The Fragility of Inducible Immune Tolerance in Type 1 Diabetes

By

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Dedication

To all patients who have experienced pregnancy complications because scientific discovery has left them behind

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Finally, my wife Catie has persisted in her marriage to me despite the number of times I left lab an hour later than my initial estimate because an experiment ran long. I'm sure Catie is ready for my PhD to be over, and I hope that this document allows for that.

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ABBREVIATIONS

MHC	Major Histocompatibility Complex
TCR	T Cell Receptor
LCMV	Lymphocytic Choriomeningitis Virus
cTEC	Cortical Thymic Epithelial Cell
mTEC	Medullary Thymic Epithelial Cell
APC	Antigen Presenting Cell
BCR	B Cell Receptor
Treg	Regulatory T Cell
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked [Syndrome]
T1D	Type 1 Diabetes
NOD	Non-Obese Diabetic [Mouse]
Idd	Insulin-Dependent Diabetes [Loci]
Breg	Regulatory B Cell
hCG	Human Chorionic Gonadotropin
PBS	Phosphate Buffered Saline
SLE	Systemic Lupus Erythematosus
B6♀B6♂	B6 female mated to B6 male
NOD♀NOD♂	NOD female mated to NOD male
B6♀NOD♂	B6 female mated to NOD male
NOD♀B6♂	NOD female mated to B6 male
T2D	Type 2 Diabetes
TMA	Tissue Microarray
ANGPT1	Angiopoietin 1
TBXA2R	Thromboxane A2 Receptor

CHAPTER I

Introduction

The immune system has evolved to maintain a constant, fragile equilibrium. Balancing on a tightrope, humans produce billions of cells that hopefully weigh the prevention of microbiological injury with avoidance of self-destruction. Avoidance of self-reactivity is called immune tolerance, the precise induction of which remains one of the greatest mysteries in immunology. The immune system must maintain tolerance throughout life to prevent autoimmune disease; interestingly, the immune system is also able to adapt and induce tolerance to foreign antigens under the very special circumstance of pregnancy. The mechanism of training the immune system to accept foreign antigens in complex, and it is not clear why individuals with autoimmunity—whose own tolerance has already failed—would be able to have successful pregnancy at all. Understanding the successes and failures of inducible immune tolerance may enable better treatment of autoimmune disease and the establishment of organ transplant without immune suppression.

While cells targeted towards pathogens can occasionally cross-react with human tissue– like when infection with Group A Streptococcus bacteria can lead to rheumatic heart disease– the immune system faces a more fundamental problem: eliminating self-reactive cells during development. The foundations of immunology rest on differentiating self from non-self. Early studies of transplantation uncovered innate immunological differences between mouse strains (Triolo, 1964). After many failed attempts to transplant tumors between different animal species, Carl Jensen in 1903 reported successful transplantation of an alveolar tumor into 19 generations of a partially inbred mouse strain, with poor results transmitting across other strains. By 1929, William Woglom concluded that an immunologic reaction led to the rejection of transplanted tumors (Woglom, 1929), creating the opportunity to understand how the immune system distinguished tissue as foreign. These discoveries enabled Paul Gorer, using three

different mouse strains, to identify genetic “antigens” that controlled reactivity to foreign sera (Gorer, 1936), and 12 years later, to name the genetic locus H-2 after the antigens previously identified (Gorer et al., 1948).

In 1972, Kindred and Weiler injected thymocytes from different mouse strains into athymic nude mice crossed with Balb/c mice and demonstrated development of antibodies only when Balb/c thymocytes were injected (Kindred and Weiler, 1972), indicating that some specificity is required to form an immune response. By 1974, the work done elucidating transplant specificity would lead to the discovery of Major Histocompatibility Complex (MHC) restriction (Zinkernagel and Doherty, 1974). Zinkernagel and Doherty would win the Nobel Prize for their 5 paragraph letter published in Nature, noticing that lymphocytic choriomeningitis virus (LCMV)-immune T cells were able to lyse LCMV-infected macrophages of the same H-2 type. The discovery of the MHC, and subsequently MHC restriction, uncovered an understanding of how leukocytes may interact to protect against pathogens, prompting questions of how lymphocytes developed such antigen specificity.

Random generation of antigen-binding regions was illustrated first in B cells with rearrangement of gene regions to create immunoglobulins. Hozumi and Tonegawa digested DNA from a mouse embryo and showed two genetic “patterns” that hybridized with V- or C-gene sequences; however, DNA from a plasmacytoma hybridized with both V-and C-gene sequences, and was a smaller size than either region alone (Hozumi and Tonegawa, 1976). They concluded that V and C regions recombine during B cell development to form the antigen-binding region of immunoglobulins.

Similar rearrangements were expected in T cells, but the membrane-bound nature of the T cell receptor (TCR) increased the difficulty of identifying genetic regions. In 1982, Meuer and colleagues demonstrated that CD8+ and CD4+ T cells recognized MHC Class I and Class II, respectively (Meuer et al., 1982), leading to an understanding of the differing functionalities between subtypes of effector T cells. The “elusive” TCR was found to be constructed of two

chains, both of which underwent rearrangement during development (Hedrick et al., 1984; Kappler et al., 1983) and allowed for binding to antigen presented on MHC (Davis and Bjorkman, 1988).

The development of T cells is dependent on interactions with peptide presented on the appropriate MHC. As alluded to by the importance of the athymic nude mouse in studying T cell biology, the thymus is required for normal T cell development. Billions of T cells expressing both CD4 and CD8 test their TCR by encountering cortical thymic epithelial cells (cTEC); rearrangement continues until cells are positively selected by successfully binding to a MHC present on cTECs (Anderson et al., 1994; Borgulya et al., 1992; Brändle et al., 1992; Jenkinson et al., 1994; Wilkinson et al., 1995). Sufficient avidity for the MHC present on cTECs is needed for the cell to continue in development (Kisielow et al., 1988).

Negative selection, the process meant to delete autoreactive T cells, occurs largely in the thymic medulla. T cells that survive positive selection increase expression of CCR7 and travel towards the CCL21-expressing medullary thymic epithelial cells (mTECs) (Takahama, 2006). In addition to mTECs, other antigen-presenting cells (APCs) reside in the thymic medulla and participate in negative selection by presenting self-antigen to the developing T cells. Studies of human thymuses from young donors identified transcripts of proteins from across the body–including tissue-specific antigens like insulin, myelin basic protein, and thyroglobulin that have been implicated in autoimmune disease (Sospedra et al., 1998). These proteins were found to be expressed by mTECs, which were found to have a “promiscuous” expression of proteins from across the body (Derbinski et al., 2001). The mechanism of broad (or promiscuous) protein expression was discovered in 2002 after investigating patients with an *AIRE* mutation; Aire expression prevented autoimmunity, and regulated thymic gene expression in mTECs (Anderson et al., 2002; Liston et al., 2003). Aire expression in the thymus promotes transcription of self-antigens from across the body, ranging from circulating antigens (like Rhesus antigen) to tissue restricted antigens (like insulin) to secluded ones like myelin-basic

protein. Patients with an Aire deficiency present with multiple autoimmune conditions, which can include Type 1 Diabetes, multiple sclerosis, vitiligo, and autoimmune thyroiditis—these diverse range of diseases provides insight into the variety of self-antigen expression that Aire expression promotes.

Initially, Aire expression–and presentation of self-antigen in the thymus–was thought to be limited to mTECs, a rare cell type solely responsible for preventing self-destruction. However, Aire is also expressed in dendritic cells (Hubert et al., 2011) and thymic B cells (Perera et al., 2013; Yamano et al., 2015). Aire expression is specific to thymic (not circulating) B cells, and cross-linking of the B Cell Receptor (BCR) downregulates Aire expression (Yamano et al., 2015).

While T cell selection was being elucidated, questions of tolerance to self-peptides were raised. As early as 1987, self-reactive T cells were found to be eliminated during selection (Kappler et al., 1987); the authors suggested that “self-MHC restriction and tolerance to self-MHC may occur almost at the same time during thymocyte maturation”. Luc Van Kaer and colleagues noted in 1994 that the avidity of the TCR:MHC connection results in disparate outcomes; TCRs that have high affinity for self-MHC are likely to be negatively selected to avoid auto-reactive cells (Ashton-Rickardt et al., 1994). TCR:MHC binding strength resembles a histogram; TCRs that are unable to bind to the peptide:MHC complex undergo rearrangement until death, whereas other cells are positively selected to become CD8+ Cytotoxic or CD4+ Helper T cells. At very high affinity for self-peptide/MHC, T cells undergo negative selection to prevent autoreactivity. Notably, even auto-reactive T cells that escape the negative selection in the thymus may not react to self-antigen present at low concentrations, a concept referred to as ‘immunologic ignorance’ (Kurts et al., 1999).

A Goldilocks group of T cells that bind slightly too strongly to self-peptide:MHC become regulatory T cells (Tregs). Tregs were first identified as CD4+CD25+ cells that have a high TCR affinity for self-peptide during thymic development (Jordan et al., 2001; Sakaguchi et al., 1995).

The search for a lineage marker for these cells included development of the scurfy mouse, with a mutation in a forkhead/winged-helix protein that researchers called scurfin; these mice developed multiorgan lymphocytic infiltrate and cytokine elevation (Brunkow et al., 2001). As the discovery of Aire was led by patients with an *AIRE* mutation, interrogation into the connotation of Foxp3 expression came from studying patients with IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), who present with various autoimmune diseases and allergies, indicating a deficit in immune regulation. The transcription factor *Foxp3* was identified in 2003 as the driver of regulatory cell activity; induction of Foxp3 in naive T cells was sufficient to induce a regulatory phenotype (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003). Treg lineage differentiation occurs in the thymic medulla (Cowan et al., 2013), largely based on interactions with mTECs presenting self-antigen (Aschenbrenner et al., 2007); increasing expression of self-antigen can increase the development of Tregs (Lin et al., 2016). Beyond Foxp3 expression, Treg survival requires signaling by IL-2 through CD25 and STAT5; otherwise, Foxp3 activates a proapoptotic pathway that causes cell death (Tai et al., 2013; Weist et al., 2015).

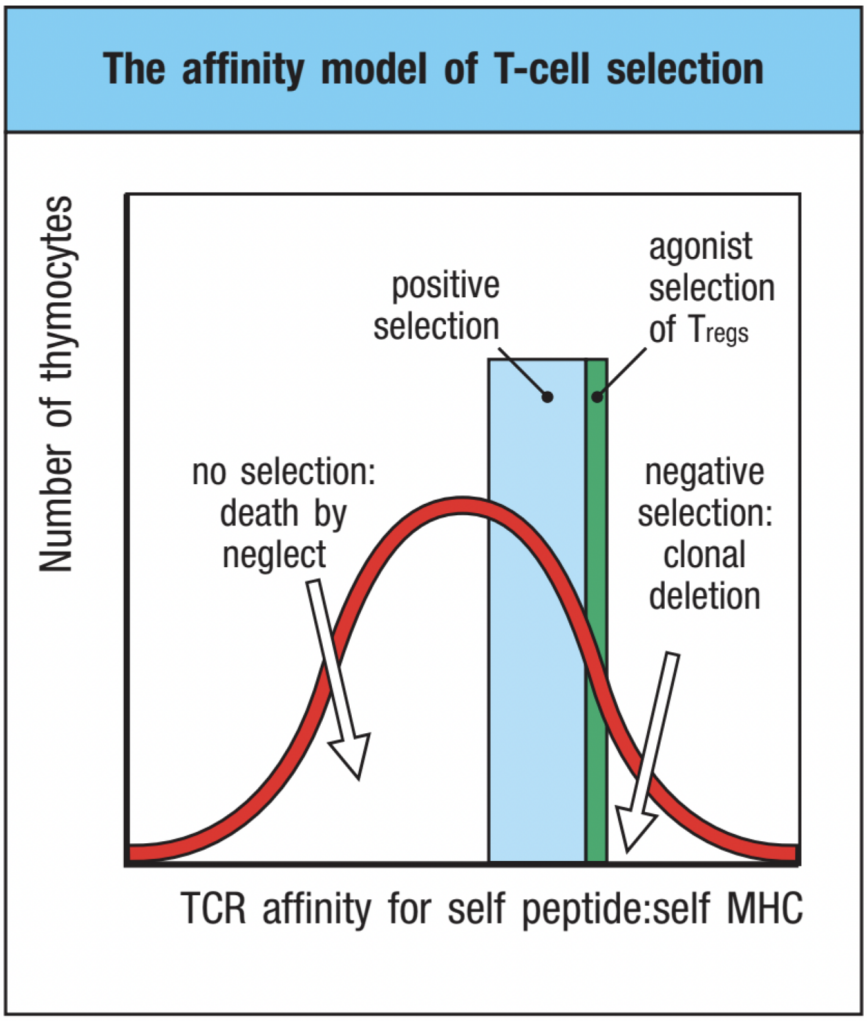


Figure 1.1. Histogram illustrating TCR:MHC binding strength and resultant effect on T cell survival. (Murphy and Weaver, 2016)

Even in healthy patients, autoreactive cells escape negative selection; Tregs are necessary to prevent autoimmunity (Danke et al., 2004). While thymically-derived Tregs can be identified by Helios expression, Tregs can also be peripherally-induced and identified by expression of Neuropilin-1 (Yadav et al., 2013). Peripherally-derived Tregs can be induced by small amounts of peptide to which a TCR has high affinity, requiring CNS1-dependent TCF-β signaling; however, peripherally-derived Tregs are found to be less suppressive than thymically-derived Tregs (Hill et al., 2007).

Similarly to T cells, B cell development occurs in a fashion to to control autoreactivity by requiring multiple checkpoints across multiple organs (Nemazee 2017). B cells are generated in the bone marrow, where V-D-J recombination occurs to create a heavy chain and V-J recombination to create a light chain—these components will form both the secreted antibody and the B cell receptor. Successful B cells undergo positive selection and migration to secondary lymphoid tissue; in contrast, B cells that bind to antigens present in the bone marrow can undergo receptor editing by downregulating the BCR and increasing RAG expression to attempt to produce a non-autoreactive BCR (Verkoczy et al., 2007). In the periphery, immature B cells are supported by a BAFF expression, which promotes B cell survival; increased levels of BAFF can be found in autoimmune disease and decrease peripheral B cell tolerance (Lesley et al., 2004). Even with so many checks on immune cell development, autoreactivity can expand into autoimmune disease.

Loss of Tolerance in Type 1 Diabetes

From the beginning of our understanding of maintaining a capable yet non-destructive immune system, diabetes has been implicated as a disease process requiring immunologic balance. Type 1 Diabetes (T1D) is characterized by autoimmune destruction of the insulin-producing beta cells in the pancreatic islets of Langerhans by T lymphocytes reactive to islet-

derived antigens (Michels et al., 2017; Nakayama et al., 2005; Skowera et al., 2008). Once a patient presents with fulminant diabetes, they are reliant on careful administration of exogenous insulin to maintain euglycemia. While the etiology of disease is beta cell destruction, sequelae extend across the body to include directly related complications like hypoglycemia and ketoacidosis, as well as indirect complications like micro- and macro-vascular disease (DiMeglio et al., 2018). A mouse model of T1D, Non-Obese Diabetic (NOD) mice, spontaneously develop overt diabetes between 12-20 weeks of age and display characteristic insulitis and microvascular disease (Anderson and Bluestone, 2005). In patients and NOD mice, over 50 loci contribute to risk, known as insulin-dependent diabetes (*Idd*) loci (Chen et al., 2018). A major contributor of genetic risk can be attributed to the MHC Class II alleles, which can interfere with lymphocyte detection of self-peptides (Chao et al., 1999).

Even before symptom onset, patients with T1D have what Eisenbarth refers to as “overt immunologic abnormalities” of multiple lymphocytes (Figure 2). These abnormalities are reflected in NOD mice, which are known to have impaired negative selection of T cells (Kishimoto and Sprent, 2001) and T effectors that resist suppression by Tregs (D’Alise et al., 2008). Even as early as 1993, T cells have been implicated in both disease pathogenesis and in rescuing adverse outcomes– transfer of IL-2 secreting CD4+ cells into thymectomized and irradiated mice was found to alleviate diabetes (Fowell and Mason, 1993). Notably, NOD Tregs

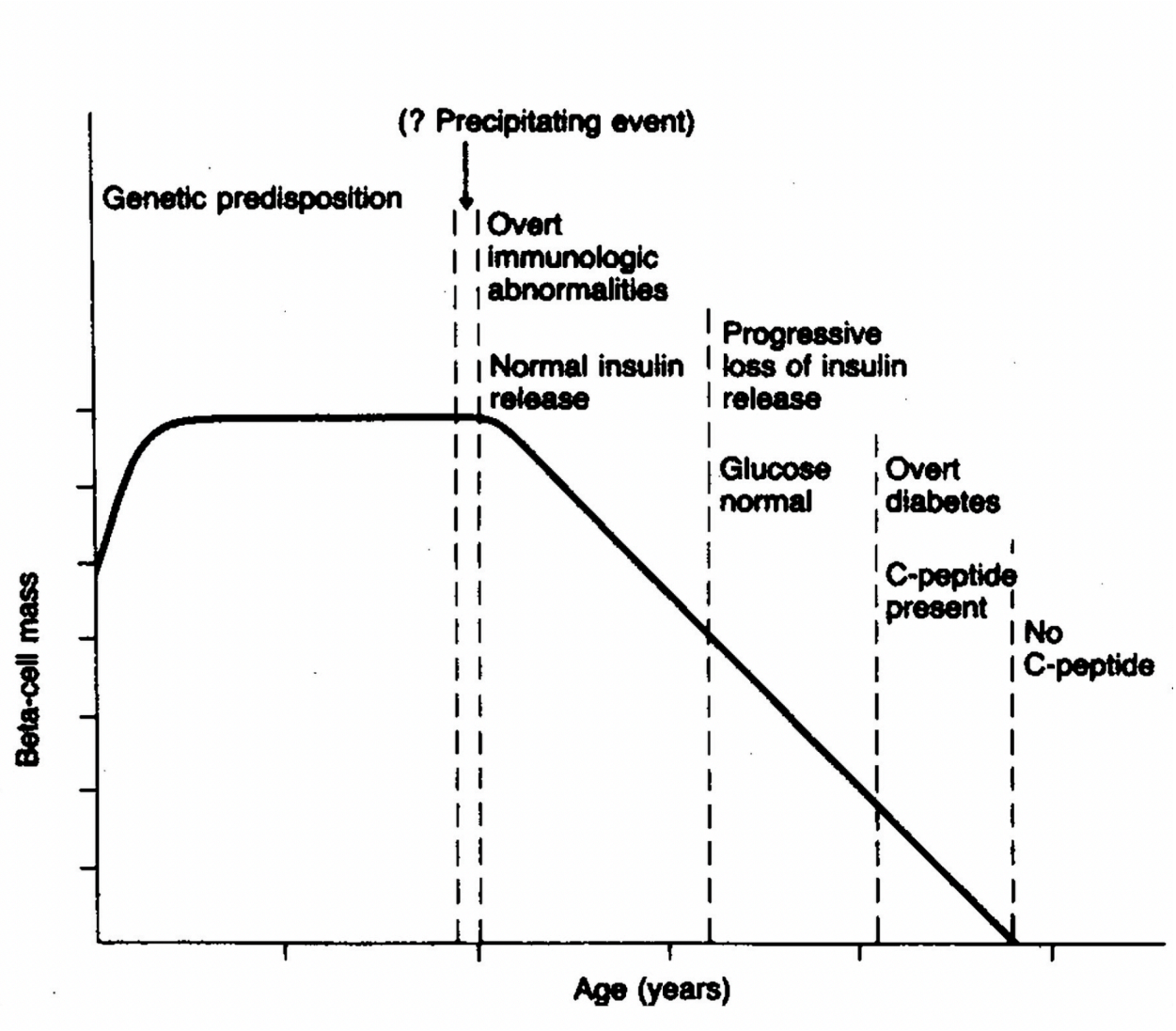
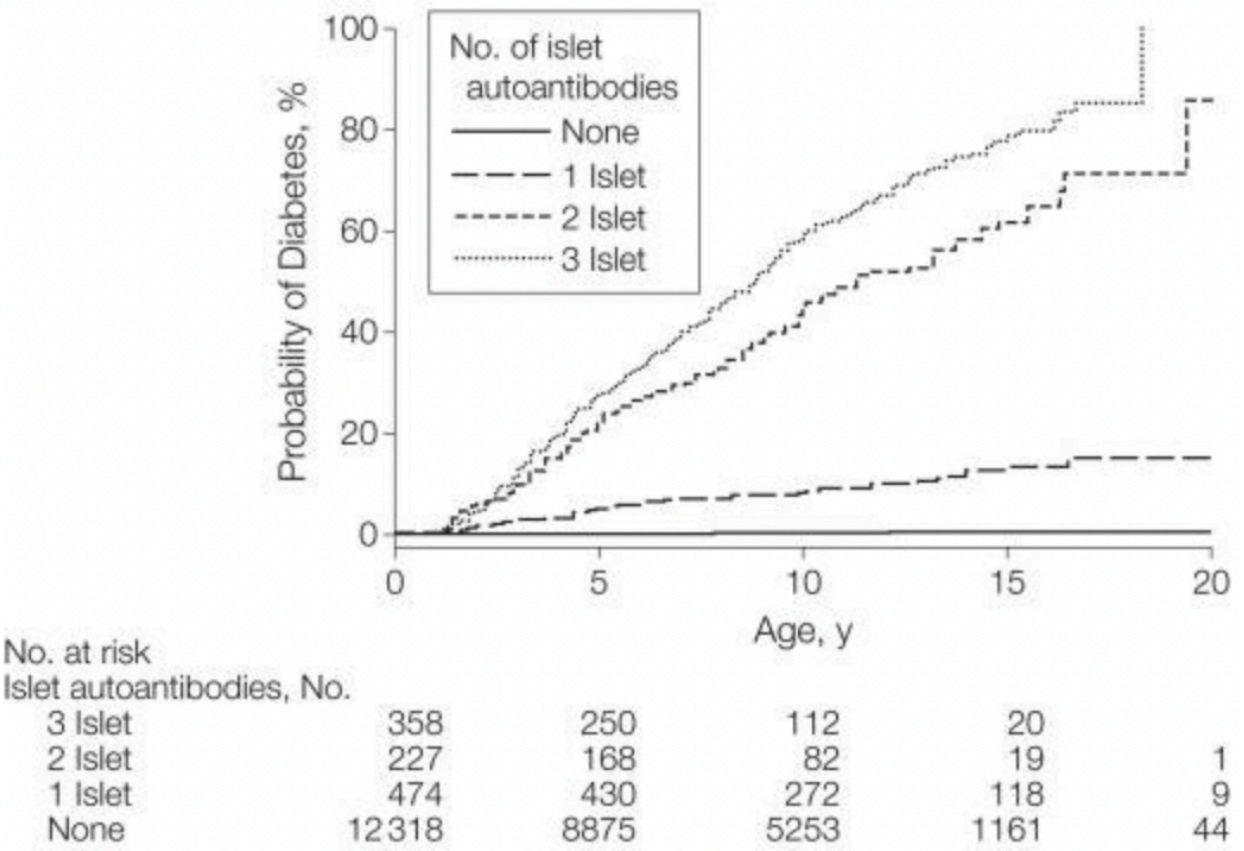


Figure 1.2. A 1986 curve suggesting a mechanism of onset of Type 1 Diabetes, both before and after fulminancy. (Eisenbarth, 1986)

develop with appropriate number; they simply fail to restrain the autoimmune destruction of beta cells (Feuerer et al., 2007; Lindley et al., 2005). Furthermore, NOD Tregs are functional–transfer of activated Tregs can suppress diabetes development in an NOD mouse that previously received islet-specific effector T cells (Tonkin and Haskins, 2009). However, in vivo, Treg function is constrained by a lack of IL-2; IL-2 abnormalities are associated with the pathogenic *Idd3* locus in NOD mice (Yamanouchi et al., 2007). Patients with long-standing T1D are also susceptible to reduced IL-2 signaling that is associated with reduced Treg function and earlier loss of Foxp3 (Yang et al., 2015).

While T1D is classically thought of as a Type 4 Hypersensitivity mediated by T cells (Justiz Vaillant et al., 2021), autoreactive B lymphocytes are required for disease (Hulbert et al., 2001; Thomas and Hulbert, 1996; Thomas et al., 2002). NOD mice deficient in B cells, known as NODμMT mice, are protected from development of diabetes (Serreze et al., 1996) and B cells are required for initiation of insulitis (Noorchashm et al., 1997). Presence of autoreactive B cells can be identified by the production of autoantibodies including anti-insulin, anti-IA2A, anti-GAD65, and anti-ZNT8 (Achenbach et al., 2005; Fousteri et al., 2017). A landmark study found that patients with two or more islet-autoantibodies will eventually progress to T1D, thus illustrating the role of B cells in predicting the onset of disease (Figure 3) (Insel et al., 2015; Ziegler et al., 2013).

However, autoreactive B cells from NOD mice are not pathogenic without T cell collaboration; chimeric animals where all B cells are NOD-derived do not develop insulitis or diabetes (Moore et al., 2005). Even in a B cell deficient NODμMT mouse, usually protected from diabetes, depletion of Tregs results in autoimmunity (Ellis et al., 2013). As B cells develop in the spleen, both follicular and marginal zone B cells are produced; CD23loCD21+IgM+ marginal zone B cells are expanded in NOD mice and infiltrate the pancreas to present antigen to CD4+ T cells (Mariño et al., 2008). NOD mice with mutated B cells that are unable to secrete



No. at risk Islet autoantibodies, No.					
3 Islet	358	250	112	20	
2 Islet	227	168	82	19	1
1 Islet	474	430	272	118	9
None	12318	8875	5253	1161	44

Figure 1.3. Patients with two or more islet auto-antibodies will eventually develop T1D. (Ziegler et al., 2013)

antibodies still develop diabetes, indicating a role for B cells in collaboration with T cells in disease pathogenesis beyond simple antibody secretion (Wong et al., 2004).

Inducible Immune Tolerance and Organ Transplantation

As transplantation set the stage for many early discoveries in immunology, it remains one of the purest tests of immune tolerance. As early as 1944, Peter Medawar was examining the difference in rejection characteristics between “autografts” and “homografts” in rabbits (Medawar, 1944), leading to the landmark discovery that tolerance to foreign tissue could be acquired (Billingham et al., 1953). Injecting splenocytes from one mouse strain to inoculate an MHC-disparate gravid uterus resulted in the fetally-tolerized mouse accepting grafts from the donor mouse strain in adulthood. Neonatal tolerance induced was specific to the donor splenocytes; these same mice were still capable of rejecting third-party grafts.

Since this Nobel Prize-winning work, attempts to develop tolerance to foreign antigens present in an organ graft has baffled immunologists. Transplanted organs face three types of graft rejection with varied timing: hyperacute, acute, and chronic (Libby and Pober, 2001; Rogers and Lechler, 2001). Hyperacute rejection begins the same day as organ transplant and is caused by preformed antibodies to the donor graft. Acute rejection begins weeks to months after transplant and is mediated by direct allorecognition; host T cells respond to MHC present on donor endothelium and donor APCs. In contrast, chronic rejection is often credited to indirect allorecognition, where donor antigens are processed and presented on host APCs to host T cells, leading to destruction of the graft. Similarly to the pathogenesis of T1D, B cells likely play a greater role in graft rejection than credited; antigen presentation by B cells is required for acute graft rejection in a heart transplant model and aid in the formation of alloreactive memory T cells (Ng et al., 2010; Noorchashm et al., 2006). Transplant recipients often face a lifetime of broad immunosuppressive treatment; dampening the immune response to the graft, however,

requires also dampening immune responses to pathogens, resulting in transplant recipients facing a higher incidence of life threatening infections (Pilch et al., 2021). Rarely, patients will develop a tolerance to their organ transplant without immunosuppression; patients who were tolerant to a kidney transplant without immunosuppression were found to have increased CD20 mRNA in their urine, as well as increased numbers of naive and transitional B cells (Newell et al., 2010). As such, preventing graft rejection requires navigating differential leukocyte activation across the lifetime of the recipient.

