting cell-cell contact as a mechanism of regulatory action by i35-Bregs that possibly occur during immune synapses. We

provided evidence that IL-12p35 and EBI3 are associated with CD81 suggesting that IL-35 could be secreted in exosomes. Additionally, CD81 may play a role in shuttling the two subunits of IL-35 during the heterodimerization process.

References

- Borg, C., Jalil, A., Laderach, D., Maruyama, K., Wakasugi, H., Charrier, S., Ryffel, B., Cambi, A., Figdor, C., Vainchenker, W., Galy, A., Caignard, A., & Zitvogel, L. (2004). NK cell activation by dendritic cells (DCs) requires the formation of a synapse leading to IL-12 polarization in DCs. *Blood*, 104(10), 3267-3275.
<https://doi.org/https://doi.org/10.1182/blood-2004-01-0380>
- Bradbury, L. E., Kansas, G. S., Levy, S., Evans, R. L., & Tedder, T. F. (1992). The CD19/CD21 signal transducing complex of human B lymphocytes includes the target of antiproliferative antibody-1 and Leu-13 molecules. *J Immunol*, 149(9), 2841-2850.
- Catalán, D., Mansilla, M. A., Ferrier, A., Soto, L., Oleinika, K., Aguillón, J. C., & Aravena, O. (2021). Immunosuppressive Mechanisms of Regulatory B Cells [Review]. *Frontiers in Immunology*, 12(654). <https://doi.org/10.3389/fimmu.2021.611795>
- Choi, J. K., Yu, C. R., Bing, S. J., Jittayasothorn, Y., Mattapallil, M. J., Kang, M., Park, S. B., Lee, H. S., Dong, L., Shi, G., Caspi, R. R., & Egwuagu, C. E. (2021). IL-27-producing B-1a cells suppress neuroinflammation and CNS autoimmune diseases. *Proc Natl Acad Sci U S A*, 118(47). <https://doi.org/10.1073/pnas.2109548118>
- Collison, L. W., Workman, C. J., Kuo, T. T., Boyd, K., Wang, Y., Vignali, K. M., Cross, R., Sehly, D., Blumberg, R. S., & Vignali, D. A. (2007). The inhibitory cytokine IL-35

contributes to regulatory T-cell function. *Nature*, 450(7169), 566-569.

<https://doi.org/10.1038/nature06306>

Dambuza, I. M., He, C., Choi, J. K., Yu, C. R., Wang, R., Mattapallil, M. J., Wingfield, P. T., Caspi, R. R., & Egwuagu, C. E. (2017). IL-12p35 induces expansion of IL-10 and IL-35-expressing regulatory B cells and ameliorates autoimmune disease. *Nat Commun*, 8(1), 719. <https://doi.org/10.1038/s41467-017-00838-4>

Devergne, O., Birkenbach, M., & Kieff, E. (1997). Epstein–Barr virus-induced gene 3 and the p35 subunit of interleukin 12 form a novel heterodimeric hematopoietin. *Proceedings of the National Academy of Sciences*, 94(22), 12041-12046.

<https://doi.org/10.1073/pnas.94.22.12041>

Egwuagu, C. E., Yu, C.-R., Sun, L., & Wang, R. (2015). Interleukin 35: Critical regulator of immunity and lymphocyte-mediated diseases. *Cytokine & growth factor reviews*, 26(5), 587-593. <https://doi.org/10.1016/j.cytogfr.2015.07.013>

Fillatreau, S., Sweeney, C. H., McGeachy, M. J., Gray, D., & Anderton, S. M. (2002). B cells regulate autoimmunity by provision of IL-10. *Nat Immunol*, 3(10), 944-950.

<https://doi.org/10.1038/ni833>

Gomez-Rodriguez, J., Wohlfert, E. A., Handon, R., Meylan, F., Wu, J. Z., Anderson, S. M., Kirby, M. R., Belkaid, Y., & Schwartzberg, P. L. (2014). Itk-mediated integration of T cell receptor and cytokine signaling regulates the balance between Th17 and regulatory T cells. *Journal of Experimental Medicine*, 211(3), 529-543.

<https://doi.org/10.1084/jem.20131459>

- Huse, M., Lillemeier, B. F., Kuhns, M. S., Chen, D. S., & Davis, M. M. (2006). T cells use two directionally distinct pathways for cytokine secretion. *Nat Immunol*, 7(3), 247-255.
<https://doi.org/10.1038/ni1304>
- Jin, G., Hamaguchi, Y., Matsushita, T., Hasegawa, M., Le Huu, D., Ishiura, N., Naka, K., Hirao, A., Takehara, K., & Fujimoto, M. (2013). B-cell linker protein expression contributes to controlling allergic and autoimmune diseases by mediating IL-10 production in regulatory B cells. *Journal of Allergy and Clinical Immunology*, 131(6), 1674-1682.e1679. <https://doi.org/10.1016/j.jaci.2013.01.044>
- Kretschmer, K., Apostolou, I., Hawiger, D., Khazaie, K., Nussenzweig, M. C., & von Boehmer, H. (2005). Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol*, 6(12), 1219-1227. <https://doi.org/10.1038/ni1265>
- Lino, A. C., Dang, V. D., Lampropoulou, V., Welle, A., Joedicke, J., Pohar, J., Simon, Q., Thalmensi, J., Baures, A., Flühler, V., Sakwa, I., Stervbo, U., Ries, S., Jouneau, L., Boudinot, P., Tsubata, T., Adachi, T., Hutloff, A., Dörner, T., Zimmer-Strobl, U., de Vos, A. F., Dahlke, K., Loh, G., Korniotis, S., Goosmann, C., Weill, J. C., Reynaud, C. A., Kaufmann, S. H. E., Walter, J., & Fillatreau, S. (2018). LAG-3 Inhibitory Receptor Expression Identifies Immunosuppressive Natural Regulatory Plasma Cells. *Immunity*, 49(1), 120-133.e129. <https://doi.org/10.1016/j.immuni.2018.06.007>
- Patterson, H. C., Kraus, M., Kim, Y. M., Ploegh, H., & Rajewsky, K. (2006). The B cell receptor promotes B cell activation and proliferation through a non-ITAM tyrosine in the Igalpha cytoplasmic domain. *Immunity*, 25(1), 55-65.
<https://doi.org/10.1016/j.immuni.2006.04.014>

- Pulecio, J., Petrovic, J., Prete, F., Chiaruttini, G., Lennon-Dumenil, A.-M., Desdouets, C., Gasman, S., Burrone, O. R., & Benvenuti, F. (2010). Cdc42-mediated MTOC polarization in dendritic cells controls targeted delivery of cytokines at the immune synapse. *Journal of Experimental Medicine*, 207(12), 2719-2732.
<https://doi.org/10.1084/jem.20100007>
- Shen, P., Roch, T., Lampropoulou, V., O'Connor, R. A., Stervbo, U., Hilgenberg, E., Ries, S., Dang, V. D., Jaimes, Y., Daridon, C., Li, R., Jouneau, L., Boudinot, P., Wilantri, S., Sakwa, I., Miyazaki, Y., Leech, M. D., McPherson, R. C., Wirtz, S., Neurath, M., Hoehlig, K., Meinl, E., Grützkau, A., Grün, J. R., Horn, K., Köhl, A. A., Dörner, T., Bar-Or, A., Kaufmann, S. H. E., Anderton, S. M., & Fillatreau, S. (2014). IL-35-producing B cells are critical regulators of immunity during autoimmune and infectious diseases. *Nature*, 507(7492), 366-370. <https://doi.org/10.1038/nature12979>
- Sullivan, J. A., Tomita, Y., Jankowska-Gan, E., Lema, D. A., Arvedson, M. P., Nair, A., Bracamonte-Baran, W., Zhou, Y., Meyer, K. K., Zhong, W., Sawant, D. V., Szymczak-Workman, A. L., Zhang, Q., Workman, C. J., Hong, S., Vignali, D. A. A., & Burlingham, W. J. (2020). Treg-Cell-Derived IL-35-Coated Extracellular Vesicles Promote Infectious Tolerance. *Cell Rep*, 30(4), 1039-1051.e1035.
<https://doi.org/10.1016/j.celrep.2019.12.081>
- Sun, L., He, C., Nair, L., Yeung, J., & Egwuagu, C. E. (2015). Interleukin 12 (IL-12) family cytokines: Role in immune pathogenesis and treatment of CNS autoimmune disease. *Cytokine*, 75(2), 249-255. <https://doi.org/10.1016/j.cyto.2015.01.030>

- Susa, K. J., Seegar, T. C., Blacklow, S. C., & Kruse, A. C. (2020). A dynamic interaction between CD19 and the tetraspanin CD81 controls B cell co-receptor trafficking. *Elife*, 9, e52337. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7228769/pdf/elife-52337.pdf>
- Thyagarajan, R., Arunkumar, N., & Song, W. (2003). Polyvalent antigens stabilize B cell antigen receptor surface signaling microdomains. *J Immunol*, 170(12), 6099-6106. <https://doi.org/10.4049/jimmunol.170.12.6099>
- Wang, R. X., Yu, C. R., Dambuza, I. M., Mahdi, R. M., Dolinska, M. B., Sergeev, Y. V., Wingfield, P. T., Kim, S. H., & Egwuagu, C. E. (2014). Interleukin-35 induces regulatory B cells that suppress autoimmune disease. *Nat Med*, 20(6), 633-641. <https://doi.org/10.1038/nm.3554>
- Yan, J., Wolff, M. J., Unternaehrer, J., Mellman, I., & Mamula, M. J. (2005). Targeting antigen to CD19 on B cells efficiently activates T cells. *International Immunology*, 17(7), 869-877. <https://doi.org/10.1093/intimm/dxh266>
- Yanaba, K., Bouaziz, J.-D., Matsushita, T., Tsubata, T., & Tedder, T. F. (2009). The Development and Function of Regulatory B Cells Expressing IL-10 (B10 Cells) Requires Antigen Receptor Diversity and TLR Signals. *The Journal of Immunology*, 182(12), 7459-7472. <https://doi.org/10.4049/jimmunol.0900270>
- Yanaba, K., Bouaziz, J. D., Haas, K. M., Poe, J. C., Fujimoto, M., & Tedder, T. F. (2008). A regulatory B cell subset with a unique CD1dhiCD5+ phenotype controls T cell-dependent inflammatory responses. *Immunity*, 28(5), 639-650. <https://doi.org/10.1016/j.immuni.2008.03.017>
- Yoshizaki, A., Miyagaki, T., DiLillo, D. J., Matsushita, T., Horikawa, M., Kountikov, E. I., Spolski, R., Poe, J. C., Leonard, W. J., & Tedder, T. F. (2012). Regulatory B cells control

T-cell autoimmunity through IL-21-dependent cognate interactions. *Nature*, 491(7423), 264-268. <https://doi.org/10.1038/nature11501>

Yuseff, M.-I., Pierobon, P., Reversat, A., & Lennon-Duménil, A.-M. (2013). How B cells capture, process and present antigens: a crucial role for cell polarity. *Nature Reviews Immunology*, 13(7), 475-486. <https://doi.org/10.1038/nri3469>

Yuseff, M. I., Pierobon, P., Reversat, A., & Lennon-Duménil, A. M. (2013). How B cells capture, process and present antigens: a crucial role for cell polarity. *Nat Rev Immunol*, 13(7), 475-486. <https://doi.org/10.1038/nri3469>

CHAPTER III: IL-35 PRODUCING B REGULATORY CELLS SECRETE IL-35 CONTAINING EXOSOMES WITH DIFFERENTIAL COMPOSITION BASED ON THE STIMULI

Abstract

Exosomes are nanovesicles produced by all cells and serve as a mechanism of intercellular communications. They can be used as biological carriers that have the advantage of passing the blood brain barrier (BBB) with minimal toxicity and immunogenicity. We have previously shown that i35-Bregs express IL-35 on the surface and that IL-35 subunits are in association with CD81 suggesting their secretion in exosomes. Here, we show that anti-IgM/anti-CD40 induced i35-Bregs produce exosomes that contain IL-35 (i35-exosomes). To provide supporting evidence that the isolated extracellular vesicles (EVs) are exosomes, we analyzed their size by using Nanoparticle Tracking Analysis and Tunable Resistive Pulse Sensing. Additionally, we examined the expression of exosome markers by Western blotting. We show that the isolated EVs are within the size range of 50-150 nm and express the exosome markers CD81, CD63, and CD9. We also show that i35-Bregs produce i35-exosomes regardless of the stimulation that was used to induce them. However, the expression of IL-35 subunits is differential based on the stimulation used to generate i35-Bregs/i35-exosomes. While i35-Bregs/i35-exosomes generated by anti-IgM+ anti-CD40 contain more IL-12p35, i35-Bregs/i35-exosomes generated by LPS stimulation are enriched with EBI3 subunit. Collectively, our data show that i35-Bregs produce IL-35 containing exosomes that represent a potential novel cell-free treatment for CNS autoimmune diseases.

Introduction

IL-35 is a potent immunosuppressive cytokine that plays a protective role in autoimmune and inflammatory diseases. However, the difficulty of *ex-vivo* production of bioactive heterodimeric IL-35 has hindered its use for immunotherapy. The two subunits of IL-12 and IL-23 are linked by a strong disulfide bond (Gubler et al., 1991; Oppmann et al., 2000). On the other hand, the two subunits of IL-27 and IL-35 are not covalently bound, rather, they are produced independently, and little is known about factors that determine their heterodimerization (Devergne et al., 1997; Pflanz et al., 2002). Our group and others detected low levels of heterodimeric IL-35 in the supernatant of cells that over express IL-35 showing the difficulty of recombinant IL-35 production (Devergne et al., 1997; Ekwuagu et al., 2015; Wang et al., 2014). Therefore, understanding the mechanism of IL-35 heterodimerization will accelerate the therapeutic use of this cytokine. To understand how the two subunits of IL-35 are assembled given that they are not covalently linked and to develop other approaches to use IL-35 as a therapy, was asked whether IL-35 subunits are packaged and secreted in exosomes.

Exosomes are a subset of extracellular vesicles (EVs) with size range (50-150 nm). They are secreted by all cells and play as a mechanism of intercellular communication. They serve as carriers of proteins, lipids, DNA, RNA, and metabolites (Kalluri & LeBleu, 2020). The membrane and cargo of exosomes resemble those of their parental cells. When exosomes are taken up by cells, the exosome membrane diffuses into the plasma membrane of the recipient cells and the exosome cargo is released into the cytoplasm resulting in phenotypical or functional changes in the recipient cells (Montecalvo et al., 2012; Parolini et al., 2009). Recently, Sullivan *et al.*, reported that EBI3 and IL-12p35 are expressed on the surface FOXP3⁺ Tregs (Sullivan et

al., 2020). In addition, FOXP3⁻ T cells that do not produce EBI3 were found to express IL-35 on the surface suggesting that they acquired IL-35 expression by taking up exosomes (Sullivan et al., 2020). In fact, they found that FOXP3⁺ Tregs produce EBI3 and IL-12p35 bound to the tetraspanin CD81 in exosomes (Sullivan et al., 2020).

Similarly, our group reported that i35-Bregs produce exosomes that contain IL-35 (i35-exosomes) (Kang et al., 2020). As mentioned in *Chapter II*, we found that IL-35 is expressed on the surface of anti-IgM/anti-CD40 induced i35-Bregs and that IL-12p35 and EBI3 are produced in association with CD81. Given that CD81 is one of the tetraspanin that are enriched in exosomes and used as exosome markers (Escola et al., 1998; Fritzsche et al., 2002), these findings suggest that IL-35 could be secreted in exosomes. Additionally, we found that IL-35 expression is polarized in i35-Bregs induced by anti-IgM (BCR)/anti-CD40. Studies suggest that B cells secrete exosomes upon BCR and/or CD40 activation (Mittelbrunn et al., 2011; Rialland et al., 2006; Yeh et al., 2015). B cell activation through the BCR by up taking an antigen from antigen presenting cells (APCs), or through CD40 during antigen presentation by B cell to T cell, leads to B cell polarization and the formation of immune synapses. Cell polarization leads to the recruitment of endosomes to the immune synapses resulting in horizontal transfer of exosomes between cells (Mittelbrunn et al., 2011)

In this study, we successfully isolated extracellular vesicles produced by anti-IgM/anti-CD40 induced i35-Bregs. We show that they are within the size range of 50-150 nm and express CD81, CD63, and CD9 suggesting that these EVs are exosomes. Additionally, we show that exosomes produced by anti-IgM/anti-CD40 induced i35-Bregs contain IL-35 (i35-exosomes).

Interestingly, we found differential expression of IL-35 subunits based on the stimulation used to generate i35-Bregs/i35-exosomes. While i35-Bregs/i35-exosomes generated by anti-IgM/anti-CD40 contain more IL-12p35, i-35Bregs/i-35 exosomes generated by LPS stimulation are enriched with EBI3 subunit. Collectively, our data show that i35-Bregs produce IL-35 containing exosomes that can be explored as a cell-free therapeutic strategy for CNS autoimmune diseases.

Materials and Methods

Mice

Wild-type C57BL/6J were purchased from Jackson Laboratory. Mice were bred, maintained, and treated in accordance with NIH Animal Care and Use Committee guidelines.

Generation of i35-Bregs

CD19⁺ B cells were isolated from spleens of C57BL/6 WT mice by using CD19 MicroBeads (Miltenyi Biotec) as per manufacturer's instructions. Briefly, the spleens were homogenized to prepare single cell suspension. The cells were washed twice with RPMI by centrifugation at 300 ×g for 10 minutes. Red blood cells were lysed by adding 1 mL of ACK buffer to pelleted splenocytes for 5 minutes at room temperature. The cells were then washed twice with RPMI by centrifugation at 300 ×g for 10 minutes and the pelleted cells were resuspended in MACs buffer (0.5% bovine serum albumin in PBS) and passed through a 40-µm pore-size cell strainer (Corning Falcon) to remove tissue fragments. The cells were counted by using Vi-Cell XR (Beckman Coulter, Indianapolis, IN). To isolate B cells, 10 µL of CD19 magnetic beads were added per 10⁷ total cells and incubated for 15 minutes at 4°C. Cells were then washed with MACs buffer and passed through LS MACS column placed in the magnetic MACs separator.

CD19⁺ B cells are retained in the magnetic column and non-B cells pass through the column. The column was then removed from the magnetic separator and flushed with MACs buffer to collect CD19⁺ B cells. B cells were washed with RPMI and resuspended in complete RPMI 1640 (Gibco) supplemented with 10% of exosomes-depleted fetal bovine serum (System Biosciences), 1% penicillin/streptomycin (Invitrogen), 1% L-glutamine (Sigma-Aldrich), 0.2% β -mercaptoethanol (Gibco), and HEPES to achieve a concentration of 2×10^6 cells/ mL. To induce i35-Bregs, B cells were stimulated with 5 μ g/mL LPS (Sigma), 1 μ g/mL anti-IgM (Jackson ImmonoResearch) + 10 μ g/mL anti-CD40 (Bio X Cell), or 5 μ g/mL anti-IgM+10 μ g/mL anti-CD40. The cells were incubated for 72 hours at 37°C in 5 % CO₂. The supernatants were collected at the end of the incubation period for exosome isolation.

Isolation of i35-Breg exosomes

Supernatants collected at 72h of i35-Breg induction were subjected to serial centrifugation steps, 300xg for 10 minutes to remove cells, 2,000xg to remove dead cells, and 10,000 xg for 30 minutes to remove cell debris and large extracellular vesicles. All centrifugation steps were performed at 4°C. The supernatants were then concentrated 4x by using Amicon Ultra centrifugal filter units (MWCO 30 kDa). Exosomes were isolated from the concentrated supernatant by using ExoQuick- ULTRA (System Biosciences) as per manufacturer's instructions. Briefly, 1 volume of ExoQuick buffer was added to 5 volumes of concentrated supernatant and incubated overnight at 4°C. The supernatants were subjected to 3,000xg for 10 minutes to precipitate the exosomes. The pelleted exosomes were resuspended in buffer B and A and passed through the purification column.

Exosomes size analysis-Nanoparticle Tracking Analysis (NTA) and Tunable Resistive Pulse Sensing (TRPS)

We used two methods to determine the size distribution of isolated exosomes, Nanoparticle Tracking Analysis (NTA) by using NanoSight system NS300 (Malvern Instrument, UK) and Tunable Resistive Pulse Sensing (TRPS) by using EXOID system (IZON, USA). For the NTA, 3 videos of 30 s each were recorded at camera level 12 and detection level of 3. The instrument was operated per manufacturer's instruction. For TRPS, the nanopore NP150 that measures particles between 70-420 nm was used at 46mm stretch. The instrument was calibrated by using CPC200 beads and operated per manufacturer's instruction.

ELISA

The number of exosomes secreted by unstimulated and stimulated B cells were determined by using ExoELISA-ULTRA Complete Kit (CD81 detection) (System Biosciences) as per manufacturer's instructions. The concentration of IL-35 in the collected exosomes were determined by using LEGEND MAX mouse IL-35 heterodimer ELISA (Biolegend) as per manufacturer's instructions.

Western blot assay

The protein amount of stimulated cell, unstimulated cell, and exosome lysates were measured by using BCA protein assay kit (Sigma) per manufacturer's instructions. Samples were treated with β -mercaptoethanol and heated at 95°C for 7 minutes. Samples were fractionated on 4-12% Sodium Dodecyl Sulfate (SDS) gels (NuPAGE). Following transfer of the proteins onto nitrocellulose membrane, the blots were blocked using 5% BSA overnight. Blots were incubated

at 4 °C overnight with primary mouse anti-CD81 (sc-166029, SantaCruz), mouse anti-CD9 (sc-13118, SantaCruz), mouse anti-CD63 (sc-5275, SantaCruz), mouse anti-EBI3(sc-166158, SantaCruz), rabbit anti-IL-12p35 (ab131039, Abcam), or goat anti- β -actin (sc1616 l, SantaCruz). The blots were then incubated with secondary antibodies conjugated with InfraRed Dye (LI-COR), donkey anti-rabbit 800, donkey anti-mouse 800, goat anti-mouse 680, or donkey anti-goat 680 and specific bands were detected using LI-COR System (LI-COR Biosciences).