Layering the immunologic challenges of tolerance to transplant with the failed tolerance in autoimmune disease creates an incredible barrier to transplant in patients with T1D or other autoimmune diseases. Ideally, patients could receive islet transplants with hope of restoring the tissue damaged by autoreactive lymphocytes; however, transplanted islets are not only subject to the alloreactivity that accompanies any organ graft but a recurrence of the autoimmunity that destroyed the endogenous islets. Successful transplantation of islets vastly improves important outcomes in patients with T1D, such as decreasing hypoglycemia unawareness (Harlan, 2016). However, islet transplants are largely insufficient to completely replace the function of endogenous islets; most patients returned to taking exogenous insulin within five years but maintained C-peptide secretion and decreased glycemic instability (Ryan et al., 2005). Transplant lifespan, even with immunosuppression, is limited by the difficulty in overcoming both allo- and auto-immunity (Rother and Harlan, 2004). Rejection can be predicted by the appearance of novel antibodies reactive to both allo- and auto-antigens; islet transplant patients without novel autoantibodies had a significantly longer graft survival than their counterparts (Piemonti et al., 2013).

The difficulty in tolerizing patients with T1D to islet transplant also extends to NOD mice, which resist tolerance induction using therapies that are successful in non-autoimmune mouse strains (Gordon et al., 2005). Treatments like ICOS/CD40L blockade, which successfully tolerizes B6 mice to MHC-mismatched islets and slows the progression of diabetes in NOD

mice, are unable to tolerize NOD mice to MHC-mismatched islet transplants (Ansari et al., 2008; Nanji et al., 2006). An additional agent, αCD45RB, is a promising tolerizing treatment; a short course is sufficient to provide specific and durable tolerance (perhaps by acting on known targets CD4+ T cells, CD8+ T cells, and NK cells) to a MHC-mismatched islet, renal, or cardiac transplant in a non-autoimmune mouse model, but not in NOD mice (Lazarovits et al., 1996; Moore et al., 2004; Stocks et al., 2016a). The tolerance induced by αCD45RB in B6 mice requires thymically-derived Tregs; thymectomized mice all reject their grafts (Deng et al., 2006).

While αCD45RB treatment is not sufficient to tolerize NOD mice to transplant, the therapy again identifies interference by B cells. Unlike their B cell-sufficient counterparts, non-autoimmune B cell-deficient B6μMT mice reject transplanted cardiac allografts without treatment; αCD45RB treatment is able to tolerize these mice to islet transplants (Deng et al., 2007; Lee et al., 2014). While B cells are required for tolerance to transplant in B6 mice, B cell-deficient NODμMT mice treated with αCD45RB are capable of being tolerized to islet grafts (Stocks et al., 2016b). These data illustrate not only differences between B cell populations in B6 and NOD mice, but opportunities to exploit B cell biology to improve inducible immune tolerance.

Immune Tolerance in Pregnancy

Another model of inducible immune tolerance is pregnancy, in which foreign fetal antigens persist in the uterus for the duration of gestation. Successful pregnancy requires not only acceptance, but protection of the often MHC-mismatched fetus. Unlike transplantation, however, pregnancy requires no exogenous immunosuppression or pre-conception MHC-matching; instead, pregnancy alters leukocyte number and function to promote tolerance over the course of gestation.

Tregs play an essential role in protecting MHC-mismatched pregnancies in mice; depletion of Tregs leads to a decreased implantation rate and increased reabsorbed

pregnancies (Robertson et al., 2018; Shima et al., 2010) In fact, Tregs reactive against the Y antigen are generated during pregnancy to protect developing male fetuses (Kahn and Baltimore, 2010). In pregnant humans compared to non-pregnant people, circulating Tregs are increased in number, peaking in the second trimester and declining postpartum (Somerset et al., 2004). Both thymically-derived and peripheral Tregs are responsible for suppressing an immune response against the fetus. Miscarriages with normal embryo karyotype had fewer Helios+ Tregs at the decidua basalis than miscarriages with abnormal embryo karyotype, indicating that in spontaneous abortions without a genetic etiology, a lack of tolerance to the developing fetus may be responsible (Inada et al., 2013, 2015). At the same time, extrathymically-derived CNS-1+ Tregs, when depleted in allogenic mouse pregnancies, are associated with increased reabsorbed fetuses and defective spiral artery remodeling (Samstein et al., 2012). Treg number has been offered as an indicator of likely miscarriage; patients with a history of miscarriage that have fewer Tregs identifiable in the first trimester are more likely to have a recurrent miscarriage (Winger and Reed, 2011). These studies indicate presence of Tregs, both circulating and at the parental:fetal interface, as an indicator of successful tolerance to foreign antigens and a predictor of healthy gestation.

Tregs are not the sole lymphocyte thought to promote tolerance to the developing fetus. B cells, particularly IL-10 secreting Regulatory B cells (Bregs), have been found in the placenta (Benner et al., 2020). Furthermore, circulating B cells are significantly lower in the third trimester of pregnancy in humans; they are thought to be migrating into the decidua to aid in alleviating inflammation (Lima et al., 2016). However, Bregs increase peripherally in early pregnancy as levels of human chorionic gonadotropin (hCG) increase; Bregs express the hCG receptor, and hCG signaling can increase IL-10 secretion (Abu-Raya et al., 2020). Bregs appear to play a significant role in tolerance induction; pregnant B6uMT mice were more susceptible to adverse outcomes with LPS (a bacterial endotoxin that binds TLR4) treatment, which was alleviated by transfer of IL-10 secreting B cells or administration of IL-10 directly (Busse et al., 2019). While

Bregs seem to play a clear role in advancing gestation, they represent a small subset of circulating B cells; overall changes to B cells in pregnancy are largely unknown.

Pregnancy in patients with autoimmune diseases requires endogenous tolerance to foreign antigens in the setting of autoimmune destruction. Interestingly, children born to a parent with T1D are more likely to have a father with T1D than a mother with T1D, indicating some protective epigenetic or immunologic factor during gestation (Jerram and Leslie, 2017). Patients with T1D face outsized pregnancy complications compared to healthy patients: a study of Swedish patients found a 20.6% rate of pregnancy-induced hypertension or preeclampsia (a condition found in pregnancy in which patients present with hypertension, headache and liver injury) vs 5% population risk. (Hanson and Persson, 1998); a study of Chinese patients found a higher rate of pregnancy loss (13.2% vs 2.9%), preeclampsia (17.74% vs 4.2%), neonatal death (5.65% vs 0.16%), and congenital malformations (8.26% vs 3.5%) compared to pregnant people without T1D (Luo et al., 2021); and a study of Dutch patients found an increased risk of preeclampsia, preterm delivery, maternal mortality, congenital malformations, perinatal mortality, and macrosomia (Evers et al., 2004). These complications in pregnancy have largely been attributed to dysglycemia; however, alterations in immunity may also contribute (Groen et al., 2019). An examination of circulating lymphocytes in peripheral blood from patients with T1D found an increased number of lymphocytes as compared to pregnant patients without T1D, as well as a lower total number of NK cells (Groen et al., 2015).

Changes in immunity that may impact gestation have been better characterized in NOD mice. Diabetic NOD syngeneic pregnancies showed compromised NK cell recruitment and poor spiral artery remodeling (Burke et al., 2007). Embryo loss in NOD pregnancies was partially alleviated by administration of Tregs and CXCL12 to aid in placental trafficking (Lin et al., 2009). Notably, treatment of NOD mice with h-CG reversed pancreatic immune infiltration and inhibited diabetes development in mice who had been pregnant (Khan et al., 2001). Decreased diabetes development was not heritable to offspring in this model; immunization of NOD mice

with insulin (and anti-insulin antibody formation) did not change the incidence of diabetes in offspring (Koczwara et al., 2004). Changes during gestation and their impact on fetal outcomes are largely unstudied, both in NOD mice and people with T1D.

Overview and Significance of the Research

The previous discussion raises complicated questions about the induction of immune tolerance, both in organ transplant and in pregnancy. While transplant and pregnancy are independently challenging, tolerance is intensified in the setting of autoimmunity like T1D. Immune pathogenesis in T1D requires intricate collaboration between B and T cells, and depletion of either immune subset impacts diabetes development and transplant acceptance. Treatments that allow for organ graft acceptance in a non-autoimmune model fail to tolerize NOD mice. Similarly, pregnant patients with T1D face adverse outcomes in pregnancy that seem unlikely to be solely related to glycemic fluctuations, as illustrated by changes in lymphocyte number and function. I sought to uncover some of the mechanisms of how inducible immune tolerance is altered by T1D, using both pregnancy and islet transplant as experimental models.

In Chapter II, I describe deleterious increases in autoimmunity as NOD mice age, revealing a narrow window in young NOD mice where mice may be able to be tolerized to organ transplant. In Chapter III, I turn to pregnancy to investigate the etiology of pregnancy loss in NOD mice and discover the impact of immunologic and vascular cross-talk via IL-6. In Chapter IV, I utilize human placentas from patients with T1D to validate my findings of an altered immunologic landscape and uncover the effect of autoimmunity on pregnancy outcomes. This work generates exciting possibilities for patients combining baseline levels of autoimmunity with the challenge of pregnancy or transplant.

CHAPTER II

IMMUNE TOLERANCE WANES AS NOD MICE AGE

Introduction

Autoimmune features develop with age in both NOD mice and people with T1D. Islet-reactive autoantibodies are found in NOD mice as early as 4 weeks of age, although they reach peak concentration between 8-12 weeks of age and portend the onset of overt diabetes (Yu et al., 2000). In patients with T1D, autoantibodies have been detected as early as 9 months of age (Yu et al., 2000). Patients with two or more islet autoantibodies are virtually guaranteed to develop hyperglycemia and life-long dependence on exogenous insulin due to beta cell destruction; the appearance of autoantibodies indicates that destructive autoimmunity has already begun (Ziegler et al., 2013). Not only does the predictive nature of autoantibodies implicate B lymphocytes in T1D development, but B lymphocyte deficient NOD (NODμMT) mice are protected from diabetes (Serreze et al., 1998). Data from our lab has shown that NODμMT mice are also capable of accepting islet transplants with α-CD45RB treatment (Figure 1A) and that the tolerance established requires Tregs (Figure 2.1B).

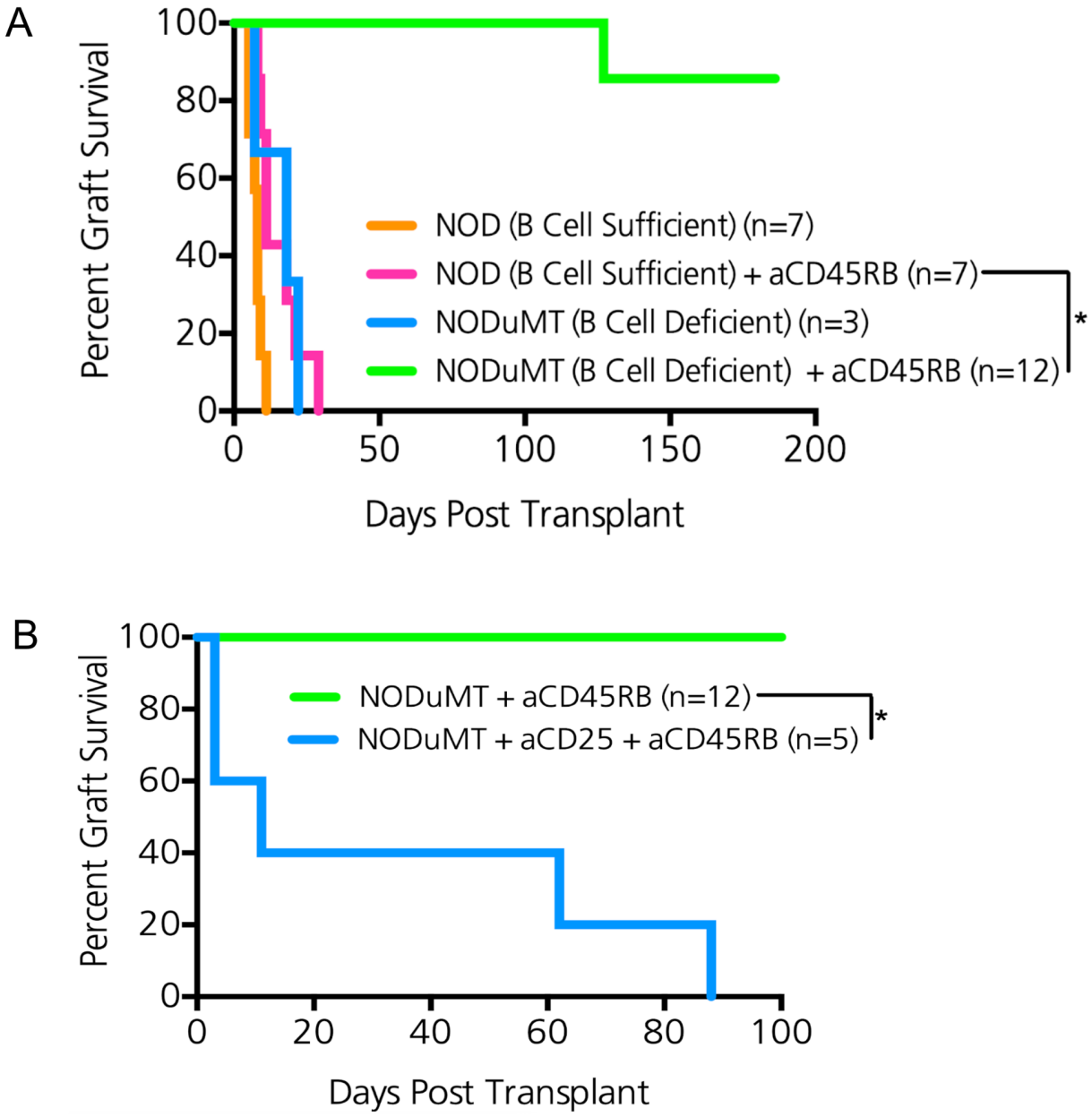


Figure 2.1. Survival curves illustrating islet graft survival in NODμMT mice. B cell-sufficient NOD mice, both untreated and treated with α-CD45RB, reject islet grafts. Untreated NODμMT also reject islet grafts, but NODμMT mice treated with α-CD45RB are able to accept islet grafts over 200 days (A). The tolerance established by α-CD45RB requires the presence of Tregs (B). Data was analyzed by log-rank statistical analysis where * indicates $p<0.05$.

One possible mechanism by which B cells are implicated in diabetes pathogenesis is through their role as thymic antigen-presenting cells. Thymic B cells support the development of thymically-derived Tregs, as discovered in BAFF transgenic mice, which have an excess of both thymic B cells and Helios+ Tregs (Walters et al., 2014). Negative selection, which occurs in the thymic medulla, requires cells to present self-antigen to developing T cells, which aids in both eliminating self-reactive T cells and creating Tregs. NOD mice are known to have a deficit in negative selection, which leads to poor deletion of islet-reactive cells (Lesage et al., 2002).

Additionally, thymic B cells have been found to express Aire (Yamano et al., 2015), which decreases in expression as mice age (Cepeda et al., 2018). Aire is required for negative selection of organ-specific T cells (Liston et al., 2003). Aire-deficient mice have a plethora of autoreactive T cells and autoantibodies; however, B cell interaction with developing T cells is necessary for autoimmune infiltration of organs (Gavanescu et al., 2008).

Once Tregs leave the thymus, they are supported by IL-2 released from neighboring cells. Low expression of CD25 (an IL-2 Receptor subunit) is associated with islet-reactive Tregs, hindering the ability of islet-reactive Tregs to respond to increased IL-2 (Hotta-Iwamura et al., 2018). Tregs with lower IL-2 sensitivity are less likely to successfully function as suppressors of the immune response, and have a more labile Foxp3 expression (Yang et al., 2015). Reduced levels of IL-2, like those found in the NOD mouse due to the *Idd3* locus, results in Tregs that have a decreased ability to protect the islet (Yamanouchi et al., 2007). IL-2 signaling can occur via three pathways within the cell, although it predominantly signals through STAT5 in Tregs (Figure 2.2) (Burchill et al., 2007; Liao et al., 2013).

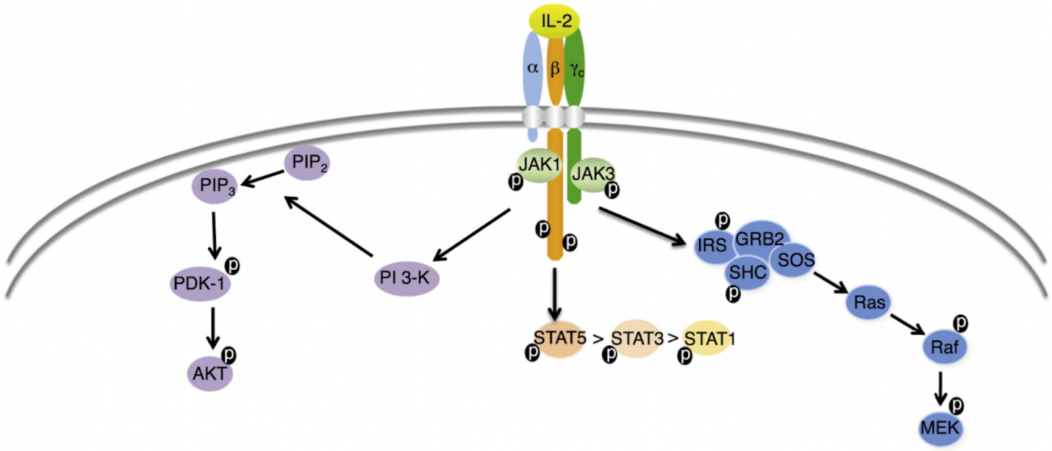


Figure 2.2. Pathways of IL-2 signaling, adapted from (Liao et al., 2013)

In this chapter, I identify some of the mechanisms by which thymic B cells may contribute to autoimmunity and transplant tolerance in NOD mice. I studied thymic B cells in both non-autoimmune B6 and diabetes-prone NOD mice to determine alterations that may be present with autoimmunity, including B cell subset differences and Aire expression; these alterations increase with age. As autoimmune features appeared to worsen in older mice, I hypothesized that the tolerizing therapy α-CD45RB may be capable of promoting tolerance in younger mice. I investigated a mechanism by which tolerance may have been promoted by examining IL-2 signaling in α-CD45RB-treated mice. Ultimately, I tested the capability of α-CD45RB to promote tolerance in both ex-vivo and in-vivo models of transplantation tolerance. This study identified an age-related decline in immune tolerance in NOD mice and found a narrow window in which mice may be able to be tolerized to islet transplant (Figure 2.3).

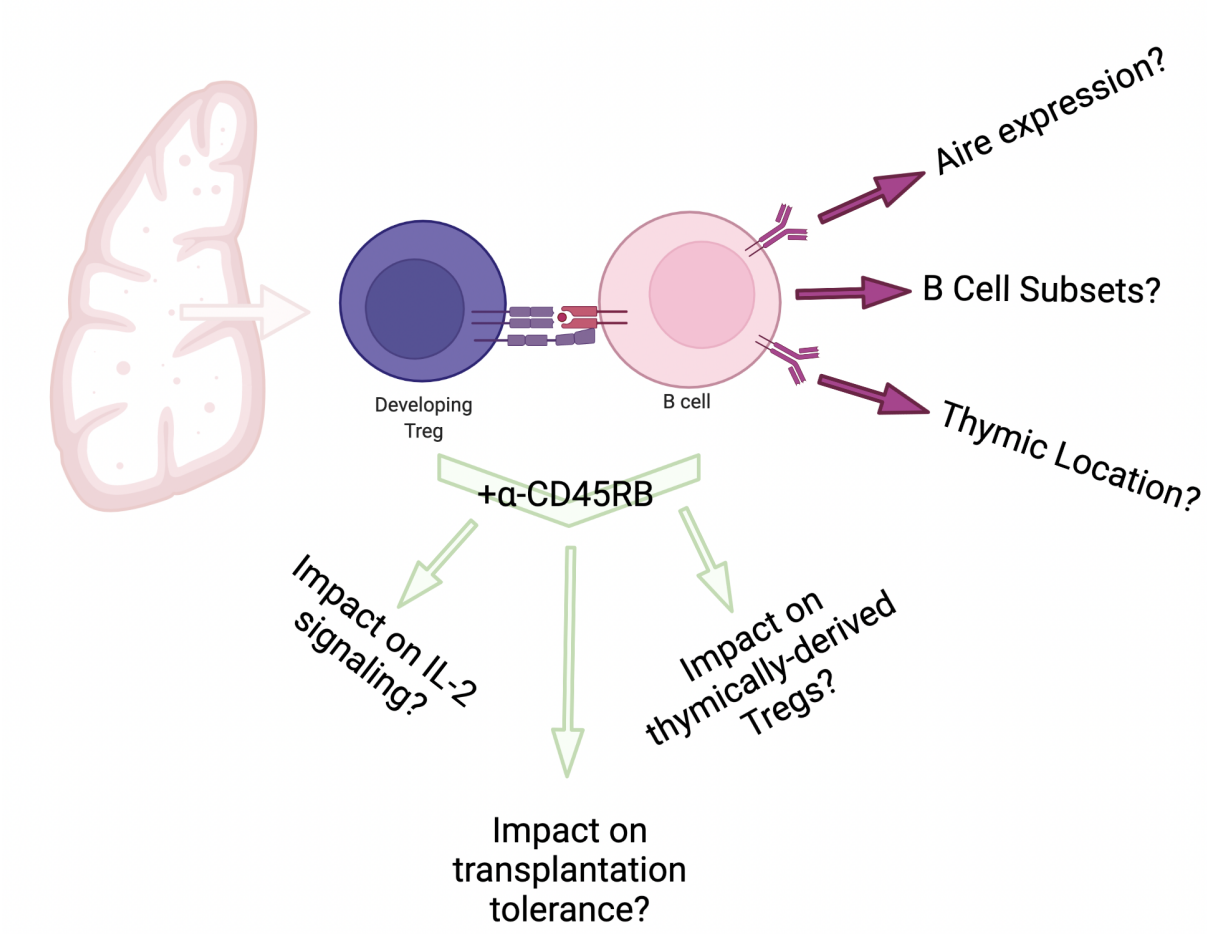


Figure 2.3. Overview figure illustrating possible characterizations and outcomes of the thymic B cell:Treg interaction.

Results

Thymic B Cells Differ as NOD Mice Age

As autoimmunity increases with age, I hypothesized that negative selection of T cells failed to appropriately prevent the escape of autoreactive cells or produce sufficiently functional Tregs as mice aged. Because of the impact of B cell depletion on transplant outcomes and diabetes development, I hypothesized that thymic B cells contribute to adverse T cell development. Because of the expected role of thymic B cells in presenting antigen to developing T cells, I began by investigating how thymic B cells in the NOD mouse differed from splenic B cells. I used a gating scheme for B cell subsetting, as outlined by Mariño and colleagues (Figure 2.4) (Mariño et al., 2008).

With confidence in the gating schemes used, I analyzed thymuses and spleens from B6 and NOD mice at 12 weeks of age to query both how the thymus differed from the spleen, but also how non-autoimmune B6 mice may differ from NOD mice (Figure 2.5A, B). In both spleen and thymus for B6 and NOD mice, the majority of cells are antibody-secreting Follicular cells, with no apparent differences between mouse strains. B cells with a Marginal Zone phenotype, implicated in disease pathogenesis in NOD mice, appear to be increased in NOD mice in both spleen and thymus, as are Marginal Zone-like precursor cells. B6 mice also appear to have an increase in B cells with a Transitional Zone 1-like phenotype in both thymus and spleen, though no statistical tests were used.

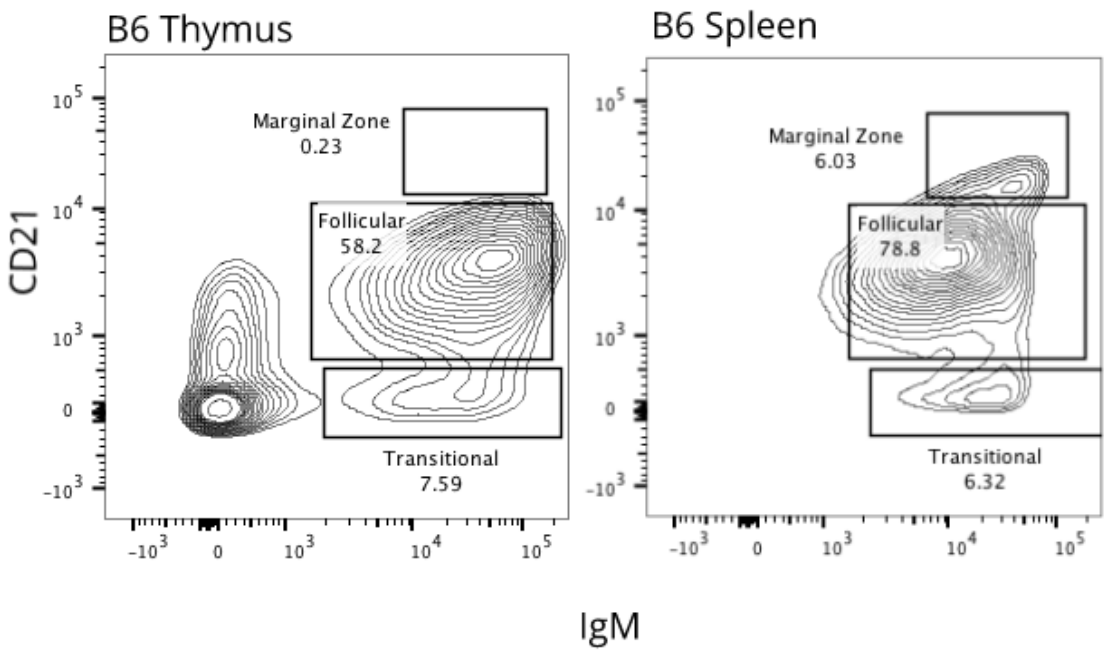


Figure 2.4. Flow cytometry plots for B6 thymus and spleen illustrating a gating strategy for B cells. Cells are stratified by CD21 and IgM expression, and then grouped by subtype.

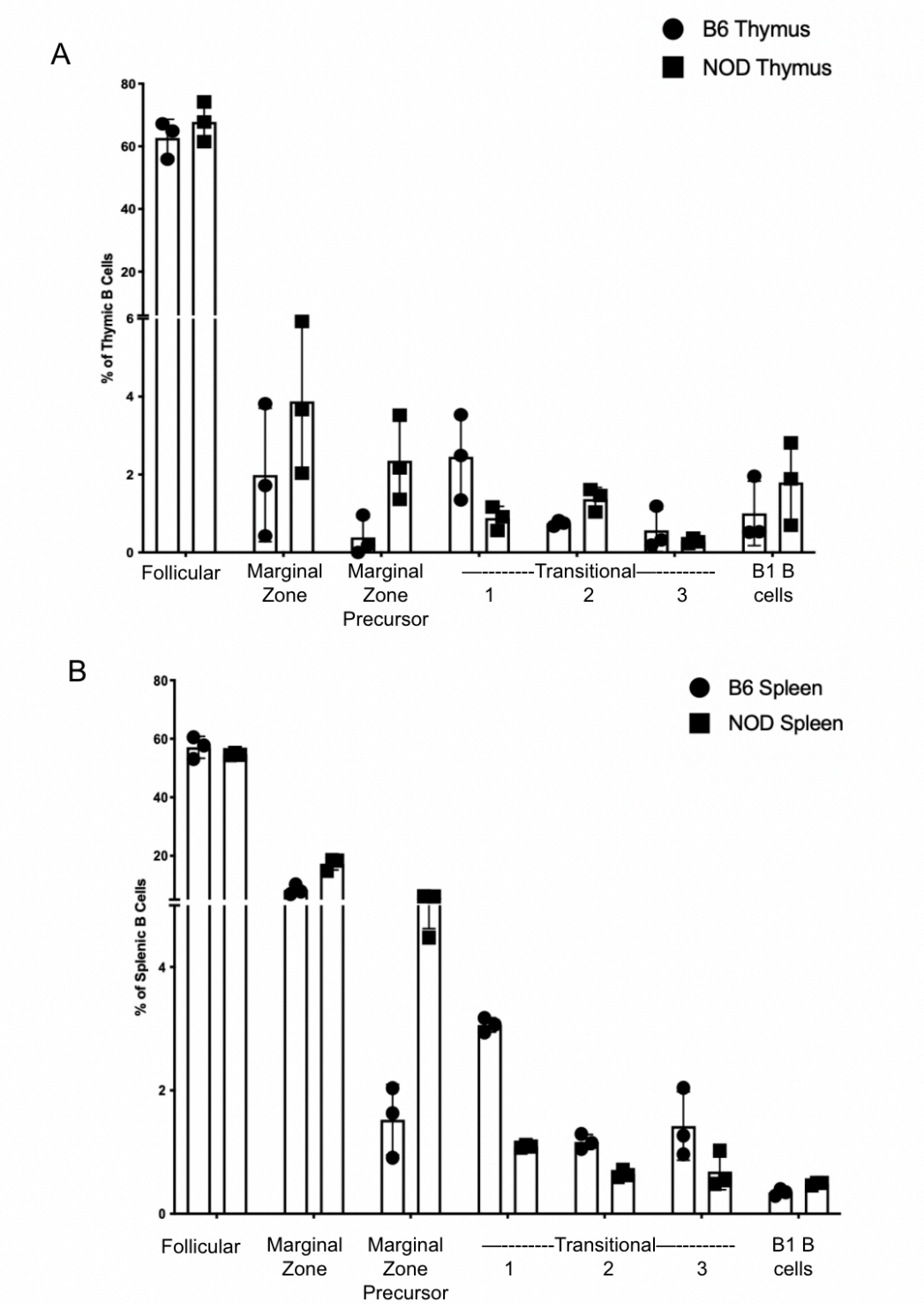


Figure 2.5. Prevalence of B cell subsets in 12-week-old B6 and NOD thymuses (A) and spleens (B) as a percentage of B220+ cells. Each point represents one spleen or thymus. Data shown is representative of 3+ experimental repetitions.