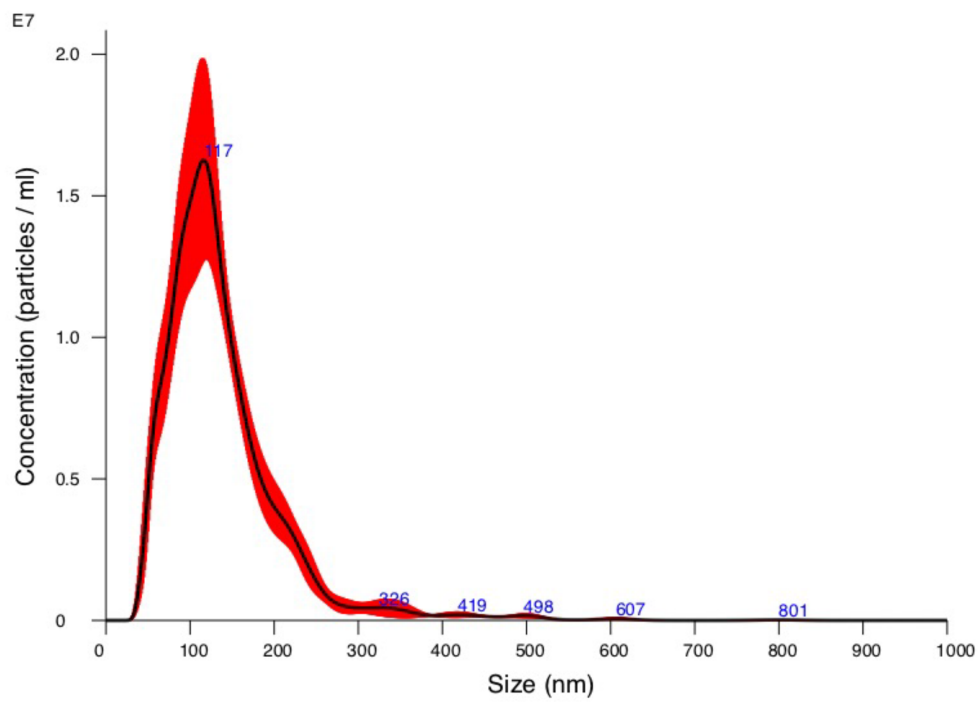
Results

Characterization of B cell-derived extracellular vesicles (EVs)

Exosomes are a subset of extracellular vesicles (EVs) that are secreted by all cells. In B cells, exosomes secretion can be induced by BCR and/or CD40 stimulation (Mittelbrunn et al., 2011; Rialland et al., 2006; Yeh et al., 2015). Additionally, in *Chapter II*, we showed that IL-12p35 and EBI3 are associated with CD81 in i35-Bregs suggesting their secretion in exosomes. Therefore, we sought to examine whether anti-IgM/anti-CD40 induced i35-Bregs secrete IL-35 containing exosomes. To isolate exosomes, B cells were stimulated with anti-IgM + anti-CD40 for 72 hours to induce i35-Bregs and the supernatant was collected at the end of the 72h incubation. Exosomes were isolated from the culture supernatant by first subjecting the supernatant to a serial centrifugal force to remove cells, dead cells and debris, and large EVs. Exosomes were then isolated using Exo-quick ULTRA, a hydrophilic polymer that precipitate exosomes. Given that cells release different type of extracellular vesicles ranging in size from 30 to 10,000 nm, more investigation is needed to support that the isolated EVs are exosomes. We examined the size of isolated EVs and analyzed them for the expression of exosome markers CD9, CD63 and CD81. To characterize the size, we used two methods, Nanoparticle Tracking

Analysis (NTA) (**Figure 10, A**) and Tunable Resistive Pulse Sensing (TRPS) (**Figure 10, B**). The two methods showed that the isolated EVs were within the size range of exosomes 50-150 nm and most of the exosomes were ~ 100 nm. To further support that the isolated EVs were exosomes, we used Western-blotting to detect the expression of exosome markers in the isolated EVs and the parental cells (i35-Bregs) as a positive control (**Figure 11**). We demonstrated that the isolated EVs express the exosome markers CD81, CD63, and CD9 (**Figure 11**). Collectively, the data support that the isolated EVs from the supernatant of anti-IgM+ anti-CD40 induced i35-Bregs were mainly exosomes.

A



B

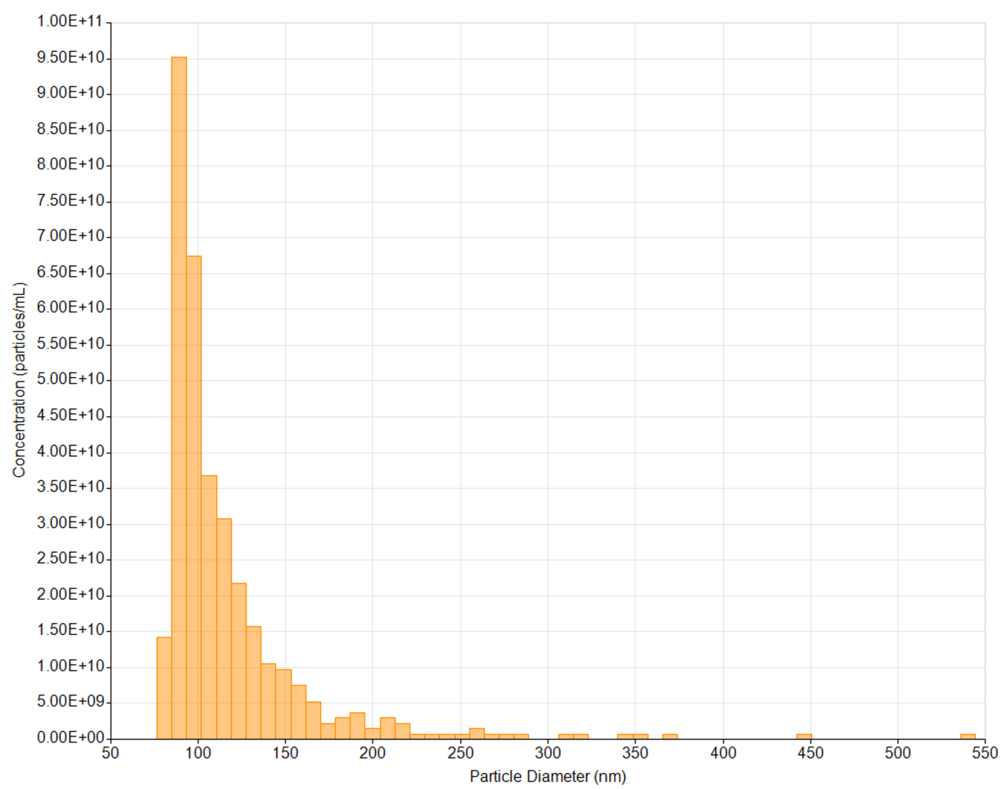


Figure 10. EVs isolated from cell supernatant of i35-Bregs are within the size range of exosomes

Exosomes were isolated from supernatant of B cells stimulated by anti-IgM/anti-CD40 for 72h by Exo-quick Ultra kit and particles were analyzed for size distribution by Nanoparticle Tracking Analysis (NTA) using nanosight and the Tunable Resistive Pulse Sensing (TRPS) technology of the EXOID instrument. **A)** NTA plot shows the size distribution of isolated EVs in nm (x axis is marked from 0 to 1000 nm) and EV-concentration is expressed as numbers of particles per mL (y axis is marked from 0 to 2 E7). **B)** EXOID plot shows the size distribution of isolated EVs in nm (x axis is marked from 0 to 550 nm) and EV-concentration is expressed as numbers of particles per mL (y axis is marked from 0 to 1 E11). Both plots show that most of the analyzed EVs are within the size range of exosomes 50-150 nm with peak diameters ~90-110 nm.

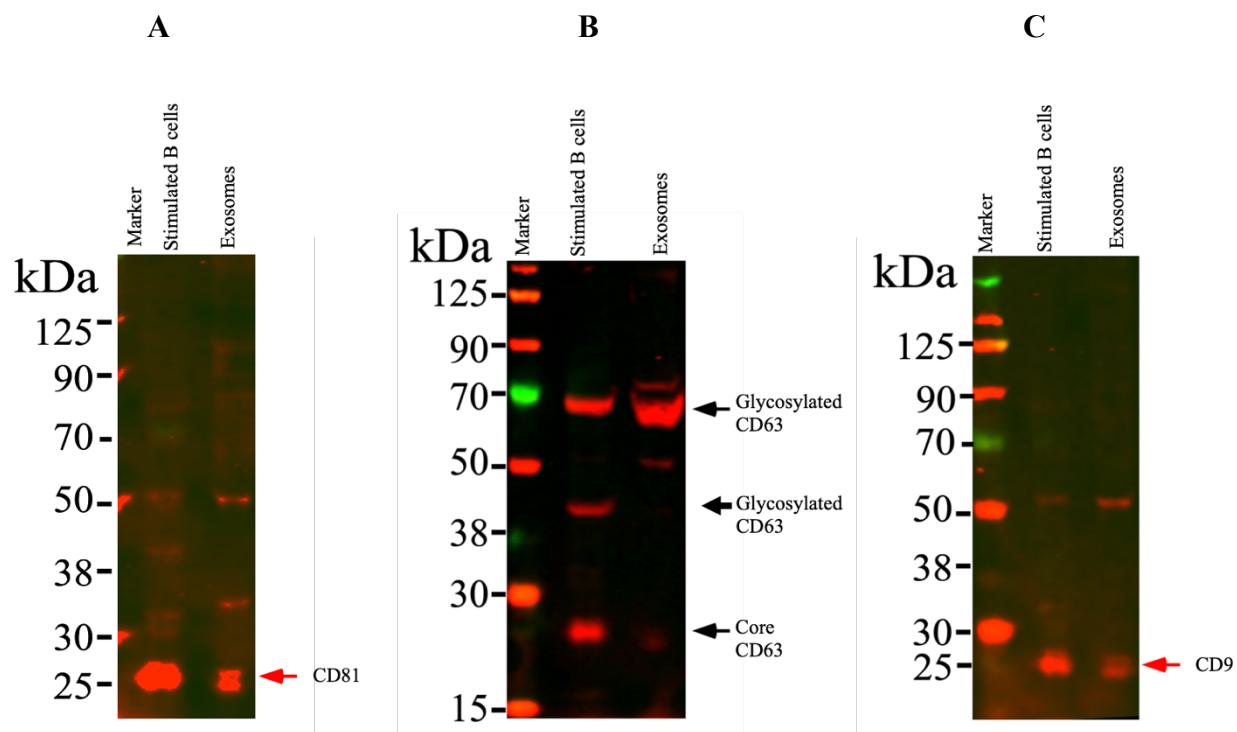


Figure 11. EVs isolated from cell supernatant of i35-Bregs express exosome markers

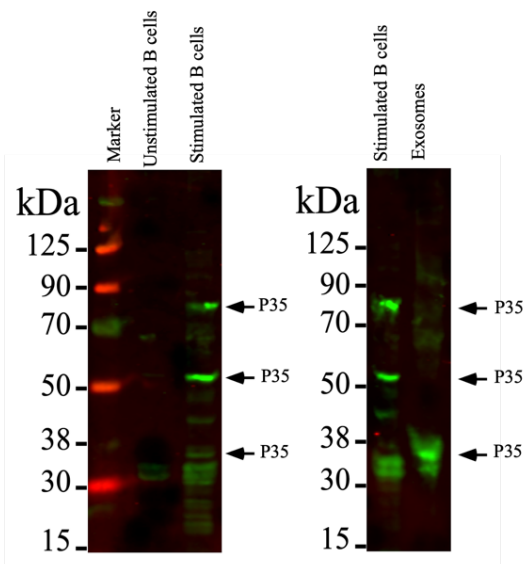
B cells were stimulated by anti-IgM/anti-CD40 for 72h and EVs were isolated from supernatant collected at 72h by Exo-quick Ultra kit. EVs were lysed and analyzed by Western-blotting side-by-side with B cell lysate as a positive control, using antibodies to CD81, CD63, CD9.

Equivalent protein amounts of each sample were loaded on the gels. **A)** Western-blot of stimulated B cells (i35-Bregs) and their secreted exosomes both express CD81 (26 kDa). **B)** Western-blot of stimulated B cells (i35-Bregs) and their secreted exosomes analyzed for the expression of CD63. While the cells express core CD63 (26 kDa) and two other forms of glycosylated CD63 (~45 kD) and (~60 kD), exosomes are enriched with the glycosylated CD63 (~60 kD). **C)** Western-blot of stimulated B cells (i35-Bregs) and their secreted exosomes both express CD9 (24 kD). The expression of exosome markers CD81, CD63, CD9 support that the isolated EVs are exosomes.

Exosomes of B cells stimulated with anti-IgM/anti-CD40 contain IL-35

We previously showed that IL-35 is expressed on the surface of BCR/CD40 induced i35-Bregs and that IL-12p35 and EBI3 are in association with CD81, suggesting its secretion in exosomes (*Chapter II*). In addition, we showed that EVs isolated from i35-Bregs are mainly exosomes based on their size and the expression of exosome markers (**Figure 10,11**). We next wanted to examine whether the isolated exosomes contain IL-35. Exosomes were isolated from culture supernatant collected at 72h of B cell stimulation with anti-IgM + anti-CD40 (i35-Bregs) and examined by Western blotting side-by side with parental cells and unstimulated B cells as a negative control. We show that stimulated B cells express both subunits, p35 (**Figure 12, A**) and EBI3 (**Figure 12, B**), compared to unstimulated B cells. Similarly, exosomes isolated from cells stimulated with anti-IgM/anti-CD40 contain both subunits (**Figure 12 A, B**), indicating that the exosomes contain IL-35. Furthermore, we quantified the exosomes based on the expression of CD81. As determined by ELISA, we found that 5×10^6 of B cells stimulated by anti-IgM/anti-CD40 produced approximately 6×10^{10} of CD81 expressing exosomes compared to 2.5×10^{10} produced by unstimulated cells (**Figure 13, A**). By using ELISA that detects heterodimeric IL-35, we found that 6×10^{10} of i35-Breg exosomes contain 500 pg of IL-35 while 6×10^{10} of exosomes from unstimulated cells show no detectable level of IL-35 (**Figure 13, B**). Collectively, our data show that i35-Bregs generated by BCR/CD40 stimulation secrete exosomes that contain IL-35 (i35-exosomes).

A



B

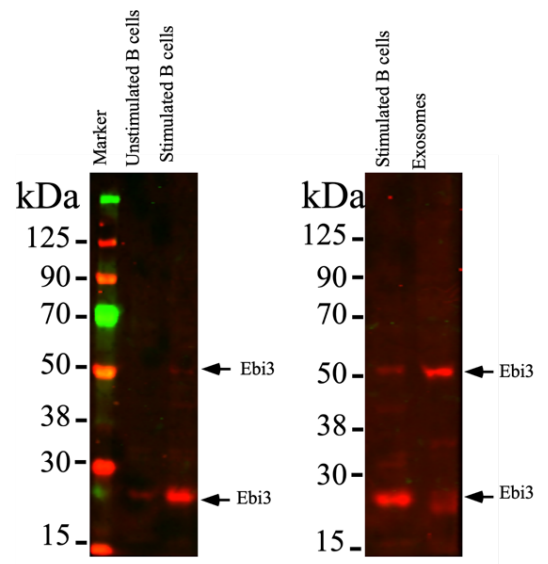
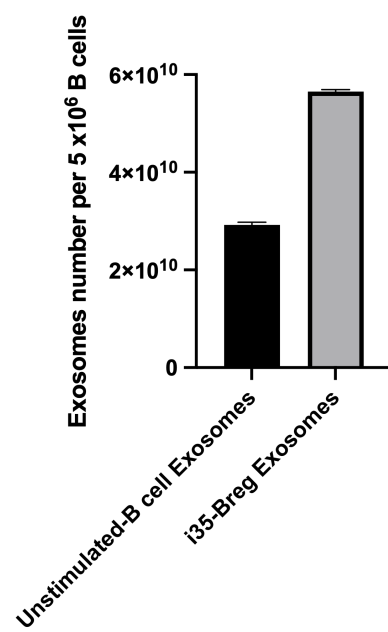


Figure 12. Exosomes of B cells stimulated with anti-IgM/anti-CD40 contain IL-35

Unstimulated B cells, B cells stimulated with anti-IgM/anti-CD40 for 72h (i35-Bregs), and exosomes isolated from the supernatant of stimulated B cells at 72h were analyzed by Western-blotting for the expression of IL-12p35 subunit *and* EBI3 subunit **A)** Left Western- blot image of unstimulated B cell side-by-side with B cells stimulated with anti-IgM/anti-CD40 (i35-Bregs) shows absence of IL-12p35 bands in unstimulated B cells and the expression of IL-12p35 in stimulated B cells, IL-12p35 monomer 35 kDa, dimer ~ 50kDa and 70 kDa. Right blot image of B cells stimulated with anti-IgM/anti-CD40 for 72h (i35-Bregs) side-by-side with their secreted exosomes shows the expression of IL-12p35 monomer 35 kDa, dimer ~ 50kDa and 70 kDa by cells and the expression of IL-12p35 monomer 35 kDa by exosomes. **B)** Left Western- blot image of unstimulated B cell side-by-side with B cells stimulated with anti-IgM/anti-CD40 (i35-Bregs) shows absence of EBI3 bands in unstimulated B cells and the expression of EBI3 in stimulated B cells, EBI3 monomer 23 kDa and dimer ~ 50kDa. Right blot image of B cells stimulated with anti-IgM/anti-CD40 for 72h (i35-Bregs) side-by-side with their secreted exosomes shows the expression of EBI3 monomer 35 kDa and dimer ~ 50kDa by cells and exosomes. Equivalent protein amounts of each sample were loaded on the gels.

A



B

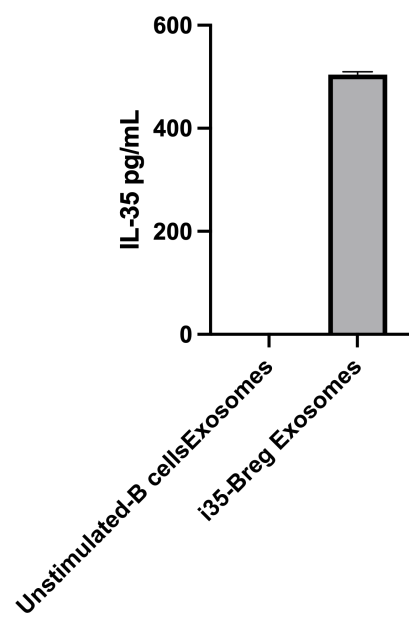
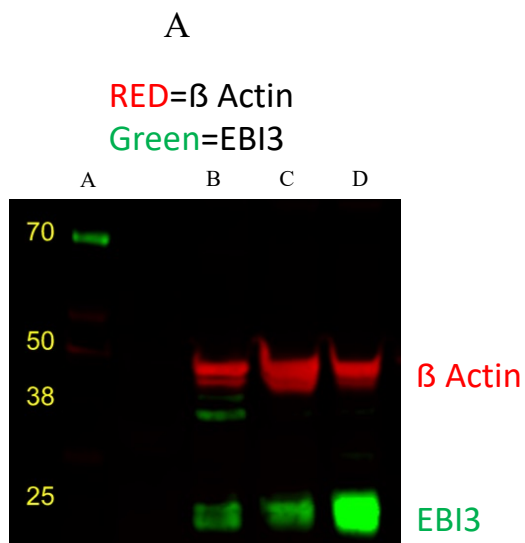


Figure 13. Exosomes of B cells stimulated with anti-IgM/anti-CD40 contain heterodimeric IL-35

CD19⁺ B cells were isolated from the spleen of C57BL/6 mice and either stimulated with 1 µg anti-IgM + 10 µg anti-CD40 to induce i35-Bregs or incubated without stimulation as a control. The supernatants were collected at 72 hours of incubation and exosomes were isolated by Exoquick Ultra kit. **A)** Exosomes isolated from 5 mL of culture supernatant of 5x 10⁶ i35-Bregs or unstimulated B cells were quantified based on the expression of CD81 by ELISA. **B)** IL-35 concentration in 6x10¹⁰ of i35-exosomes or exosomes of unstimulated B cells as measured by heterodimeric IL-35 ELISA. Data represents the mean of two replicates.

Differential expression of IL-35 subunits based on the stimulation used to generate i35-Bregs/i35-exosomes

Then, we asked whether the secretion of i35-exosomes is a characteristic of i35-Bregs induced by anti-IgM/anti-CD40, or if other stimuli could induce IL-35-containing exosomes. We found a different expression level of IL-35 subunits based on the stimulus that was used. i35-Bregs generated by anti-IgM/anti-CD40 showed a higher expression of IL-12p35 subunit compared to i35-Bregs generated by LPS, which were more enriched with EBI3 subunit (**Figure 14**). Similarly, exosomes from anti-IgM/anti-CD40 stimulated B cells contains more IL-12p35 compared to exosomes from LPS stimulated B cells, which contains more EBI3 (**Figure 15**). While our data confirmed that i35-exosomes are generated by i35-Bregs, regardless of the stimuli, the differential expression of IL-35 subunits raises the question whether these exosomes will have different immunomodulatory effect on recipient cells?



Samples

- A. Marker
- B. B cells stimulated with 1 μg anti-IgM/10 μg anti-CD40
- C. B cells stimulated with 5 μg anti-IgM/10 μg anti-CD40
- D. B cells stimulated LPS

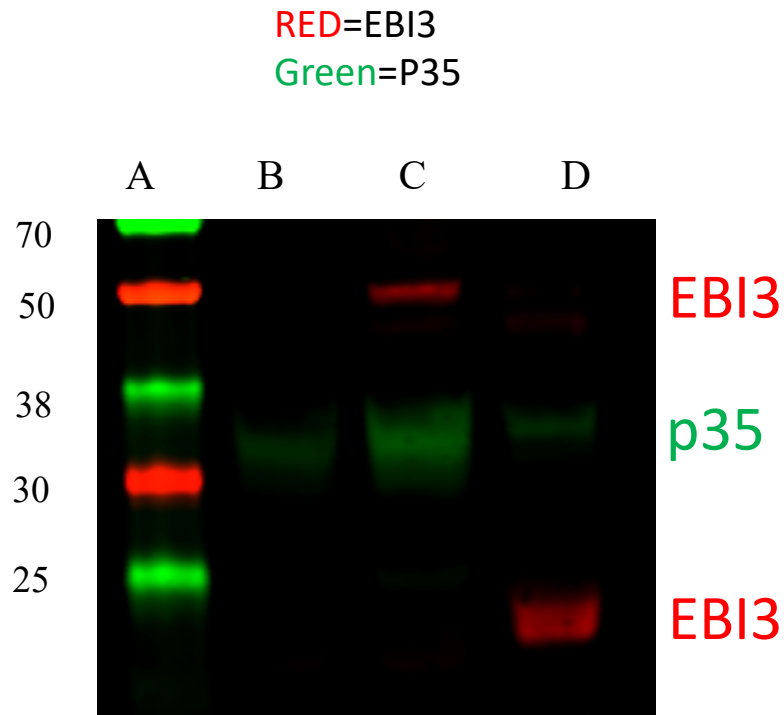


Samples

- A. Marker
- B. B cells stimulated with 1 μg anti-IgM/10 μg anti-CD40
- C. B cells stimulated with 5 μg anti-IgM/10 μg anti-CD40
- D. B cells stimulated LPS

Figure 14. Differential expression of IL-35 subunits based on the stimulation used to generate i-35Bregs

B cells stimulated with 1 μ g anti-IgM+10 μ g anti-CD40, 5 μ g anti-IgM+10 μ g anti-CD40, or LPS for 72h (i35-Bregs) were analyzed by Western-blotting for the expression of EBI3, IL-12p35, and β -actin which was used as a loading control **A)** Western-blot image shows the expression of EBI3 monomer 23 kDa and β -actin 42 kDa by B cells stimulated with 1 μ g anti-IgM+10 μ g anti-CD40, 5 μ g anti-IgM+10 μ g anti-CD40, or LPS. B cells stimulated with LPS show a higher expression of EBI3 subunit compared with B cells stimulated with anti-IgM+ anti-CD40 **B)** Western-blot image shows the expression of IL-12p35 monomer 35 kDa, dimer ~ 50kDa and 70 kDa , and β -actin 42 kDa by B cells stimulated with 1 μ g anti-IgM+10 μ g anti-CD40, 5 μ g anti-IgM+10 μ g anti-CD40, or LPS. B cells stimulated with 1 μ g anti-IgM+10 μ g anti-CD40 show a higher expression of IL-12p35 dimer ~ 50 kDa compared with B cells stimulated with LPS. Data is representative of at least two independent experiments.



Samples:

- A. Marker
- B. Exosomes from unstimulated B cells
- C. Exosomes from B cells stimulated with 1 μ g anti-IgM/10 μ g anti-CD40
- D. Exosomes from B cells stimulated with LPS

34 μ g of lysed exosomes were used

Figure 15. Differential expression of IL-35 subunits based on the stimulation used to generate i35-exosomes

Exosomes were isolated from culture supernatants collected at 72h of unstimulated B cells or B cells stimulated with 1 μ g anti-IgM+10 μ g anti-CD40 or LPS. Exosomes were lysed and analyzed by Western-blotting for the expression of EBI3 and IL-12p35 subunits. Western-blot image show the expression of IL-12p35 (35 kDa) and EBI3 (monomer 23 kDa and dimer ~ 50 kDa) as analyzed in all samples side-by-side. The presence of IL-12p35 band in exosomes from unstimulated cells is due to the high amount of protein loaded in this experiment 34 μ g. IL-12p35 band is absent when a lower amount of protein is used (data not shown) suggesting that IL-12p35 is constitutively expressed but at a lower level. The EBI3 band is absent in exosomes from unstimulated cells. In addition, the image shows a higher expression of IL-12p35 subunit in exosomes from B cells stimulated with 1 μ g anti-IgM+10 μ g anti-CD40 as compared to exosomes from B cells stimulated with LPS. On the other hand, exosomes from B cells stimulated with LPS are enriched with the EBI3 subunit as compared to exosomes from B cells stimulated 1 μ g anti-IgM+10 μ g anti-CD40. Equivalent protein amounts of each sample were loaded on the gels (34 μ g of exosome lysate as determined by BCA protein quantification assay). Data is representative of two independent experiments.

Discussion

In this study we examined the hypothesis that i35-Bregs induced by anti-IgM/anti-CD40 secrete IL-35-containing exosomes. Some studies have shown that the secretion of exosomes is induced in B cells by BCR and/or CD40 stimulation (Arita et al., 2008; Mittelbrunn et al., 2011; Muntasell et al., 2007; Rialland et al., 2006; Yeh et al., 2015). During the activation of B cells by the BCR or CD40 and the formation of immune synapses, the endosome gets recruited to the synapse area resulting in a polarized secretion of exosomes (Arita et al., 2008; Muntasell et al., 2007; Yuseff et al., 2013). In *Chapter II*, we have shown that i35-Bregs induced by anti-IgM/anti-CD40 stimulation express IL35 on the surface and that IL-12p35 and EBI3 are associated with the tetraspanin CD81. We also noticed that IL35 expression is polarized in i35-Bregs induced by anti-IgM/anti-CD40. Given that exosome synthesis starts with inward budding of the plasma membrane and that exosomes are enriched with CD81(Perez-Hernandez et al., 2013), our data suggest that IL-35 could be secreted in exosomes by anti-IgM/anti-CD40 induced i35-Bregs.

In this report, we show that i35-Bregs produce IL-35 containing exosomes. To isolate exosomes, we collected the supernatant of B cells stimulated by anti-IgM+anti-CD40 at 72h post-stimulation. The supernatant was subjected to a serial centrifugation to remove cells, dead cells and debris, and large microvesicles. Exosomes were isolated by using Exo-quick ULTRA, a hydrophilic polymer that precipitate exosomes. Given that exosomes are differentiated from other extracellular vesicles by size (Zhang et al., 2018), we examined the size of the isolated exosomes by using Nanoparticle Tracking Analysis and Tunable Resistive Pulse Sensing (**Figure 10**). We found that the isolated EVs are within the size range of exosomes 50-150 nm

and most of them are around 100 nm suggesting that the isolated EVs are exosomes (**Figure 10**).

Exosomes are enriched with the tetraspanins CD81, CD63, and CD9 (Perez-Hernandez et al., 2013). These tetraspanins also are often used as exosomes markers that differentiate them from other subsets of EVs (Perez-Hernandez et al., 2013) . We found that i35-Bregs and their derived exosomes express CD81, CD63, and CD81 (**Figure 11**). These findings further support that the isolated EVs are exosomes. Our findings are in line with other studies that investigated B cell exosomes. For example, CD81 and CD63 were found to be enriched in exosomes from EBV-transformed human B-cell line (Admyre et al., 2007; Escola et al., 1998). Additionally, CD81 and CD9 were detected in exosomes from mouse B cell line (Muntasell et al., 2007). We also quantified exosomes based on the expression of CD81 and found that anti-IgM+anti-CD40 stimulated B cells produce more exosomes compared to unstimulated B cells (**Figure 13**). These findings further support that exosome secretion by B cells is induced by B cell activation.

Recently, CD9 was identified as a marker of mouse and human IL-10 producing Bregs (Braza et al., 2015; Brosseau et al., 2018). Adoptive transfer of CD9⁺ B cells ameliorated allergic airway inflammation by inhibiting Th1 and Th17 through the production of IL-10 (Braza et al., 2015). In this report, we show that i35-Bregs express CD9 as well (**Figure 11, C**). However, analyzing anti-IgM/anti-CD40 stimulated B cells by flow cytometry is needed to determine whether CD9 is enriched in i35-Bregs compared to non-IL-35 producing cells. The other tetraspanins, CD63 and CD81, were reported to have functions in B cells. CD63 was found to be involved in the regulation of CXCR1 in B cells (Yoshida et al., 2011). CD81 is bound to CD19 in B cells and it lowers the threshold for B cell response by regulating the expression of CD19

(Maecker & Levy, 1997). In *Chapter II*, we showed that EBI3 and IL-12p35 are associated with CD81 in i35-Bregs suggesting IL-35 secretion in exosomes. In line with our previous report (Kang et al., 2020), here, we showed that i35-Bregs secrete exosomes that contain IL-35 (**Figure 12, Figure 13**). By using co-immunoprecipitation, Sullivan *et al.*, found that EBI3 and IL-12p35 are secreted bound to CD81 in exosomes produced by IL-35 producing Tregs (Sullivan et al., 2020). Similarly, our group found that IL-12p35 and EBI3 get precipitated with CD81 (data not shown) suggesting that IL-12p35 and EBI3 are bound to CD81 in exosomes produced by i35-Bregs. However, our study does not exclude the secretion of IL-35 containing microvesicles by i35-Bregs as they are removed by our exosome isolation protocol at 10,000xg.

The release of i35-exosomes indicates that i35-Bregs could induce “infectious tolerance” in which non-IL-35 producing cells uptake i35-exosomes resulting in acquiring of IL-35 expression on the surface. This mechanism will increase the number of IL-35 expressing cells and disseminate the suppression effect of i35-Bregs. In fact, Sullivan *et al.*, found that the rare IL35-producing Tregs secrete IL-35 containing exosomes (Sullivan et al., 2020). These exosomes were up taken by non-IL-35 producing cells resulting in increasing the number of IL-35-surface expressing cells up to 25-100 folds (Sullivan et al., 2020). These IL-35 cross-dressed cells acquired regulatory function and were able to suppress inflammatory cells. They also found that “infectious tolerance” is IL-35 dependent meaning that exosomes from regulatory cells could inhibit inflammatory cells (primary suppression) without IL-35, however, only exosomes that contain IL-35 could inhibit inflammatory cells (primary suppression) and turn them into regulatory cells with the ability to suppress other cells (secondary suppression) (Sullivan et al., 2020). Our data suggest the ability of i35-Bregs to disseminate immunosuppression through the

secretion of i35-exosomes which add an advantage to their use for immunotherapy. Recently, the role of i35-Bregs in cancer has emerged (Mirlekar et al., 2020). It is possible that the secretion of i35-exosomes is one of the mechanisms by which i35-Bregs limit anti-tumor immunity

The finding that IL-35 is mainly secreted in exosomes and not as a water-soluble protein indicates that IL-35 is unconventional cytokine. We and others detected low levels of heterodimeric IL-35 in the supernatant of cells that over express IL-35 (Devergne et al., 1997; Egwuagu et al., 2015; Wang et al., 2014). The reasons behind the difficulty of detecting heterodimeric IL-35 in the supernatant are not clear and were mainly attributed to the instability of EBI3:IL-12p35 bond. It is possible that IL-35 being mainly secreted in exosomes contributes to the difficulty of producing significant amount of recombinant IL-35. Because the majority of IL-35 will be associated with exosome proteins or expressed on the plasma membrane of the cells. Another possibility is that the overexpression of CD81 is required to overexpress heterodimeric IL-35. This possibility is valid in case CD81 is indispensable for IL-35 heterodimerization process which remains to be elucidate.

Given that exosomes secretion can be induced by BCR and/or CD40 stimulation as we have discussed, we wanted to answer the question of whether i35-exosome production is a characteristic of BCR/CD40 induced i35-Bregs, or if i35-Bregs induced by other stimuli also produce i35-exosomes. We generated i35-Bregs by anti-IgM/anti-CD40 or LPS stimulation and we collected the supernatant for exosome isolation at 72h. Interestingly, we found that the expression of IL-12p35 and EBI3 in i35-Bregs differs based on the stimulation used to induce i35-Bregs (**Figure 14**). LPS generated i35-Bregs are enriched with EBI3 subunit compared to

anti-IgM/anti-CD40 generated i35-Bregs, which contained more IL-12p35 (**Figure 14**). While the expression of both IL-12p35 and EBI3 is required to upregulate IL-35, the two subunits are regulated and produced independently. Our data suggest that EBI3 and IL-12p35 are not necessarily transcribed/translated at similar levels, rather their expression might be differential dependent on the type of the stimulation. Pflanz *et al.*, reported that human monocytes stimulated with LPS showed upregulation of both EBI3 and IL-12p35 mRNA within 3 hours (Pflanz et al., 2002). IL-12p35 peak expression was at 12 hours and returned to baseline at 24 hours (Pflanz et al., 2002). On the other hand, EBI3 peak expression was around 24 hours and remained above the baseline after 72 hours (Pflanz et al., 2002). In all time points, the expression of EBI3 was higher than the expression of IL-12p35 (Pflanz et al., 2002). These findings further support the differential transcription/expression of IL-35 subunits.

We also found that i35-Bregs induced by anti-IgM/anti-CD40 or LPS produced i35-exosomes (**Figure 15**). However, similar to stimulated B cells, exosomes from anti-IgM/anti-CD40 stimulated B cells contained more IL-12p35 compared to exosomes from LPS stimulated B cells, which were enriched with EBI3 (**Figure 15**). Some studies showed that the EBI3 and IL-12p35 have intrinsic effects. For example, treatment of EAE or EAU mice with IL-12p35 only ameliorated the disease (Choi et al., 2017; Dambuza et al., 2017). Moreover, Dambuza et al. showed that EBI3 has inhibitory effect on T cell proliferation (Dambuza et al., 2017). Given that the single subunits of IL-35 can act independently (Choi et al., 2017; Dambuza et al., 2017; Ma et al., 2019; Takeda et al., 2014), the differential expression of IL-35 subunits in exosomes raises the question of whether these exosomes will have different immunomodulatory effect on recipient cells? To better understand how EBI3 and IL-12p35 are regulated, we will need to

identify signaling pathways that exclusively promote the expression of a single subunit and signals that promote the expression of both subunits, simultaneously. One of the important unanswered questions regarding IL-35 biology is what are the factors that contribute to IL-35 heterodimerization? Since our data showed that the expression of EBI3 and p35 could be differential based on the stimulation used to induce i35-Bregs (**Figure 14**), we raise the question of whether the concentration of IL-35 individual subunits influence the formation of heterodimeric IL-35. If yes, what is the optimal condition to maximize the formation of heterodimeric IL-35? Equal concentrations of the two subunits, higher concentration of IL-12p35 than EBI3, or higher concentration of EBI3 than IL-12p35? To accurately answer these questions, there is a need to develop an accurate method to detect and measure the concentration of heterodimeric IL-35. Collectively, our data show that i35-Bregs secrete i35-exosomes regardless of the stimulation used to induce them. However, the type of the stimulation impacts the subunits concentration in exosomes. Given the advantages of exosomes as biological carriers, i35-exosomes can be explored as alternative therapeutic strategy of using recombinant IL-35.

References

- Admyre, C., Bohle, B., Johansson, S. M., Focke-Tejkl, M., Valenta, R., Scheynius, A., & Gabrielsson, S. (2007). B cell-derived exosomes can present allergen peptides and activate allergen-specific T cells to proliferate and produce TH2-like cytokines. *J Allergy Clin Immunol*, 120(6), 1418-1424. <https://doi.org/10.1016/j.jaci.2007.06.040>
- Arita, S., Baba, E., Shibata, Y., Niino, H., Shimoda, S., Isobe, T., Kusaba, H., Nakano, S., & Harada, M. (2008). B cell activation regulates exosomal HLA production. *Eur J Immunol*, 38(5), 1423-1434. <https://doi.org/10.1002/eji.200737694>
- Braza, F., Chesne, J., Durand, M., Dirou, S., Brosseau, C., Mahay, G., Cheminant, M. A., Magnan, A., & Brouard, S. (2015). A regulatory CD9(+) B-cell subset inhibits HDM-induced allergic airway inflammation. *Allergy*, 70(11), 1421-1431. <https://doi.org/10.1111/all.12697>
- Brosseau, C., Durand, M., Colas, L., Durand, E., Foureau, A., Cheminant, M. A., Bouchaud, G., Castan, L., Klein, M., Magnan, A., & Brouard, S. (2018). CD9(+) Regulatory B Cells Induce T Cell Apoptosis via IL-10 and Are Reduced in Severe Asthmatic Patients. *Front Immunol*, 9, 3034. <https://doi.org/10.3389/fimmu.2018.03034>
- Choi, J. K., Dambuza, I. M., He, C., Yu, C. R., Uche, A. N., Mattapallil, M. J., Caspi, R. R., & Egwuagu, C. E. (2017). IL-12p35 Inhibits Neuroinflammation and Ameliorates Autoimmune Encephalomyelitis. *Front Immunol*, 8, 1258. <https://doi.org/10.3389/fimmu.2017.01258>
- Dambuza, I. M., He, C., Choi, J. K., Yu, C. R., Wang, R., Mattapallil, M. J., Wingfield, P. T., Caspi, R. R., & Egwuagu, C. E. (2017). IL-12p35 induces expansion of IL-10 and IL-35-

- expressing regulatory B cells and ameliorates autoimmune disease. *Nat Commun*, 8(1), 719. <https://doi.org/10.1038/s41467-017-00838-4>
- Devergne, O., Birkenbach, M., & Kieff, E. (1997). Epstein–Barr virus-induced gene 3 and the p35 subunit of interleukin 12 form a novel heterodimeric hematopoietin. *Proceedings of the National Academy of Sciences*, 94(22), 12041-12046. <https://doi.org/10.1073/pnas.94.22.12041>
- Egwuagu, C. E., Yu, C.-R., Sun, L., & Wang, R. (2015). Interleukin 35: Critical regulator of immunity and lymphocyte-mediated diseases. *Cytokine & growth factor reviews*, 26(5), 587-593. <https://doi.org/10.1016/j.cytogfr.2015.07.013>
- Escola, J. M., Kleijmeer, M. J., Stoorvogel, W., Griffith, J. M., Yoshie, O., & Geuze, H. J. (1998). Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. *J Biol Chem*, 273(32), 20121-20127. <https://doi.org/10.1074/jbc.273.32.20121>
- Fritzsche, B., Schwer, B., Kartenbeck, J., Pedal, A., Horejsi, V., & Ott, M. (2002). Release and intercellular transfer of cell surface CD81 via microparticles. *J Immunol*, 169(10), 5531-5537. <https://doi.org/10.4049/jimmunol.169.10.5531>
- Gubler, U., Chua, A. O., Schoenhaut, D. S., Dwyer, C. M., McComas, W., Motyka, R., Nabavi, N., Wolitzky, A. G., Quinn, P. M., Familletti, P. C., & et al. (1991). Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor. *Proc Natl Acad Sci U S A*, 88(10), 4143-4147. <https://doi.org/10.1073/pnas.88.10.4143>
- Kalluri, R., & LeBleu, V. S. (2020). The biology, function, and biomedical applications of exosomes. *Science*, 367(6478). <https://doi.org/10.1126/science.aau6977>

- Kang, M., Choi, J. K., Jittayasothorn, Y., & Egwuagu, C. E. (2020). Interleukin 35-Producing Exosomes Suppress Neuroinflammation and Autoimmune Uveitis. *Front Immunol*, 11, 1051. <https://doi.org/10.3389/fimmu.2020.01051>
- Ma, N., Fang, Y., Xu, R., Zhai, B., Hou, C., Wang, X., Jiang, Z., Wang, L., Liu, Q., Han, G., & Wang, R. (2019). Ebi3 promotes T- and B-cell division and differentiation via STAT3. *Mol Immunol*, 107, 61-70. <https://doi.org/10.1016/j.molimm.2019.01.009>
- Maecker, H. T., & Levy, S. (1997). Normal lymphocyte development but delayed humoral immune response in CD81-null mice. *J Exp Med*, 185(8), 1505-1510. <https://doi.org/10.1084/jem.185.8.1505>
- Mirlekar, B., Michaud, D., Lee, S. J., Kren, N. P., Harris, C., Greene, K., Goldman, E. C., Gupta, G. P., Fields, R. C., Hawkins, W. G., DeNardo, D. G., Rashid, N. U., Yeh, J. J., McRee, A. J., Vincent, B. G., Vignali, D. A. A., & Pylayeva-Gupta, Y. (2020). B cell-Derived IL35 Drives STAT3-Dependent CD8(+) T-cell Exclusion in Pancreatic Cancer. *Cancer Immunol Res*, 8(3), 292-308. <https://doi.org/10.1158/2326-6066.Cir-19-0349>
- Mittelbrunn, M., Gutiérrez-Vázquez, C., Villarroya-Beltri, C., González, S., Sánchez-Cabo, F., González, M. Á., Bernad, A., & Sánchez-Madrid, F. (2011). Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells. *Nature Communications*, 2(1), 282. <https://doi.org/10.1038/ncomms1285>
- Montecalvo, A., Larregina, A. T., Shufesky, W. J., Stolz, D. B., Sullivan, M. L., Karlsson, J. M., Baty, C. J., Gibson, G. A., Erdos, G., Wang, Z., Milosevic, J., Tkacheva, O. A., Divito, S. J., Jordan, R., Lyons-Weiler, J., Watkins, S. C., & Morelli, A. E. (2012). Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. *Blood*, 119(3), 756-766. <https://doi.org/10.1182/blood-2011-02-338004>

- Muntasell, A., Berger, A. C., & Roche, P. A. (2007). T cell-induced secretion of MHC class II-peptide complexes on B cell exosomes. *Embo j*, 26(19), 4263-4272.
<https://doi.org/10.1038/sj.emboj.7601842>
- Oppmann, B., Lesley, R., Blom, B., Timans, J. C., Xu, Y., Hunte, B., Vega, F., Yu, N., Wang, J., Singh, K., Zonin, F., Vaisberg, E., Churakova, T., Liu, M.-r., Gorman, D., Wagner, J., Zurawski, S., Liu, Y.-J., Abrams, J. S., Moore, K. W., Rennick, D., de Waal-Malefyt, R., Hannum, C., Bazan, J. F., & Kastelein, R. A. (2000). Novel p19 Protein Engages IL-12p40 to Form a Cytokine, IL-23, with Biological Activities Similar as Well as Distinct from IL-12. *Immunity*, 13(5), 715-725. [https://doi.org/https://doi.org/10.1016/S1074-7613\(00\)00070-4](https://doi.org/https://doi.org/10.1016/S1074-7613(00)00070-4)
- Parolini, I., Federici, C., Raggi, C., Lugini, L., Palleschi, S., De Milito, A., Coscia, C., Iessi, E., Logozzi, M., Molinari, A., Colone, M., Tatti, M., Sargiacomo, M., & Fais, S. (2009). Microenvironmental pH is a key factor for exosome traffic in tumor cells. *J Biol Chem*, 284(49), 34211-34222. <https://doi.org/10.1074/jbc.M109.041152>
- Perez-Hernandez, D., Gutiérrez-Vázquez, C., Jorge, I., López-Martín, S., Ursa, A., Sánchez-Madrid, F., Vázquez, J., & Yáñez-Mó, M. (2013). The Intracellular Interactome of Tetraspanin-enriched Microdomains Reveals Their Function as Sorting Machineries toward Exosomes*. *Journal of Biological Chemistry*, 288(17), 11649-11661.
<https://doi.org/https://doi.org/10.1074/jbc.M112.445304>
- Pflanz, S., Timans, J. C., Cheung, J., Rosales, R., Kanzler, H., Gilbert, J., Hibbert, L., Churakova, T., Travis, M., Vaisberg, E., Blumenschein, W. M., Mattson, J. D., Wagner, J. L., To, W., Zurawski, S., McClanahan, T. K., Gorman, D. M., Bazan, J. F., de Waal Malefyt, R., Rennick, D., & Kastelein, R. A. (2002). IL-27, a heterodimeric cytokine

composed of EBI3 and p28 protein, induces proliferation of naive CD4⁺ T cells.

Immunity, 16(6), 779-790. [https://doi.org/10.1016/s1074-7613\(02\)00324-2](https://doi.org/10.1016/s1074-7613(02)00324-2)

Rialland, P., Lankar, D., Raposo, G., Bonnerot, C., & Hubert, P. (2006). BCR-bound antigen is targeted to exosomes in human follicular lymphoma B-cells. *Biol Cell*, 98(8), 491-501.