Thymic B cells are thought to function as antigen-presenting cells, aiding in negative selection of developing T cells. Accordingly, thymic B cells have been found to express Aire, which allows for the presentation of self-antigen. I hypothesized that NOD B cells may have disturbed Aire expression, which may allow for poor Treg development. In thymuses from 10-week-old B6 and NOD mice, I compared Aire expression in CD19+IgK+ B cells to CD45-EpCAM+UEA1+ mTECs, which are thought to be the main Aire expressing cell type (Figure 2.6A, B). I found that NOD thymuses have an overall deficit in Aire+ cells, as both B cells and mTECs showed lower Aire expression than their B6 counterparts. Notably, both NOD and B6 B cells appeared to have higher Aire expression than mTECs, solidifying the role of B cells as important antigen presenting cells for negative selection.

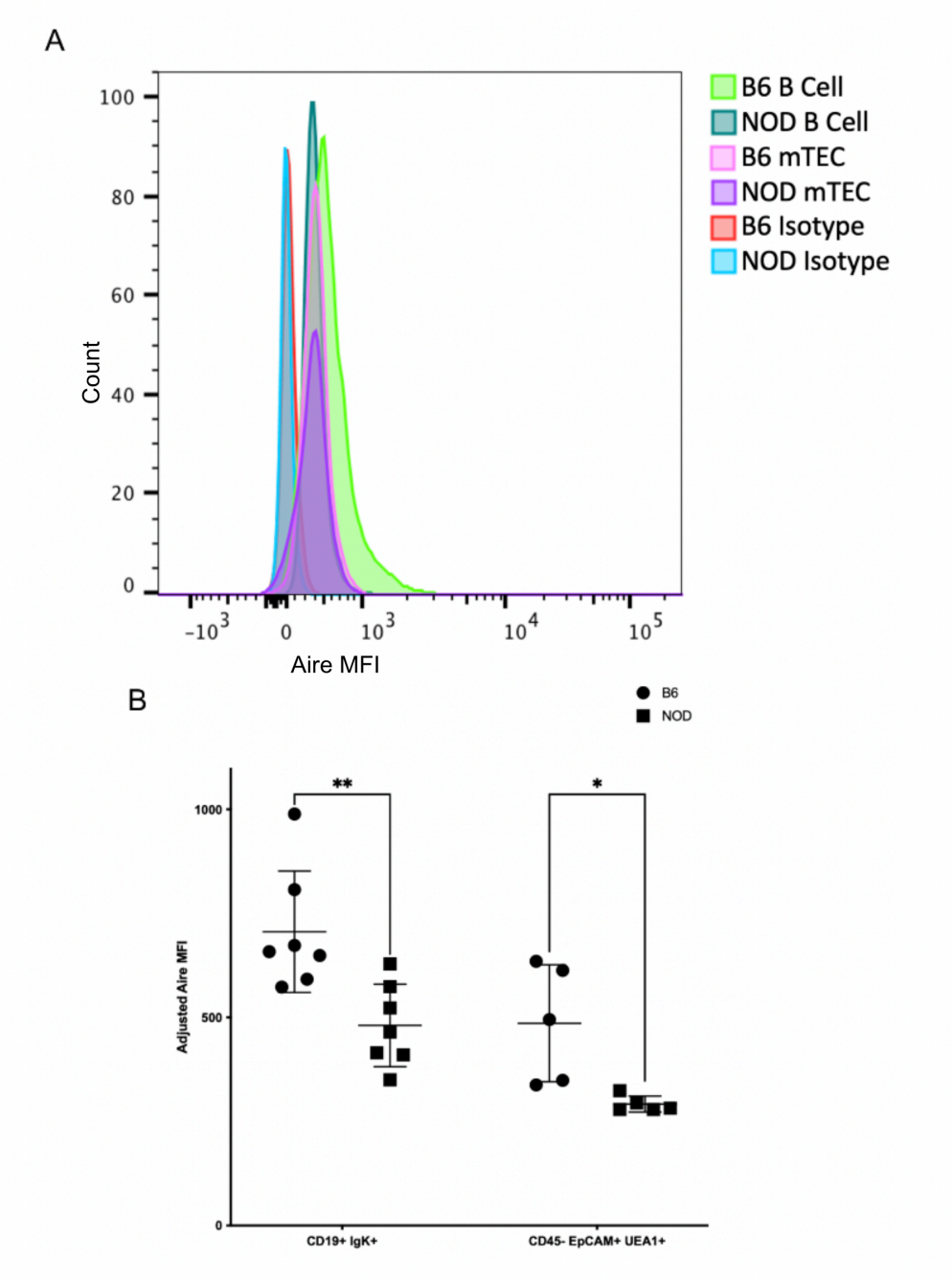


Figure 2.6. Aire expression in 10-week-old B6 and NOD thymocytes, both CD19+IgK+ B cells and CD45-EpCAM+UEA1+ mTECs. Histogram showing expression compared to isotype control (A) and graphed adjusted MFI (B). Each point represents one thymus. Data shown represents 3+ experiments. Results were analyzed using a t-test, where * indicates $p<0.05$ and ** indicates $p<0.01$.

Having identified alterations in B6 and NOD thymic B cell subsets, I hypothesized that changes in B cells in the NOD mice may become increasingly apparent as NOD mice age and develop an increasing burden of autoimmunity. Using histopathology to examine location and presence of thymic B220+ B cells in B6 and NOD thymuses at both 3 and 20 weeks of age, I found that differences develop with age (Figure 2.7A, B). B6 and NOD mice have similar thymic B cell compositions at 3 weeks of age; most of the B220+ cells are well-spaced throughout the thymic medulla, where negative selection occurs. In contrast, by 20 weeks of age, some cell clumps are seen in the B6 thymus. However, in the NOD thymus, enormous clusters of B220+ cells are clumped at the cortico-medullary junction. The changes in B cell presence in the 20-week-old NOD thymus may reflect the increased burden of autoimmunity seen in the older NOD mouse.

These data illustrate a thymic B cell compartment that reflects pathology found in splenic B cells and is exacerbated as mice age. The clustering of B cells in the 20-week-old NOD thymus may reflect a germinal center; regardless, the increased number of marginal-zone-like and possible autoreactive B lymphocytes that reflect the abnormal cell composition seen in the NOD spleen suggest a contribution of thymic B lymphocytes to autoimmunity.

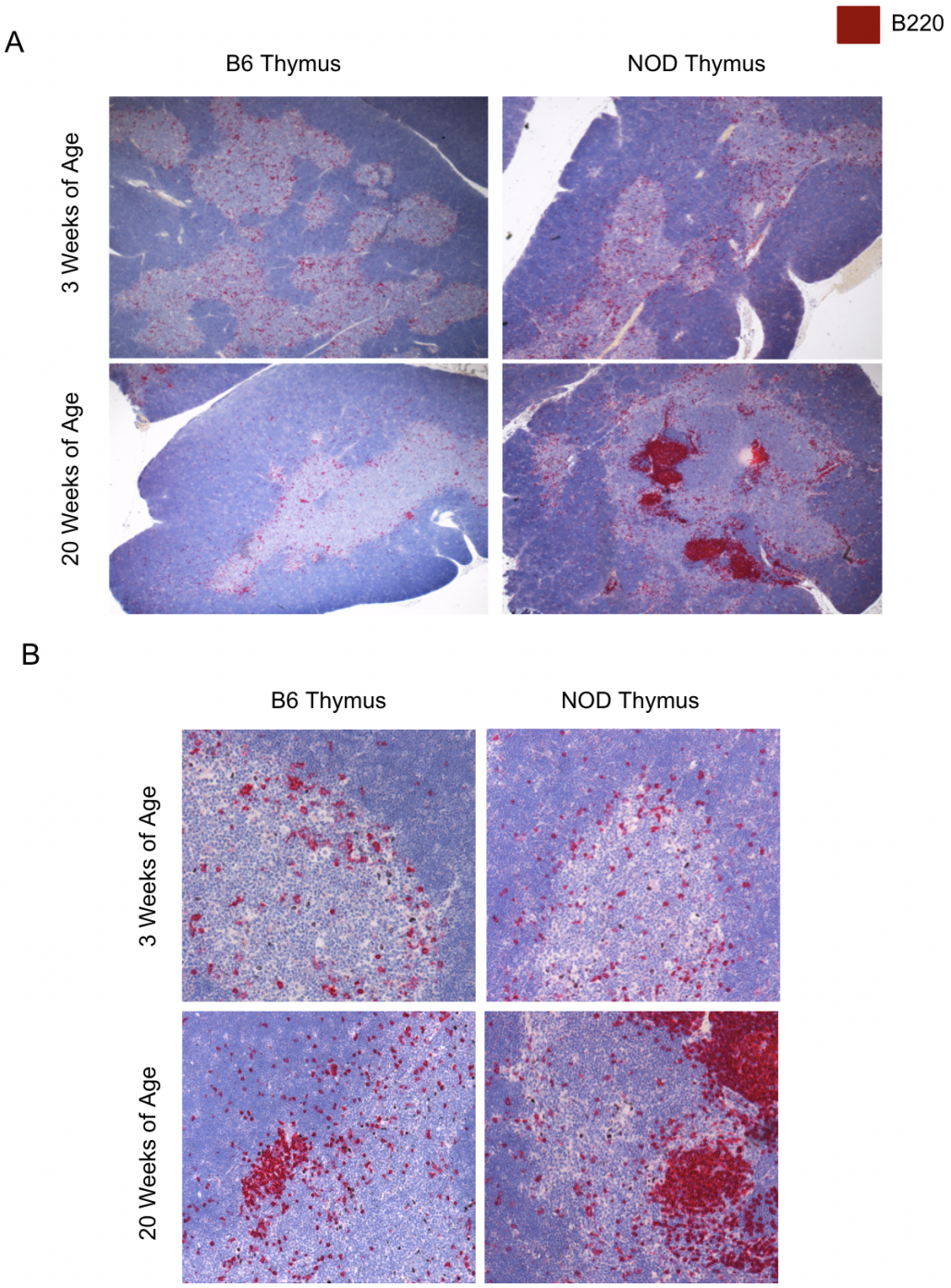


Figure 2.7. Histopathologic image of B6 and NOD thymuses at both 3 and 20 weeks of age. B220 staining reveals dispersed B cells at 3 weeks of age in both B6 and NOD mice, with predominance in the thymic medulla. By 20 weeks of age, the B6 mouse has slight clustering of B cells, whereas the NOD mouse has enormous groups of B220+ cells. Shown at lower magnification overview (A) and higher magnification (B).

Age Controls the Impact of α-CD45RB Treatment on Splenic Lymphocytes

As age appears to be associated with abnormalities in thymic B cells and these B cells may negatively impact the ability of NOD mice to be tolerized to transplant using α-CD45RB, I hypothesized that α-CD45RB treatment may be more effective at tolerance induction in younger mice. I treated 4- and 6-week-old B6 and NOD mice with a 7-day course of α-CD45RB. This treatment revealed that α-CD45RB in the 4-week-old mice expanded splenic Foxp3+Helios+ Tregs, indicating that α-CD45RB was able to increase the number of thymically-derived Tregs (Figure 2.8). The degree of increase, however, was dependent on the age of the mice. B6 mice at both 4 and 6 weeks of age had a large increase in Tregs, with an even greater response at 6 weeks of age. In contrast, NOD mice had a large increase at 4 weeks of age; by 6 weeks of age, the response to α-CD45RB is weaker. While no statistical tests were used to analyze this data, the change may indicate that the capacity of the thymus to generate new Tregs in response to α-CD45RB treatment decreases with age.

As the Treg response to α-CD45RB is altered with age, I also examined the B cell response, querying whether α-CD45RB treatment might diminish pathogenic Marginal Zone B cells in the spleen in young mice. Both B6 and NOD mice had an increase in Follicular B cells with increasing age, but the Follicular B cell percentage did not appear to be altered with α-CD45RB treatment (Figure 2.9A). In contrast, NOD mice at both 4 and 6 weeks had an increase in Marginal Zone and Marginal Zone precursor B cells compared to B6 mice, but α-CD45RB treatment decreased the percentages to those comparable to B6 mice (Figure 2.9B).

In 4-week-old NOD mice, α-CD45RB treatment appears to promote tolerogenic changes like an increase in thymically-derived Tregs and a decrease in pathogenic Marginal Zone B cells. By 6 weeks, the effects seem to be present but slightly diminished. These data illustrate that α-CD45RB can remedy some of the preliminary effects of autoimmunity when administered at a young age.

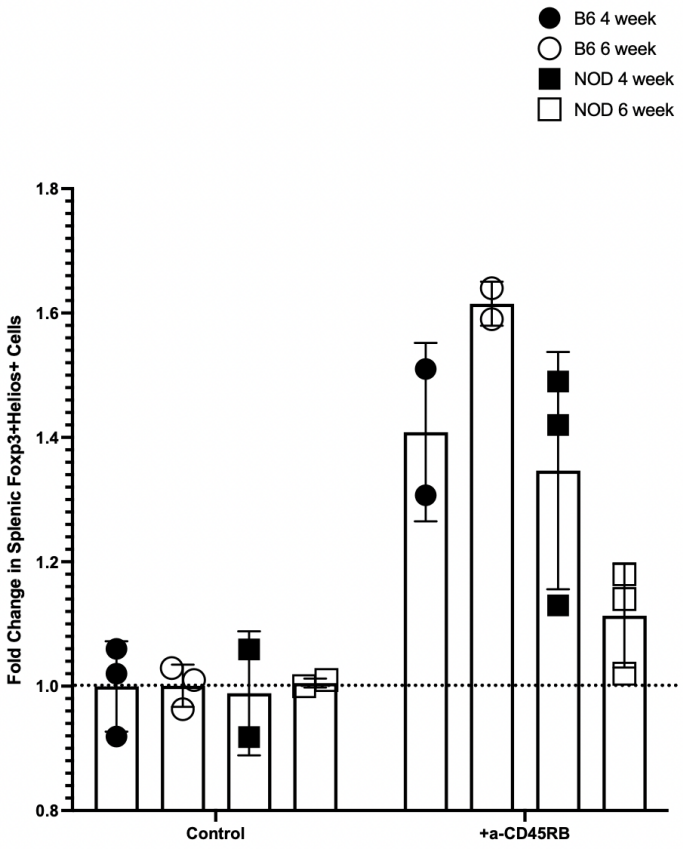


Figure 2.8. Fold Change in splenic Foxp3+Helios+ Tregs from untreated control mice to mice treated with α-CD45RB. Each dot represents one spleen. Data shown is representative of 3+ experiments.

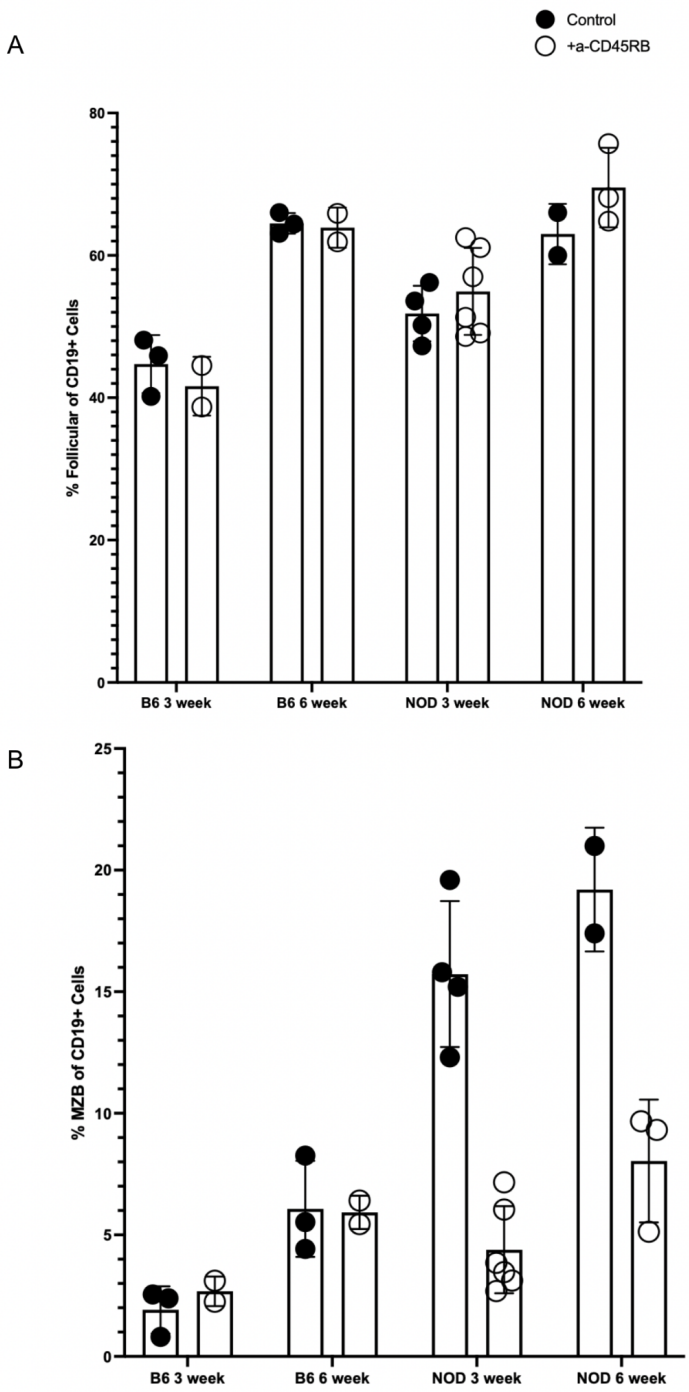


Figure 2.9. Percentage of CD19+ cells that are Follicular (A) or Marginal Zone/Marginal Zone Precursors (B) in both B6 and NOD mice of different ages. α-CD45RB has little effect on Follicular B cells and decreases Marginal Zone B cells in the NOD mouse. Each dot represents one spleen. Data shown is representative of 3+ experiments.

α-CD45RB Increases IL-2 Responsiveness in B6 Mice but not NOD mice

After identifying that α-CD45RB increased thymically-derived Tregs in both B6 and NOD mice, I hypothesized that α-CD45RB may be acting through increasing IL-2 responsiveness in Tregs. While most IL-2 signaling happens through STAT5, I chose to examine three possible signaling pathways by incubating splenocytes in 20 ng/mL IL-2 for 0, 15, 30, or 45 minutes and measuring phosphorylation of STAT5, STAT3, AKT, and MEK. α-CD45RB increased phosphorylation of STAT5 in B6 splenocytes as compared to untreated B6 splenocytes, but did not have the same effect on NOD splenocytes (Figure 2.10A). Phosphorylation of STAT3 and MEK seem to be increased at baseline in both untreated and α-CD45RB-treated B6 splenocytes, but are unaffected by treatment (Figure 2.10B, C). Stimulation with IL-2 did not reveal a clear pattern in phosphorylation of AKT (Figure 2.10D). It appears that while the 6-week-old NOD mouse expands thymically-derived Tregs in response to α-CD45RB treatment, the function of α-CD45RB is not through increasing IL-2 signaling capabilities.

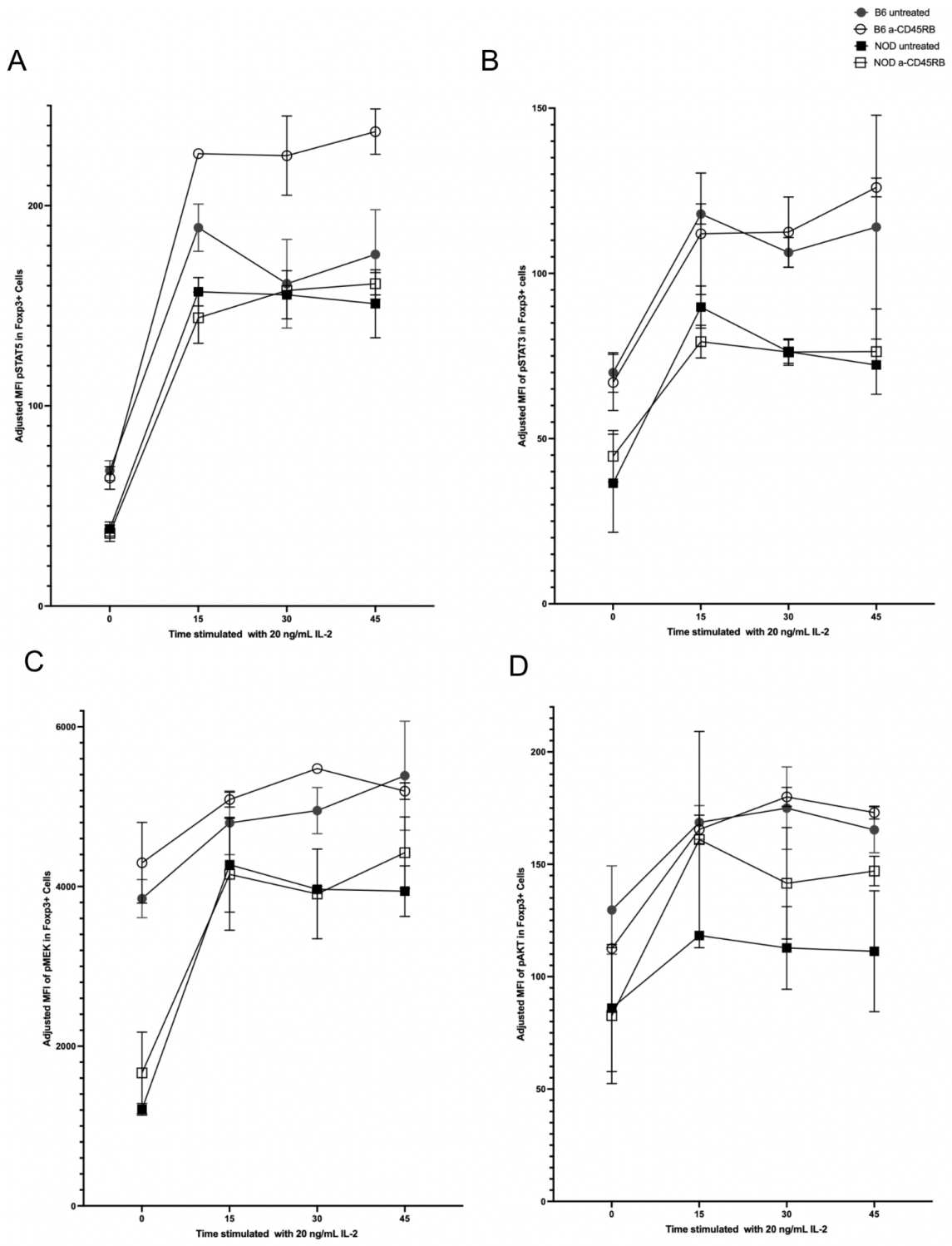


Figure 2.10. Adjusted MFI of phosphorylated STAT5 (A), STAT3 (B), MEK (C), and AKT (D) in 6-week-old B6 and NOD splenocytes stimulated with 20 ng/mL IL-2 for 0, 15, 30, and 45 minutes. 3 spleens per condition were used.

Young NOD Mice are Able to be Tolerized to Foreign Antigen in an Ex-Vivo Model of Transplantation Tolerance

While neither α-CD45RB nor any other treatment has ever led to organ allograft acceptance in the NOD mouse, all mice used for previous transplantation experiments were over 8 weeks of age. Given the expansion of thymically-derived Tregs and decrease in Marginal Zone B cells in both 4- and 6-week-old NOD mice, I hypothesized that intervening at a younger age may allow for transplant acceptance. In order to test this hypothesis, I used the Mixed Lymphocyte Reaction (MLR), an ex vivo model of transplant tolerance. First, I injected T cell-depleted C3H splenocytes into both B6 and NOD mice at different ages, and then treated half of the mice with a course of α-CD45RB. After completion of α-CD45RB, I set up the assay using MHC-mismatched splenocytes as foreign antigens. To test tolerance to the injected C3H cells, both B6 and NOD cells were mixed with C3H cells as antigens. Additionally, to test specificity of the tolerance to C3H, B6 and NOD cells were mixed with each other. In order to test viability of the splenocytes, an additional group of cells were stimulated with αCD3/αCD28. The assay was analyzed using flow cytometry (Figure 2.11A, B).

B6 mice at 3, 6, and 8 weeks of age were all able to be successfully tolerized to C3H cells using α-CD45RB treatment; treatment led to all groups showing a decrease in the percentage of Cell Trace Violet low cells, meaning that proliferation to foreign antigen decreased (Figure 2.12A, B). NOD mice at 3 and 6 weeks of age showed a similar decrease in proliferating cells after α-CD45RB treatment, but 8-week-old NOD mice had a nonsignificant change in CD4+ cells and an increase in CD8+ cell proliferation with α-CD45RB treatment.

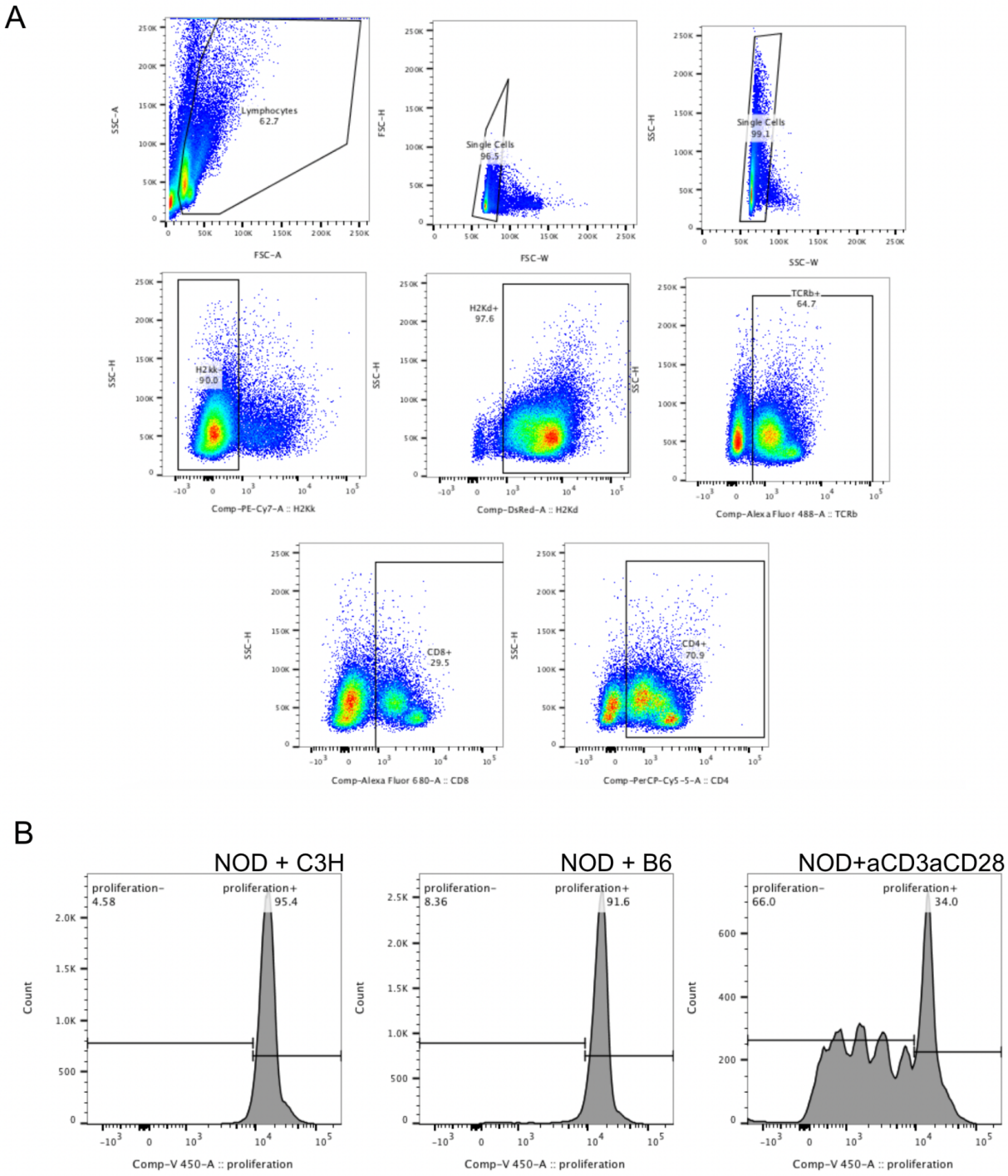


Figure 2.11. Gating scheme for Mixed Lymphocyte Reaction (MLR) for an NOD mouse. Cell populations were isolated to be H2Kk negative, H2Kd positive, then assessed for presence of T cell markers (A). Cell Trace Violet example staining illustrates the peaks of proliferating cells that are Cell Trace Violet low (B).