<https://doi.org/10.1042/bc20060027>

Sullivan, J. A., Tomita, Y., Jankowska-Gan, E., Lema, D. A., Arvedson, M. P., Nair, A., Bracamonte-Baran, W., Zhou, Y., Meyer, K. K., Zhong, W., Sawant, D. V., Szymczak-Workman, A. L., Zhang, Q., Workman, C. J., Hong, S., Vignali, D. A. A., & Burlingham, W. J. (2020). Treg-Cell-Derived IL-35-Coated Extracellular Vesicles Promote Infectious Tolerance. *Cell Rep*, 30(4), 1039-1051.e1035.

<https://doi.org/10.1016/j.celrep.2019.12.081>

Takeda, A., Hasegawa, E., Fukuhara, T., Hirakawa, S., Yamada, H., Yang, Y., Yoshimura, T., Hisatomi, T., Oshima, Y., Yoshida, H., Sonoda, K. H., & Ishibashi, T. (2014). EBI3 is pivotal for the initiation of experimental autoimmune uveitis. *Exp Eye Res*, 125, 107-113.

<https://doi.org/10.1016/j.exer.2014.06.004>

Wang, R. X., Yu, C. R., Dambuza, I. M., Mahdi, R. M., Dolinska, M. B., Sergeev, Y. V., Wingfield, P. T., Kim, S. H., & Egwuagu, C. E. (2014). Interleukin-35 induces regulatory B cells that suppress autoimmune disease. *Nat Med*, 20(6), 633-641.

<https://doi.org/10.1038/nm.3554>

Yeh, Y.-Y., Ozer, H. G., Lehman, A. M., Maddocks, K., Yu, L., Johnson, A. J., & Byrd, J. C. (2015). Characterization of CLL exosomes reveals a distinct microRNA signature and enhanced secretion by activation of BCR signaling. *Blood*, 125(21), 3297-3305.

<https://doi.org/10.1182/blood-2014-12-618470>

- Yoshida, N., Kitayama, D., Arima, M., Sakamoto, A., Inamine, A., Watanabe-Takano, H., Hatano, M., Koike, T., & Tokuhisa, T. (2011). CXCR4 expression on activated B cells is downregulated by CD63 and IL-21. *J Immunol*, 186(5), 2800-2808. <https://doi.org/10.4049/jimmunol.1003401>
- Yuseff, M.-I., Pierobon, P., Reversat, A., & Lennon-Duménil, A.-M. (2013). How B cells capture, process and present antigens: a crucial role for cell polarity. *Nature Reviews Immunology*, 13(7), 475-486. <https://doi.org/10.1038/nri3469>
- Zhang, H., Freitas, D., Kim, H. S., Fabijanic, K., Li, Z., Chen, H., Mark, M. T., Molina, H., Martin, A. B., Bojmar, L., Fang, J., Rampersaud, S., Hoshino, A., Matei, I., Kenific, C. M., Nakajima, M., Mutvei, A. P., Sansone, P., Buehring, W., Wang, H., Jimenez, J. P., Cohen-Gould, L., Paknejad, N., Brendel, M., Manova-Todorova, K., Magalhães, A., Ferreira, J. A., Osório, H., Silva, A. M., Massey, A., Cubillos-Ruiz, J. R., Galletti, G., Giannakakou, P., Cuervo, A. M., Blenis, J., Schwartz, R., Brady, M. S., Peinado, H., Bromberg, J., Matsui, H., Reis, C. A., & Lyden, D. (2018). Identification of distinct nanoparticles and subsets of extracellular vesicles by asymmetric flow field-flow fractionation. *Nature Cell Biology*, 20(3), 332-343. <https://doi.org/10.1038/s41556-018-0040-4>

CHAPTER IV: IL-35 CONTAINING EXOSOMES SUPPRESS ENCEPHALITOGENIC T CELLS AND INDUCE REGULATORY LYMPHOCYTES

Abstract

Multiple Sclerosis is an autoimmune disease of the central nervous system in which Th1 and Th17 cells play a major role in axonal demyelination via the production of their signature cytokines, IFN γ and IL-17, respectively. Regulatory cells and their suppressive cytokines, on the other hand, inhibit inflammatory cells and mitigate the disease. Previous studies supported the potential use of IL-35 and i35-Bregs as therapeutics for autoimmune diseases. Given that exosomes pass the blood brain barrier (BBB) and show minimal immunogenicity and toxicity, i35-exosomes represent a potential novel cell-free treatment for central nervous system (CNS) autoimmune diseases. Here, we show that IL-35 inhibits encephalitogenic T cells further supporting its suppressive effect in autoimmune diseases. Lymphoid cells were obtained from EAE mice and stimulated with MOG35-55 in the presence of IL-35 or PBS. IL-35 treated cells showed a decrease in the frequency of IL-17 producing T cells and IFN γ producing T cells compared to PBS treated cells. The IL-35 inhibition of encephalitogenic T cells was associated with expansion of IL-10 and IL-35 producing regulatory T and B cells. We also show that i35-exosomes mimic the suppressive effect of IL-35 *in vitro*. Treatment of encephalitogenic T cells with i35-exosomes suppressed IFN- γ and IL-17 production in response to MOG35-55 re-stimulation. Additionally, i35-exosomes mediated the expansion of IL-10 and IL-35 producing regulatory lymphocytes. *In vivo*, however, i35-exosomes did not show therapeutic efficacy in EAE based on the protocol used in this study. Taken together, our findings provide additional evidence of the suppressive role of IL-35 in EAE and show that i35-exosomes is a promising

therapeutic for CNS autoimmune diseases, however, some challenges still need to be addressed for their use *in vivo*.

Introduction

MS is an autoimmune neurodegenerative disease of the CNS. The pathology is driven, in part, by Th1 and Th17 cells that infiltrate the CNS and produce large amounts of the inflammatory cytokines, IFN γ and IL-17 (Kuchroo et al., 1993; Park et al., 2005). The immune attacks against the myelin sheath eventually results in neuronal damage making MS the most common cause of disability among young people (Browne et al., 2014). The currently available treatments for MS are mainly disease modifying therapies with significant side effects (Rommer & Zettl, 2018). Therefore, there is a need for developing alternative therapeutic approaches. A major therapeutic goal is to suppress inflammation or antagonize pathogenic cells that mediate CNS damages. One therapeutic approach is to use inhibitory cytokines that suppress expansion of pathogenic inflammatory cells.

IL-35 is a heterodimeric cytokine composed of IL-12p35 and EBI3 subunits. It has emerged as a potent immunosuppressive cytokine that plays a role in regulating autoimmune and inflammatory diseases (Collison et al., 2010; Collison et al., 2007; Shen et al., 2014; Wang et al., 2014). Our group previously showed that IL-35 treatment induces the expansion of a population of B regulatory cells that produce IL-35 (i35-Bregs) resulting in the amelioration of experimental autoimmune uveitis (EAU), an animal model of uveitis that shares immunological and pathological features with Experimental autoimmune encephalomyelitis (EAE) (Wang et al., 2014). In line with these findings, other studies have supported the regulatory role of IL-35 in EAE (Choi et al., 2017; Shen et al., 2014; Xie et al., 2016). Shen *et al.*, found that mice with B-

cell-restricted deficiency in one of IL-35 subunits, IL-12p35 or EBI3, developed an exacerbated EAE (Shen et al., 2014). The mice with B cells deficient in IL-12p35 showed an increase in the infiltration of inflammatory cells into the CNS and an increase in the production of IL-17 and IFN- γ by T cells, as compared to mice with wild-type (WT) B cells (Shen et al., 2014). Moreover, mice that lack IL-12R β 2 (a receptor of IL-35) developed severe EAE with increased T cell proliferation and infiltration into the CNS compared to WT mice (Xie et al., 2016). Taken together, these findings support the protective role of IL-35 in EAE and suggest the therapeutic potential of IL-35 in treating EAE/MS. In fact, our group previously showed that treatment with the IL-12p35 subunit reduced the severity of EAE, in part, by inhibiting Th1 and Th17, reducing cell infiltration into the CNS, and expanding B and T regulatory cells that produce IL-10 and IL-35 (Choi et al., 2017). This study further supported the protective role of IL-35 in EAE.

However, there are some obstacles to using IL-35 and i35-Breg therapy for CNS autoimmune diseases. It is difficult to produce heterodimeric IL-35 given the instability of IL-12p35:EBI3 complex *ex-vivo*. While i35-Breg treatment may provide some advantages such as the production of bioactive IL-35 *in vivo*, there is a need to produce a high amount of autologous i35-Bregs to avoid an allogeneic immune response to the transferred cells. Therefore, alternative approaches are needed to take advantage of the anti-inflammatory effect of IL-35.

Exosomes are small extracellular vesicles (40-160 nm in diameter) generated by all cells and contain proteins, DNA, RNA, and metabolites (Kalluri & LeBleu, 2020; Ridder et al., 2014). They can modulate the function and phenotype of recipient cells and have the advantage of being less immunogenic compared to the cells (Kalluri & LeBleu, 2020; Zhu et al., 2017). In *Chapter III*, we showed that i35-Bregs produce exosomes that contain IL-35. The production of IL-35 in

biological carriers that pass the BBB makes i35-exosomes an attractive therapeutic strategy for CNS inflammatory diseases. Our group previously showed that mice treated with i35-exosomes developed a mild EAU with a decrease in retinal pathology, a decrease in Th17 cell response, and an increase in IL-10 and IL-35 producing Tregs, as compared to PBS treated mice (Kang et al., 2020). Given the pathophysiological similarities between EAU and EAE, we sought to examine whether i35-exosomes will suppress EAE.

In this study, we show that heterodimeric IL-35 inhibits IL-17 and IFN γ production in response to MOG35-55 re-stimulation. Additionally, we show that IL-35 inhibits encephalitogenic T cells is, in part, by inducing the expansion of IL-10 and IL-35 producing Tregs and Bregs. Similarly, i35-exosomes inhibit encephalitogenic T cells *in vitro*, in part, by inducing the expansion of regulatory lymphocytes. Unexpectedly, i35-exosomes treatment has no effect on clinical EAE possibly due ineffective dose or route of administration that were used in this study. Collectively, these findings further support the suppressive role of IL-35 in EAE and show that i35-exosomes is a promising therapeutic for EAE/MS, however, some challenges still need to be addressed for their use *in vivo*.

Material and Methods

Mice

Wild-type C57BL/6J were purchased from Jackson Laboratory. Mice were maintained and treated in accordance with NIH Animal Care and Use Committee guidelines.

Generation of i35-Bregs for i35-exosome collection

The spleen of C57BL/6J WT mice were collected for B cell isolation. Single cell suspension was prepared by placing the spleens on cell strainers (Corning Falcon) and manually homogenizing the spleens using the plunger of a disposable 1 mL syringe. Cells were then washed twice with RPMI at 300xg for 10 minutes. Red blood cells were lysed by adding 5 mL of ACK buffer to pelleted splenocytes and incubating for 5 minutes at room temperature (RT). To remove ACK buffer, cells were washed twice with RPMI at 300xg for 10 minutes. Cells were resuspended in MACs buffer (0.5% bovine serum albumin in PBS) and B cells were sorted using CD19 MicroBeads (Miltenyi Biotec) as per manufacturer's instructions. Briefly, cells were first counted by using Vi-Cell XR (Beckman Coulter, Indianapolis, IN) to determine the amount of CD19 MicroBeads to be added to the cells. 10 μ L of CD19 MicroBeads were added per 10^7 of total cells and incubated for 15 minutes at 4°C. The cells were washed with MACs buffer, and B cells were isolated by passing the cells: beads mixture through LS MACs column placed in MACs magnetic separator. LS MACs columns were removed from the magnetic separator and flushed with MACs buffer to collect the bound B cells. B cells were counted by Vi-Cell XR (Beckman Coulter, Indianapolis, IN) and resuspended in complete RPMI 1640 (Gibco) supplemented with 10% exosomes-depleted fetal bovine serum (System Biosciences), 1% penicillin/streptomycin (Invitrogen), 1% L-glutamine (Sigma-Aldrich), 0.2% β -mercaptoethanol (Gibco), and HEPES to

achieve 2×10^6 cells/ mL. To induce i35-Bregs, B cells were stimulated with 1 $\mu\text{g/mL}$ anti-IgM (Jackson ImmunoResearch) + 10 $\mu\text{g/mL}$ anti-CD40 (Bio X Cell) and incubated at 37°C in 5 % CO₂ for 72 hours.

Exosomes isolation

i35-Bregs were induced as mentioned above and the supernatant was collected at the end of the 72h incubation. The collected supernatant was subjected to serial centrifugation steps at 4°C, 300xg for 10 minutes to remove cells, 2,000xg to remove dead cells, and 10,000 xg for 30 minutes to remove cell debris and large extracellular vesicles. The supernatant was then concentrated 4x by using Amicon Ultra centrifugal filter units (MWCO 30 kDa). The concentrated supernatant was subjected to 10,000xg for 30 minutes to remove any aggregates. Exosomes were isolated from the concentrated supernatant by using ExoQuick- ULTRA (System Biosciences) as per manufacturer's instructions. Briefly, 1 volume of ExoQuick buffer was added to 5 volumes of concentrated supernatant and incubated overnight at 4°C. The supernatants were subjected to 3,000xg for 10 minutes and the pelleted exosomes were resuspended and passed through the purification column. The protein concentration of exosomes was determined by using BCA protein assay kit (Sigma) per manufacturer's instructions. Exosomes were characterized as mentioned in *Chapter III*. Briefly, the concentration of exosomes was determined by using ExoELISA-ULTRA Complete Kit (CD81 detection) (System Biosciences) as per manufacturer's instructions. The presence of IL-35 in the exosomes was confirmed by using Western-blotting to detect IL-12p35 and EBI3 and heterodimeric ELISA.

EAE induction

For EAE induction, 6-8 weeks old female C57BL/6 mice were immunized with 200 µg MOG35-55 (Bio basic, Canada) emulsified in complete Freund's adjuvant (Sigma) containing 400 µg Mycobacterium tuberculosis strain H37RA. Each mouse received 0.05 ml of emulsion subcutaneously at each flank of hind legs and 0.1 ml at the base of the tail. Mice received 300 ng of pertussis toxin (Sigma) intraperitoneally at day 0 and day 2 of immunization.

EAE *ex-vivo* experiments

EAE was induced in 6-8 weeks old female C57BL/6 mice as mentioned above. On day 10 post immunization, immunization draining lymph nodes (inguinal and iliac) were collected. Lymph nodes were placed on cell strainers (Corning Falcon) and homogenized by gentle rubbing with the plunger of a disposable 1 mL syringe. The cell strainers were washed with RPMI and the effluent single cell suspensions were collected. The cells were washed twice with RPMI by centrifugation at 300 ×g for 10 minutes and then counted by using Vi-Cell XR (Beckman Coulter, Indianapolis, IN). The cells were resuspended in complete RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (Hyclone), 1% penicillin/streptomycin (Invitrogen), 1% L-glutamine (Sigma-Aldrich), 0.2% β-mercaptoethanol (Gibco), and HEPES to achieve 2 × 10⁶ cell/mL. To examine the effect of IL-35, lymphoid cells were re-stimulated with 20 µg of MOG 35-55 and treated with PBS as a control or 50 ng of IL-35 prepared by our group as mentioned previously (Wang et al., 2014). The cells were incubated at 37°C in 5 % CO₂ for 72 hours. Similarly, to examine the effect of i35-exosomes, lymphoid cells from EAE mice were re-stimulated with 20 µg of MOG 35-55 and treated with PBS as a control or ~4 × 10¹⁰ of i35-exosomes (~80 µg of total protein containing ~ 500 pg of IL-35). The cells were incubated at 37°C in 5 % CO₂ for 72 hours. At the end of the 72h of stimulation, cells were stimulated with

PMA (20 ng/mL)/ionomycin (1 μ M) in the presence of GolgiStop for 4 hours at 37°C in order to be stained for flow cytometry analysis.

Flow cytometry

After stimulation with PMA (20 ng/mL)/ionomycin (1 μ M) in the presence of GolgiStop for 4 hours, cells were washed twice with PBS. Then, the cells were stained with Fixable Viability Dye eFluor™ 450 (eBioscience) (diluted as 1:1000 in PBS) for 5 minutes at RT. After washing the cells twice with complete media at 300xg for 10, the cells were stained for surface markers, anti-CD4 (APC-Cy7) and anti-CD-19 (BV510) for 30 minutes at 4 °C. After washing twice with FACS buffer (0.5% bovine serum albumin in PBS), intracellular cytokine staining was performed using the BD Biosciences Cytofix/Cytoperm kit as per manufacturer's instructions. Briefly, the cells were resuspended in Cytofix/Cytoperm solution and incubated for 20 minutes at 4 °C. After washing twice with Cytofix/Cytoperm washing buffer, the cells were either stained for anti-IL-17 (PerCP) and anti-IFN γ (APC), or, anti-IL-10 (PE), anti-EBI-3 (PerCP) and anti-IL-12p35 (APC) overnight at 4 °C. The cells were then washed twice with Cytofix/Cytoperm washing buffer at 300xg for 10, resuspended in FACS buffer, and the data were acquired by CytoFlex flow cytometer. The data analysis was performed using FlowJo™ v10.8.1 software (Tree Star Inc., Ashland, OR, USA) following the gating strategy in (**Figure 16**).

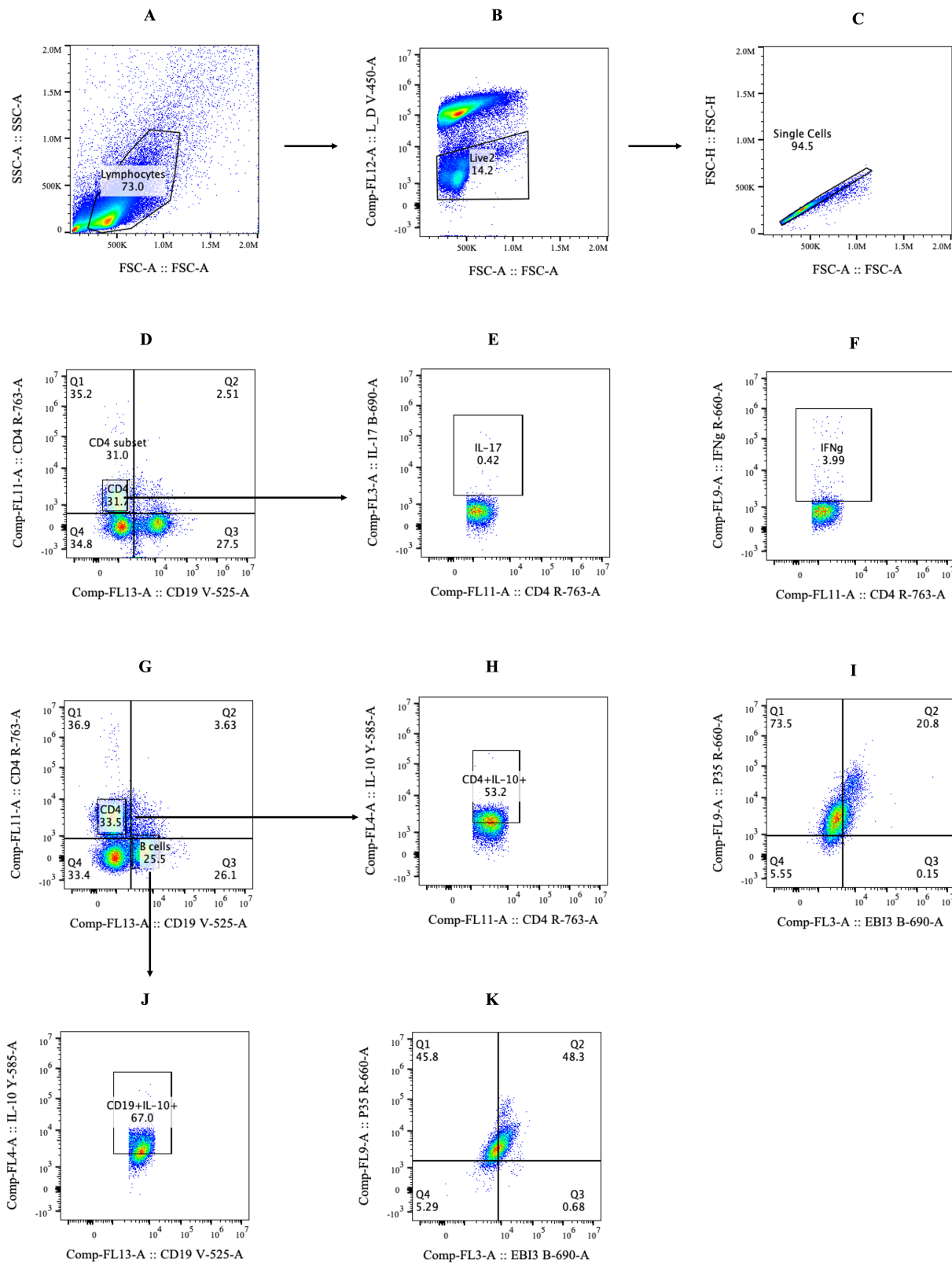


Figure 16. Sequential gating strategy to identify inflammatory T cells and regulatory lymphocytes.

EAE was induced in C57BL/6 mice by immunization with MOG35-55 emulsified in complete

Freund's adjuvant. Lymphoid cells were isolated on Day 10 post immunization and re-stimulated with MOG35-55 *in vitro* for 72 hours in the presence of IL-35. The cells were then stimulated with PMA/ionomycin in the presence of GolgiStop for 4 hours and stained for intracellular detection of inflammatory or regulatory cytokines by flow cytometry. The data were analyzed using FlowJo software as follows. **A)** Lymphocytes were first selected based on the forward scatter- area (FSC-A) vs side scatter-area (SSC-A). **B)** Viable cells were selected based on FSC-A vs Fixable Viability Dye eFluor™ 450 as cells that are negative for Viability Dye eFluor™ 450. **C)** Single cells were gated based on FSC-A vs forward scatter- height (FSC-H). **D)** Cells were gated based on CD19 vs CD4 to identify B cells and T helper cells. CD4⁺T cells were gated for the expression of IL-17 **(E)** and IFN γ **(F)**. To identify regulatory lymphocytes, cells were first gated as in **(A-D)**. CD4⁺ T cells from **(G)** were gated based on CD4 vs IL-10 to identify IL-10 producing T cells **(H)** and IL-12p35 vs EBI3 **(I)** to identify IL-35 producing Tregs as IL-12p35⁺EBI3⁺. CD19⁺ B cells from **(G)** were gated based on CD19 vs IL-10 to identify IL-10 producing B cells **(J)** and IL-12p35 vs EBI3 **(K)** to identify i35-Bregs as IL-12p35⁺EBI3⁺. The same gating strategy were used to identify inflammatory T cells and regulatory lymphocytes in cells that were treated with i35-exosomes or PBS.

***In vivo* treatment of EAE mice with i35-exosomes and clinical scoring**

EAE was induced in 6-8 weeks old female C57BL/6 mice as described earlier. For *in vivo* treatment, mice received $\sim 4 \times 10^{10}$ of i35-exosomes ($\sim 80 \mu\text{g}$ of total protein containing $\sim 500 \text{ pg}$ of IL-35 in total volume of $100 \mu\text{l}$) via the retro-orbital vein every other day starting from the day of immunization (day 0) until (day 12), $n=8$ mice. Mice treated with PBS were used as controls, $n=8$ mice. Mice were graded daily for 18 days by a masked observer based on 0-5 scale as follows; (0) normal; (1) limp tail; (2) hind limb weakness; (3) hind limb paralysis; (4) hind limb and fore limb paralysis; (5) moribund or death. On day 18, mice were euthanized and immunization draining lymph nodes (inguinal and iliac), spleen and CNS were collected for analysis by flow cytometry.

Flow cytometry analyses of splenocytes and lymphoid cells collected from EAE mice

After euthanizing the mice of the *in vivo* experiment, spleens and draining lymph nodes were collected and homogenized as mentioned previously. Single cells suspensions were prepared in complete RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (Hyclone), 1% penicillin/streptomycin (Invitrogen), 1% L-glutamine (Sigma-Aldrich), 0.2% β -mercaptoethanol (Gibco), and HEPES to achieve 2×10^6 cell/mL. Cells were stimulated with PMA (20 ng/mL)/ionomycin (1 μM) in the presence of GolgiStop for 4 hours at 37°C in 5 % CO_2 followed by staining for flow cytometry analysis. The cells were washed twice with PBS at 300xg for 10 minutes and then stained with Fixable Viability Dye eFluor™ 450 (eBioscience) (dilutes as 1:1000 in PBS) for 5 minutes at RT. The cells were washed twice with complete RPMI and stained for surface markers anti-CD4 (APC-Cy7) and anti-CD-19 (BV510) for 30 minutes at 4°C . Then intracellular staining was performed with anti-IL-17 (PerCP) and anti-

IFN γ (APC) as mentioned earlier. Data were acquired by CytoFlex flow cytometer and analysis was performed using FlowJo™ v10.8.1 software (Tree Star Inc., Ashland, OR, USA) following the gating strategy in (**Figure 16, A-F**).

Isolation of brain and spinal cord infiltrating lymphocytes using percoll gradients

After euthanizing the mice of the *in vivo* experiment, the brain and spinal cord were harvested and chopped into 5 mm length pieces before transferring to c-tubes (miltenyibiotec) containing 5 mL of incomplete RPMI and 1 mg/mL of collagenase D (Roche). Tissues were digested with gentleMACS™ Octo Dissociator (miltenyibiotec) for 1 hour at 37 C. The digested tissues were filtered through 70 μ M cell strainer (Corning Falcon) first and then through 40 μ M strainer (Corning Falcon). After washing with RPMI, cells were resuspended in 5 mL of 30% stock isotonic percoll (GE Healthcare) and slowly layered above 5 mL of 70% SIP. Samples were centrifuged at 500xg for 30 minutes at 18°C with no brake. The 70%-30% interphase containing cells were collected and washed twice with 8 ml of 1X HBSS at 500xg for 7 minutes at 18°C. The 30% and 70% SIP were prepared with 1X HBSS without Ca⁺⁺ and Mg⁺⁺ from 100% SIP (9 parts of percoll with one part of 10X HBSS without Ca⁺⁺ and Mg⁺⁺). Isolated PBMCs were then resuspended in complete RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (Hyclone), 1% penicillin/streptomycin (Invitrogen), 1% L-glutamine (Sigma-Aldrich), 0.2% β -mercaptoethanol (Gibco), and HEPES, and stimulated with PMA (20 ng/mL)/ionomycin (1 μ M) in the presence of GolgiStop for 4 hours at 37°C in 5 % CO₂. Cells were then washed twice with PBS and stained with Fixable Viability Dye eFluor™ 450 (eBioscience) (diluted as 1:1000 in PBS) for 5 minutes at RT. After staining for surface markers with anti-CD4 (APC-Cy7), anti-CD45 (FITC), and anti-CD19 (BV510), intracellular staining was performed with anti-IL-17

(PerCP) and anti-IFN γ (APC) as mentioned earlier. Data were acquired by CytoFlex flow cytometer and analyzed using FlowJo™ v10.8.1 software (Tree Star Inc., Ashland, OR, USA).

Statistical Analysis

Statistical analysis was performed using Prism 9 software. Student's *t*-test was performed to determine differences between groups. Non-parametric Mann-Whitney U test was used to compare the clinical scores of EAE mice. *P* value of <0.05 was considered statistically significant.

Results

IL-35 suppresses encephalitogenic T cells and expands regulatory T cells

IL-35 suppresses autoimmune diseases such as uveitis (Wang et al., 2014). The role of IL-35 in EAE was supported by the finding that mice with IL-35 deficient B cells developed exacerbated EAE (Shen et al., 2014). In addition, treatment with IL-12p35 subunit ameliorated EAE (Choi et al., 2017). Before examining the effect of i35-exosomes in EAE, we sought to examine whether heterodimeric IL-35 would inhibit encephalitogenic T cells, IFN γ or IL-17 producing T cells, that have been implicated as drivers of MS/EAE pathogenesis (Pierson et al., 2012). Lymphoid cells were isolated from mice immunized with MOG35-55 on day 10 post immunization. The cells were re-stimulated with MOG35-55 *in vitro* and either treated with PBS as a control or with 50 ng of recombinant IL-35 produced by our group as described previously (Wang et al., 2014). We found that IL-35 significantly inhibited IFN γ producing T cells compared to PBS treated cells (**Figure 17**). Similarly, IL-35 significantly inhibited IL-17 producing T cells compared to PBS treated cells (**Figure 17**). Given that IL-35 induces the expansion of regulatory T cells (Collison et al., 2010; Wang et al., 2014), we examined whether IL-35 inhibited encephalitogenic T cells, in part, by inducing the expansion of Tregs in response to MOG35-55 re-stimulation. We found that IL-35 significantly induced the expansion of IL-10 producing T cells and IL-35 producing T cells as compared to PBS (**Figure 18**). These results are consistent with previous reports showing that IL-35 induces regulatory T cells (Collison et al., 2010). Collectively, our findings support the suppressive role of IL-35 in EAE by showing that it inhibits encephalitogenic T cells and expands regulatory T cells.

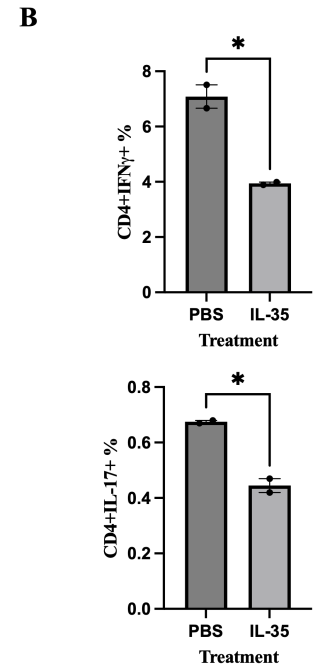
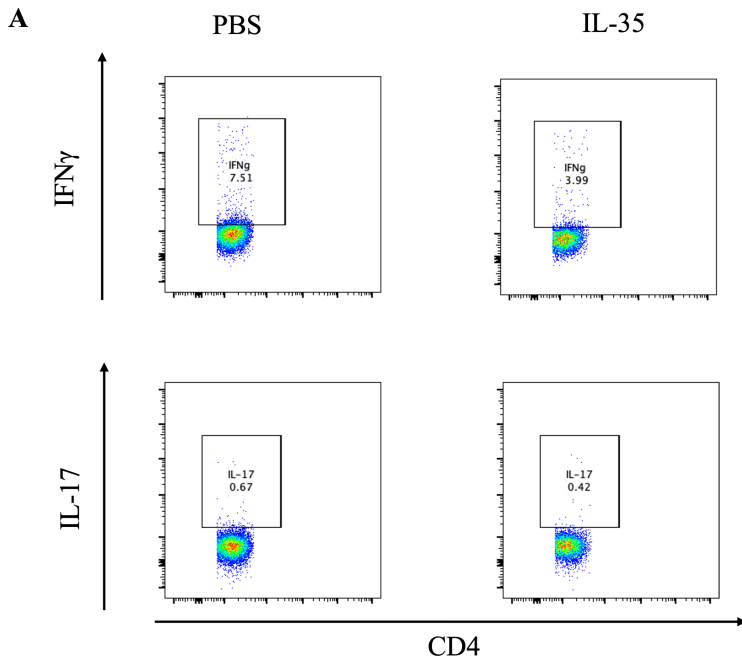


Figure 17. IL-35 suppresses encephalitogenic T cells

C57BL/6 mice were immunized with 100 µg of MOG35-55 emulsified in CFA to induce EAE. Lymphoid cells were isolated from immunization draining lymph nodes (inguinal and lumbar) on day 10 post immunization and were re-stimulated with MOG35-55, *in vitro* for 72h, in the presence of IL-35 (50 ng) or PBS, as a control. For intracellular cytokine analyses by flow cytometry, cells were then stimulated with PMA/I in the presence of GolgiStop for 4 hours and then stained for flow cytometry analysis. **A)** Representative flow cytometry plots of intracellular cytokine staining analysis. Gates indicate percent of T cells (identified as CD4⁺) expressing IFNγ or IL-17. **B)** Frequency of CD4⁺IFNγ⁺ and CD4⁺IL-17⁺ from **(A)**. *P* values were calculated using Student's *t* test, **p*<0.05. Error bars indicate SEM. Data represent the mean of two replicates.

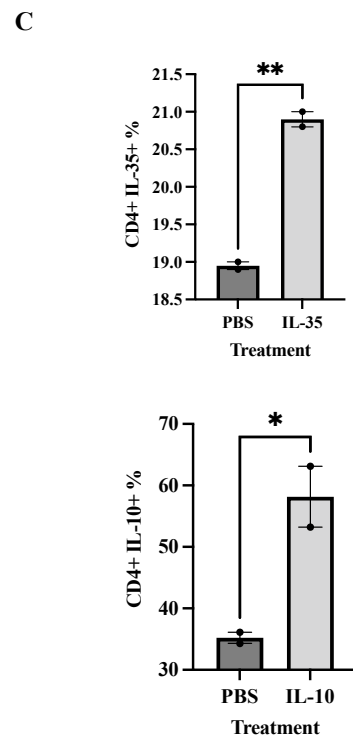
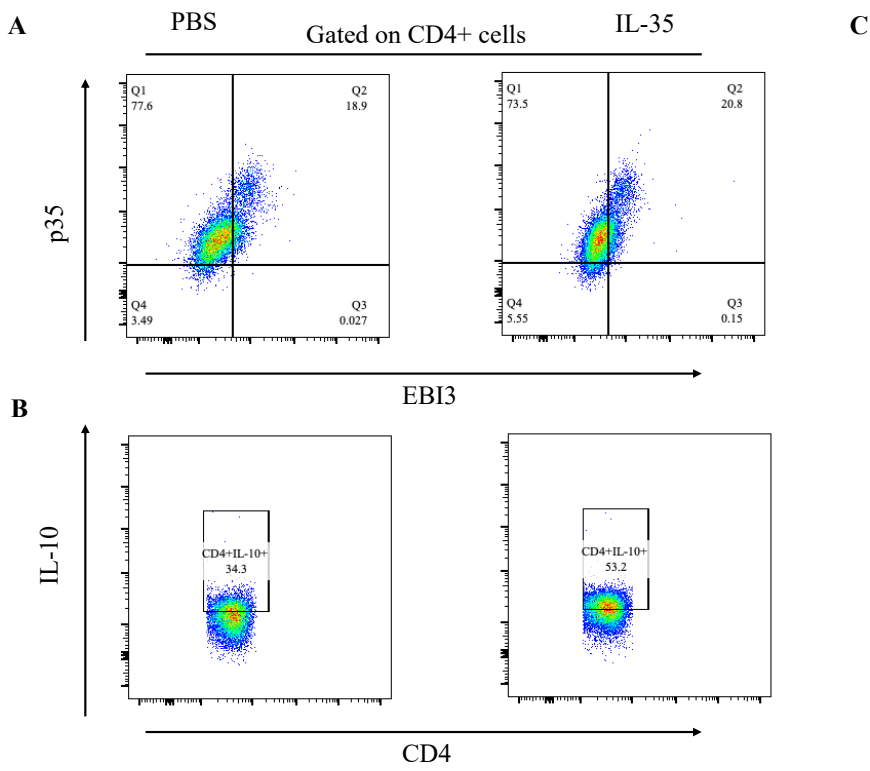


Figure 18. IL-35 expands regulatory T cells in EAE

Lymphoid cells isolated from EAE mice on day 10 post immunization were re-stimulated with MOG35-55, *in vitro* for 72h, in the presence of IL-35 (50 ng) or PBS, as a control. For intracellular cytokine analyses by flow cytometry, cells were then stimulated with PMA/I in the presence of GolgiStop for 4 hours and then stained and analysed by flow cytometry. **A)** Representative flow cytometry plots of intracellular cytokine staining analysis. T cells were identified as CD4⁺ and then gated for IL-12p35 and EBI3. Quadrants indicate percent of T cells IL-12p35⁻EBI3⁻, IL-12p35⁺EBI3⁻, IL-12p35⁻EBI3⁺, or IL-12p35⁺EBI3⁺. **B)** Representative flow cytometry plots of intracellular cytokine staining analysis. Gates indicate percent of T cells (identified as CD4⁺) expressing IL-10. **C)** Frequency of CD4⁺IL-12p35⁺EBI3⁺ (IL-35 producing Tregs) from the upper right quadrants of **(A)** and CD4⁺IL-10⁺ from **(B)**. *P* values were calculated using Student's *t* test, **p*<0.05, ***p*<0.01. Error bars indicate SEM. Data represent the mean of two replicates.

IL-35 induces IL-10 regulatory B cells and i35-Bregs in EAE

We have shown earlier that IL-35 inhibits encephalitogenic T cells and expands regulatory T cells (**Figure 17, Figure 18**). In our previous study, we showed that IL-35 induced the expansion of IL-35 and IL-10 producing regulatory B cells *in vitro* and *in vivo* resulting in amelioration of uveitis (Wang et al., 2014). Therefore, we examined whether IL-35 treatment induces the expansion of regulatory B cells in lymphoid cells isolated from EAE mice and re-stimulated *in vitro* with MOG 35-55 for 72h. As expected, IL-35 treatment increased the frequency of i35-Bregs from ~ 40% to 48% and IL-10 producing Bregs from ~ 50% to 67% (**Figure 19**). Interestingly, IL-35 increased IL-10 producing Bregs and IL-10 producing Tregs at a similar magnitude, up to 20%, compared to PBS treated cells (**Figure 18, Figure 19**). On the other hand, IL-35 treatment increased the frequency of i35-Bregs by ~10% and IL-35 producing Tregs by only 2%, compared to PBS treated cells (**Figure 18, Figure 19**). These results may suggest that B cells are more responsive to IL-35 compared to T cells.

Additionally, our data showed an enrichment of regulatory B cells in immunization draining lymph nodes of EAE mice. IL-10 producing regulatory B cells represented ~50% of total B cells (**Figure 19, B**), while IL-10 producing Tregs represented ~35% of total T cells in the lymph nodes (**Figure 18**). Moreover, i35-Bregs represented ~40% of total B cells (**Figure 19, A**) in immunization draining lymph nodes, while IL-35 producing Tregs represented ~18% of total T cells (**Figure 18**). In summary, the frequency of regulatory B cells out of total B cells is higher than the frequency of Tregs out of total T cells in the lymph nodes of EAE mice. Collectively, our findings support the suppressive effect of IL-35 in EAE, by inducing the expansion of regulatory B cells.

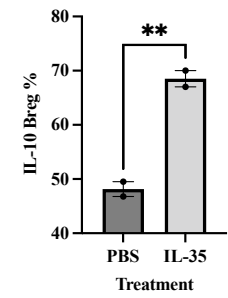
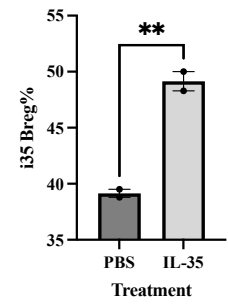
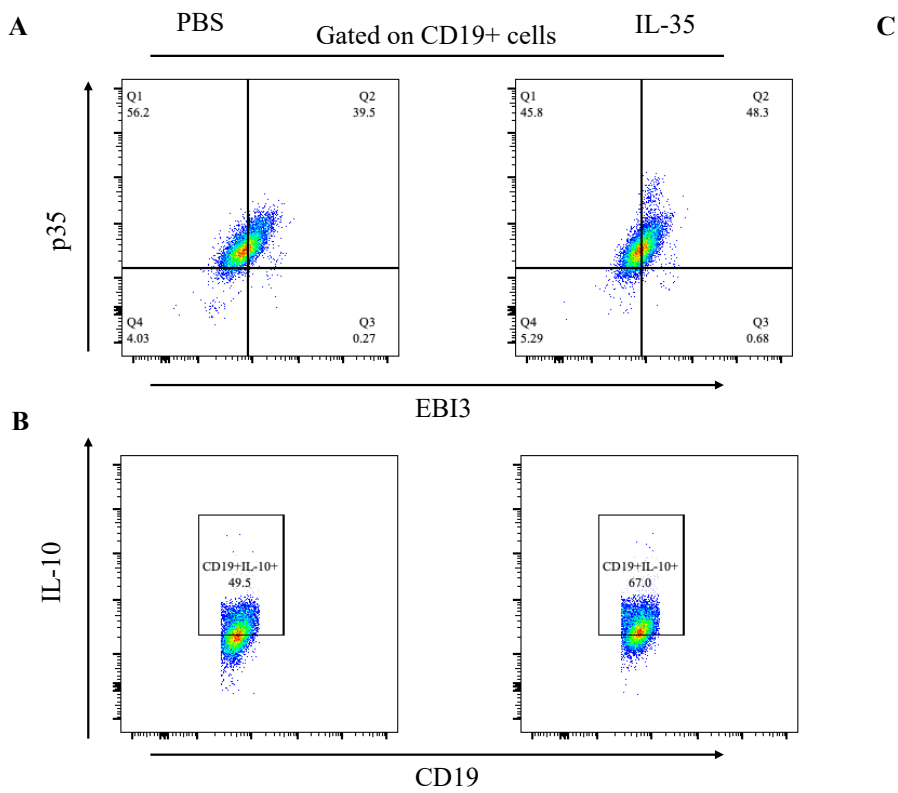


Figure 19. IL-35 induces IL-10 regulatory B cells and i35-Bregs in EAE

C57BL/6 mice were immunized with 100 µg of MOG35-55 emulsified in CFA to induce EAE. Lymphoid cells were isolated from immunization draining lymph nodes (inguinal and lumbar) on day 10 post immunization and were re-stimulated with MOG35-55, *in vitro* for 72h, in the presence of IL-35 (50 ng) or PBS, as a control. For intracellular cytokine analyses by flow cytometry, cells were then stimulated with PMA/I in the presence of GolgiStop for 4 hours and then stained and analysed by flow cytometry **A)** Representative flow cytometry plots of intracellular cytokine staining analysis. B cells were identified as CD19⁺ and then gated for IL-12p35 and EBI3. Quadrants indicate percent of B cells IL-12p35⁻EBI3⁻, IL-12p35⁺EBI3⁻, IL-12p35⁻EBI3⁺, or IL-12p35⁺EBI3⁺. **B)** Representative flow cytometry plots of intracellular cytokine staining analysis. Gates indicate percent of B cells (identified as CD19⁺) expressing IL-10. **C)** Frequency of CD19⁺IL-12p35⁺EBI3⁺ from the upper right quadrants of **(A)** and CD19⁺IL-10⁺ from **(B)**. *P* values were calculated using Student's *t* test, ***p*<0.01. Error bars indicate SEM. Data represent the mean of two replicates.

i35-exosomes suppress encephalitogenic T cells

A recent report showed that IL-35 containing exosomes secreted by Tregs have an immunosuppressive effect and induce infectious tolerance (Sullivan et al., 2020). Similarly, IL-35 containing exosomes produced by i35-Bregs suppressed uveitis, in part, by inhibiting inflammatory T cells (Kang et al., 2020). Given that our data showed that IL35 suppressed encephalitogenic T cells, we hypothesized that i35-exosomes will suppress encephalitogenic T cells. Lymphoid cells isolated from mice immunized with MOG35-55 were re-stimulated with MOG35-55 *in vitro* and treated with 80 µg of i35-exosomes $\sim 4 \times 10^{10}$ particles containing ~ 500 pg of IL-35 or PBS for 72h. Intracellular staining of T cells was performed and analyzed by flow cytometry. We found that i35-exosomes significantly suppressed IFN γ producing T cells ($\sim 3\%$) of total T cells compared to control ($\sim 8\%$) (**Figure 20**). Similarly, i35-exosomes significantly suppressed IL-17 producing T cells ($\sim 0.8\%$) compared to control ($\sim 1.5\%$) (**Figure 20**). Collectively, these data show a suppressive effect of i35-exosomes on encephalitogenic T cells and, therefore, suggest that i35-exosomes have the potential to be used as an alternative to IL-35 in the treatment of autoimmune diseases.