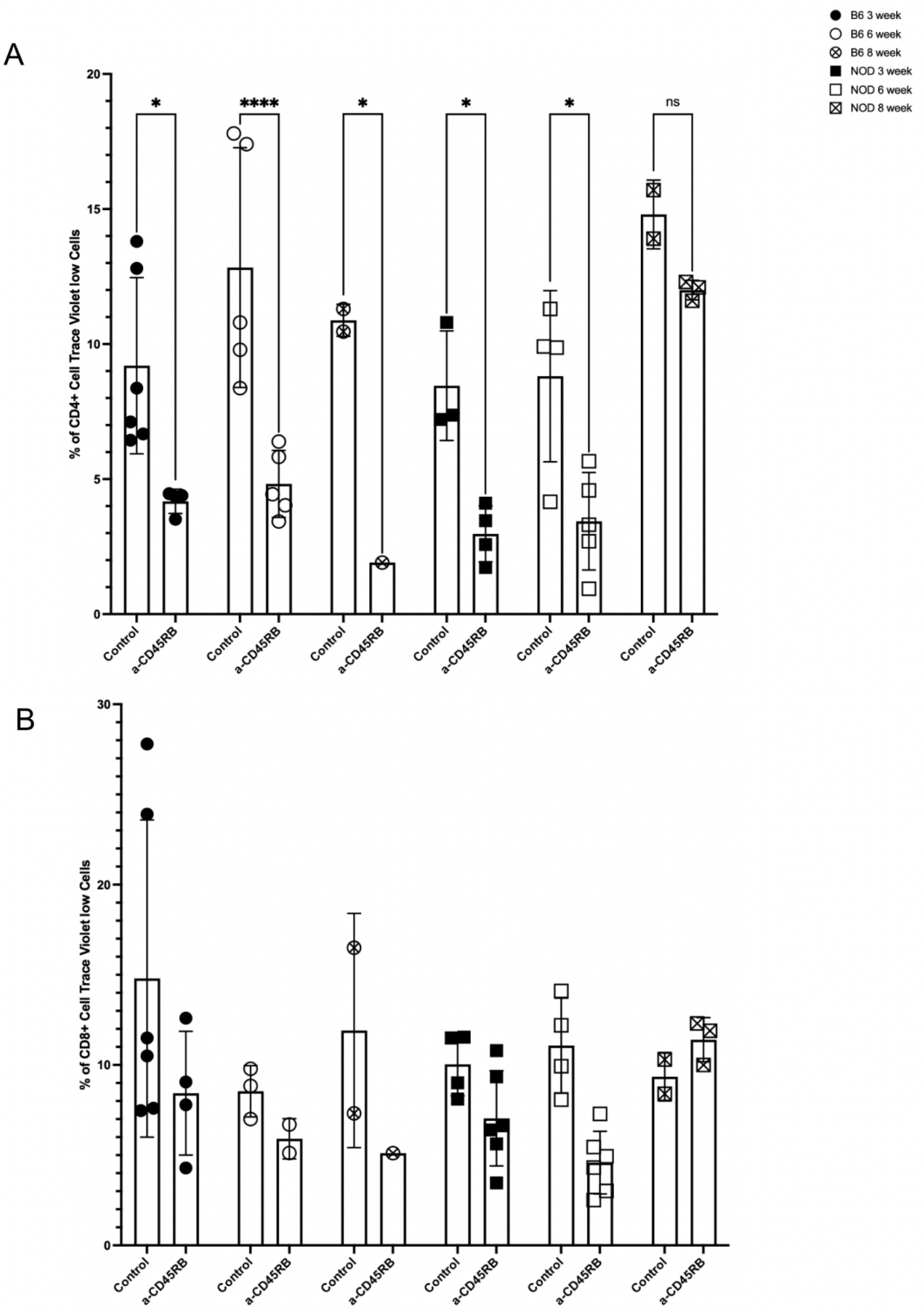


Figure 2.12. Percentage of CD4+ (A) and CD8+ (B) Cell Trace Violet low cells (amount of proliferating cells) to C3H antigen in 3-, 6-, and 8-week-old B6 and NOD mice. Each point represents one spleen. Data shown is representative of 3+ experiments. Data was analyzed by individual t test, where * indicates $p<0.05$ and **** indicates $p<0.001$.

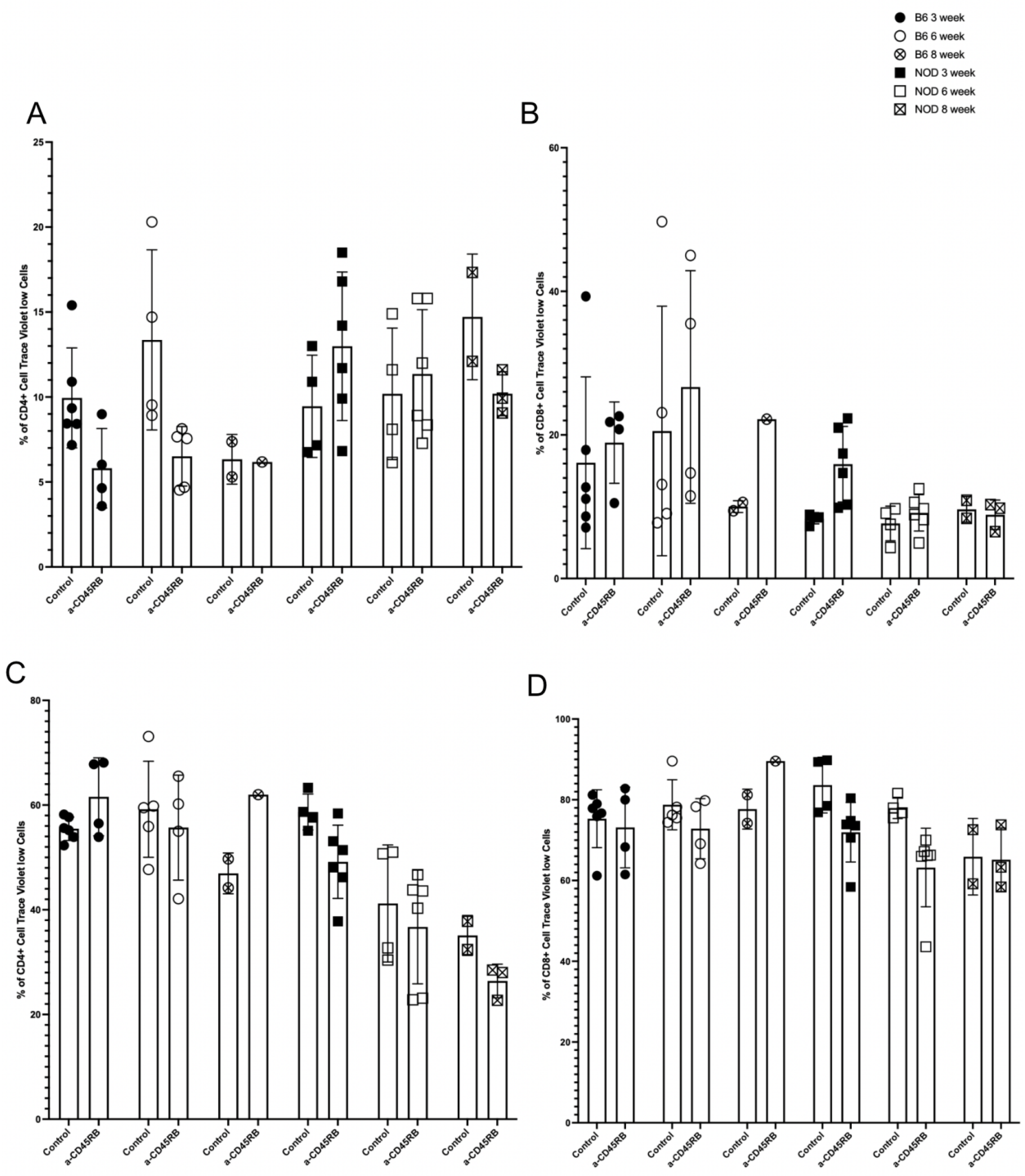


Figure 2.13. Percentage of CD4+ (A, C) and CD8+ (B, C) Cell Trace Violet low cells (amount of proliferating cells) to B6 or NOD antigen (A, B) or αCD3/αCD28 (C, D) in 3-, 6-, and 8-week-old B6 and NOD mice. Each point represents one spleen. Data shown is representative of 3+ experiments.

Testing the specificity of the tolerance to C3H antigens revealed no off-target tolerance to NOD (with B6 responder cells) or B6 (with NOD responder cells) antigens (Figure 2.13A, B). Additionally, the majority of splenocytes in all age groups responded to αCD3/αCD28 stimulation, indicating that the cells were not failing to respond to C3H antigens because they were unwell. These data show that NOD mice are capable of being tolerized to foreign antigen with early intervention at a young age; furthermore, the tolerance established is specific, and is not attributable to cell death in the treatment group.

Tolerance to Islet Transplant May Be Possible in Young NOD Mice

NOD mice at 3 and 6 weeks of age appeared to be capable of tolerance to foreign antigens with α-CD45RB treatment in an ex-vivo model, the Mixed Lymphocyte Reaction. While older NOD mice have never been successfully tolerized to foreign antigens in an organ transplant, there have not been reports of attempts at transplantation tolerance in young NOD mice.

NOD mice at 3 weeks of age were treated with streptozotocin to induce diabetes, and islet transplant under the kidney capsule with MHC-mismatched C3H islets was performed a week later in hyperglycemic mice. 5 transplanted mice were treated with a standard course of α-CD45RB, and 2 were left untreated. Blood glucose measurements taken at least every 3 days shows that young mice treated with α-CD45RB largely lost islet grafts faster than untreated NOD mice (Figure 2.14). Four of the five mice treated with α-CD45RB lost the grafts as quickly as 4 days after transplant, while experiencing the initial euglycemia that indicates islet engraftment.

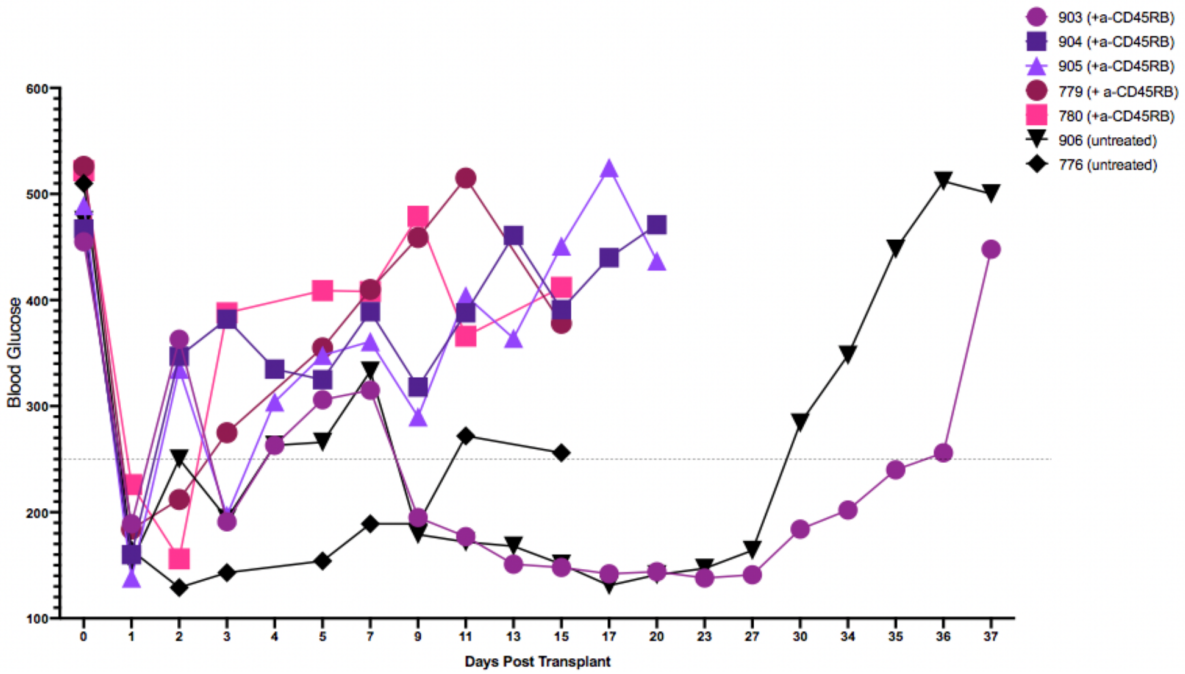


Figure 2.14. Blood glucose curves for 4-week-old NOD mice receiving islet grafts under the kidney capsule. Mice that received α-CD45RB are shown in color, and untreated mice are in black. A dotted line represents a blood glucose of 250; two consecutive values over 250 indicate loss of the islet graft. Each number represents one animal.

After the surprising response to α-CD45RB treatment, kidneys containing islet grafts were sent for histopathologic examination to further assess the mechanism of graft loss. Histologic examination revealed that the untreated islet graft was surrounded by lymphocytes, indicating that the reason for rejection was likely immunologic (Figure 2.15A). However, in the α-CD45RB treated mouse, the islet graft is surrounded by hemorrhagic material (Figure 2.15B). A closer examination shows the presence of hemorrhagic clots and hemosiderin-laden macrophages (Figure 2.15C). However, the vast lymphocytic presence seen in the untreated mouse is absent, indicating that the α-CD45RB treatment may have induced tolerance, but engraftment failed because α-CD45RB induced hemorrhage in the kidneys.

As α-CD45RB did not fail to induce tolerance for an immunologic reason, I hypothesized that tolerance may be possible towards transplant into a different site. NOD mice at 3 weeks of age were treated with streptozotocin to induce diabetes, and islet transplant into the pinna of the ear with MHC-mismatched C3H islets was performed a week later in hyperglycemic mice. Unfortunately, transplant into the ear pinna was received similarly to transplant under the kidney capsule; α-CD45RB treatment led to islet loss after a brief euglycemic period (Figure 2.16). While transplant loss appeared to not be of an immunologic etiology, it is still unknown whether α-CD45RB is able to tolerize young mice to islet transplant; however, α-CD45RB treatment appeared to remedy some of the adverse effects of autoimmunity (Figure 2.17).

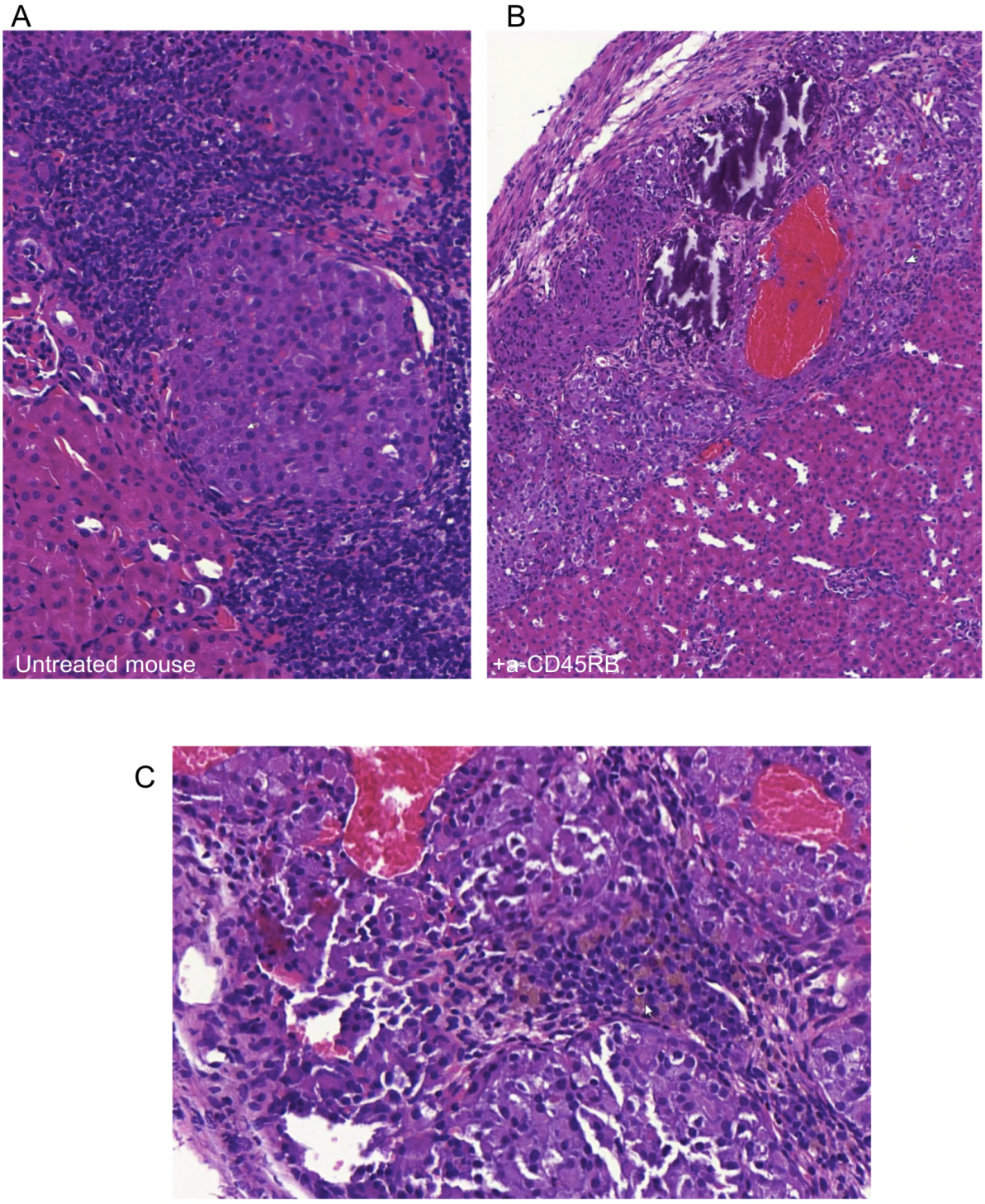


Figure 2.15. Histologic images of kidney tissue containing islet grafts. The islet graft in the NOD mouse untreated with α-CD45RB is surrounded by lymphocytes (A). The graft in the NOD mouse treated with α-CD45RB has few visible lymphocytes but is surrounded by hemorrhage (B). A higher magnitude image of the α-CD45RB-treated islet graft shows hemorrhagic material and hemosiderin-laden macrophages (C).

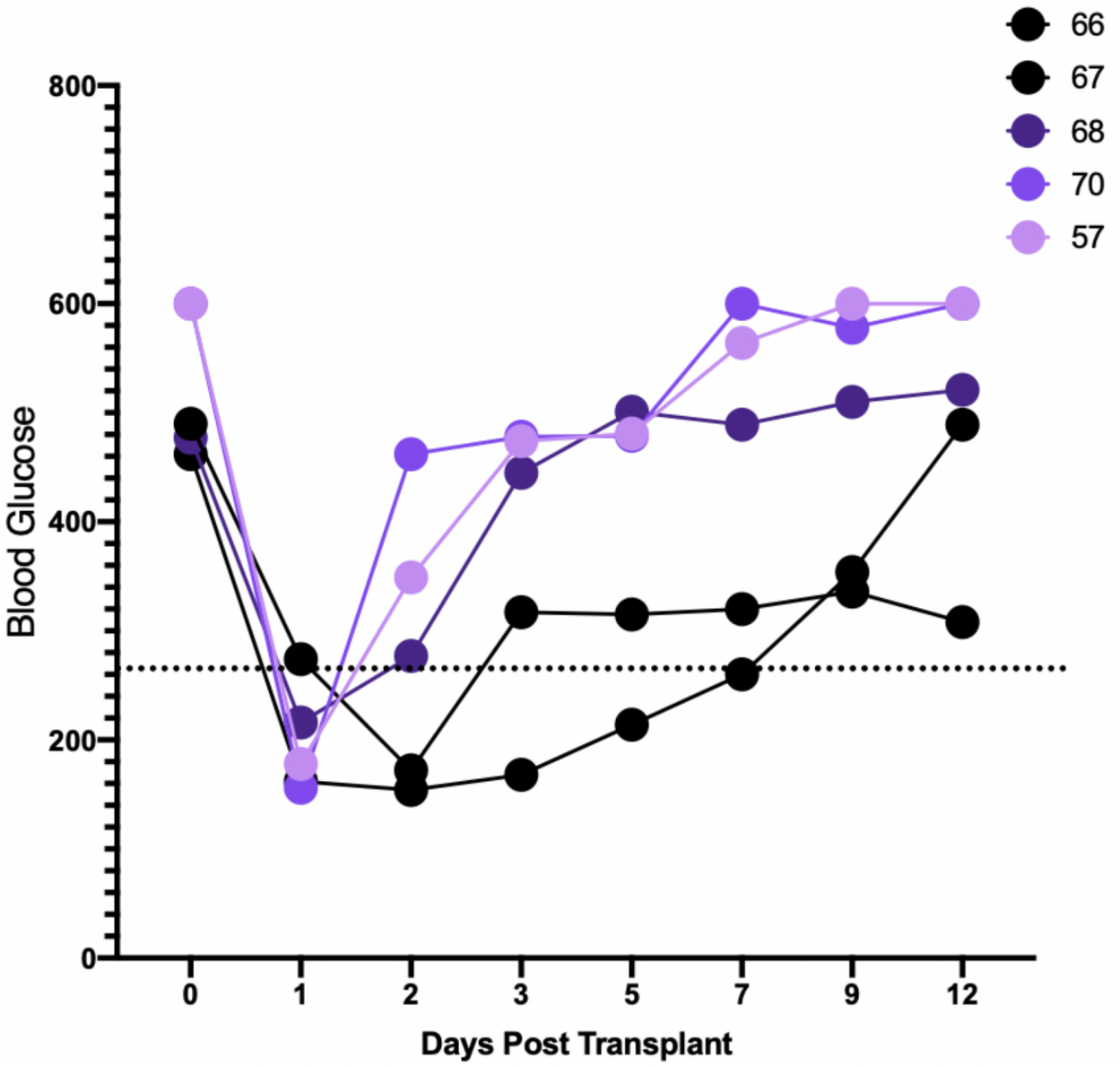


Figure 2.16. Blood glucose curves for 4-week-old NOD mice receiving islet grafts into the pinna of the ear. Mice that received α-CD45RB are shown in color, and untreated mice are in black. A dotted line represents a blood glucose of 250; two consecutive values over 250 indicate loss of the islet graft. Each number represents one animal.

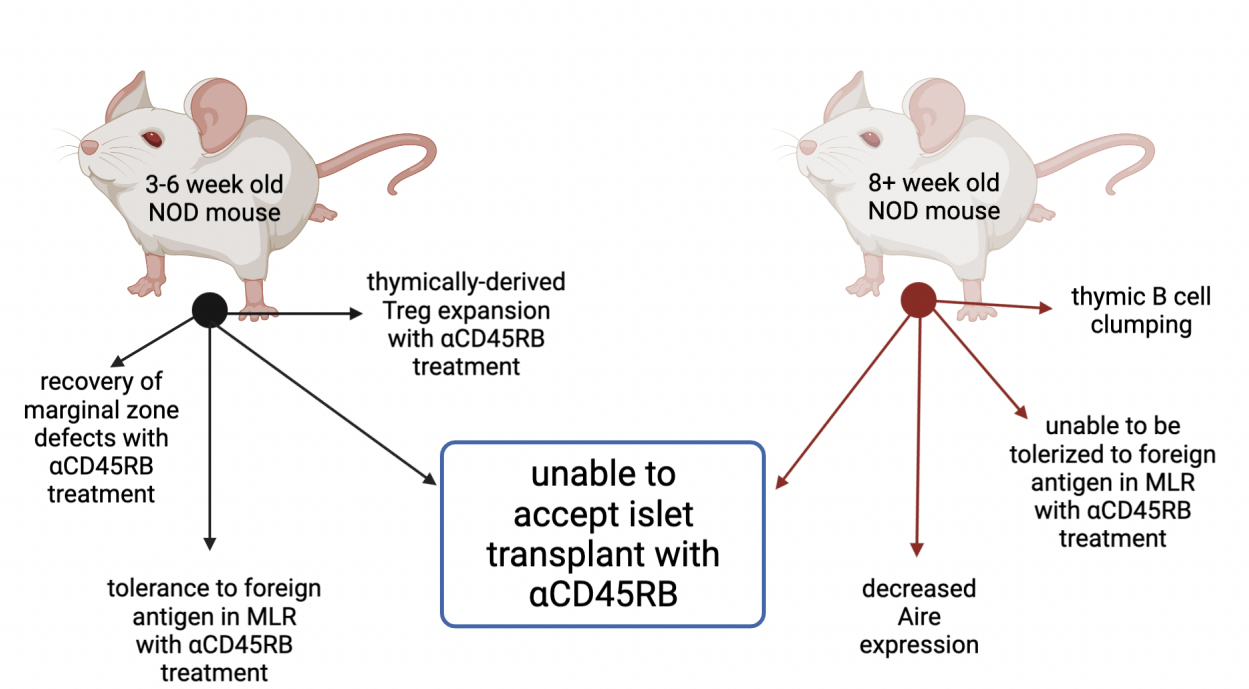


Figure 2.17. Overview of discoveries from Chapter II.

Conclusion

The increasing burden of autoimmunity as NOD mice age revealed opportunities for early intervention to establish a more permanent tolerance. I discovered that thymic B cells reflect disparities found in splenic B cells in NOD mice, including a notable expansion of pathogenic Marginal Zone-like B cells. Increased thymic B lymphocytes have also been found in other autoimmune conditions, including myasthenia gravis and systemic lupus erythematosus (SLE). While thymic B cells are meant to be presenting self-antigen for Treg development, the increased number of B cells in these autoimmune conditions seems to lead to an increase in Tregs that still fail to protect from autoimmune attack.

In the 20-week-old NOD thymus, B cells appeared clumped together as if in a germinal center. Although these cells were not verified to be germinal center B cells, thymic B cells have been known to create germinal centers in both T1D and other autoimmune diseases like SLE (Hidalgo et al., 2020). Additionally, germinal centers in the NOD thymus are thought to produce autoantibodies that bind to mTECs and lead to apoptosis of mTECs, therefore indirectly decreasing the cells that are capable of aiding in negative selection (Pinto et al., 2018). While I did not measure autoantibodies in the NOD thymus, it does appear that the increase in thymic B lymphocytes with increased age is not incidental, and may correlate with the decreased expansion of thymically-derived Tregs in older α-CD45RB-treated mice. Furthermore, while B cells are present in similar numbers to the non-autoimmune B6 mice, their Aire expression is significantly decreased. Further investigation into how B lymphocytes interact with developing Tregs would uncover the role of the expanded thymic B cell population.