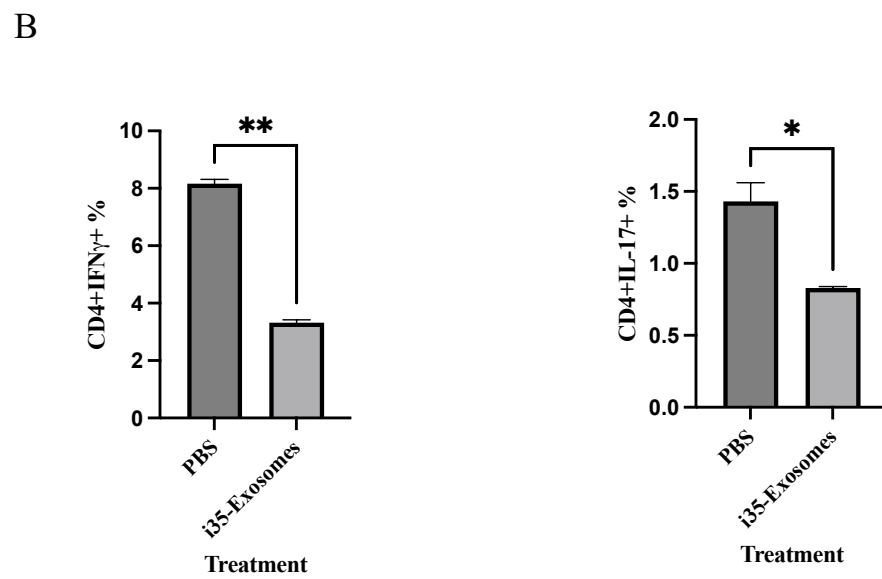
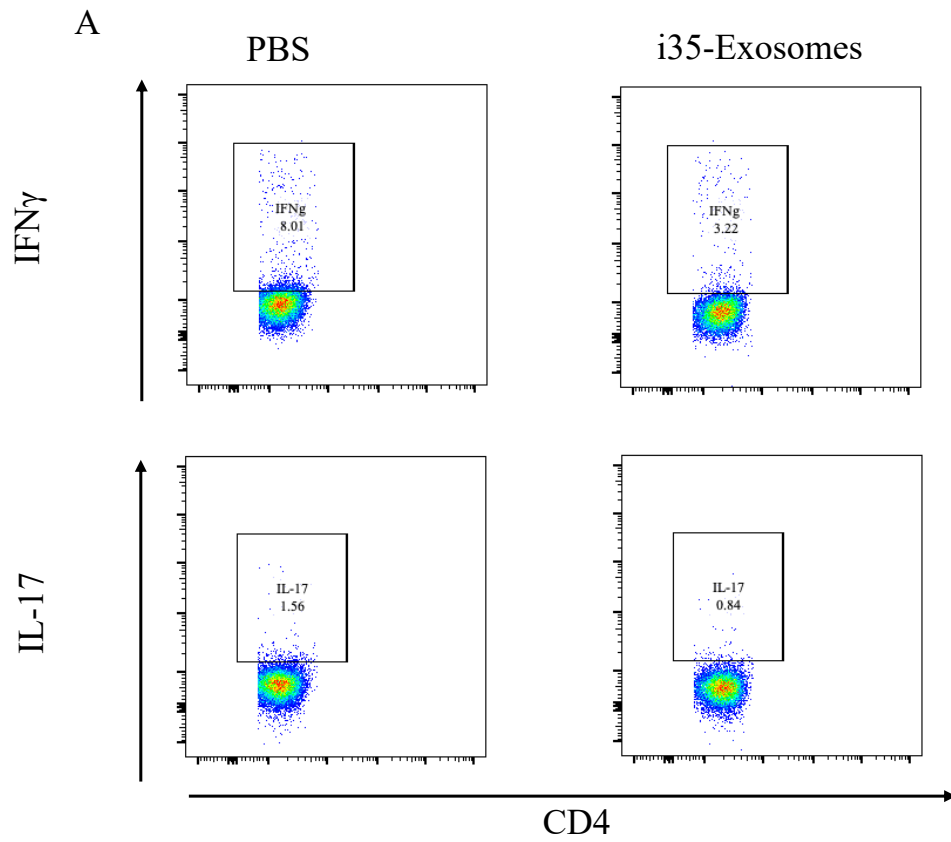


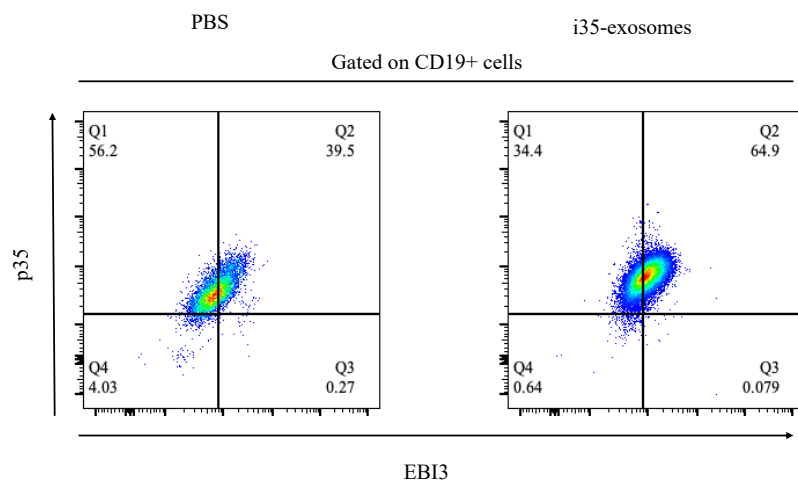
Figure 20. i35-exosomes suppress encephalitogenic T cells

C57BL/6 mice were immunized with 100 µg of MOG35-55 emulsified in CFA to induce EAE. Lymphoid cells were isolated from immunization draining lymph nodes (inguinal and lumbar) on day 10 post immunization and were re-stimulated with MOG35-55, *in vitro* for 72h, in the presence of i35-exosomes or PBS, as a control. For intracellular cytokine analyses by flow cytometry, cells were then stimulated with PMA/I in the presence of GolgiStop for 4 hours and then stained and analysed by flow cytometry. **A)** Representative flow cytometry plots of intracellular cytokine staining analysis. Gates indicate percent of T cells (identified as CD4⁺) expressing IFN γ or IL-17. **B)** Frequency of CD4⁺IFN γ ⁺ and CD4⁺IL-17⁺ from **(A)**. Data represent the mean of two replicates. *P* values were calculated using Student's *t* test, **p*<0.05, ***p*<0.01. Error bars indicate SEM. Data represent the mean of two independent experiments.

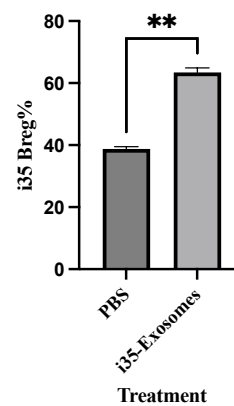
i35-exosomes induce IL-35 and IL-10 producing B cells in EAE

To identify possible mechanisms by which i35-exosomes suppressed encephalitogenic T cells, we examined whether similar to IL-35, i35-exosomes will induce the expansion of regulatory B cells. Lymphoid cells isolated from mice immunized with MOG35-55 on day 10 were re-stimulated with MOG35-55 *in vitro* and treated with 80 ug of i35-exosomes $\sim 4 \times 10^{10}$ particles containing ~ 500 pg of IL-35 or PBS for 72h. Intracellular staining of B cells was performed and analyzed by flow cytometry. We found that i35-exosomes significantly expanded i35-Bregs by $\sim 25\%$ compared to PBS treated cells (**Figure 21, A-B**). Additionally, i35-exosomes significantly expanded IL-10 producing Bregs by $\sim 20\%$ compared to PBS treated cells (**Figure 21, C-D**). These data suggest that i35-exosomes inhibit encephalitogenic T cells, in part, by inducing the expansion of regulatory B cells.

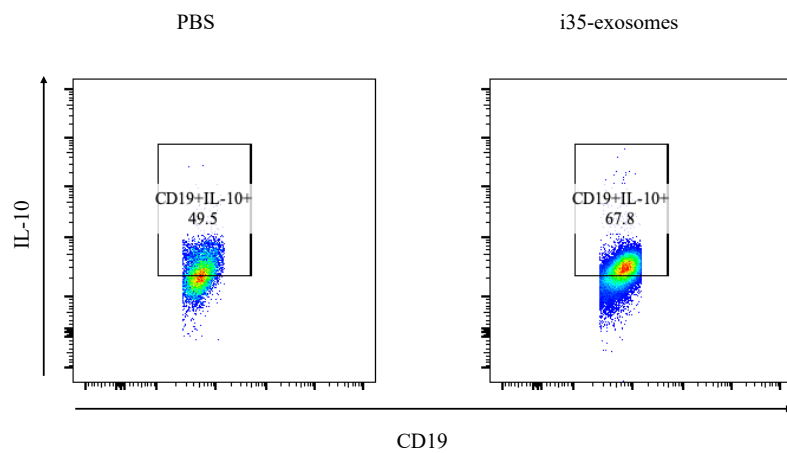
A



B



C



D

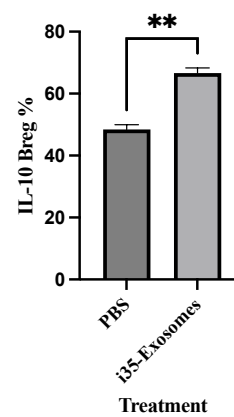


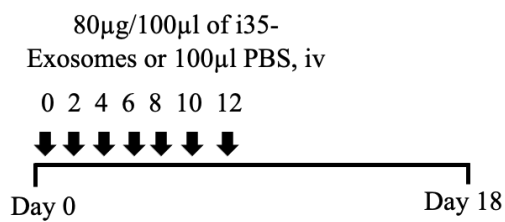
Figure 21. i35-exosomes induce IL-35 and IL-10 producing B cells in EAE

C57BL/6 mice were immunized with 100 µg of MOG35-55 emulsified in CFA to induce EAE. Lymphoid cells were isolated from immunization draining lymph nodes (inguinal and lumbar) on day 10 post immunization and were re-stimulated with MOG35-55, *in vitro* for 72h, in the presence of i35-exosomes or PBS, as a control. For intracellular cytokine analyses by flowcytometry, cells were then stimulated with PMA/I in the presence of GolgiStop for 4 hours and then stained and analysed by flowcytometry. **A)** Representative flow cytometry plots of intracellular cytokine staining analysis. B cells were identified as CD19⁺ and then gated for IL-12p35 and EBI3. **B)** Frequency of CD19⁺IL-12p35⁺EBI3⁺ from the upper right quadrants of **(A)**. **C)** Representative flow cytometry plots of intracellular cytokine staining analysis. Gates indicate percent of B cells (identified as CD19⁺) expressing IL-10. **D)** Frequency of CD19⁺IL-10⁺ from **(C)**. *P* values were calculated using Student's *t* test, ***p*<0.01. Error bars indicate SEM. Data represent the mean of two independent experiments.

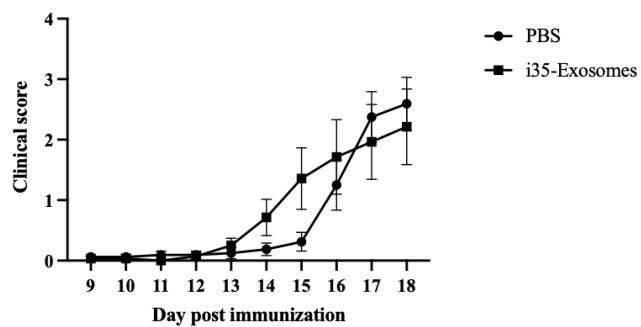
Treatment with i35-exosomes has no significant effect on ongoing EAE

IL-35 has been reported to suppress autoimmune diseases and to play a protective role in EAE (Shen et al., 2014; Wang et al., 2014). Given that exosomes produced by i35-Bregs contain IL-35 and suppress encephalitogenic T cells *in vitro* (**Figure 20**), we hypothesized that treatment with i35 exosomes will ameliorate EAE. To test this hypothesis, EAE was induced by active immunization with MOG 35-55. Mice were treated with $\sim 4 \times 10^{10}$ i35-exosomes (80 μ g of total protein containing ~ 500 pg of IL-35 in 100 μ l total volume) intravenously at day 0 and every other day until day 12 (**Figure 22, A**). The mice were graded daily for signs of paralysis until day 18 post-immunization. Unexpectedly, we found there is no significant difference in the clinical scores between i35-exosome treated mice and PBS treated mice (**Figure 22, B**). Additionally, we found no significant difference in the disease onset between i35-exosome treated mice and PBS treated mice (**Figure 22, C**). Collectively, these data show that i35-exosomes had no therapeutic effect on clinical EAE, using the current experimental protocol.

A



B



C

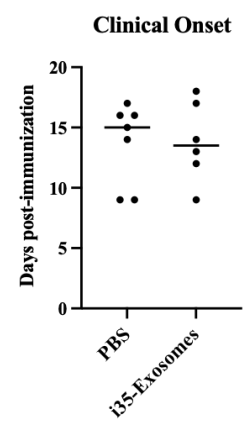


Figure 22. Treatment with i35-exosomes has no significant effect on EAE disease

Mice were immunized with 100 µg of MOG35-55 emulsified in CFA to induce EAE and either received i35-exosome treatment (i35-exosomes, n=8) or PBS as a control (PBS, n=8). **A)** Schematic of the treatment protocol indicates that mice either received i35-exosomes 80 µg of exosome total protein in 100 µl of PBS $\sim 4 \times 10^{10}$ exosomes containing ~ 500 pg of IL-35/ dose) or PBS intravenously (iv). **B)** EAE clinical score of i35-exosomes treated mice (i35-exosomes) and PBS treated mice (PBS) starting from day 9 to day 18 post immunization. Differences between groups were calculated using Non-parametric Mann-Whitney U test and it showed no significant difference between the treated and control group. **C)** Day of EAE onset was compared between i35-exosomes treated mice (i35-exosomes) and PBS treated mice (PBS). Mice that did not develop the disease were excluded from the analysis. Differences between groups were calculated using student *t* test which showed no significant difference between the treated and control group.

Mice treated with i35-exosomes showed no significant difference in frequency of inflammatory T cells compared to control mice

We wanted to investigate whether i35-exosomes would reduce the frequency of pro-inflammatory T cells in EAE mice, regardless of the clinical scores. CNS were collected from EAE mice at day 18 post immunization, and lymphocytes were isolated and analyzed by flow cytometry. We found no significant differences in the frequency of Th1 and Th17 cells in the CNS of i35-exosome treated mice compared to controls (**Figure 23, A**). Similarly, lymph nodes and spleens were collected from these mice and analyzed for intracellular expression of IL-17 and IFN γ . Mice treated with i35-exosomes showed no significant difference in the frequency of Th1 and Th17 in spleen and lymph nodes, compared to controls (**Figure 23 B, C**). Collectively, these data correspond with the clinical data in showing that i35-exosomes had no therapeutic effect on EAE, using the current experimental methods.

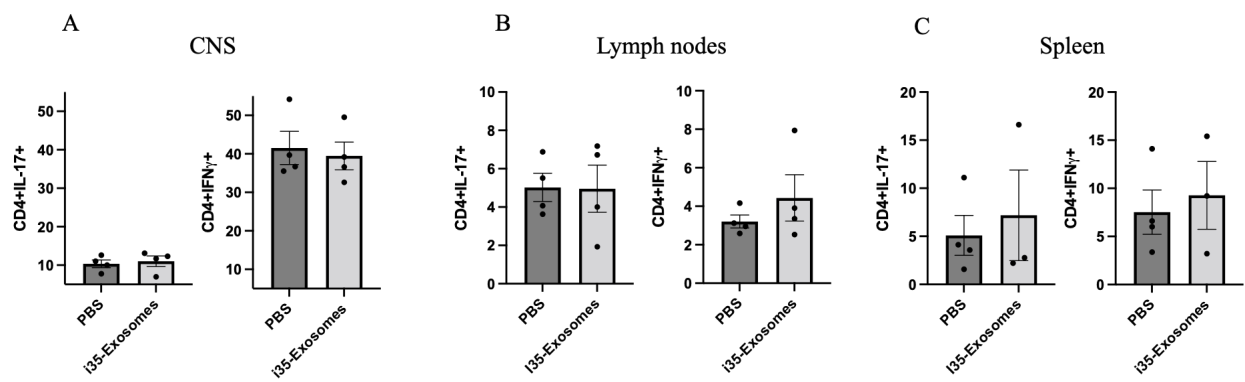


Figure 23. Mice treated with i35-exosomes showed no significant difference in frequency of inflammatory T cells compared to control mice

On day 18 post immunization, CNS, lymph nodes and spleens were collected from EAE mice treated with i35-exosomes (n=8) or PBS (n=8). The tissues of every two mice with a similar clinical score were pooled together. The cells were stimulated with PMA/I for 4 hours and analyzed for intracellular expression of IFN γ and IL17 in T cells by flow cytometry. **A)** Frequency of IL17 producing T cells and IFN γ producing T cells in the CNS, **B)** Frequency of IL17 producing T cells and IFN γ producing T cells in lymph nodes, and **C)** Frequency of IL17 producing T cells and IFN γ producing T cells in spleen. *P* values were calculated using Student's *t* test. Error bars indicate SEM.

Discussion

CNS autoimmune diseases are characterized by infiltration of inflammatory cells into the CNS. The pathology of Multiple Sclerosis is mainly driven by Th1 and Th17 cells that produce the pro-inflammatory cytokines IFN γ and IL-17, respectively (Kuchroo et al., 1993; Park et al., 2005). A major therapeutic goal is to suppress pathogenic cells and reduce inflammatory cytokines that mediate CNS autoimmune diseases. Accumulating evidence supports the protective role of regulatory B cells in MS (Blumenfeld-Kan et al., 2019; Duddy et al., 2007; Hirotani et al., 2010) and EAE (Fillatreau et al., 2002; Shen et al., 2014; Wolf et al., 1996). One of the mechanisms by which Bregs suppress inflammation is through the production of IL-35 (Shen et al., 2014; Wang et al., 2014). In this report, we further support the suppressive effect of IL-35 in CNS autoimmune diseases by showing that treatment of lymphoid cells from EAE mice with IL-35 or IL-35 containing exosomes resulted in inhibition of encephalitogenic T cells and expansion of regulatory cells. By using *in vitro* and *in vivo* studies, we evaluated the potential and challenges of using i35-exosomes as a novel cell free therapy for MS/EAE.

The role of IL-35 in EAE was supported by the finding that mice with IL-35 deficient B cells developed exacerbated EAE (Shen et al., 2014). In addition, treatment with IL-12p35 subunit ameliorated EAE (Choi et al., 2017). However, the effect of heterodimeric IL-35 on EAE has not been tested. To examine the effect of heterodimeric IL-35 in EAE, we immunized mice with MOG 35-55 and collected draining lymph nodes by day 10 post immunization. We performed *in vitro* studies in which lymphoid cells were re-stimulated with MOG 35-55 in the presence of PBS as a control or recombinant IL-35 (rIL-35) for 72h. We found that IL-35 treatment reduced the frequency of IFN γ producing T cells and IL-17 producing T cells (**Figure 17**). These findings are in line with our previous report showing that the treatment of EAU mice,

a model of uveitis that share pathological features with EAE, with IL-35 resulted in inhibition of IFN γ producing T cells and IL-17 producing T cells (Wang et al., 2014). Similarly, treatment of EAU mice with i35-Bregs that were expanded *ex-vivo* suppressed uveitis by inhibiting Th1 and Th17 cells (Wang et al., 2014). Moreover, IL-12p35 treatment suppressed EAE and EAU in which both models showed a decrease in the frequency of IL-17 and IFN γ producing T cells as compared to PBS treated mice (Choi et al., 2017; Dambuza et al., 2017). Collectively, our findings further support the suppressive effect of IL-35 on IL-17 producing and IFN- γ producing T cells by showing that it inhibits encephalitogenic T cells.

Additionally, we found that lymphoid cells that were re-stimulated with MOG 35-55 in the presence of rIL-35 showed an increase of IL-10 producing Tregs, IL-35 producing Tregs, IL-10 producing Bregs, and i35-Bregs as compared to cells treated with PBS (**Figure 18, Figure 19**). These data suggest that IL-35 inhibits encephalitogenic T cells, in part, by expanding IL-10 and IL-35 producing regulatory T and B cells. These data are consistent with previous reports showing that IL-35 induces regulatory T cells and B cells (Collison et al., 2010; Wang et al., 2014). Collison et al, showed that treatment of naïve human or mouse T cells with rIL-35 induced the expansion of regulatory T cells that produce IL-35 (Collison et al., 2010). Additionally, Wang et al., showed that stimulating B cells with LPS in the presence of rIL-35 increased the percentage of IL-35 producing Bregs from 7% (LPS alone) to 35% (LPS+rIL-35) (Wang et al., 2014). IL-35 treatment also induced the expansion of IL-10 producing Bregs (Wang et al., 2014). While 7% of i35-Bregs produce IL-10 in response to LPS stimulation alone, 17% of i35-Bregs produce IL-10 in response to LPS stimulation in the presence of rIL-35 (Wang et al., 2014). Moreover, treatment of EAU mice with rIL-35 induced the expansion of IL-10

producing Bregs *in vivo* (Wang et al., 2014). Furthermore, treatment of EAU mice with i35-Bregs therapy induced the expansion of IL-10 and IL-35 producing Bregs, and *Foxp3*⁺ Tregs (Wang et al., 2014). While the expansion of regulatory cells could inhibit encephalitogenic T cells, it is possible that IL-35 acts directly on encephalitogenic T cells and suppresses them. It was found that rIL-35 converted conventional T cells into IL-35 producing Tregs that mediated immune suppression (Collison et al., 2010). Collectively, our findings further support the suppressive effect of IL-35 in autoimmune diseases by showing that it suppresses encephalitogenic T cells and expands regulatory cells.

Interestingly, we found that lymph nodes of EAE mice are enriched with IL-10 Bregs and i35-Bregs (**Figure 19**). A previous report showed that IL-10 producing plasmablasts are generated in the lymph nodes upon EAE induction, and they are not of splenic B lineage cells (Matsumoto et al., 2014). These cells were mainly located in the extrafollicular region with dendritic cells between T cell zones and B cell follicles, and inhibited dendritic cell ability to generate encephalitogenic T cells by producing IL-10 (Matsumoto et al., 2014). However, it remains to be elucidated whether i35-Bregs in the lymph nodes of EAE mice are of splenic B lineage cells or generated in the lymph nodes. Additionally, identifying mechanisms by which i35-Bregs regulate encephalitogenic T cells in lymph nodes of EAE mice is of interest.

While our results further suggest IL-35 potential to treat MS/EAE, the difficulty of *ex-vivo* production of bioactive heterodimeric IL-35 has impeded its use for immunotherapy. On the other hand, i35-Breg provides an advantage over therapeutic administration of IL-35 because it proliferates *in vivo* and can sustain delivery of IL-35 in target tissues. Indeed, the use of i35-

Bregs generated *in vitro* was effective in ameliorating EAU (Wang et al., 2014). However, there are some obstacles to use i35-Breg therapy for CNS autoimmune diseases. To use i35-Breg in patients, there is a need to produce a high amount of autologous i-35 Bregs, to avoid allogeneic immune response to transferred cells. Another big challenge in treating CNS diseases is the need for a therapy that can passively pass the BBB without compromise it. Therefore, alternative approaches are needed to take advantage of the anti-inflammatory effect of IL-35.