I discovered that in the time before NOD mice are overburdened with autoimmunity, they appear to respond to α-CD45RB treatment. α-CD45RB increased thymically-derived Tregs and reduced the percentage of pathogenic Marginal Zone B lymphocytes. When I investigated whether this expansion of Tregs was due to enhanced sensitivity to IL-2 signaling, I found no changes in NOD splenocytes with α-CD45RB treatment, even at a young age. Interestingly,

these data show that B6 splenocytes, both treated and untreated, have a higher level of phosphorylation of STAT5, STAT3, and MEK at baseline, even before additional IL-2 stimulation. This indicates increased baseline IL-2 signaling; this, in conjunction with increased receptiveness to stimulation with IL-2, corroborates findings from the literature about IL-2 related deficits in the NOD mouse. As the mechanism of α-CD45RB is still unknown, future studies might interrogate the mechanism by which this therapy is able to promote tolerogenic changes in young mice.

While α-CD45RB was able to tolerize young mice to foreign antigens in an ex-vivo model of transplantation tolerance, the transplants failed in vivo. Islet loss from both the pinna of the ear and under the kidney capsule occurred very quickly, within the first week of transplant and before completion of the course of α-CD45RB. As α-CD45RB treated mice lost grafts before their untreated counterparts, it seems unlikely that the loss was due to an immunologic etiology. While untreated islet grafts were surrounded by lymphocytes on histologic examination, the α-CD45RB-treated graft showed hemorrhage and hemosiderin-laden macrophages. It seems likely that α-CD45RB interferes with revascularization of the islet graft in young mice. The standard transplant location, under the kidney capsule, is in a very vascular area that is still developing in young mice. I hoped that switching to a site requiring less surgical intervention would alleviate concerns of vascular interference, but mice with transplants into the pinna of the ear also lost grafts quickly. Future studies may attempt transplant into other locations, as it seems that young mice may still be able to be tolerized to islet transplant. Additionally, other tolerizing agents that have failed to tolerize older NOD mice may be attempted; the unknown mechanism of α-CD45RB may mean the treatment is not appropriate for animals undergoing puberty.

In this chapter, I uncovered a window in which NOD mice may be susceptible to tolerizing interventions. If unchecked, an autoimmune cascade results in B cells that both produce autoantibodies and present antigen to destructive T lymphocytes, leading to demolition

of the pancreatic islets of Langerhans. However, intervention before 6 weeks of age leads to recovery of defects in B lymphocyte subsets and the ability to expand thymically-derived Tregs with α-CD45RB treatment. The presence of an age window in which mice may be tolerized to foreign antigen brings to mind the Nobel Prize-winning work of Billingham, Brent, and Medawar showing that mice can accept foreign antigens when tolerized in utero (Billingham et al., 1953). If mice are tolerizable in utero, at what stage does progression of autoimmunity permanently impact antigen tolerance? Furthermore, what natural changes occur during pregnancy to facilitate this tolerance? This work has identified a promising opportunity to slow autoimmunity in NOD mice and offered new questions about the timing of inducible immune tolerance.

CHAPTER III

FRAGILE TOLERANCE TO PREGNANCY IN NOD MICE IS DISRUPTED BY IL-6

Introduction

Similar to transplant, pregnancy is another test of inducible immune tolerance. Pregnancy requires endogenous acceptance of and tolerance to MHC-mismatched foreign fetal antigens over the course of gestation. This requirement can coexist with autoimmunity; it is possible to simultaneously accept foreign fetal tissue and experience autoreactivity towards endogenous organs.

This careful inducible immune tolerance to the developing fetus requires multiple immune adaptations. Tregs play a tremendous role in protecting the fetus; they have been found to adapt to the uterine environment and predict risk of spontaneous abortion (Wienke et al., 2020; Winger and Reed, 2011). Administration of IL-2 can reduce adverse pregnancy outcomes in an abortion-prone mouse model (Chen et al., 2013). Tregs present at the placental:uterine interface are incredibly specific; depletion of uterine Tregs reactive to the Y antigen results in selective destruction of male fetuses (Kahn and Baltimore, 2010).

Much less is known about the functionality of B cells in the placenta. Pregnant patients in the third trimester and immediately postpartum have decreased circulating B cells compared to non-pregnant patients (Lima et al., 2016). This may be attributed to the only known role for B cells in pregnancy: producing tolerogenic IL-10 (Benner et al., 2020; Busse et al., 2019). In both mice and humans, IL-10 secreting B cells have been found in the placenta, even colocalized with Tregs. While it seems unlikely that the sole function of B cells in pregnancy is IL-10 production, other roles have not been identified.

The tolerance necessary in pregnancy is challenged in T1D. Studies of patients with T1D show an increased likelihood of adverse pregnancy outcomes; however, it is difficult to tease apart the effects of hyperglycemia and those of autoimmunity (Evers et al., 2004; Groen et al., 2019; Hanson and Persson, 1998; Luo et al., 2021). Optimizing glycemia reduces risk of vascular complications like preeclampsia and pregnancy-induced hypertension, as well as other complications like preterm birth and intrauterine fetal demise; however, the absolute risk compared to patients without autoimmunity is not clear (Maresh et al., 2015).

The NOD mouse offers an opportunity to investigate autoimmunity before the onset of hyperglycemia. Previous studies done examining pregnancy in the NOD mouse have focused on the impact of diabetes on gestation (Albaghdadi and Kan, 2012; Koczwara et al., 2004) or have used diabetic NOD mice. Data from The Jackson Laboratory shows that NOD mice have similar numbers of live pups as non-autoimmune B6 mice, but investigation into the actual phenotype of pregnancy is understudied (Figure 3.1).

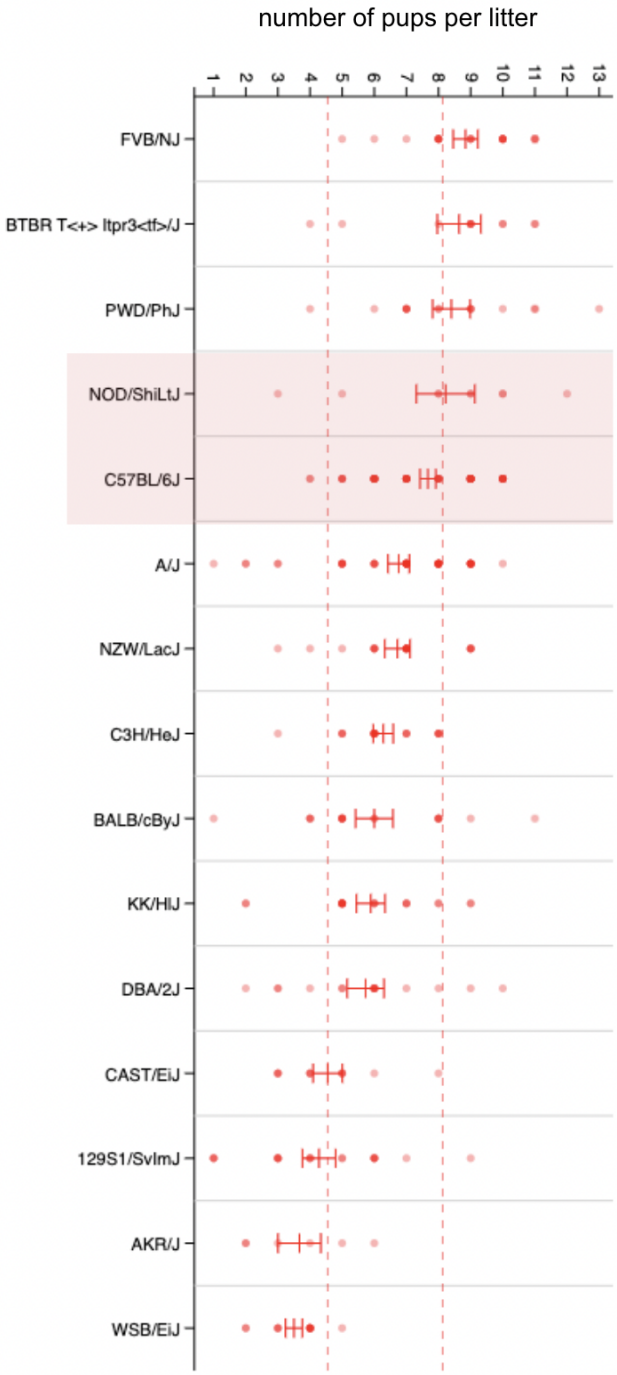


Figure 3.1. Common inbred mouse strains with litter size information shown. B6 and NOD mice are highlighted in pink. Graph adapted from (Mouse Phenome Database at The Jackson Laboratory, 2010).

In this chapter, I examine the effects of unchecked autoimmunity on pregnancy by studying euglycemic NOD mice during the pre-diabetic, autoimmune interval. I phenotype the lymphocyte presence in the placenta in both syngeneic and allogeneic pairings of B6 and NOD mice to uncover the effect of MHC-mismatch on gestational tolerance. I impede the function of Tregs by both depleting with a monoclonal antibody against CD25, and using an IL-2 neutralizing antibody. I investigate amniotic fluid cytokine levels, and uncover differences between mouse strains in all IL-6 family members; when IL-6 is neutralized, pregnancy outcomes improve in NOD mice. This chapter illustrates contributions of autoimmunity to adverse pregnancy outcomes (Figure 3.2).

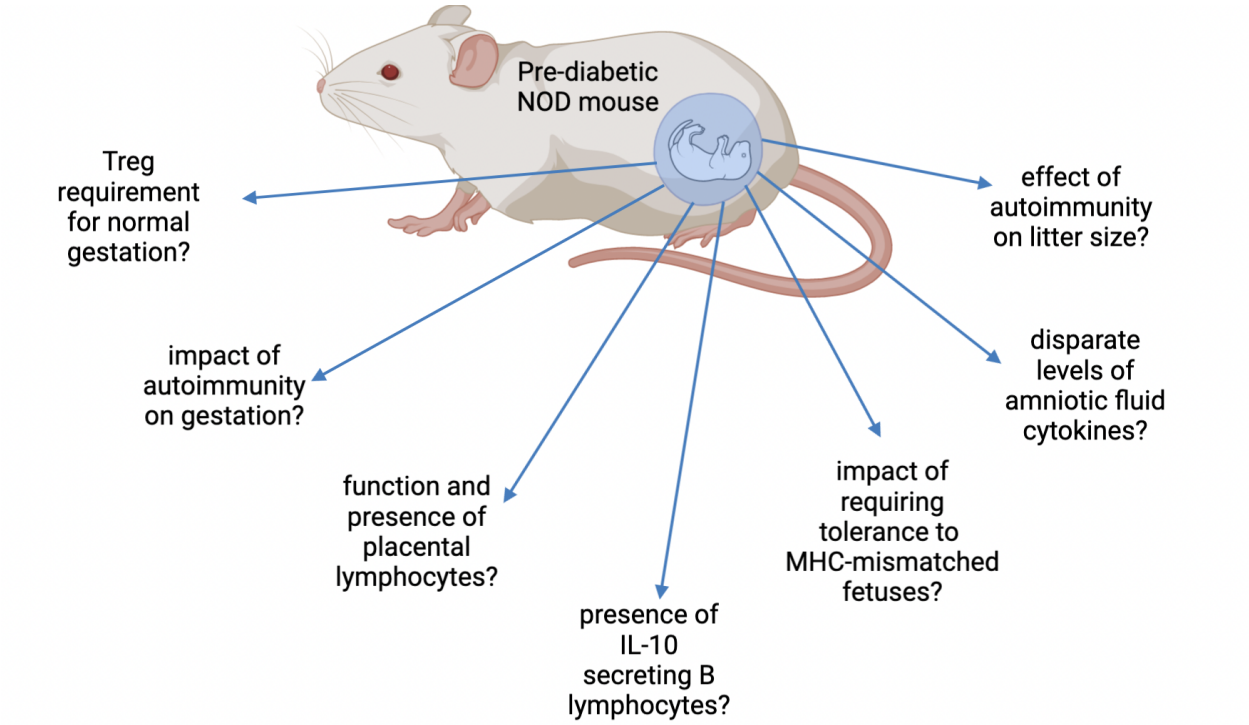


Figure 3.2. Overview figure illustrating possible sequelae of autoimmunity during pregnancy.

Results

NOD Mice Have Poor Pregnancy Outcomes

Given the increased pregnancy complications in patients with T1D, I hypothesized that the autoimmunity would have an independent effect on pregnancy outcomes. I chose to examine pre-diabetic NOD mice in order to remove any effects of hyperglycemia on pregnancy; however, mice used in these experiments were over 8 weeks of age, so autoimmune characteristics were present. Litters from B6 females mated with B6 males (B6♀B6♂) were examined at day 17.5 of gestation and compared to NOD females mated with NOD males (NOD♀NOD♂). NOD♀NOD♂ litters had an increased percentage of reabsorbed and abnormal fetuses compared to B6♀B6♂ litters; 97% of B6♀B6♂ fetuses were normal whereas only 79% of NOD♀NOD♂ were normal (Figure 3.3).

I investigated whether the difference in percentage of normal-appearing pups may be due to alterations in the lymphocyte presence in the placenta. I digested each placenta individually, and analyzed them separately by flow cytometry. As a percentage of total cells in the placenta, NOD♀NOD♂ have far fewer B lymphocytes than B6♀B6♂ placentas, as well as a decrease in CD8+ T cells (Figure 3.4A). The deficit in B lymphocytes was confirmed by histopathologic analysis (Figure 3.4B, C).

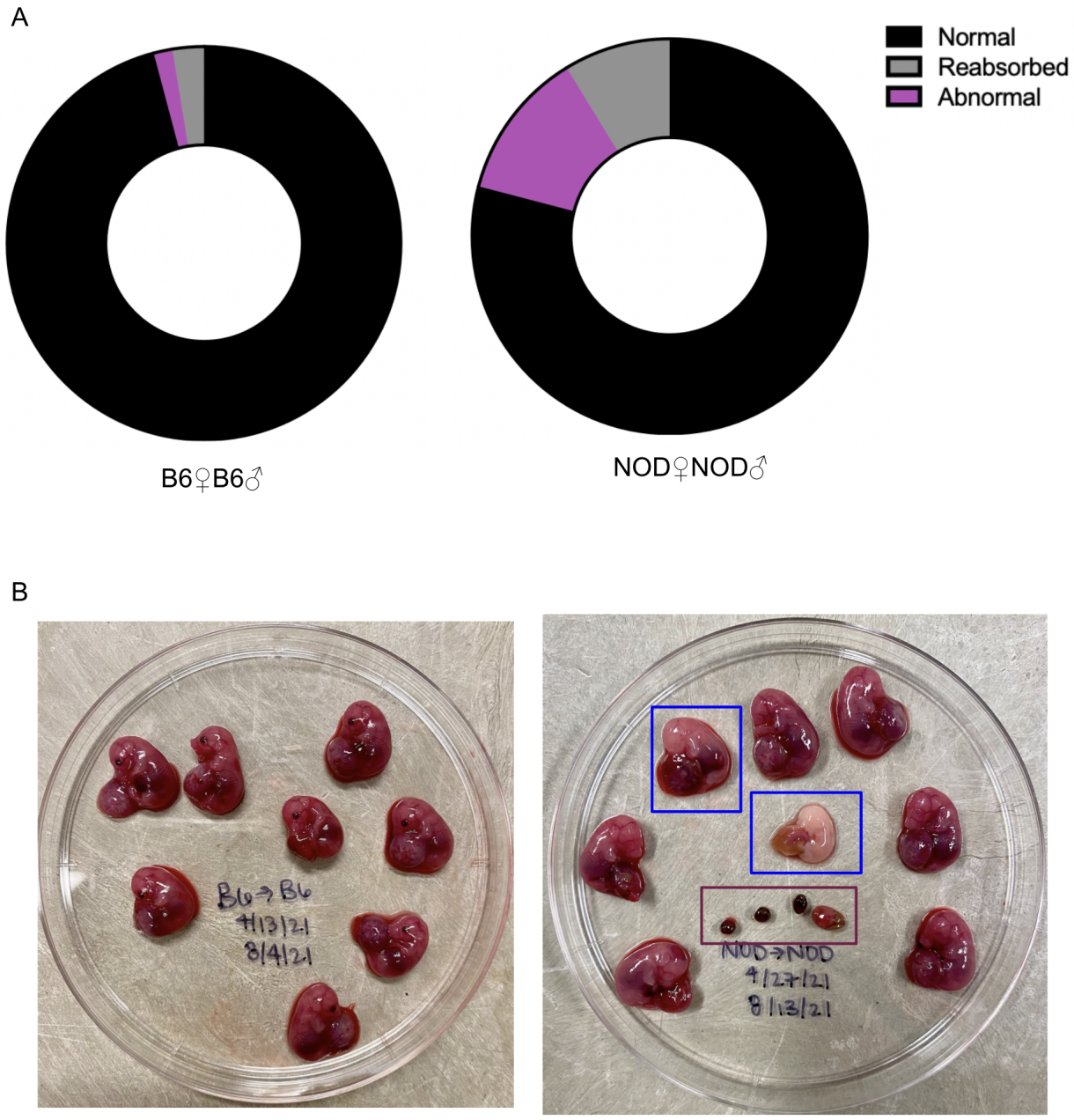


Figure 3.3. Composite pregnancy outcomes for B6 (20 pregnancies, 97% successful) and NOD (19 pregnancies; 79% successful) mice, showing an increase in both reabsorbed and abnormal fetuses in the NOD mouse (A). Representative images of B6 (left) and NOD (right) litters. Outlined in blue are two abnormal fetuses (small and pale) The NOD litter also has four reabsorbed fetuses, outlined in maroon (B).

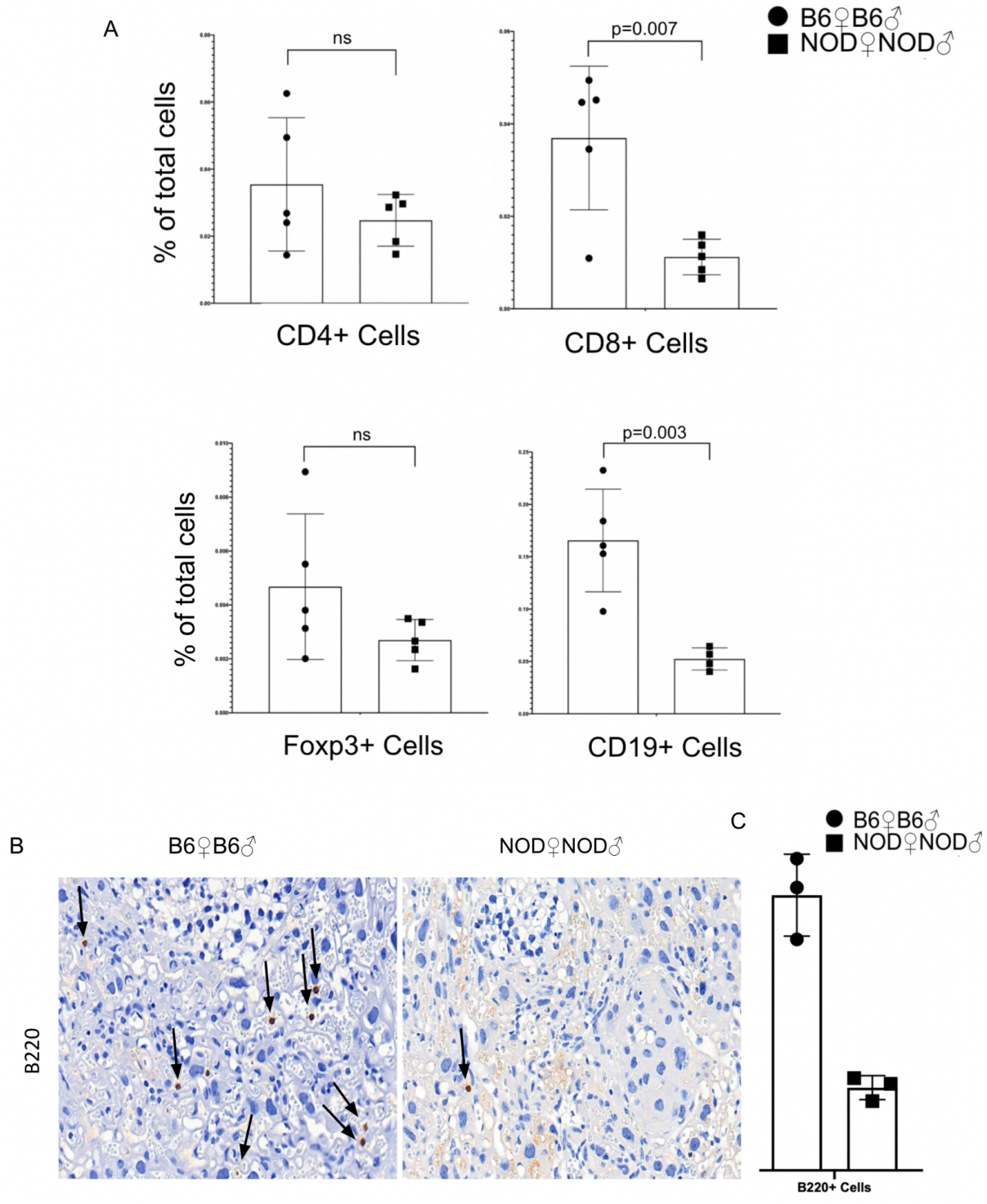


Figure 3.4. Immune cells as a percentage of total cells in the B6 and NOD placenta. Each point is an average of all the placentas harvested from that mouse (A) B cell presence was confirmed via IHC staining of B6♀B6♂ (left) and NOD♀NOD♂ (right) placentas for B220. Arrows illustrate positive cells (B) Graphical representation of number of B220+ cells per 1.2 mm2 field of view. Each point represents the average of three random 1.2 mm2 circles. NOD mice have decreased B220+ B cells. (C) Data shown is representative of multiple experiments and analyzed by individual t-tests.

Poor Pregnancy Outcomes in NOD Mice are Worsened by Allogeneic Pairings

NOD mice seem to have an increase in poor pregnancy outcomes even without the additional challenge of MHC-mismatch. However, to truly assess the impact of autoimmunity while controlling for potential strain-specific differences in the pups, I assessed the impact of autoimmunity in gestation by crossing B6 and NOD mice to create genetically identical litters. Mice were either paired as B6 females mated with NOD males (B6♀NOD♂) or NOD females mated with B6 males (NOD♀B6♂). Litters from these pairings differ only by the uterine environment in which pups are gestated. I hypothesized that an allogeneic mating would increase reabsorbed and abnormal fetuses gestated in an NOD uterine environment. The NOD (NOD♀B6♂) uterine environment resulted in increased reabsorbed and abnormal pups as compared to B6♀NOD♂ litters (Figure 3.5A, B). Whereas 98% of B6♀NOD♂ fetuses were normal, only 67% of NOD♀B6♂ fetuses appeared normal.

Flow cytometric evaluation of placental immune cells revealed persistent alterations in the immune cell compartment. Similar to syngeneic pairings, NOD♀B6♂ placentas had a lower percentage of CD8+ cells of total cells and an even greater decrease in CD19+ cells than B6♀NOD♂ (Figure 3.6A). Histologic examination corroborated the decrease in B cells associated with the NOD maternal environment (Figures 3.6B, C).

I assessed whether the alterations in immune cells were due to maternal or fetal abnormalities by staining for the B6 MHC Class I, H2Kb, on immune cells in allogeneic NOD♀B6♂ pairs. In placenta, as validated with uterine-draining lymph nodes, PBMCs, and spleen, all immune cells were H2Kd+, indicating that the cells were maternal and not fetal in origin (Figure 3.7). These data show that changes in maternal lymphocytes are associated with the abnormal gestational environment in NOD mice that results in larger numbers of abnormal and reabsorbed pups.

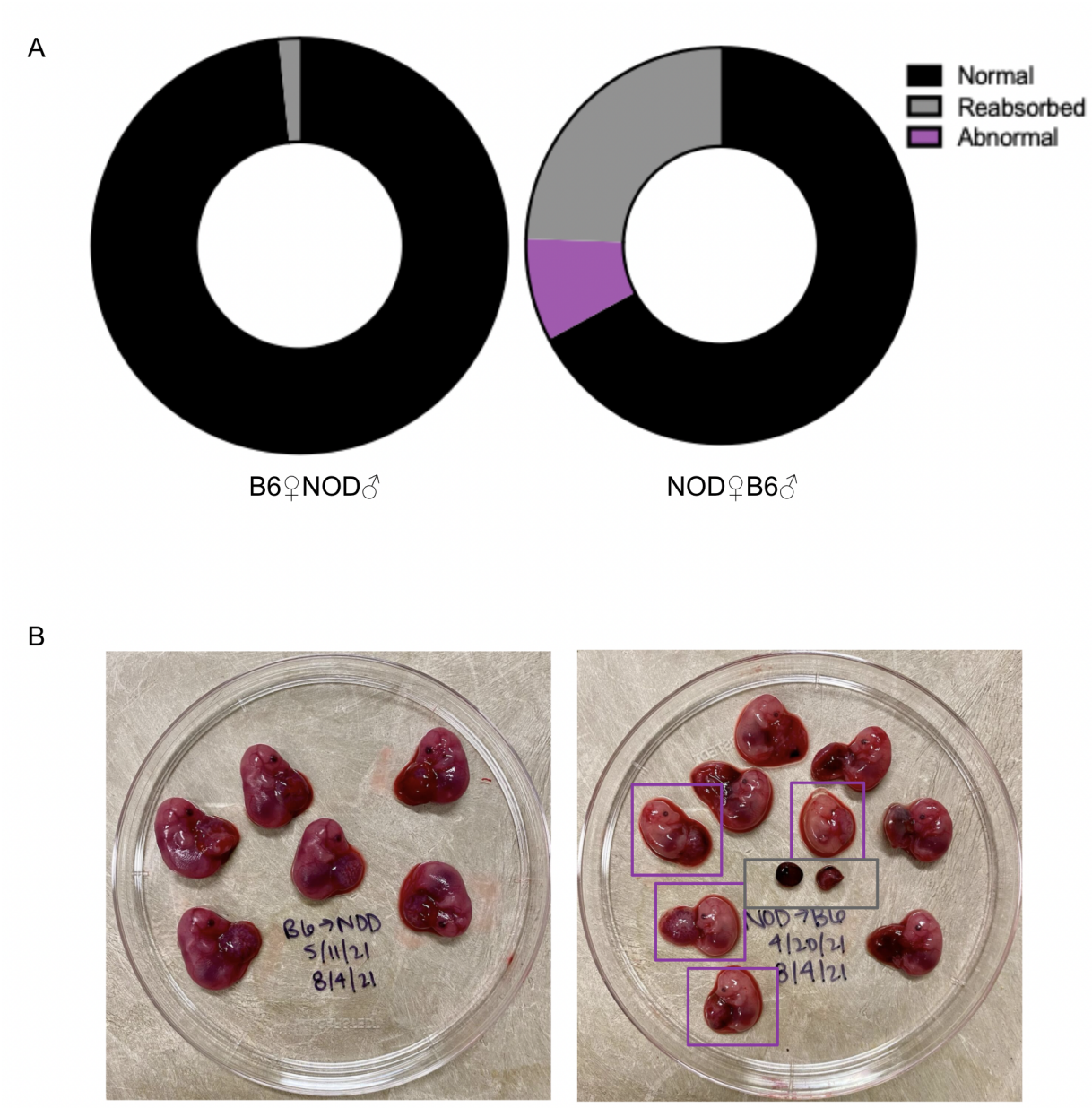


Figure 3.5. Composite pregnancy outcomes for B6 (20 pregnancies; 98% normal) and NOD (13 pregnancies; 67% normal) mice, showing an increase in both reabsorbed and abnormal fetuses in the NOD mouse (A). Representative images of B6 (left) and NOD (right) litters. Outlined in maroon are four abnormal fetuses (small and pale) The NOD litter also has two reabsorbed fetuses, outlined in grey (B).

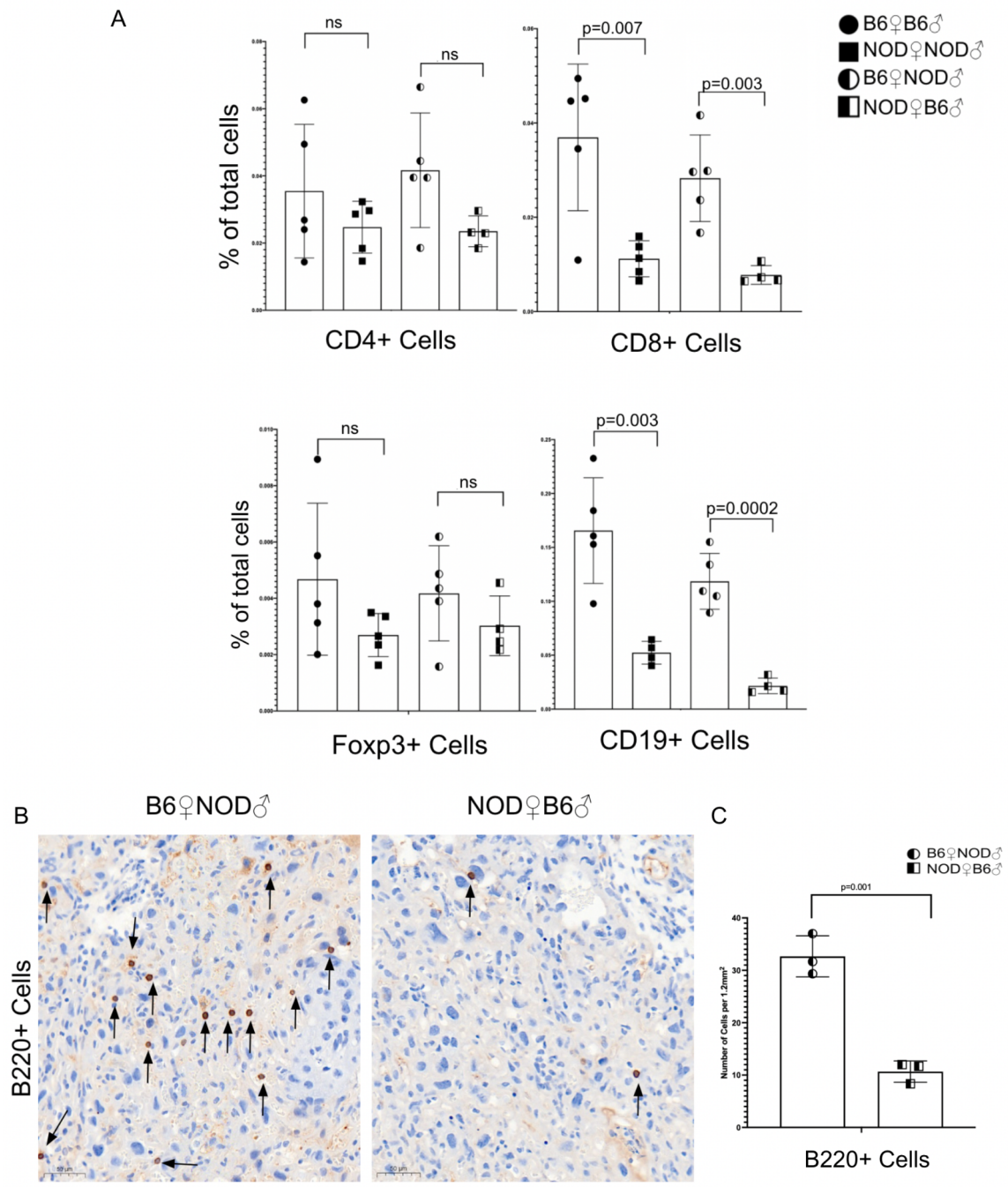


Figure 3.6. Immune cells as percentage of total cells in the B6 and NOD placenta, shown with B6♀B6♂ and NOD♀NOD♂ pairings (A). IHC staining of B6♀NOD♂ (left) and NOD♀B6♂ (right) placentas for B220. Arrows illustrate positive cells (B). Graphical representation of number of B220+ cells per 1.2 mm2 field of view. Each point represents the average of three random 1.2 mm2 circles. NOD mice have decreased B220+ B cells (C). Data shown is representative of 3+ experiments and analyzed by individual t-test.

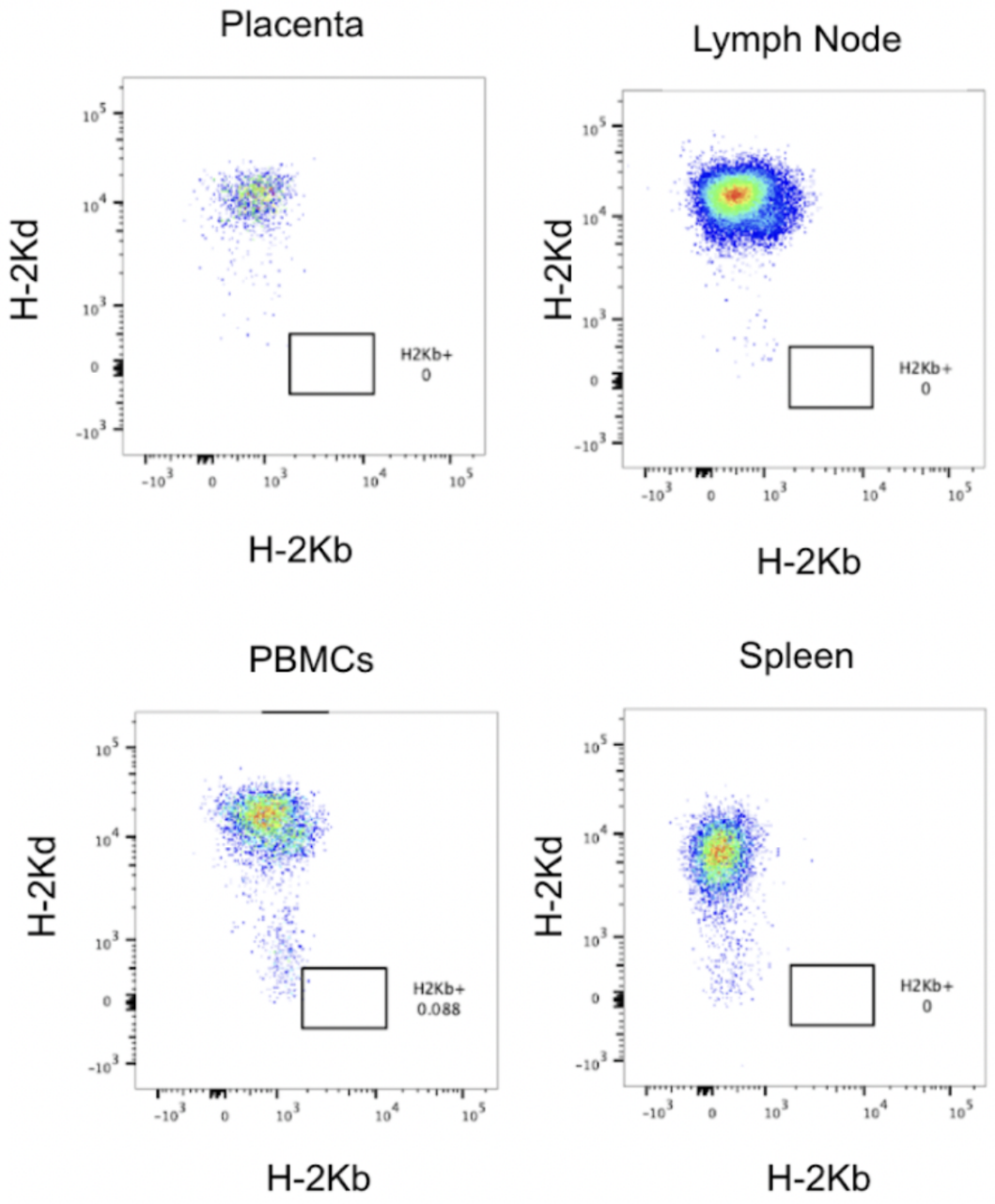


Figure 3.7. Lymphocytes in the placental of NOD♀B6♂ mice are maternal. Flow plots from a NOD♀B6♂litter with H2kd (NOD MHC Class I) vs H2Kb (B6 MHC Class I) show that in Placenta, Lymph Nodes, PBMCs, and spleen, all cells are H2Kd+ and therefore maternally-derived.

B Cell Subsetting in the NOD Placenta Reveals Sweeping B Cell Deficits

Both NOD♀NOD♂ and NOD♀B6♂ placentas seem to have a deficit of B cells compared to B6 placentas. In other organs, NOD mice have an expansion of pathogenic B cells with a marginal zone-like phenotype. I performed B cell subsetting in the placenta to identify whether any specific B cell type was responsible for the B cell deficit (Figure 3.8). NOD♀B6♂ placentas appear to have deficits in both Follicular and Marginal Zone-like B cells, with an almost undetectable level of Marginal Zone-like B cells. As such, the deficit in NOD mice cannot be attributed to a specific cell subset.

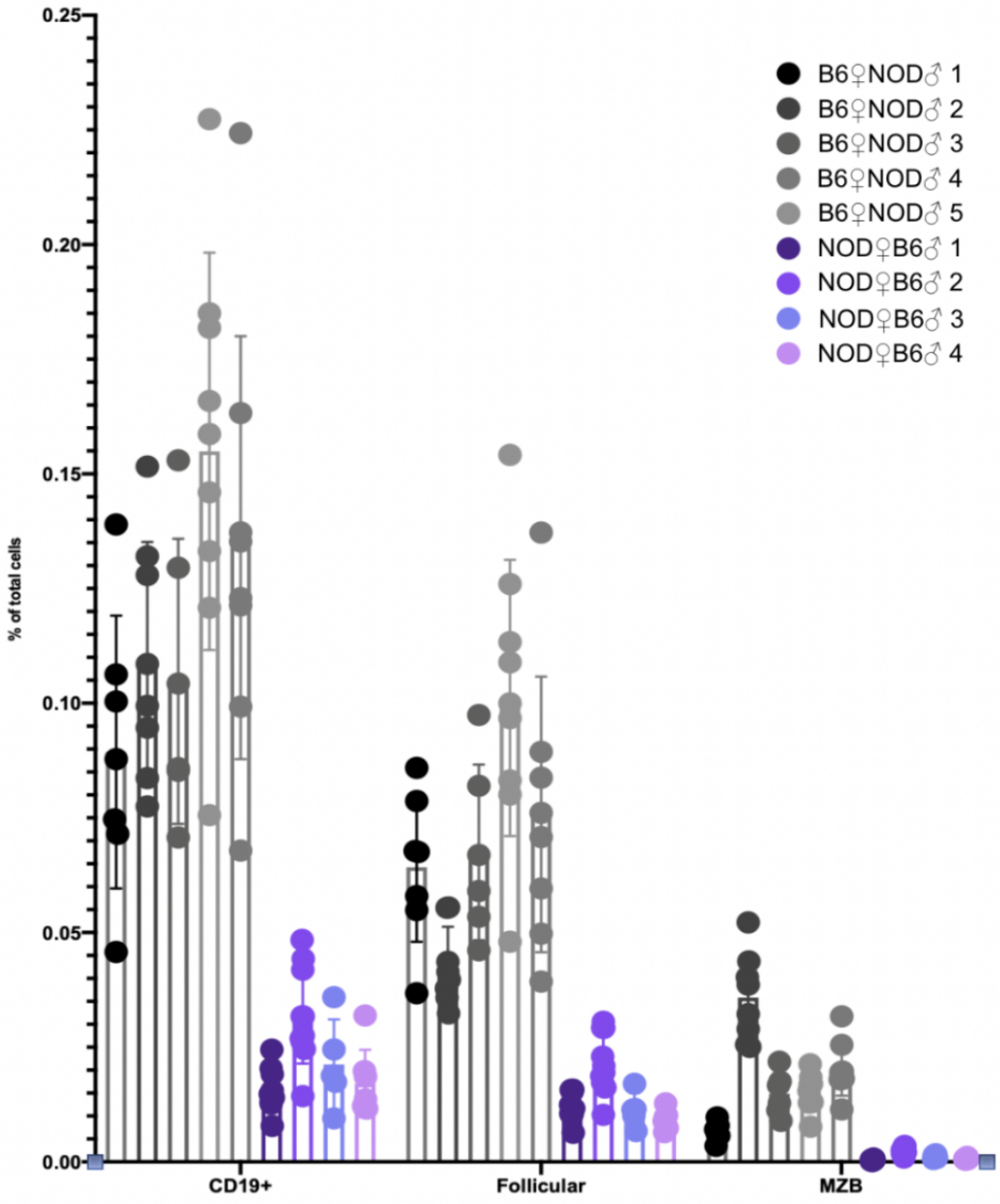


Figure 3.8. B cell subsetting reveals an overall decrease in CD19+ cells in NOD♀B6♂ litters (purple), with corresponding decreases in both follicular and marginal zone-like B cells. Each dot represents one placenta, with individual mice as different columns. Data shown represents 3+ experiments.

Gestation in NOD Mice is Extremely Vulnerable to Treg Depletion

Although NOD and B6 placentas did not show a significant difference in Tregs as a percentage of total cells, Tregs remain a crucial cell type for gestational tolerance. The similar number of Tregs in the NOD placenta may reflect the Treg deficit in the NOD mouse–a deficit not of number, but of functionality. To examine the impact of Tregs on protecting the developing fetus, B6 and NOD mice were treated with an αCD25 monoclonal antibody to deplete Tregs on days 5 and 10 of gestation. I found that NOD mice are extremely susceptible to Treg depletion; 96% of pups from B6♀NOD♂ pairs were normal and only 19% of pups from NOD♀B6♂ pairs were normal (Figure 3.9).

Interestingly, the percentage of Tregs of CD45+ cells in the placenta are different between B6♀NOD♂ and NOD♀B6♂ αCD25-treated placentas. While data from the spleen indicates that circulating Tregs were reduced from untreated mice for both B6♀NOD♂ and NOD♀B6♂ litters, the B6♀NOD♂ placentas have an increase in Tregs from the untreated mouse, whereas the NOD♀B6♂ have a decrease (Figure 3.10). These data indicate that the B6 mouse is able to prioritize Tregs in the uterus when Tregs are partially depleted peripherally; the NOD mouse appears to lack this capacity, resulting in fetuses that are poorly tolerated (abnormal or reabsorbed).

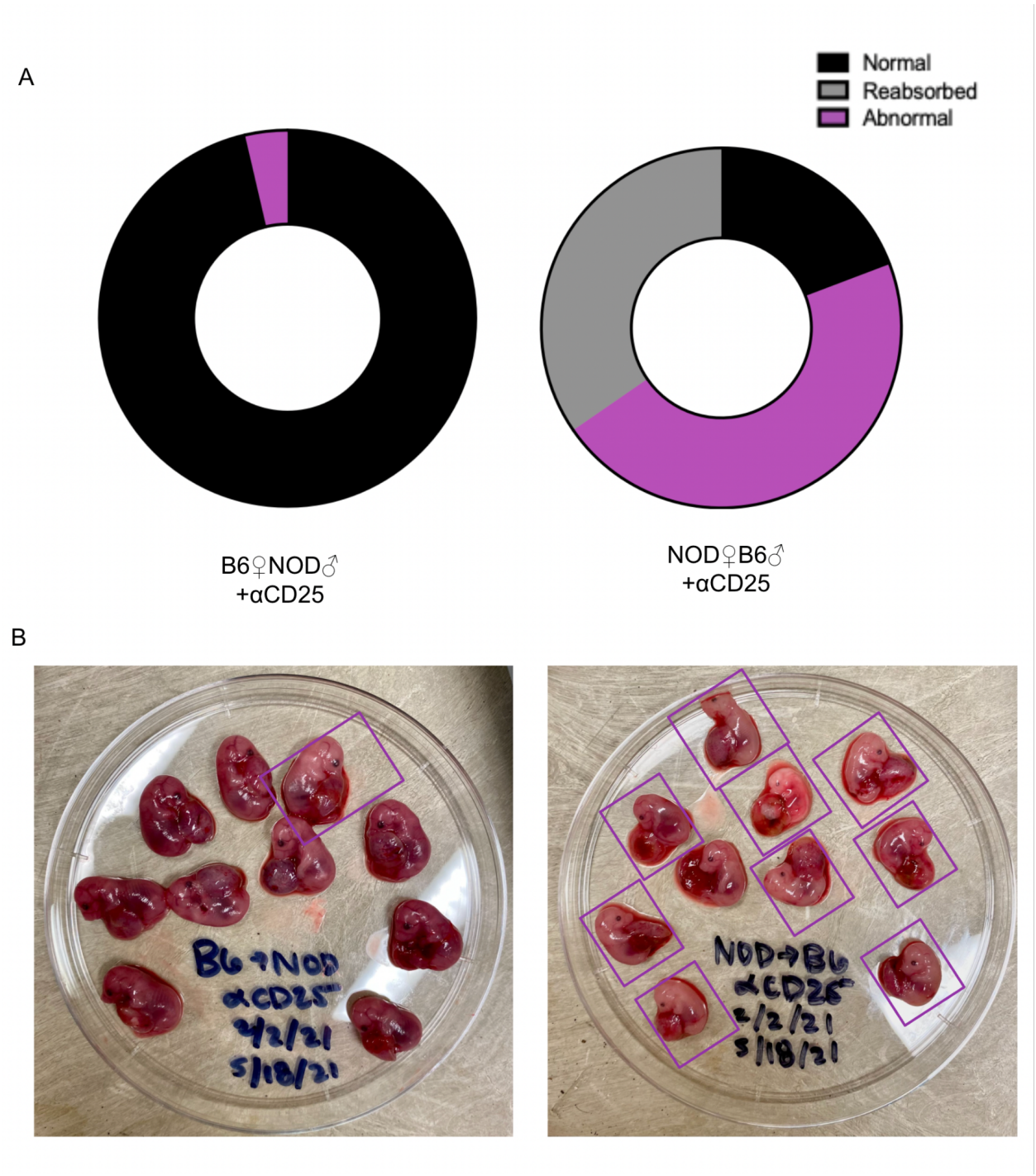


Figure 3.9. Composite pregnancy outcomes for B6 (12 pregnancies; 96% successful) and NOD (3 pregnancies; 19% successful) mice treated with αCD25, showing an overwhelming increase in both reabsorbed and abnormal fetuses in the NOD mouse (A). Representative images of B6 (left) and NOD (right) litters. Outlined in maroon is one abnormal fetus in the B6 litter (pale) and nine abnormal fetuses for the NOD litter (all very small with one purulent fetus) (B).

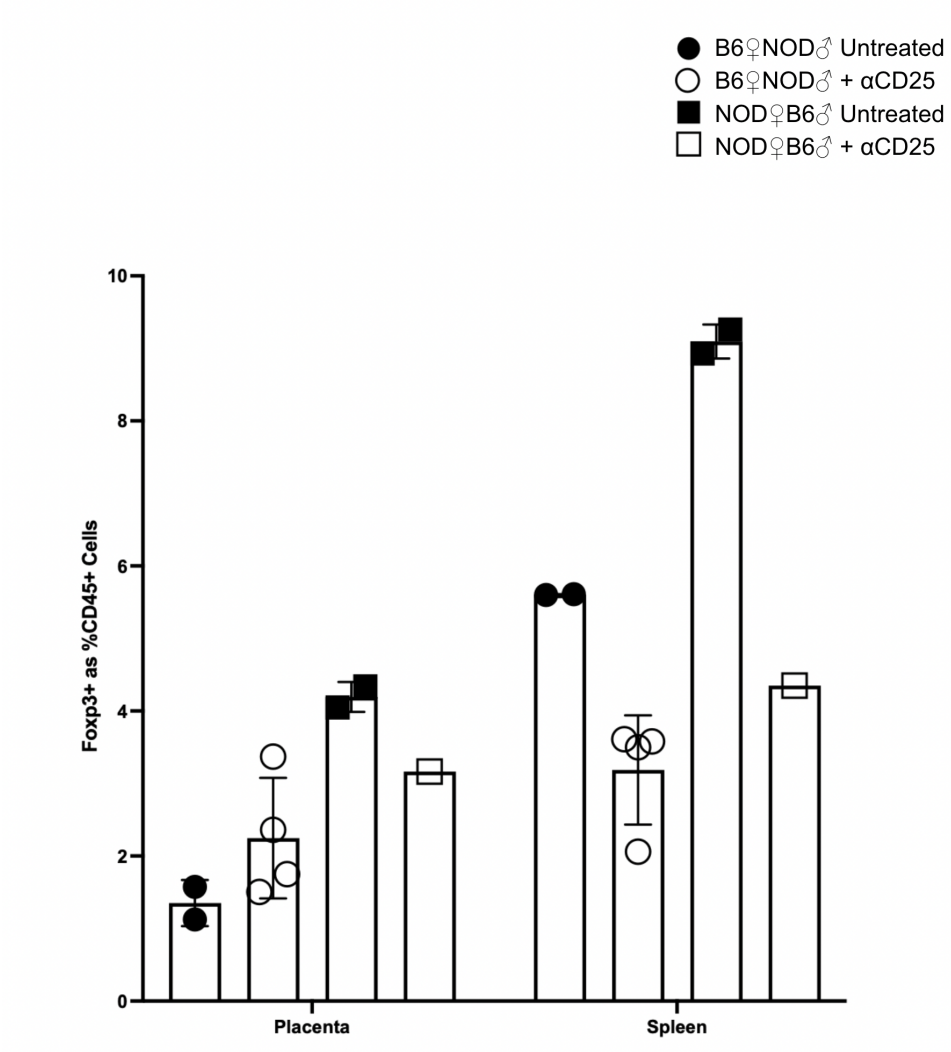


Figure 3.10. Foxp3+ cells as a percentage of CD45 positive cells in both placenta and spleen. Each dot represents an average of all placentas from that mouse. Splenic Foxp3+ cells show a decrease with αCD25 treatment for both B6 and NOD mice. However, B6 mice have an increase in placental Tregs from baseline, whereas NOD mice have a decrease.

Gestation in NOD Mice is Negligibly Impacted by IL-2 Neutralization

The staggering deficits induced by αCD25 created a model that was difficult to analyze because of the fetal loss in NOD mice. I next chose to treat with a monoclonal antibody that neutralizes IL2 (αIL-2), which should hinder Treg function and survival, but perhaps allow for more persistent cell survival than αCD25 depletion. αIL-2 treatment had a much more modest effect; 85% of pups from B6♀NOD♂ pairs were normal and 72% of pups from NOD♀B6♂ pairs were normal (Figure 3.11). αIL-2 neutralization had a greater impact on B6♀NOD♂ pairs than αCD25 treatment, indicating that Tregs from the B6 mouse are capable of functioning with lower numbers, but are reliant on a larger IL-2 cytokine presence than NOD mice. In contrast, NOD mice, which are at baseline affected by poor IL-2 sensitivity, seemed to be unaffected by IL-2 neutralization.

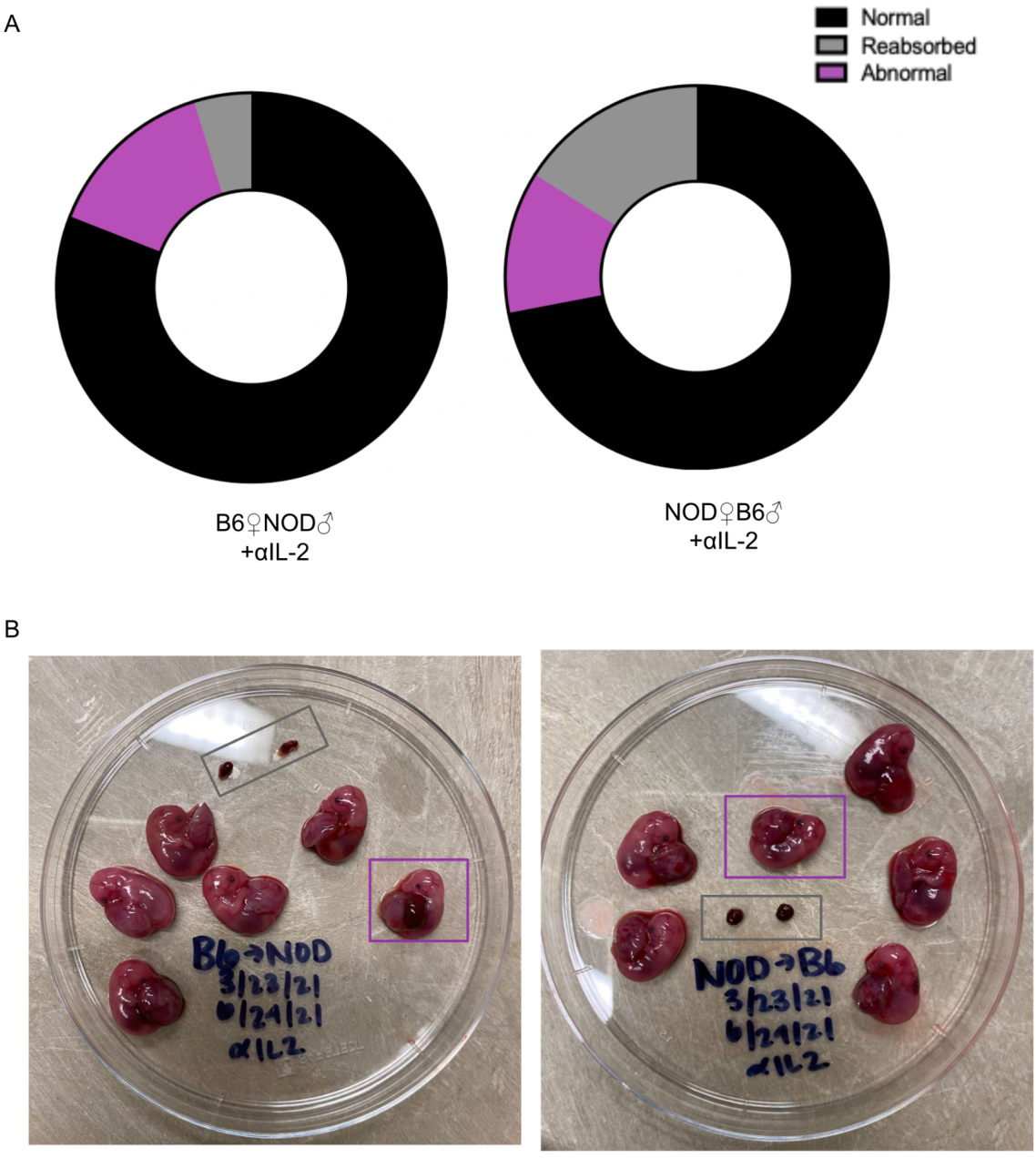


Figure 3.11. Composite pregnancy outcomes for B6 (3 pregnancies; 85% successful) and NOD (3 pregnancies; 72% successful) mice treated with αIL-2, showing similar reabsorbed and abnormal fetuses between mouse strains (A). Representative images of B6 (left) and NOD (right) litters. Outlined in maroon is one abnormal fetus in the B6 litter (maroon box) with two reabsorbed fetuses (grey box) and one abnormal fetus in the NOD litter (maroon box) with two reabsorbed fetuses (grey box) (B).

Cytokine Secretion Varies Between B6 and NOD Uterine Environments

Both αIL-2 and αCD25 treatments targeted Tregs present in the placenta. However, other placental lymphocytes were different between mouse strains, indicating that either cytokines secreted by those lymphocytes or supporting those lymphocytes may be altered as well. I performed an unbiased cytokine array for 32 cytokines in amniotic fluid from syngeneic B6♀B6♂ and NOD♀NOD♂ pairs, as well as allogeneic B6♀NOD♂ and NOD♀B6♂ pairs (Figure 3.12A, B).

Amniotic fluid from the NOD uterus (both NOD♀NOD♂ and NOD♀B6♂) contained increased IL-6 family members, including IL-6, LIF, and MCP-1 (Figure 13A). Increased IL-6 secretion into NOD amniotic fluid was confirmed by ELISA, with significant increases in NOD♀NOD♂ compared to B6♀B6♂ pairs as well as NOD♀B6♂ compared to B6♀NOD♂ (Figure 3.13B).