Exosomes are small extracellular vesicles (50-150 nm in diameter) that readily cross the BBB (Ridder et al., 2014), and therefore they are desirable vehicles to deliver biologics into CNS tissues. Given that exosomes generated by i35-Bregs contain IL-35, we hypothesized that i35-exosomes will suppress encephalitogenic T cells. We used *in vitro* studies in which lymphoid cells isolated from EAE mice were re-stimulated with MOG 35-55 and treated with PBS as a control or i35-exosomes. We found that, similar to IL-35, i35-exosomes suppressed encephalitogenic T cells as indicated by a decrease in the frequency of IL-17 producing T cells and IFN γ producing T cells as compared to PBS treated cells (**Figure 20**). These data are in line with our previous report showing that i35-exosomes suppress T cell proliferation and IFN γ production by cells stimulated with anti-CD3/CD28 antibodies *in vitro* (Kang et al., 2020). Additionally, i35-exosomes suppressed Th17 cells in EAU model (Kang et al., 2020). Our current findings provide additional evidence of the regulatory effect of i35-exosomes by showing that they suppress encephalitogenic T cells.

The suppression of encephalitogenic T cells by i35-exosomes was associated with the expansion of IL-10 Bregs and i35-Bregs (**Figure 21**). While the inhibition of encephalitogenic T cells could be due to the expansion of regulatory B cells, our study does not exclude the

involvement of other mechanisms. For example, i35-exosomes could be taken up by antigen presenting cells (APCs), turning them into tolerogenic APCs with reduced capacity to induce encephalitogenic T cells. Another mechanism could be that i35-exosomes directly inhibit encephalitogenic T cells by loading their RNA cargo that modulate T cell effector functions. In fact, we found that i35-exosomes are enriched with miRNAs (**Figure 25**). miRNAs of exosome could play a regulatory function. For example, Okoye *et al.*, found that RNAs were transferred from Treg cells to conventional T effector cells by exosomes. Specifically, Treg exosomes inhibited Th1 response, in part, by transferring Let-7b miRNA that targets signaling molecules involved in Th1 proliferation and cytokine secretion (Okoye et al., 2014). Whether i35-exosomes contain miRNAs that suppress effector T cells remains to be elucidated. In conclusion, i35-exosomes showed an ability to inhibit encephalitogenic T cells and expand regulatory lymphocytes supporting their potential to be used as a treatment for EAE/MS.

We next examined the effect of i35-exosomes on clinical EAE. EAE mice were treated either with PBS as a control or i35-exosomes via intravenous injection every other day starting from day 0 to day 12 post immunization. Surprisingly, we did not find a therapeutic effect of i35-exosome treatment *in vivo* in our experimental setting (**Figure 22, Figure 23**). While using exosomes *in vivo* presented different challenges, one possible reason of our unexpected results could be the route of administration. We used intravenous injection (iv) starting at the day of disease induction. A study showed that exosomes administered by iv led to a higher accumulation of the exosomes in the liver, compared to when they were administered by intraperitoneal injection (ip) (Wiklander et al., 2015). During the first week of the EAE induction, most of the immune reactions happen in the lymph nodes, and not in the blood. It is

possible that exosomes administered by ip injection will have a better access to immunization draining lymph nodes than iv injection. In the previous report from our lab, Kang *et al.*, showed that i35-exosomes ameliorate EAU (Kang et al., 2020). Given that i35-exosomes were administered via the retro-orbital vein, it is possible that the retro-orbital injection led to a better infiltration of i35-exosomes into the eye resulting in a local suppressive effect of EAU. Such possibility suggests that injecting i35-exosomes directly into the CNS via intracerebroventricular or intrathecal injections might result in a better outcome in EAE. Therefore, the fate of i35-exosomes after the injection needs to be identified by trafficking analysis. Such studies will help in determining the biodistribution of i35-exosomes using different route of administration and could help in identifying the cellular targets of i35-exosomes.

Another issue could be the dose or the concentration of IL-35 in exosomes. While kang *et al.*, showed that 2×10^{10} of exosomes contain 20 ng of IL-35, my data show that $\sim 4 \times 10^{10}$ of exosomes contain around 500 pg of IL-35. One possible reason could be the different assays used to quantify exosomes. Here, we used ELISA that quantify exosomes based on the expression of CD81 while Kang *et al.*, used Exocet assay that quantify exosomes based on the activity of AChE, an enzyme enriched in most exosomes. Engineered exosomes could be one solution to adjust IL-35 dosing. In addition, engineered exosomes could also be designed to target specific type of cells. In conclusion, the use of i35-exosomes is a promising therapeutic strategy for EAE/MS, however, some challenges still need to be addressed for their use *in vivo*.

References

- Blumenfeld-Kan, S., Staun-Ram, E., & Miller, A. (2019). Fingolimod reduces CXCR4-mediated B cell migration and induces regulatory B cells-mediated anti-inflammatory immune repertoire. *Mult Scler Relat Disord*, 34, 29-37.
<https://doi.org/10.1016/j.msard.2019.06.016>
- Browne, P., Chandraratna, D., Angood, C., Tremlett, H., Baker, C., Taylor, B. V., & Thompson, A. J. (2014). Atlas of Multiple Sclerosis 2013: A growing global problem with widespread inequity. *Neurology*, 83(11), 1022-1024.
<https://doi.org/10.1212/wnl.0000000000000768>
- Choi, J. K., Dambuza, I. M., He, C., Yu, C. R., Uche, A. N., Mattapallil, M. J., Caspi, R. R., & Egwuagu, C. E. (2017). IL-12p35 Inhibits Neuroinflammation and Ameliorates Autoimmune Encephalomyelitis. *Front Immunol*, 8, 1258.
<https://doi.org/10.3389/fimmu.2017.01258>
- Collison, L. W., Chaturvedi, V., Henderson, A. L., Giacomini, P. R., Guy, C., Bankoti, J., Finkelstein, D., Forbes, K., Workman, C. J., Brown, S. A., Rehg, J. E., Jones, M. L., Ni, H. T., Artis, D., Turk, M. J., & Vignali, D. A. (2010). IL-35-mediated induction of a potent regulatory T cell population. *Nat Immunol*, 11(12), 1093-1101.
<https://doi.org/10.1038/ni.1952>
- Collison, L. W., Workman, C. J., Kuo, T. T., Boyd, K., Wang, Y., Vignali, K. M., Cross, R., Sehy, D., Blumberg, R. S., & Vignali, D. A. (2007). The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature*, 450(7169), 566-569.
<https://doi.org/10.1038/nature06306>

- Dambuza, I. M., He, C., Choi, J. K., Yu, C. R., Wang, R., Mattapallil, M. J., Wingfield, P. T., Caspi, R. R., & Egwuagu, C. E. (2017). IL-12p35 induces expansion of IL-10 and IL-35-expressing regulatory B cells and ameliorates autoimmune disease. *Nat Commun*, 8(1), 719. <https://doi.org/10.1038/s41467-017-00838-4>
- Duddy, M., Niino, M., Adatia, F., Hebert, S., Freedman, M., Atkins, H., Kim, H. J., & Bar-Or, A. (2007). Distinct effector cytokine profiles of memory and naive human B cell subsets and implication in multiple sclerosis. *J Immunol*, 178(10), 6092-6099. <https://doi.org/10.4049/jimmunol.178.10.6092>
- Fillatreau, S., Sweeney, C. H., McGeachy, M. J., Gray, D., & Anderton, S. M. (2002). B cells regulate autoimmunity by provision of IL-10. *Nat Immunol*, 3(10), 944-950. <https://doi.org/10.1038/ni833>
- Hirotsu, M., Niino, M., Fukazawa, T., Kikuchi, S., Yabe, I., Hamada, S., Tajima, Y., & Sasaki, H. (2010). Decreased IL-10 production mediated by Toll-like receptor 9 in B cells in multiple sclerosis. *J Neuroimmunol*, 221(1-2), 95-100. <https://doi.org/10.1016/j.jneuroim.2010.02.012>
- Kalluri, R., & LeBleu, V. S. (2020). The biology, function, and biomedical applications of exosomes. *Science*, 367(6478). <https://doi.org/10.1126/science.aau6977>
- Kang, M., Choi, J. K., Jittayasothorn, Y., & Egwuagu, C. E. (2020). Interleukin 35-Producing Exosomes Suppress Neuroinflammation and Autoimmune Uveitis. *Front Immunol*, 11, 1051. <https://doi.org/10.3389/fimmu.2020.01051>
- Kuchroo, V. K., Martin, C. A., Greer, J. M., Ju, S. T., Sobel, R. A., & Dorf, M. E. (1993). Cytokines and adhesion molecules contribute to the ability of myelin proteolipid protein-

- specific T cell clones to mediate experimental allergic encephalomyelitis. *J Immunol*, 151(8), 4371-4382.
- Matsumoto, M., Baba, A., Yokota, T., Nishikawa, H., Ohkawa, Y., Kayama, H., Kallies, A., Nutt, S. L., Sakaguchi, S., Takeda, K., Kurosaki, T., & Baba, Y. (2014). Interleukin-10-producing plasmablasts exert regulatory function in autoimmune inflammation. *Immunity*, 41(6), 1040-1051. <https://doi.org/10.1016/j.immuni.2014.10.016>
- Okoye, I. S., Coomes, S. M., Pelly, V. S., Czieso, S., Papayannopoulos, V., Tolmachova, T., Seabra, M. C., & Wilson, M. S. (2014). MicroRNA-containing T-regulatory-cell-derived exosomes suppress pathogenic T helper 1 cells. *Immunity*, 41(1), 89-103. <https://doi.org/10.1016/j.immuni.2014.05.019>
- Park, H., Li, Z., Yang, X. O., Chang, S. H., Nurieva, R., Wang, Y. H., Wang, Y., Hood, L., Zhu, Z., Tian, Q., & Dong, C. (2005). A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol*, 6(11), 1133-1141. <https://doi.org/10.1038/ni1261>
- Pierson, E., Simmons, S. B., Castelli, L., & Goverman, J. M. (2012). Mechanisms regulating regional localization of inflammation during CNS autoimmunity. *Immunological reviews*, 248(1), 205-215. <https://doi.org/10.1111/j.1600-065X.2012.01126.x>
- Ridder, K., Keller, S., Dams, M., Rupp, A.-K., Schlaudraff, J., Del Turco, D., Starmann, J., Macas, J., Karpova, D., Devraj, K., Depboylu, C., Landfried, B., Arnold, B., Plate, K. H., Höglinger, G., Sülthmann, H., Altevogt, P., & Momma, S. (2014). Extracellular Vesicle-Mediated Transfer of Genetic Information between the Hematopoietic System and the Brain in Response to Inflammation. *PLOS Biology*, 12(6), e1001874. <https://doi.org/10.1371/journal.pbio.1001874>

- Rommer, P. S., & Zettl, U. K. (2018). Managing the side effects of multiple sclerosis therapy: pharmacotherapy options for patients. *Expert Opinion on Pharmacotherapy*, 19(5), 483-498. <https://doi.org/10.1080/14656566.2018.1446944>
- Shen, P., Roch, T., Lampropoulou, V., O'Connor, R. A., Stervbo, U., Hilgenberg, E., Ries, S., Dang, V. D., Jaimes, Y., Daridon, C., Li, R., Jouneau, L., Boudinot, P., Wilantri, S., Sakwa, I., Miyazaki, Y., Leech, M. D., McPherson, R. C., Wirtz, S., Neurath, M., Hochlig, K., Meinl, E., Grützkau, A., Grün, J. R., Horn, K., Köhl, A. A., Dörner, T., Bar-Or, A., Kaufmann, S. H. E., Anderton, S. M., & Fillatreau, S. (2014). IL-35-producing B cells are critical regulators of immunity during autoimmune and infectious diseases. *Nature*, 507(7492), 366-370. <https://doi.org/10.1038/nature12979>
- Sullivan, J. A., Tomita, Y., Jankowska-Gan, E., Lema, D. A., Arvedson, M. P., Nair, A., Bracamonte-Baran, W., Zhou, Y., Meyer, K. K., Zhong, W., Sawant, D. V., Szymczak-Workman, A. L., Zhang, Q., Workman, C. J., Hong, S., Vignali, D. A. A., & Burlingham, W. J. (2020). Treg-Cell-Derived IL-35-Coated Extracellular Vesicles Promote Infectious Tolerance. *Cell Rep*, 30(4), 1039-1051.e1035. <https://doi.org/10.1016/j.celrep.2019.12.081>
- Wang, R. X., Yu, C. R., Dambuza, I. M., Mahdi, R. M., Dolinska, M. B., Sergeev, Y. V., Wingfield, P. T., Kim, S. H., & Egwuagu, C. E. (2014). Interleukin-35 induces regulatory B cells that suppress autoimmune disease. *Nat Med*, 20(6), 633-641. <https://doi.org/10.1038/nm.3554>
- Wiklander, O. P. B., Nordin, J. Z., O'Loughlin, A., Gustafsson, Y., Corso, G., Mäger, I., Vader, P., Lee, Y., Sork, H., Seow, Y., Heldring, N., Alvarez-Erviti, L., Smith, C. I. E., Le Blanc, K., Macchiarini, P., Jungebluth, P., Wood, M. J. A., & Andaloussi, S. E. (2015).

Extracellular vesicle in vivo biodistribution is determined by cell source, route of administration and targeting. *Journal of extracellular vesicles*, 4, 26316-26316.

<https://doi.org/10.3402/jev.v4.26316>

Wolf, S. D., Dittel, B. N., Hardardottir, F., & Janeway, C. A., Jr. (1996). Experimental autoimmune encephalomyelitis induction in genetically B cell-deficient mice. *J Exp Med*, 184(6), 2271-2278. <https://doi.org/10.1084/jem.184.6.2271>

Xie, C., Ciric, B., Yu, S., Zhang, G. X., & Rostami, A. (2016). IL-12R β 2 has a protective role in relapsing-remitting experimental autoimmune encephalomyelitis. *J Neuroimmunol*, 291, 59-69. <https://doi.org/10.1016/j.jneuroim.2015.12.009>

Zhu, X., Badawi, M., Pomeroy, S., Sutaria, D. S., Xie, Z., Baek, A., Jiang, J., Elgamal, O. A., Mo, X., Perle, K., Chalmers, J., Schmittgen, T. D., & Phelps, M. A. (2017). Comprehensive toxicity and immunogenicity studies reveal minimal effects in mice following sustained dosing of extracellular vesicles derived from HEK293T cells. *J Extracell Vesicles*, 6(1), 1324730. <https://doi.org/10.1080/20013078.2017.1324730>

CHAPTER V: SUMMARY AND CONCLUSIONS

The goal of this research was to better understand IL-35 biology and to develop an innovative approach to use IL-35 as an immunosuppressive therapy for CNS autoimmune diseases. Our findings provide insights on regulatory mechanisms exert by i35-Bregs and the potential use of their derived exosomes as a therapeutic carrier of IL-35. We hypothesized that *IL-35 is expressed on the surface of i35-Bregs in association with CD81 and secreted in exosomes that can be used to treat CNS autoimmune diseases.*

This hypothesis was tested by accomplishing the following specific aims.

1. Optimize the generation of i35-Bregs
2. Characterize IL-35 expression in i35-Bregs
 - A. Examine the expression of IL-35 on the surface of i35-Bregs
 - B. Examine the association of IL35 subunits with CD81
3. Isolate and characterize i35-exosomes
 - A. Isolate exosomes produced by i35-Bregs
 - B. Determine their size and the expression of exosome markers
 - C. Examine the expression of IL-35 in isolated exosomes
4. Determine the effect of i35-exosome treatment in EAE
 - A. Examine the effect of IL-35 on inflammatory and regulatory cells from EAE mice *in vitro*
 - B. Examine the effect of i35-exosomes on inflammatory and regulatory cells from EAE mice *in vitro*
 - C. Examine the effect of i35-exosomes treatment on clinical EAE

Despite the advancement in our understanding of functional properties of regulatory B cells, little is known about the requirements of their development *in vivo*. The lack of knowledge of signals required to polarize B cells toward regulatory phenotype has hindered their expansion *ex-vivo* for therapeutic purposes. The current understanding of regulatory B cell induction implicates the involvement of inflammatory cues such as activating B cells through TLRs, BCR, and/or CD40. While such signals were better studied in IL-10 producing Bregs (Fillatreau et al., 2002; Mauri et al., 2003; Mielle et al., 2018; Poe et al., 2011; Yanaba et al., 2009), little is known about signals involved in i35-Breg development.

Here, we examined combinations of different stimuli to determine the most optimal condition to induce i35-Bregs. We found that stimulating CD19⁺ B cells with anti-IgM/anti-CD40 resulted in the highest frequency of i35-Bregs (~32-44%) compared to LPS ~13%, LPS/CD40 ~13%, or anti-CD40 ~ 26%. We also found that weaker BCR signal as mimicked by stimulating B cells with 1 µg anti-IgM+ 10 µg anti-CD40 resulted in a higher frequency of i35-Bregs ~44% compared to B cells stimulated with 10 µg anti-IgM+ 10 µg anti-CD40 ~32%. By using proximity ligation assay, we showed that i35-Bregs induced by anti-IgM/anti-CD40 stimulation indeed produce heterodimeric IL-35. When we looked to IL-10-producing Bregs, however, we found that anti-IgM/anti-CD40 stimulation did not induced the expansion of IL-10 producing Bregs. In line with previous studies, we showed that LPS is a potent inducer of IL-10 Bregs. Collectively, our data show that anti-IgM/anti-CD40 signaling in B cells from naïve mice specifically induce the expansion of i35-Bregs and bypass the induction of IL-10 production. Additionally, our data suggest that the strength of BCR signaling may play a role in favoring i35-

Breg induction. Further investigation is needed to elucidate the importance of BCR signaling in the development of i35-Bregs *in vivo*. Another important question that needs to be answered is whether IL-35 producing Breg is a distinct subset from IL-10 producing Bregs? While our current data show that i35-Bregs and IL-10 producing Bregs are not induced at the same proportion by the different stimuli, we have previously shown that a percentage of i35-Bregs produce IL-10 as well (Wang et al., 2014). One possibility that some Bregs are single producers while other can be dual producers suggesting that they are distinct subsets. Another possibility that regulatory B cells are plastic and capable of producing different immunosuppressive cytokines based on the context.

Unlike the other cytokines of the IL-12 family, the two subunits that make IL-35, IL-12p35 and EBI3, are not covalently linked (Devergne et al., 1997). They are secreted independently, and little is known about the mechanism that leads to their heterodimerization (Devergne et al., 1997). The paucity of heterodimeric IL-35 in the supernatant of cells that overexpress IL-35 was mainly attribute to the instability of IL-12p35:EBI3 bound (Devergne et al., 1997; Egwuagu et al., 2015; Wang et al., 2014). Here, we showed that IL-35 is expressed on the surface of i35-Bregs in colocalization with the tetraspanin CD81. By using proximity ligation assay, we showed that IL-12p35 and EBI3 are in association with CD81. The expression of IL-35 on the surface of i35-Bregs suggests that i35-Bregs could exert their regulatory function in a cell: cell contact manner. It is possible that IL-35 bind to IL-35 receptors at the time that i35-Breg is in a contact with other cells (**Figure 24**). In fact, our data showed that the expression of IL-35 in anti-IgM/anti-CD40 induced i35-Bregs is polarized. This observation suggests that such contact could happen during the formation of immune synapses with APCs or T cells (**Figure**

24). Additionally, this finding suggests that IL-35 could be directed to be released into the synapse area providing a more directed and robust effect on the recipient cells. The association of IL-12p35 and EBI3 with CD81 provide an insight on how the two subunits interact with each other *in vivo* given that one main function of CD81 is to coordinate protein trafficking. In a similar phenomenon, CD81 is bound to CD19 and regulates its trafficking to the cell membrane where CD19 is released from CD81 upon BCR activation (Shoham et al., 2003; Susa et al., 2020). Identification of signals that induce the release of IL-12p35 and EBI3 from CD81 will reveal an important step in the process of IL-35 heterodimerization. Collectively, our data support our hypothesis that IL-35 is expressed on the surface of i35-Bregs in association with CD81. Our findings provide insight on i35-Breg mechanisms of action and IL-35 heterodimerization.

IL-35 as an immunosuppressive cytokine with an ability to convert conventional cells into regulatory cells is an attractive therapy for autoimmune and inflammatory diseases (Collison et al., 2010; Wang et al., 2014). However, the difficulty to produce heterodimeric IL-35 in large amounts has impeded its use as a therapy. Therefore, alternative approaches are needed to take advantage of the immunosuppressive properties of IL-35. Here, we successfully isolated exosomes from the supernatant of i35-Bregs induced by anti-IgM/anti-CD40 stimulation as indicated by their size and the expression of exosome markers CD81, CD63, and CD9. We also showed that exosomes secreted by anti-IgM/anti-CD40 induced i35-Bregs contain IL-35 as indicated by Western-blot and ELISA data. Given that exosomes secretion can be induced by BCR and/or CD40 stimulation in B cells (Mittelbrunn et al., 2011; Rialland et al., 2006; Yeh et al., 2015), we asked whether i35-Bregs induced by other stimuli could also produce i35-

exosomes. We induced i35-Bregs either by anti-IgM/anti-CD40 or LPS stimulation and isolated exosomes from the supernatants. We first looked to the expression of IL-12p35 and EBI3 in parental cells by Western blotting. We found that i35-Bregs induced by LPS stimulation are enriched with EBI3. On the other hand, i35-Bregs induced by anti-IgM/anti-CD40 express more IL-12p35 than LPS induced i35-Bregs. We also found a similar pattern in the isolated exosomes. While exosomes secreted by LPS induced i35-Bregs are enriched with the EBI3 subunit, exosomes secreted by anti-IgM/anti-CD40 induced i35-Bregs are enriched with the IL-12p35 subunit. Exosome secretion by i35-Bregs is an indirect mechanism by which i35-Bregs could regulate other cells and disseminate their suppressive effect. i35-exosomes can be taken up by other cells leading to suppressive effect on the recipient cells (primary immunosuppression). Cells that uptake i35-exosomes would acquire IL-35 expression on the surface and act like regulatory cells themselves that suppress other cells (secondary immunosuppression) (**Figure 24**). The ability of IL-35 to turn conventional cells into regulatory cells is termed “infectious tolerance” and it was reported to be induced by IL-35 containing exosomes produced by Tregs (Sullivan et al., 2020). The finding that IL-35 secreted mainly in exosomes advances our understanding of IL-35 biology and provides a possible explanation of the difficulty of producing heterodimeric IL-35.