In order to investigate lymphocyte secretion of IL-6, as well as IL-2 and IL-10 (two cytokines not detected on the multiplex array but that have been shown to function in gestational tolerance), I performed a cytokine secretion assay on CD45+ cells from placenta, thymus, and spleen from B6♀NOD♂ and NOD♀B6♂ pairs (Figure 3.14). Overall, it appears that there are more CD45+ cells secreting IL-2, IL-6, and IL-10 in the NOD♀B6♂ placenta than the B6♀NOD♂. Levels of all three cytokines appear similarly between mouse strains in the thymus. Notably, the B6 spleen has many more CD45+ cells that are IL-2-secreting than the NOD spleen. Cytokine secretion from TCRb+, B220+, and CD68+ cells in the B6♀NOD♂ and NOD♀B6♂ placenta showed an increase in IL-6 from all cell types, with little difference in IL-2 or IL-10 (Figure 3.15).

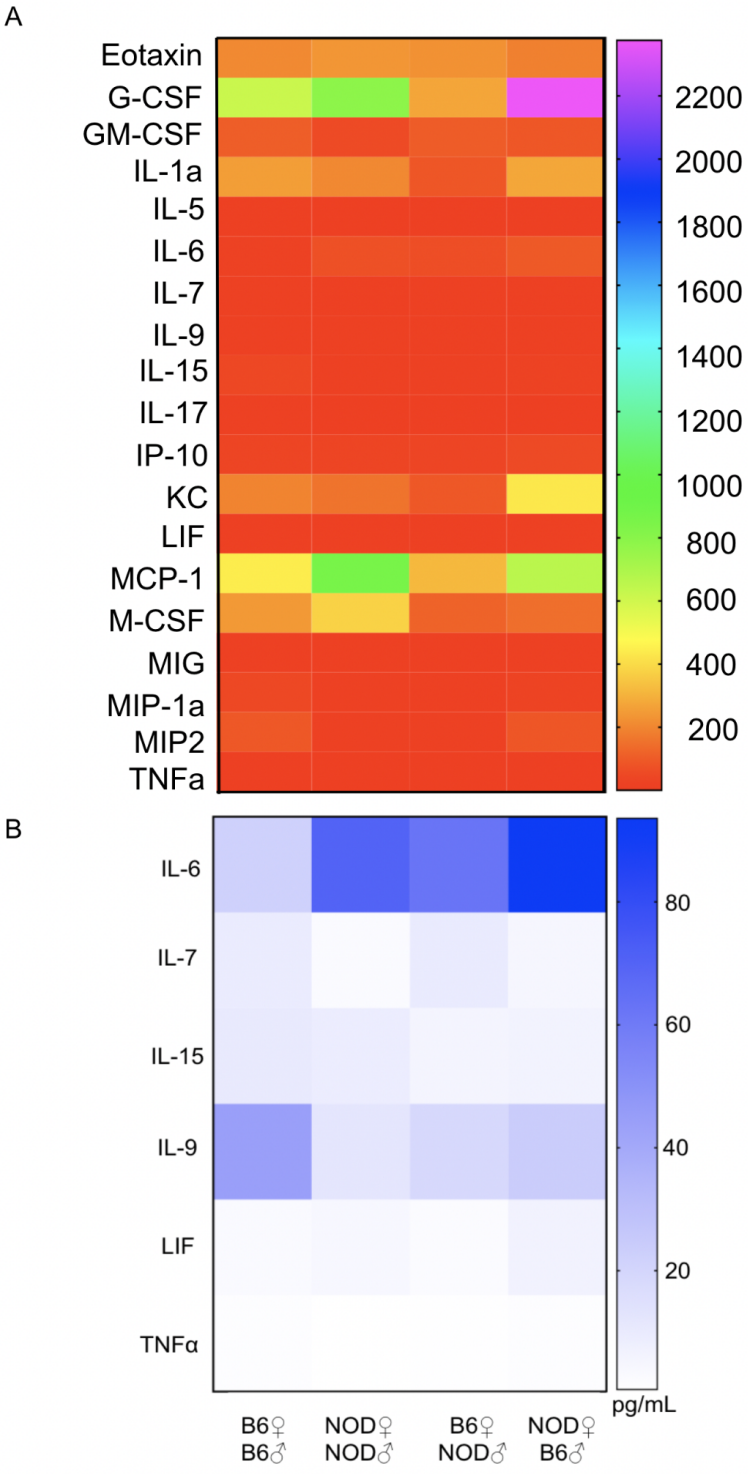


Figure 3.12. All detectable cytokines from amniotic fluid in an unbiased multiplex cytokine array (A) with selected cytokines shown with a different scale (B).

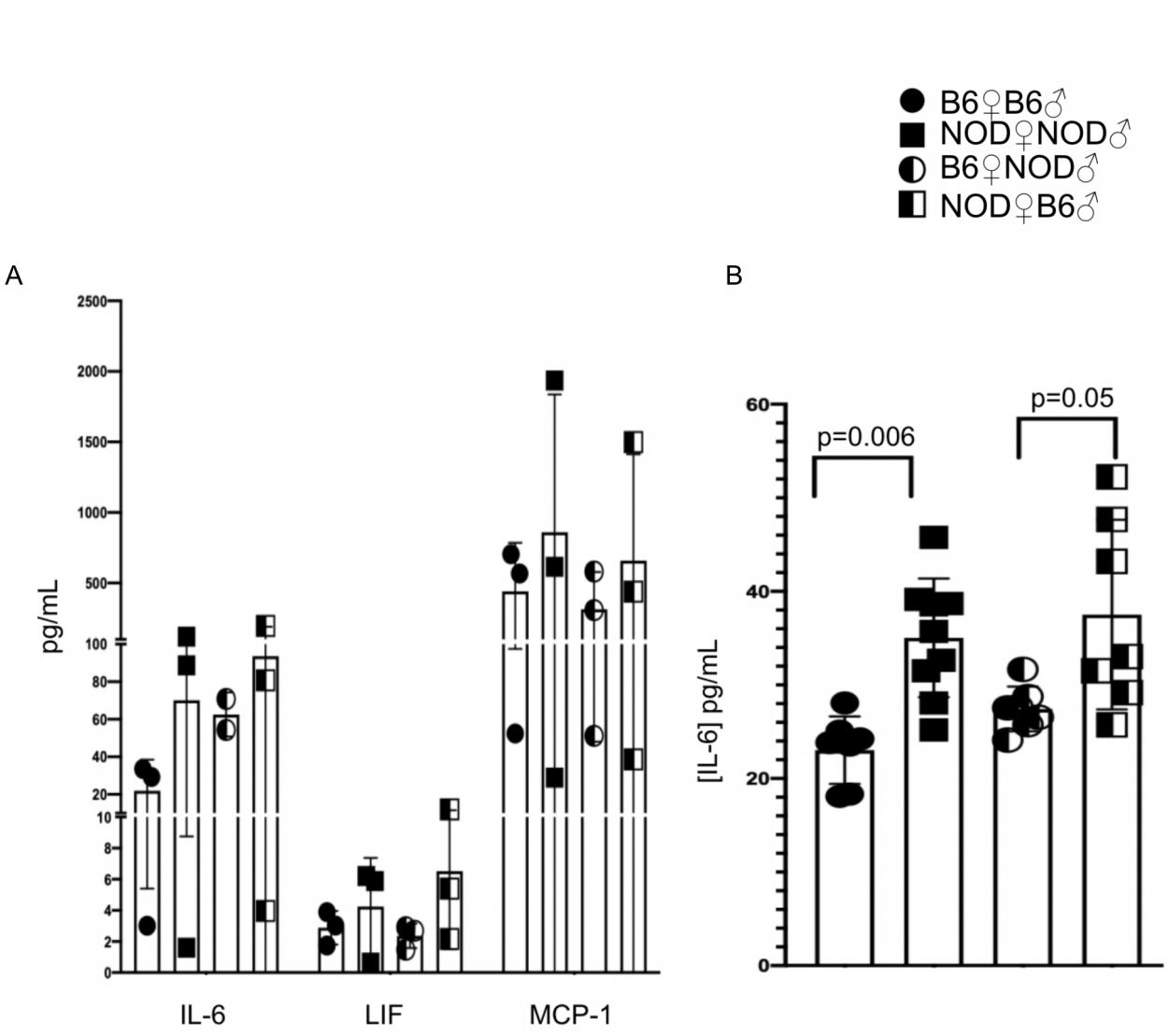


Figure 3.13. Concentrations of IL-6 family members from cytokine array. NOD♀NOD♂and NOD♀B6♂ litters have increases in IL-6, LIF, and MCP-1 (A). The increase in IL-6 in amniotic fluid from NOD mice is confirmed via ELISA (B). Each dot represents amniotic fluid from one mouse. Data was analyzed by individual t-test.

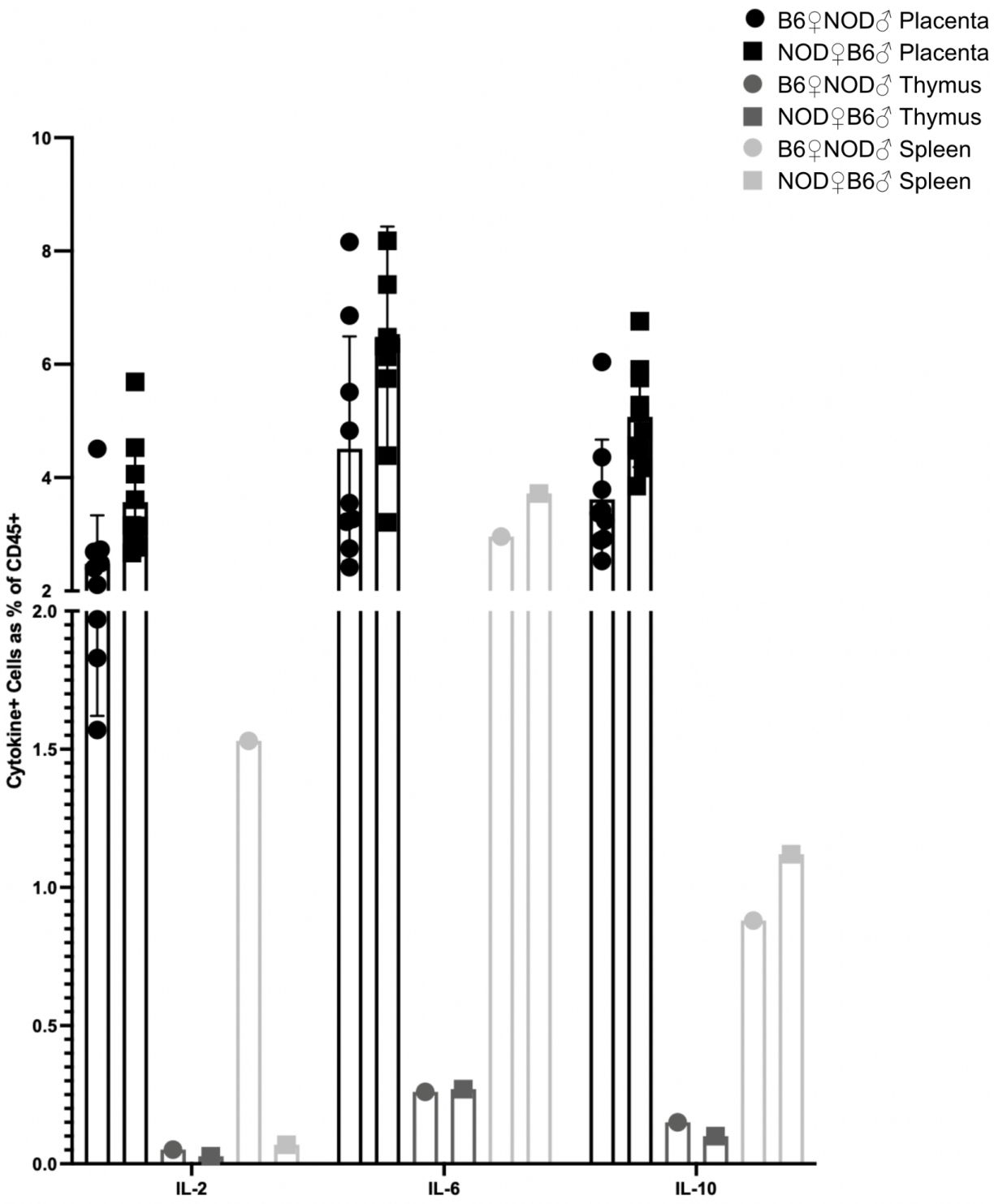


Figure 3.14. Percent of CD45+ cells that are positive for IL-2, IL-6, and IL-10 in B6 and NOD placenta, thymus, and spleen. Each dot represents one placenta, thymus, or spleen.

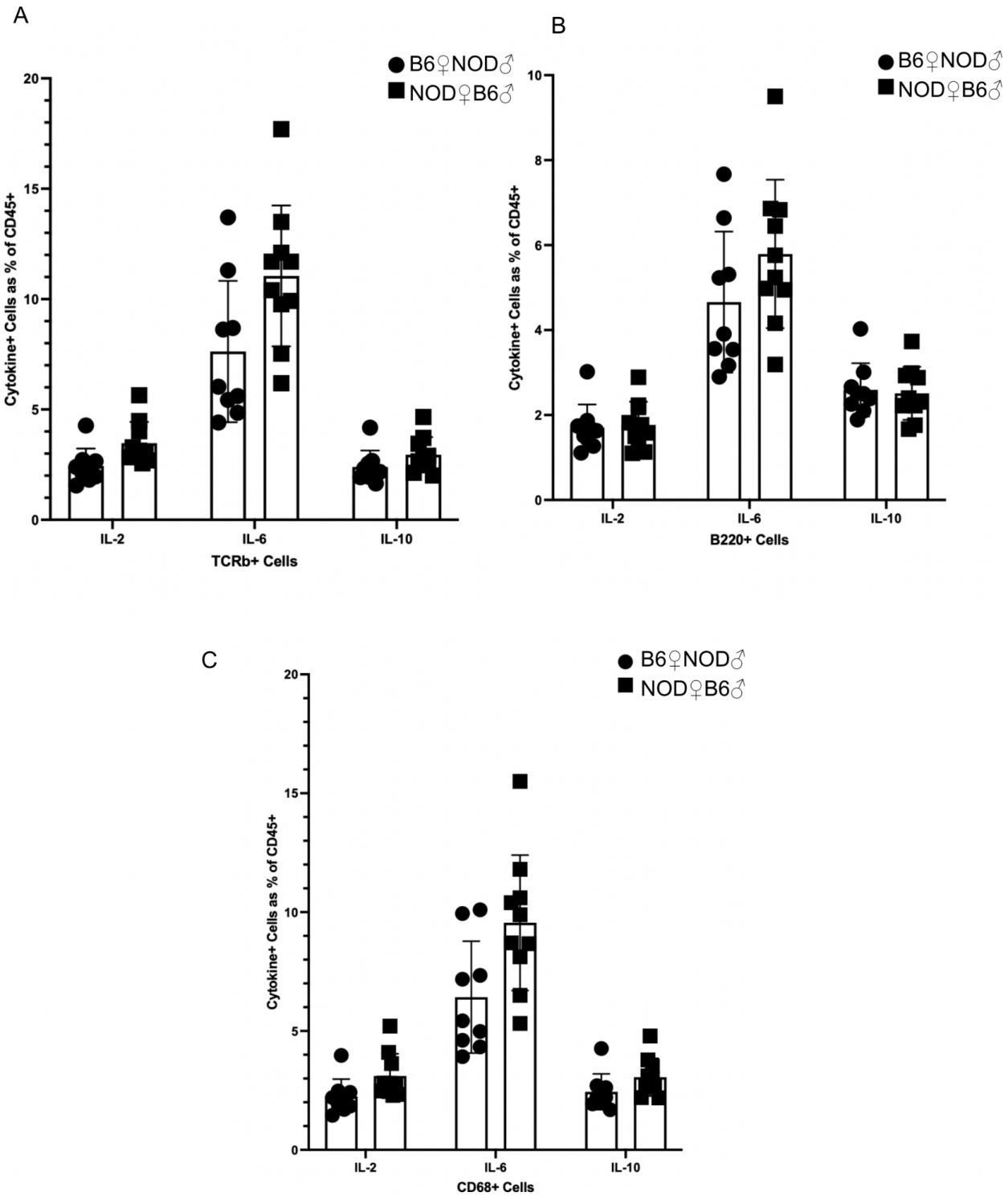


Figure 3.15. Percent of TCRb+ (A), B220+ (B), and CD68+ (C) cells that are positive for IL-2, IL-6, and IL-10 in B6 and NOD placenta. Each dot represents one placenta. Data shown is representative of 3 experiments.

Given both the increase in IL-6 seen across cell types from the NOD♀B6♂ placentas and the increased IL-6 in amniotic fluid, I investigated other cell types to identify the predominant source of IL-6 secretion in the placenta. In a cytokine secretion assay, I found that in both B6♀NOD♂ and NOD♀B6♂ placentas, the vast majority of CD31+ cells were positive for IL-6 (Figure 3.16).

CD31+ cells comprise the plurality of IL-6 secreting cells in both B6♀NOD♂ and NOD♀B6♂ placentas (Figure 3.17A). IL-6 is increased both in count and MFI in the NOD♀B6♂ placentas (Figure 3.17B). While several cytokines are found to be altered in the NOD amniotic fluid, IL-6 is secreted by multiple cells at a higher level than in B6 mice. In particular, CD31+ cells in the NOD placenta appear to be a major secretor of IL-6, edging the NOD IL-6 levels higher than those in the B6 placenta or amniotic fluid.

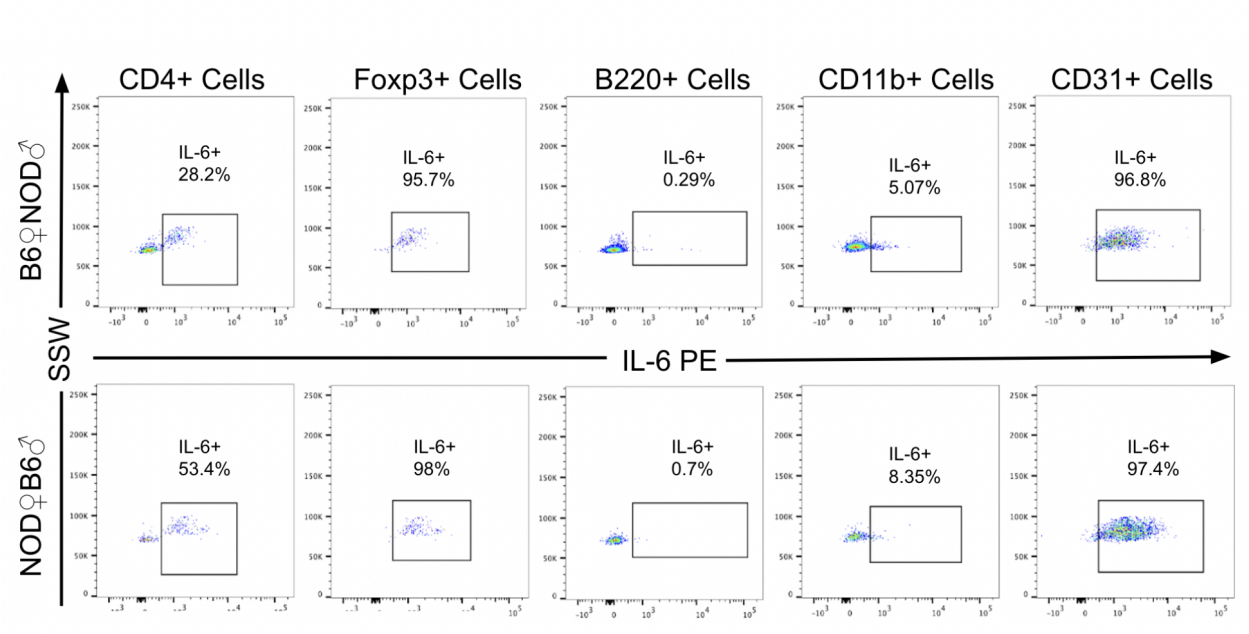


Figure 3.16. Flow cytometry plots indicating IL-6 positive cells in both B6♀NOD♂ and NOD♀B6♂ placentas.

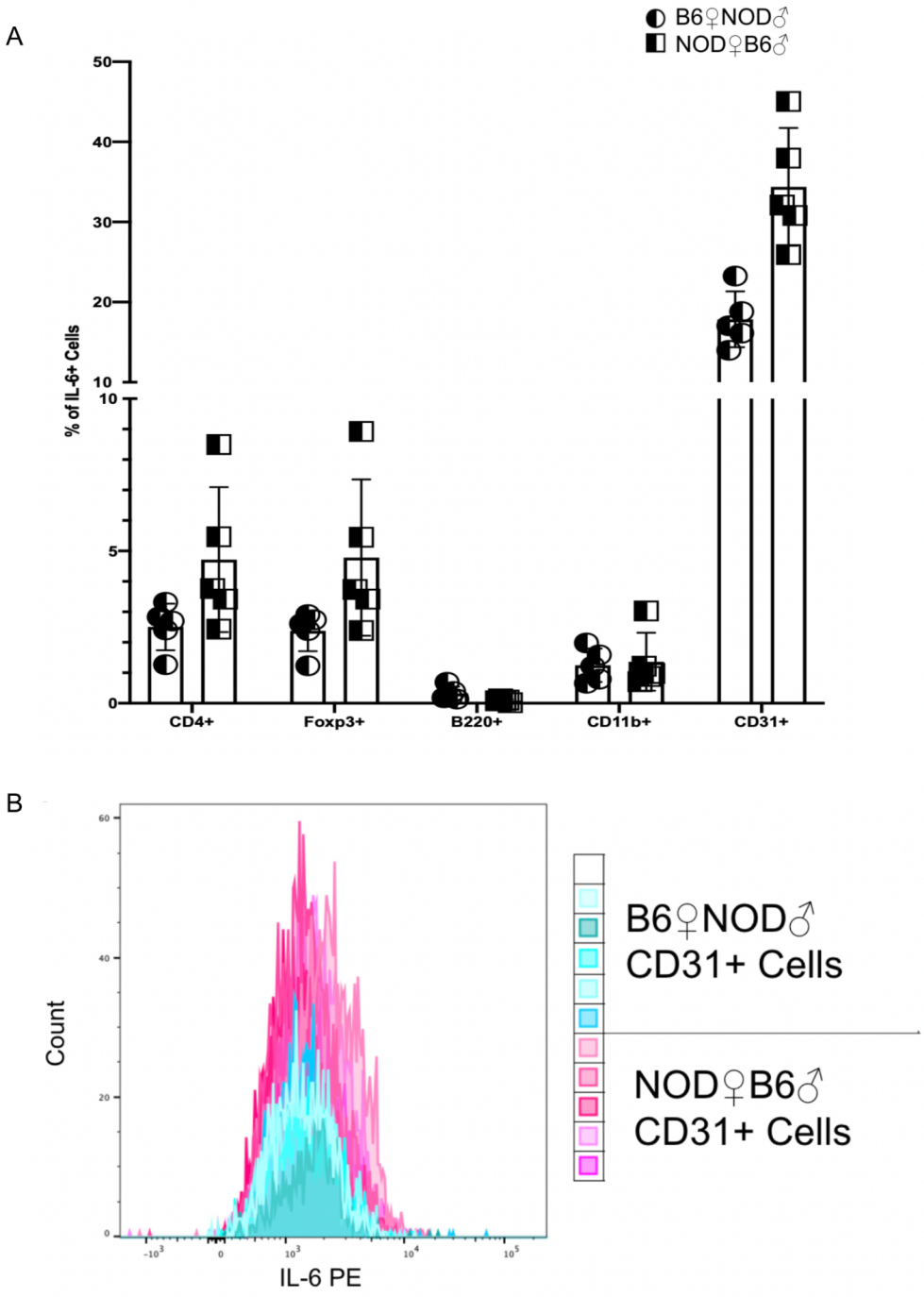


Figure 3.17. Percentage of IL-6+ cells across different cell types. The plurality of IL-6+ cells are CD31+ both B6♀NOD♂ and NOD♀B6♂ placentas (A). Each dot represents one placenta. NOD♀B6♂ placentas have an increase in CD31+ cells that are IL-6+ by both count and MFI (B).

Neutralization of IL-6 Improves Outcomes in NOD Pregnancies

Aberrant IL-6 levels are associated with several pregnancy-related diseases and can be harmful when insufficient or in excess. I investigated the impact of altering IL-6 levels by administering an IL-6 neutralizing antibody throughout gestation. The impact of IL-6 neutralization varied greatly with uterine environment; outcomes worsened for B6♀NOD♂, whereas NOD♀B6♂ litters benefitted from decreased IL-6, with fewer reabsorbed fetuses and abnormal pups (Figure 3.18A, B). While 64% of fetuses from B6♀NOD♂ pairings treated with αIL-6 appeared normal, 93% of fetuses NOD♀B6♂ treated with αIL-6 appeared normal. I verified that αIL-6 neutralized amniotic fluid IL-6 by performing an ELISA on amniotic fluid, which demonstrated normalization of IL-6 levels across mouse strains treated with αIL-6 (Figure 3.19).

Increased IL-6 levels in NOD♀B6♂ mice correlate with a significant increase in the IL-6 secreting CD31+ cells compared to B6♀NOD♂ pairs (Figure 3.20A), but αIL-6 did not alter the number of CD31+ cells in either B6♀NOD♂ or NOD♀B6♂ pairs. CD31 has been well established as a regulator of leukocyte transmigration that aids in the emigration of inflammatory leukocytes into target tissue. I found decreased DX5+ NK cells in NOD♀B6♂ placentas compared to B6♀NOD♂ placentas (Figure 3.20B); this alteration was partially restored by administration of αIL-6. I conclude that the increased IL-6 from CD31+ cells in NOD mice has a harmful effect on gestation; furthermore, the increased CD31+ cell number correlates with a decrease in NK cells. These changes may explain some of the increased burden of autoimmunity in NOD pregnancy (Figure 3.21).

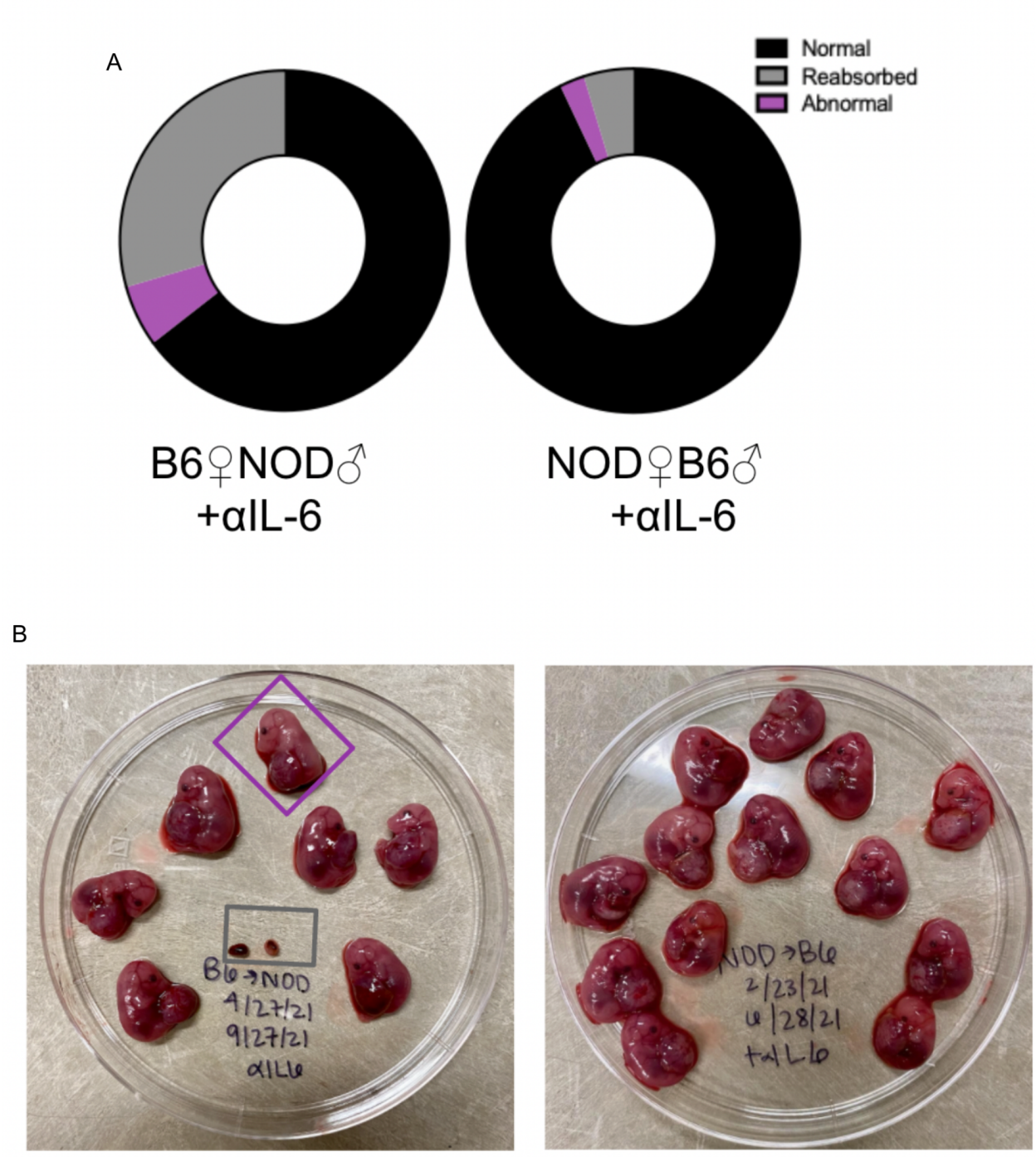


Figure 3.18. Composite pregnancy outcomes for B6 (8 pregnancies; 64% successful) and NOD (10 pregnancies; 93% successful) mice treated with αIL-6. αIL-6 treatment worsened outcomes in the B6 mouse while improving NOD outcomes (A). Representative images of B6 (left) and NOD (right) litters. Outlined in maroon is one abnormal fetus in the B6 litter (maroon box) with two reabsorbed fetuses (grey box), while the NOD litter contained 13 healthy pups (B).