Exosomes are nanovesicles that can cross the BBB and serve as a carrier to deliver biologics into the CNS (Alvarez-Erviti et al., 2011; Ridder et al., 2014; Wiklander et al., 2015; Yuan et al., 2017). Therefore, we wanted to explore i35-exosomes as a novel cell free therapy for CNS autoimmune diseases by using the experimental autoimmune encephalomyelitis (EAE) as model. By using EAE *ex-vivo* studies, we examined the effect of IL-35 on encephalitogenic T

cells (MOG35-55 specific T cells) and regulatory lymphocytes. We found that lymphoid cells from EAE mice re-stimulated with MOG 35-55 *ex-vivo* and treated with IL-35 showed a decrease in the frequency of IL-17 and IFN γ producing T cells compared to PBS treated cells. Additionally, we showed that the inhibition of IL-17 and IFN γ producing T cells was associated with the expansion of IL-10 and IL-35 producing T and B cells. Our data add to the cumulative evidence of the suppressive effect of IL-35 in CNS autoimmune diseases. We next examined the effect of i35-exosomes on lymphoid cells from EAE mice *ex-vivo*. Similar to IL-35, we found that i35-exosomes inhibited IL-17 and IFN γ producing T cells. Additionally, we showed that i35-exosomes expanded IL-10 and IL-35 producing B cells. These data suggest that i35-exosomes inhibit encephalitogenic T cells, in part, by expanding regulatory cells. Furthermore, these findings support that i35-exosome treatment is a potential therapeutic strategy for EAE/MS. We next examined the effect of i35-exosomes on clinical EAE. EAE mice were treated with i35-exosomes at the day of disease induction (day 0) and every other day until (day 12). We did not find a significant difference in clinical scores of i35-exosomes treated mice and control mice. Studying i35-exosomes fate *in vivo* will help in optimizing the dose and the route of administration for a better therapeutic outcome.

In summary, this work shows that BCR/CD40 signals promote the expansion of i35-Bregs and that weak BCR signals favor the induction of i35-Bregs. Additionally, we showed that IL-35 is expressed on the surface of i35-Bregs in colocalization with CD81 in a polarized phenotype. We provided additional evidence that IL-12p35 and EBI3 are in association with CD81. We showed that i35-Bregs secrete IL-35 containing exosomes regardless of the stimulation used to induce them. However, i35-exosomes from LPS-induced i35-Bregs are

enriched with EBI3 subunit while i35-exosomes from anti-IgM/anti-CD40 induced i35-bregs are enriched with IL-12p35 subunit. Finally, we showed that IL-35 and similarly i35-exosomes inhibit encephalitogenic T cells and expand regulatory lymphocytes *ex-vivo*.

In conclusion, our study underscores the importance of BCR signaling in the induction of i35-Bregs. Additionally, our data suggest different mechanisms of actions by which i35-Bregs could exert their regulatory effect i.e cell-cell contact and exosome secretion (**Figure 24**). We provided evidence of the association of IL-35 subunits with CD81 which provide insights on IL-35 heterodimerization process. We showed that i35-exosomes could be explored as a potential novel cell free therapy for CNS autoimmune diseases.

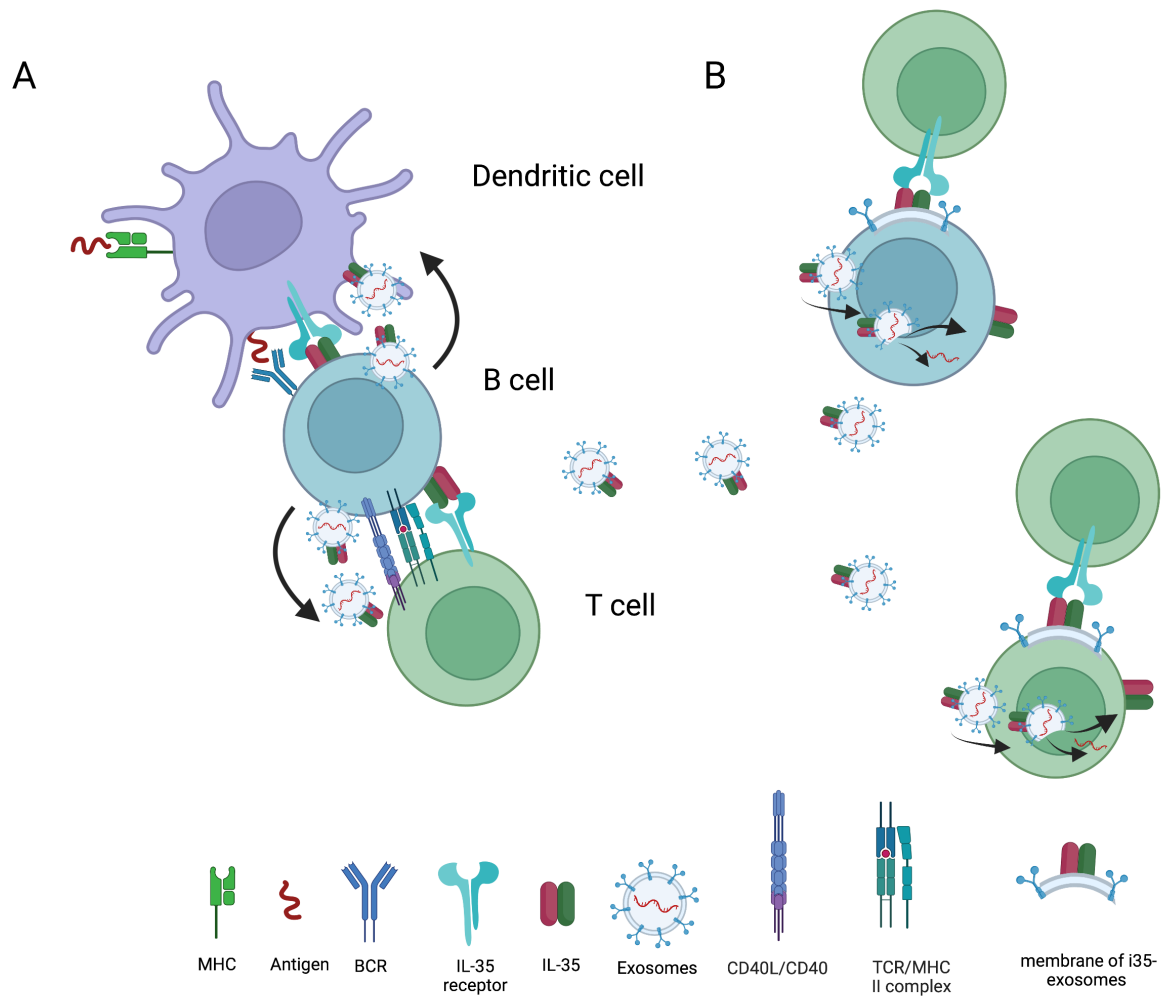


Figure 24. Proposed model of i35-Breg regulatory mechanisms

A) The surface expression of IL-35 by i35-Bregs suggest that i35-Bregs could exert their regulatory function via cell-cell contact mechanism (s). The polarized expression of IL-35 by i35-Bregs suggests that such contact could happen during the formation of immune synapses. Upon interacting with APCs (purple), to capture an antigen, or T cells (green), to present an antigen, B cells (blue) undergo morphological changes, leading to the formation of immune synapses. IL-35, expressed on the surface of i35-Bregs, interacts with IL-35 receptor on the surface of APC or T cells, leading to suppressive effect on APCs and T cells (primary suppression). In another mechanism of inhibition, i35-Bregs secrete i35-exosomes that are captured by APCs or T cells during the formation of immune synapses. Upon capturing the i35-exosomes, APCs and T cells acquire IL-35 expression on their surface, enabling them to function as inhibitory cells (secondary suppression). **B)** Similarly, i35-exosomes can be taken up by distant cells leading to immunosuppression of these cells (primary suppression) and turning them into IL-35 surface expressing cells. IL-35 surface expression could be due to the diffusion of i35-exosome membrane into the plasma membrane of recipient cells during uptake of the exosomes. Another possibility is that i35-exosomes are totally internalized, and IL-35 is redirected to be expressed on the surface. Cells acquired IL-35 surface expression can act as regulatory cells and suppress other cells (secondary suppression). The effect of RNA cargo of i35-exosomes on recipient cells remains to be elucidated. This image was created with BioRender.com

References

- Alvarez-Erviti, L., Seow, Y., Yin, H., Betts, C., Lakhal, S., & Wood, M. J. A. (2011). Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nature Biotechnology*, 29(4), 341-345. <https://doi.org/10.1038/nbt.1807>
- Collison, L. W., Chaturvedi, V., Henderson, A. L., Giacomini, P. R., Guy, C., Bankoti, J., Finkelstein, D., Forbes, K., Workman, C. J., Brown, S. A., Rehg, J. E., Jones, M. L., Ni, H. T., Artis, D., Turk, M. J., & Vignali, D. A. (2010). IL-35-mediated induction of a potent regulatory T cell population. *Nat Immunol*, 11(12), 1093-1101. <https://doi.org/10.1038/ni.1952>
- Devergne, O., Birkenbach, M., & Kieff, E. (1997). Epstein–Barr virus-induced gene 3 and the p35 subunit of interleukin 12 form a novel heterodimeric hematopoietin. *Proceedings of the National Academy of Sciences*, 94(22), 12041-12046. <https://doi.org/10.1073/pnas.94.22.12041>
- Egwuagu, C. E., Yu, C.-R., Sun, L., & Wang, R. (2015). Interleukin 35: Critical regulator of immunity and lymphocyte-mediated diseases. *Cytokine & growth factor reviews*, 26(5), 587-593. <https://doi.org/10.1016/j.cytogfr.2015.07.013>
- Fillatreau, S., Sweeney, C. H., McGeachy, M. J., Gray, D., & Anderton, S. M. (2002). B cells regulate autoimmunity by provision of IL-10. *Nat Immunol*, 3(10), 944-950. <https://doi.org/10.1038/ni833>
- Mauri, C., Gray, D., Mushtaq, N., & Londei, M. (2003). Prevention of arthritis by interleukin 10-producing B cells. *J Exp Med*, 197(4), 489-501. <https://doi.org/10.1084/jem.20021293>

- Mielle, J., Audo, R., Hahne, M., Macia, L., Combe, B., Morel, J., & Daien, C. (2018). IL-10 Producing B Cells Ability to Induce Regulatory T Cells Is Maintained in Rheumatoid Arthritis [Original Research]. *Frontiers in Immunology*, 9. <https://doi.org/10.3389/fimmu.2018.00961>
- Mittelbrunn, M., Gutiérrez-Vázquez, C., Villarroja-Beltri, C., González, S., Sánchez-Cabo, F., González, M. Á., Bernad, A., & Sánchez-Madrid, F. (2011). Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells. *Nature Communications*, 2(1), 282. <https://doi.org/10.1038/ncomms1285>
- Poe, J. C., Smith, S. H., Haas, K. M., Yanaba, K., Tsubata, T., Matsushita, T., & Tedder, T. F. (2011). Amplified B lymphocyte CD40 signaling drives regulatory B10 cell expansion in mice. *PLoS One*, 6(7), e22464. <https://doi.org/10.1371/journal.pone.0022464>
- Rialland, P., Lankar, D., Raposo, G., Bonnerot, C., & Hubert, P. (2006). BCR-bound antigen is targeted to exosomes in human follicular lymphoma B-cells. *Biol Cell*, 98(8), 491-501. <https://doi.org/10.1042/bc20060027>
- Ridder, K., Keller, S., Dams, M., Rupp, A.-K., Schlaudraff, J., Del Turco, D., Starmann, J., Macas, J., Karpova, D., Devraj, K., Depboylu, C., Landfried, B., Arnold, B., Plate, K. H., Höglinger, G., Sülthmann, H., Altevogt, P., & Momma, S. (2014). Extracellular Vesicle-Mediated Transfer of Genetic Information between the Hematopoietic System and the Brain in Response to Inflammation. *PLOS Biology*, 12(6), e1001874. <https://doi.org/10.1371/journal.pbio.1001874>
- Shoham, T., Rajapaksa, R., Boucheix, C., Rubinstein, E., Poe, J. C., Tedder, T. F., & Levy, S. (2003). The Tetraspanin CD81 Regulates the Expression of CD19 During B Cell

- Development in a Postendoplasmic Reticulum Compartment. *The Journal of Immunology*, 171(8), 4062-4072. <https://doi.org/10.4049/jimmunol.171.8.4062>
- Sullivan, J. A., Tomita, Y., Jankowska-Gan, E., Lema, D. A., Arvedson, M. P., Nair, A., Bracamonte-Baran, W., Zhou, Y., Meyer, K. K., Zhong, W., Sawant, D. V., Szymczak-Workman, A. L., Zhang, Q., Workman, C. J., Hong, S., Vignali, D. A. A., & Burlingham, W. J. (2020). Treg-Cell-Derived IL-35-Coated Extracellular Vesicles Promote Infectious Tolerance. *Cell Rep*, 30(4), 1039-1051.e1035. <https://doi.org/10.1016/j.celrep.2019.12.081>
- Susa, K. J., Seegar, T. C., Blacklow, S. C., & Kruse, A. C. (2020). A dynamic interaction between CD19 and the tetraspanin CD81 controls B cell co-receptor trafficking. *Elife*, 9, e52337. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7228769/pdf/elife-52337.pdf>
- Wang, R. X., Yu, C. R., Dambuza, I. M., Mahdi, R. M., Dolinska, M. B., Sergeev, Y. V., Wingfield, P. T., Kim, S. H., & Egwuagu, C. E. (2014). Interleukin-35 induces regulatory B cells that suppress autoimmune disease. *Nat Med*, 20(6), 633-641. <https://doi.org/10.1038/nm.3554>
- Wiklander, O. P., Nordin, J. Z., O'Loughlin, A., Gustafsson, Y., Corso, G., Mäger, I., Vader, P., Lee, Y., Sork, H., Seow, Y., Heldring, N., Alvarez-Erviti, L., Smith, C. I., Le Blanc, K., Macchiarini, P., Jungebluth, P., Wood, M. J., & Andaloussi, S. E. (2015). Extracellular vesicle in vivo biodistribution is determined by cell source, route of administration and targeting. *J Extracell Vesicles*, 4, 26316. <https://doi.org/10.3402/jev.v4.26316>
- Yanaba, K., Bouaziz, J.-D., Matsushita, T., Tsubata, T., & Tedder, T. F. (2009). The Development and Function of Regulatory B Cells Expressing IL-10 (B10 Cells) Requires

Antigen Receptor Diversity and TLR Signals. *The Journal of Immunology*, 182(12), 7459-7472. <https://doi.org/10.4049/jimmunol.0900270>

Yeh, Y.-Y., Ozer, H. G., Lehman, A. M., Maddocks, K., Yu, L., Johnson, A. J., & Byrd, J. C. (2015). Characterization of CLL exosomes reveals a distinct microRNA signature and enhanced secretion by activation of BCR signaling. *Blood*, 125(21), 3297-3305. <https://doi.org/10.1182/blood-2014-12-618470>

Yuan, D., Zhao, Y., Banks, W. A., Bullock, K. M., Haney, M., Batrakova, E., & Kabanov, A. V. (2017). Macrophage exosomes as natural nanocarriers for protein delivery to inflamed brain. *Biomaterials*, 142, 1-12. <https://doi.org/10.1016/j.biomaterials.2017.07.011>

APPENDIX

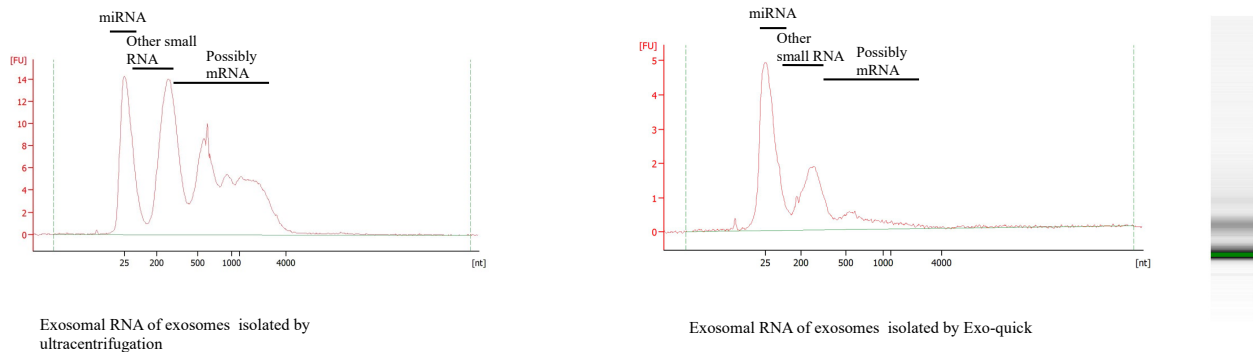


Figure 25. Assessment of Exosomal RNA by Agilent Analyzer

i35-Bregs were generated by anti-IgM/anti-CD40 stimulation for 72h. Supernatants were collected at the end of the incubation and exosomes were isolated by ultracentrifugation or Exo-quick. RNAs were extracted from exosomes by ExoQuick® Exosome RNA Column Purification Kit (System Bioscience) and analyzed by Agilent Analyzer. Exosomal RNA shows enrichment of miRNAs.

CURRICULUM VITAE

SAHAR ALHAKEEM

EDUCATION

- PhD National Eye Institute/National Institute of Health** 2020-2022
As part of Graduate Partnerships Program (GPP)
Supervisor: Charles Egwuagu
Dissertation: “Characterization of B Regulatory Cell-Derived-Interleukin-35 Containing Exosomes and Their Immunosuppressive Effect”
- Health Sciences, University of Wisconsin-Milwaukee, USA** 2016-2022
Emphasis: Immunology
Supervisor: Jeri Lyons & Janis Eells
- MS Biomedical Sciences, University of Wisconsin-Milwaukee, USA** 2013-2015
Emphasis: Infection and Immunity
Supervisor: Wail Hassan
Thesis: “Functional and Phenotypic Analysis of B- and T-Helper Cells in Rhesus Macaques and African Green Monkeys”
- BS Clinical Laboratory Sciences, University of Dammam, SA** 2007-2011
First Class Honors
Research project: “Evaluation of Vancomycin Therapeutic Drug Monitoring Process at King Fahad’s Hospital, Saudi Arabia”

RESEARCH EXPERIENCE

- Pre-doctoral Research Fellow, National Eye Institute** 2020-2022
Advisor: Charles Egwuagu
- Optimized the generation of IL-35 producing B regulatory cells
 - Isolated and characterized IL-35 containing exosomes
 - Investigated the effect of i35-Exosome therapy on Experimental Autoimmune Encephalomyelitis (EAE)
 - Examined the effect of STAT3-Specific single domain nanobody on EAE
- Graduate Researcher, University of Wisconsin-Milwaukee** 2016-2020
PhD Advisor: Jeri-Anne Lyons, Janis Eells
- Investigated the therapeutic effect of photobiomodulation on optic neuritis by using EAE as a model

- Investigated whether gender influences the amelioration effect of photobiomodulation on EAE by assessing cytokine production and EAE clinical scores
- Assisted in conducting experiments to investigate the source of IL-10 seen in EAE as a response to photobiomodulation therapy
- Participated in examining the applicability of using 830 nm as compared to 670 nm for treating EAE
- Managed animal research colony

Graduate Researcher, University of Wisconsin-Milwaukee

2014-2015

MS Advisor: Wail Hassan

- Identified immunological differences among B and T cell populations of two non-human primate models of SIV/HIV infection
- Participated in cloning a chimeric CD4-immunoglobulin with neutralizing activity against SIV/HIV
- Supervised undergraduate students and taught them a variety of research techniques

PUBLICATIONS

Mbanefo, Evaristus C., Ming Yan, Minkyung Kang, **Sahar A. Alhakeem**, Yingyos Jittayasothorn, Cheng-Rong Yu, Ashutosh Parihar, Sunanda Singh, and Charles E. Egwuagu. "STAT3-Specific single domain nanobody inhibits expansion of pathogenic Th17 responses and suppresses uveitis in mice." *Frontiers in immunology* (2021): 3645.

Egwuagu, Charles E., **Sahar A. Alhakeem**, and Evaristus C. Mbanefo. "Uveitis: molecular pathogenesis and emerging therapies." *Frontiers in Immunology* (2021): 1293.

PRESENTATIONS

Laboratory of Immunology Seminar Series/ National Eye Institute 25-June-2021
 "IL-35-containing exosomes (i35-Exosomes): Characterization and therapeutic effect on Experimental Autoimmune Encephalomyelitis (EAE)"

Laboratory of Immunology Seminar Series/ National Eye Institute 6-May-2022
 "i35-Bregs secrete IL-35-containing exosomes that induce regulatory lymphocytes and suppress encephalitogenic T cells"

GRANTS AND AWARDS

NIH Intramural Training Pre-Doctoral Award
 National Eye Institute, National Institute of Health

2020-2022

UWM Chancellor's Graduate Student Award
 Department of Biomedical Sciences, College of Health Sciences, University of Wisconsin-Milwaukee

2022

Student Research Grant Award

2019

For the proposal entitled “The Therapeutic Effect of Photobiomodulation on Optic Neuritis in a Model of Multiple Sclerosis”

College of Health Sciences’ Research Growth Committee, University of Wisconsin-Milwaukee

Student Assistantship Award 2019

Department of Biomedical Sciences, University of Wisconsin-Milwaukee

Full Study Abroad Scholarship to obtain PhD degree 2015-2019

King Saud Bin Abdulaziz University for Health Sciences, SA

Full Study Abroad Scholarship to obtain Master’s degree 2013-2015

King Saud Bin Abdulaziz University for Health Sciences, SA

Academic Excellence Award received for 4 consecutive years 2007-2010

University of Dammam, SA

TEACHING EXPERIENCE

University of Wisconsin-Milwaukee, USA Spring 2019

Teaching Assistant, Department of Biomedical Sciences
“Human Pathophysiology” and “Molecular Diagnostic Lab”

University of Wisconsin-Milwaukee, USA Fall 2017-2018

Lecturer/Teaching fellow, College of Health Sciences
“New Student Seminar in Health Professions”

King Saud Bin Abdulaziz University for Health Sciences, SA 2011-2013

Teaching Assistant

CLINICAL EXPERIENCE

Johns Hopkins Aramco Health Care, SA Sep 2010-Sep 2011

Clinical Laboratory Intern

Rotated in clinical diagnostic labs (Phlebotomy and blood sample collection, General Chemistry, Blood Bank, Microbiology, Hematology Lab, Histology and Cytology, Special Chemistry and Serology, Molecular Diagnostic)

King Fahad Hospital of the University and Regional Laboratory, SA Sep 2009-Sep 2010

Clinical Laboratory Trainee

Rotated in clinical diagnostic labs (Blood Bank, General/ Special Chemistry, Microbiology, Immunology, Serology and Virology, Histology and Cytology, Hematology)

PROFESSIONAL ACTIVITIES

- The NEI Intramural Research Seminar Series** 2022
National Eye Institute/National Institute of Health
Host: Prepare introduction of the speaker, introduce the speaker and moderate the questions
- Health Sciences PhD Fall Gathering** 2018
University of Wisconsin-Milwaukee
Invited to be on the student panel to discuss the preliminary exam process and give tips for preparation and success with PhD milestones
- UWM Undergraduate Research Symposium** 2018
University of Wisconsin-Milwaukee
Oral Presentation Judge; evaluate oral presentations and nominate award winners