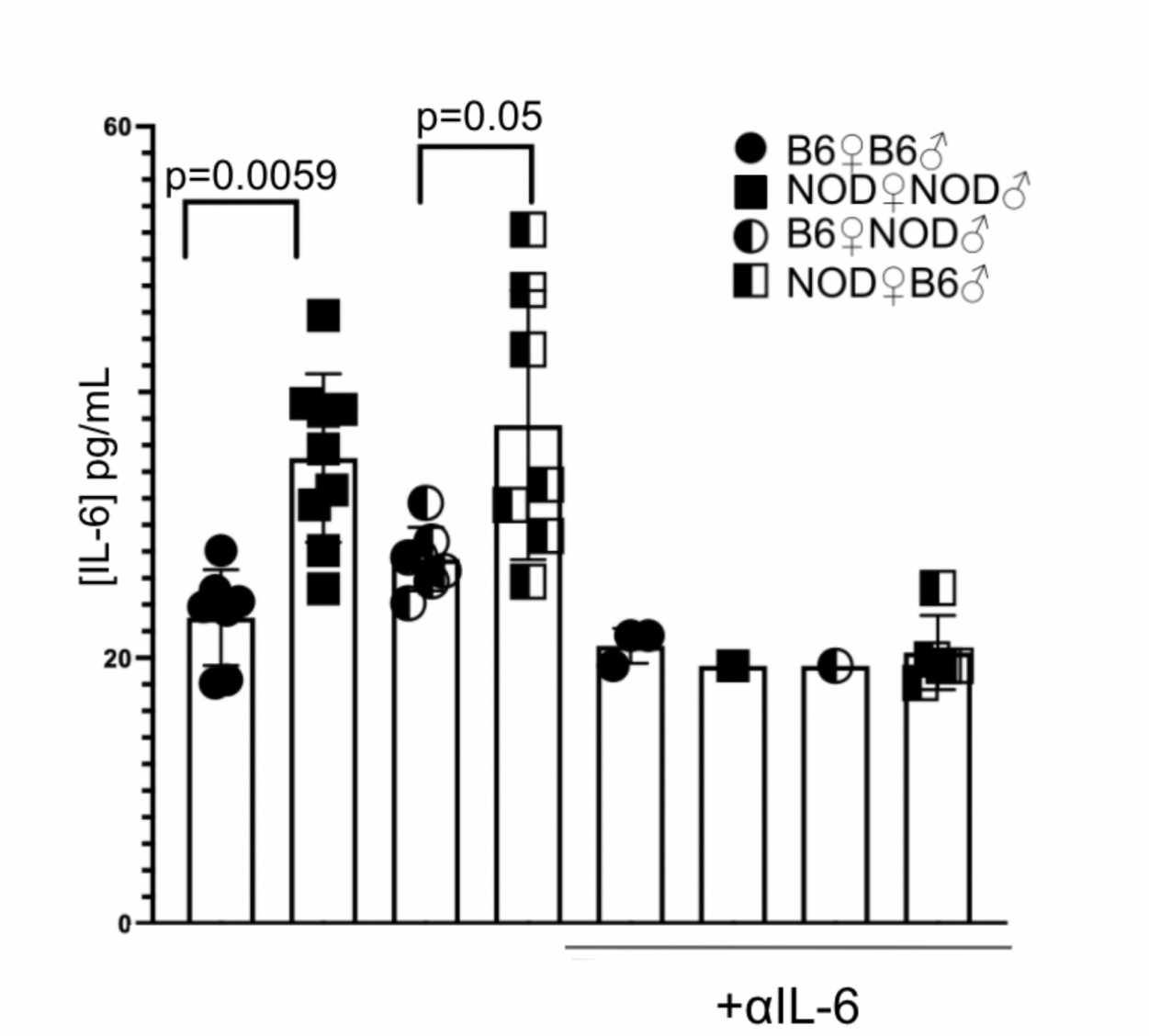


Figure 3.19. Administration of αIL-6 decreased IL-6 levels in the amniotic fluid equally across all mouse pairings. Each point represents amniotic fluid from one mouse. Data was analyzed by individual t-test.

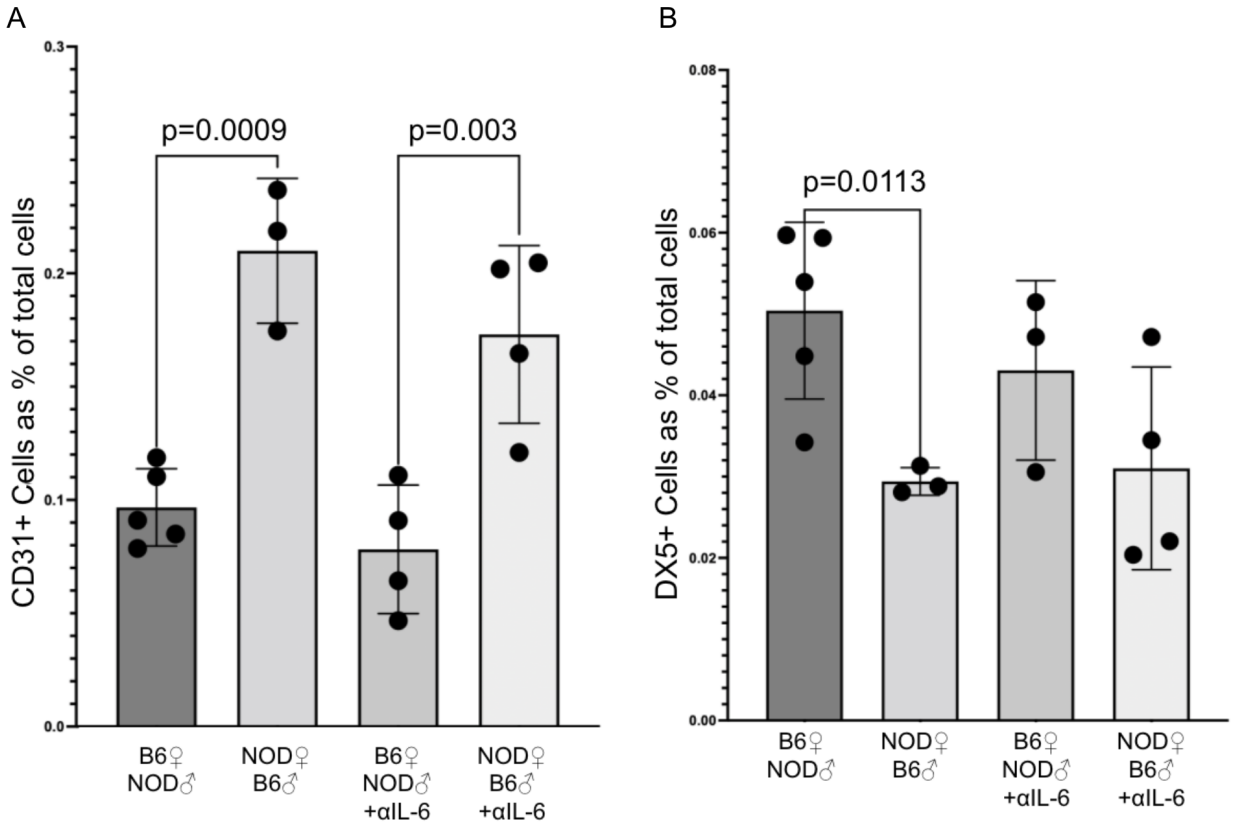


Figure 3.20. Both NOD♀B6♂ without treatment and treated with αIL-6 had a significant increase in placental CD31+ endothelial cells relative to B6♀NOD♂ mice (A). NOD♀B6♂ mice had significantly fewer placental DX5+ NK cells relative to B6♀NOD♂ mice, which may have normalized with αIL-6 treatment (B). Each dot represents an average of all placentas from one mouse. Data was analyzed by individual t-test.

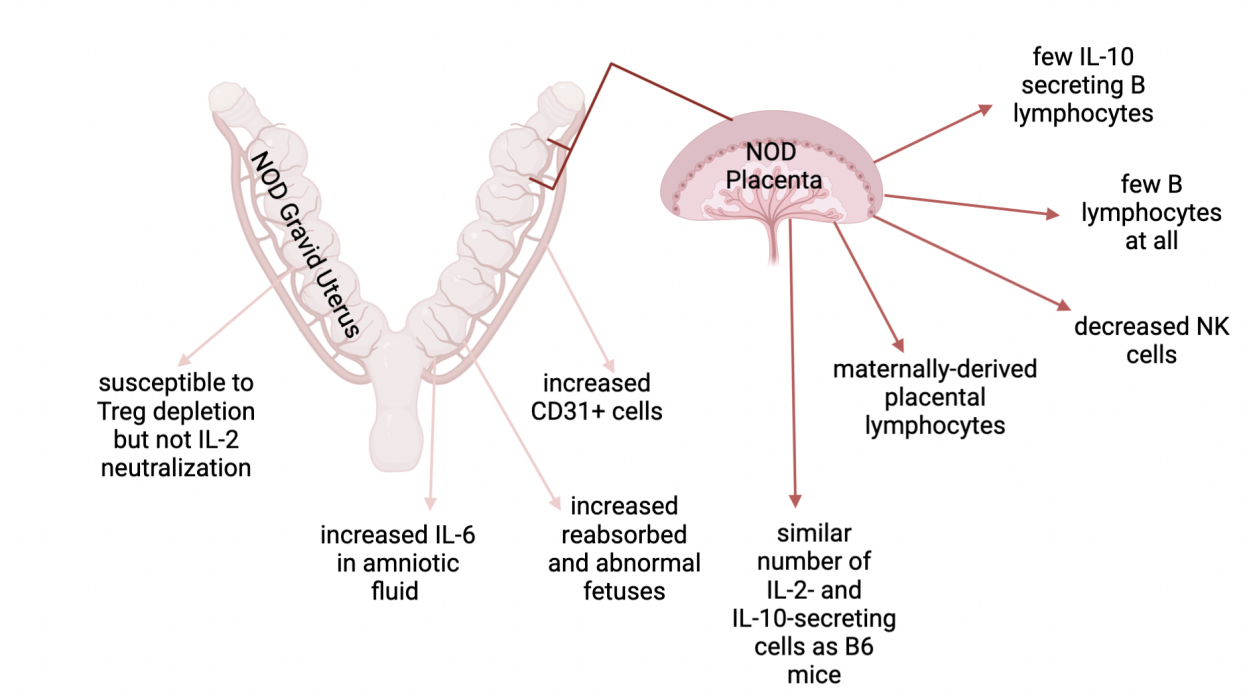


Figure 3.21. Overview of discoveries from Chapter III.

Conclusions

While B6 and NOD mice may have similar numbers of healthy, live-born pups, the number of live pups does not reflect the turbulent intrauterine environment in the NOD mouse. In this chapter, I show that NOD mice have an increased prevalence of abnormal and reabsorbed fetuses. Using flow cytometry, immunohistochemistry, and ELISA, I found that genetically identical fetuses between B6♀NOD♂ and NOD♀B6♂ pairs have disparate outcomes determined by the uterine environment, illustrated by alterations in placental lymphocytes and cytokines.

Tregs are required to protect the developing fetus during gestation. Interestingly, I show different results with depleting Tregs in number and inhibiting their function. Treatment with αCD25 dramatically worsened NOD♀B6♂ pregnancies, while IL-2 neutralization with αIL-2 impacted B6♀NOD♂ litters more than NOD♀B6♂ litters. This speaks to the adaptations in the NOD mouse to signal appropriately with low levels of IL-2 at baseline, as I confirmed in Chapter II; IL-2 neutralization did not register as significantly different. In contrast, the NOD mouse appears to be reliant on the highest possible number of Tregs to balance the relatively low functioning. Future studies into the mechanism by which αCD25 treatment increased placental Tregs in B6♀NOD♂ litters may reveal an opportunity for a treatment paradigm which could improve both pregnancy and transplant outcomes in NOD mice.

Notably, NOD♀NOD♂ and NOD♀B6♂ mice had decreased placental B lymphocytes, with deficits in both Follicular and Marginal Zone B lymphocytes. As discussed in Chapter II, Marginal Zone B lymphocytes are pathogenic in NOD mice and present autoantigen to T cells. The absence of these diabetogenic B lymphocytes from the placenta underlies the tolerogenic tendencies of the uterus and gestation-associated lymphocytic changes. Previous work proposed that B cells function in the placenta predominantly as anti-inflammatory secretors of IL-10 (Benner et al., 2020; Busse et al., 2019), but the B lymphocytes found in the NOD uterus

do not appear to be secreting IL-10. As B lymphocytes play an important role in the pathogenesis of T1D, it is tempting to speculate that their absence from the placenta is required for the fragile tolerance developed. Future studies to further characterize both the phenotype and function of the missing B cells in NOD mice would not only deepen our understanding of how B cells could negatively impact pregnancy in T1D, but could identify a mechanism by which autoreactive or pathogenic cells are trafficked away from an organ of interest.

Interestingly, our studies also implicated a common culprit in adverse pregnancy outcomes, IL-6. Both insufficient and excess IL-6 have been associated with poor prognosis, and increased levels of IL-6 family members like LIF are required for pregnancy (Dimitriadis et al., 2005). Notably, IL-6 secretion from endothelial cells is increased in patients with preeclampsia (Benyo et al., 2001; Wang et al., 2021), which decreases the activity of Foxp3+ CD4+ Tregs (Bettelli et al., 2006; Goodman et al., 2009). Localizing the increased IL-6 secretion to CD31+ cells, which are found to be increased in NOD♀B6♂, may explain the increased levels of IL-6 present in NOD♀B6♂ amniotic fluid. CD31 on endothelial cells aids in leukocyte transmigration; the presence of increased CD31+ cells in NOD mice may promote the translocation of inflammatory cells into the placenta and worsen pregnancy outcomes (Dasgupta et al., 2009; Rijcken et al., 2007; Woodfin et al., 2007). In contrast, decidual DX5+ NK cells are beneficial in pregnancy and aid in remodeling spiral arteries (Fraser et al., 2015). NOD mice may be subject to a deleterious cycle in which inflammatory IL-6 secreting CD31+ cells increase due to a lack of sufficient NK cells. Future studies examining spiral artery remodeling, including the role of IL-6 and NK cells, would benefit our understanding of neovascularization in T1D.

While I identified gestational complications in the NOD mouse that correlate with findings in patients with T1D, our studies were limited by several factors. I was unable to capture data

from reabsorbed mouse fetuses; the data may represent a survival bias in fetuses that survived to preterm gestation. Furthermore, all placentas from each individual mouse were averaged together; some of the diversity in phenotypes of individual mouse placentas may reflect the diversity in disease onset or severity in adult mice. Because fetal mice were sacrificed for placental examination, I was unable to follow them to adulthood to monitor for diabetes onset. Autoimmune features are more present in female NOD mice than male NOD mice, which minimizes the effect of paternal autoimmunity on this study that was designed to investigate the effects of autoimmunity on the uterine environment in the presence of genetically identical pups.

In this chapter, I examined the effect of autoimmunity on gestation by studying pre-diabetic NOD mice. In both syngeneic and allogeneic pregnancies, NOD mice had worse outcomes than non-autoimmune B6 mice. While NOD placentas had lymphocyte alterations like decreased CD8+ T cells and B cells, the increased presence of IL-6 was responsible for some of the poor pregnancy outcomes. Neutralization of IL-6 alleviated some disparity in placental NK cells, potentially interrupting the devastating cycle of decreased spiral artery remodeling that contributes to vascular disease. I discovered an independent effect of autoimmunity on pregnancy that should be considered in patients with T1D.

CHAPTER IV

THE IMPACT OF TYPE 1 DIABETES ON PREGNANCY EXTENDS BEYOND HYPERGLYCEMIA

Introduction

Pregnancy in patients with T1D is associated with increased risks of complications, including premature delivery, congenital malformations, perinatal death, and perigestational mortality (Evers et al., 2004). Pregnancy in T1D comes with especially increased risk of vascular complications like preeclampsia and pregnancy-induced hypertension; these complications are only occasionally associated with A1c (Holmes et al., 2011; Weissgerber and Mudd, 2015). While A1c does correlate with increased adverse outcomes, rates of preterm birth are higher in patients with T1D than Type 2 Diabetes (T2D), indicating an excess effect of T1D (Murphy et al., 2021). While risks increase with hyperglycemia (Davidson et al., 2020), they arise alongside immune alterations that may independently contribute to these risks.

In healthy pregnant individuals, peripheral blood lymphocytes decrease; however, in T1D, patients exhibit similar pre-and peri-gestational lymphocyte counts (Groen et al., 2015). Successful pregnancy in healthy patients reflects a careful balance in which Tregs protect the developing fetus, while activated T cells can initiate labor (Arenas-Hernandez et al., 2019). Immune evolution during pregnancy initiates the development of pregnancy-specific regulatory cells; for instance, depletion of Tregs reactive to male-specific antigen causes specific destruction of male fetuses (Kahn and Baltimore, 2010). Cord blood from infants delivered by a parent with T1D contains increased Tregs, suggesting an increased need for regulatory cells to dampen the immune response (Luopajärvi et al., 2012). However, no examination has been

done of placentas from patients with T1D to determine whether peripheral changes mirror those at the actual parental:fetal interface.

In this chapter, I performed an examination of placental tissue from patients with T1D, T2D, and healthy controls. I utilized immunohistochemistry, both with single stains and in multiplex, to uncover the immunologic landscape of the placenta and tease apart effects of hyperglycemia vs autoimmunity. Additionally, with collaborators, I created a retrospective study of the Electronic Health Record (EHR) at Vanderbilt University Medical Center to identify pregnancy outcomes in patients with T1D as compared to patients without T1D. This chapter provides an unprecedented view of how autoimmunity impacts human pregnancy (Figure 4.1).

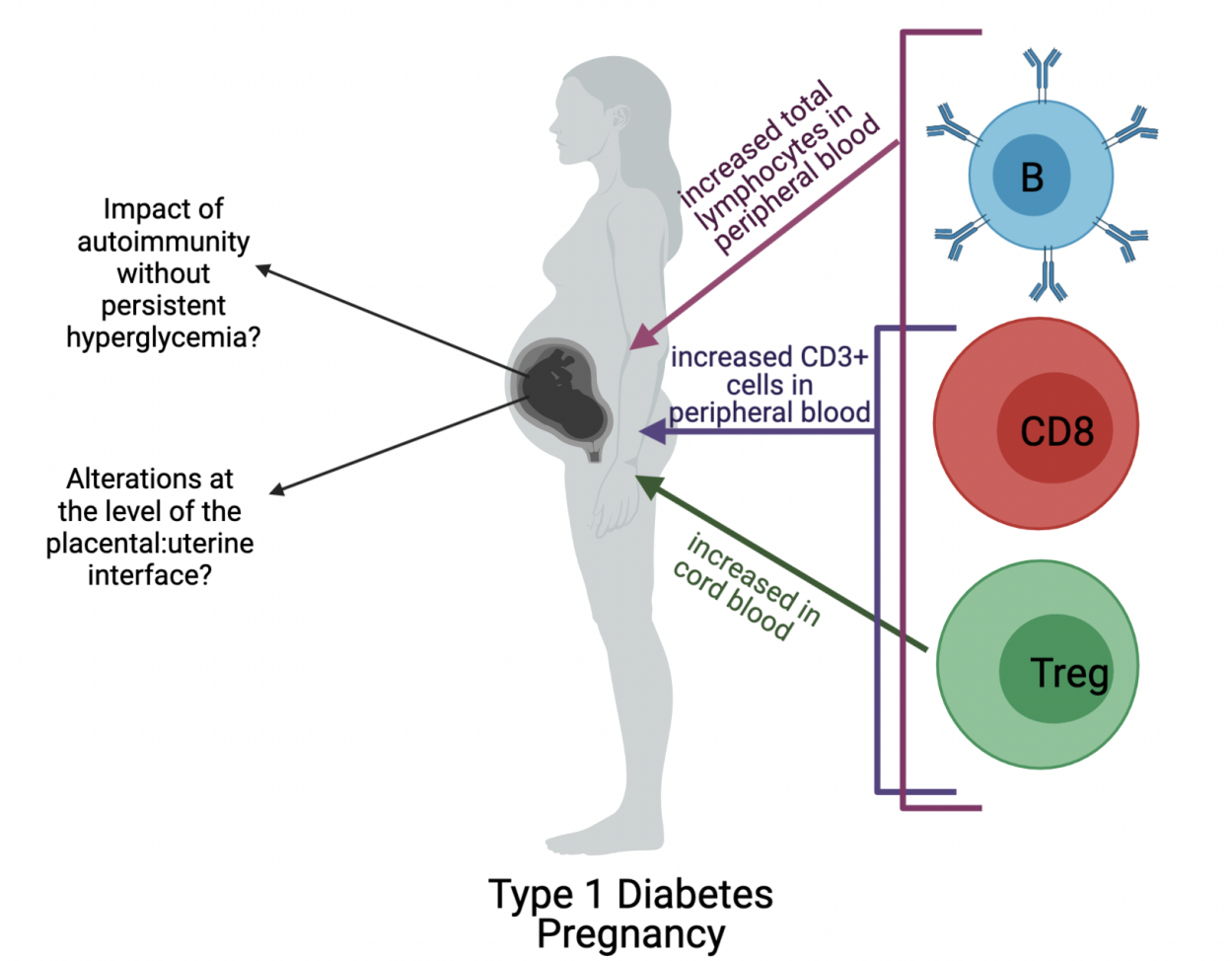


Figure 4.1. Overview figure illustrating unknown impacts of Type 1 Diabetes on pregnancy.

Results

Placentas From Patients with Type 1 Diabetes Are Immunologically Different than Healthy Controls

As discussed in Chapter III, pregnancy in pre-diabetic NOD mice is associated with worse outcomes than in non-autoimmune B6 mice. I hypothesized that patients with T1D are also affected by the effects of autoimmunity on pregnancy. Retrospectively studying autoimmunity without hyperglycemia in pregnancy is challenging, as most patients presenting to the hospital are classified as having or not having T1D. I chose to study placentas from patients with a range of A1c values in pregnancy, to provide a broad view of possible additive effects of hyperglycemia. In collaboration with a pathologist, I selected patients with T1D whose first A1c of pregnancy ranged from normal (4.9) to high (10.2) who delivered by Cesarean Section and had their placenta sent for histopathologic analysis. I matched this T1D cohort with patients with T2D (A1c range 5.6-12.1) to serve as controls for hyperglycemia and patients with no diabetes as an additional control. Four cores from each placenta were identified by a pathologist and placed into a tissue microarray containing cores from 17 patients with T1D, 18 patients with T2D, and 17 healthy controls (Figure 4.2). H&E staining did not reveal any immediate differences between groups (Figure 4.3).

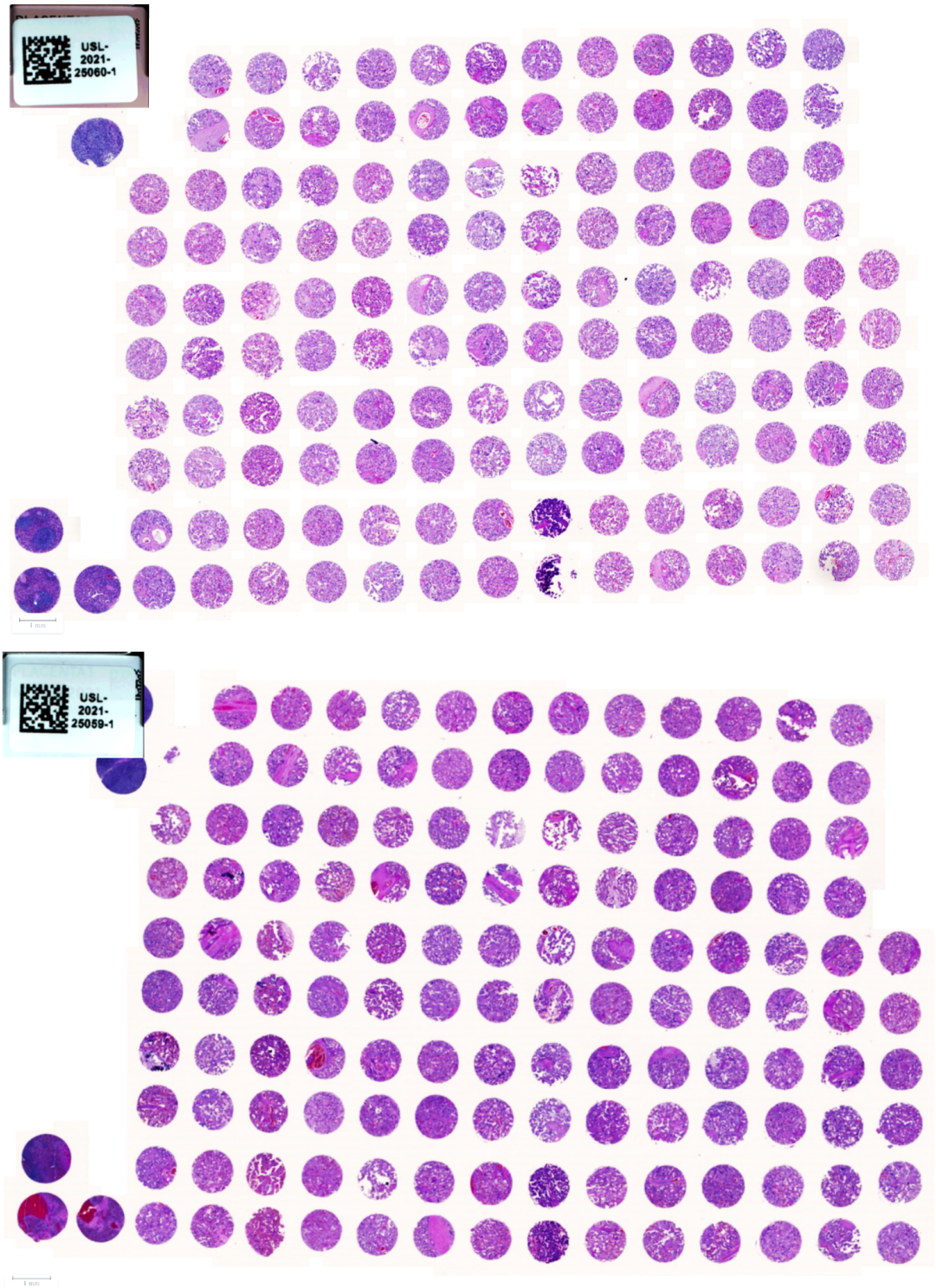


Figure 4.2. H&E staining illustrating placenta TMA construction. Each TMA slide has 2 cores per patient, along with 3 control cores each from both lymph node and spleen.

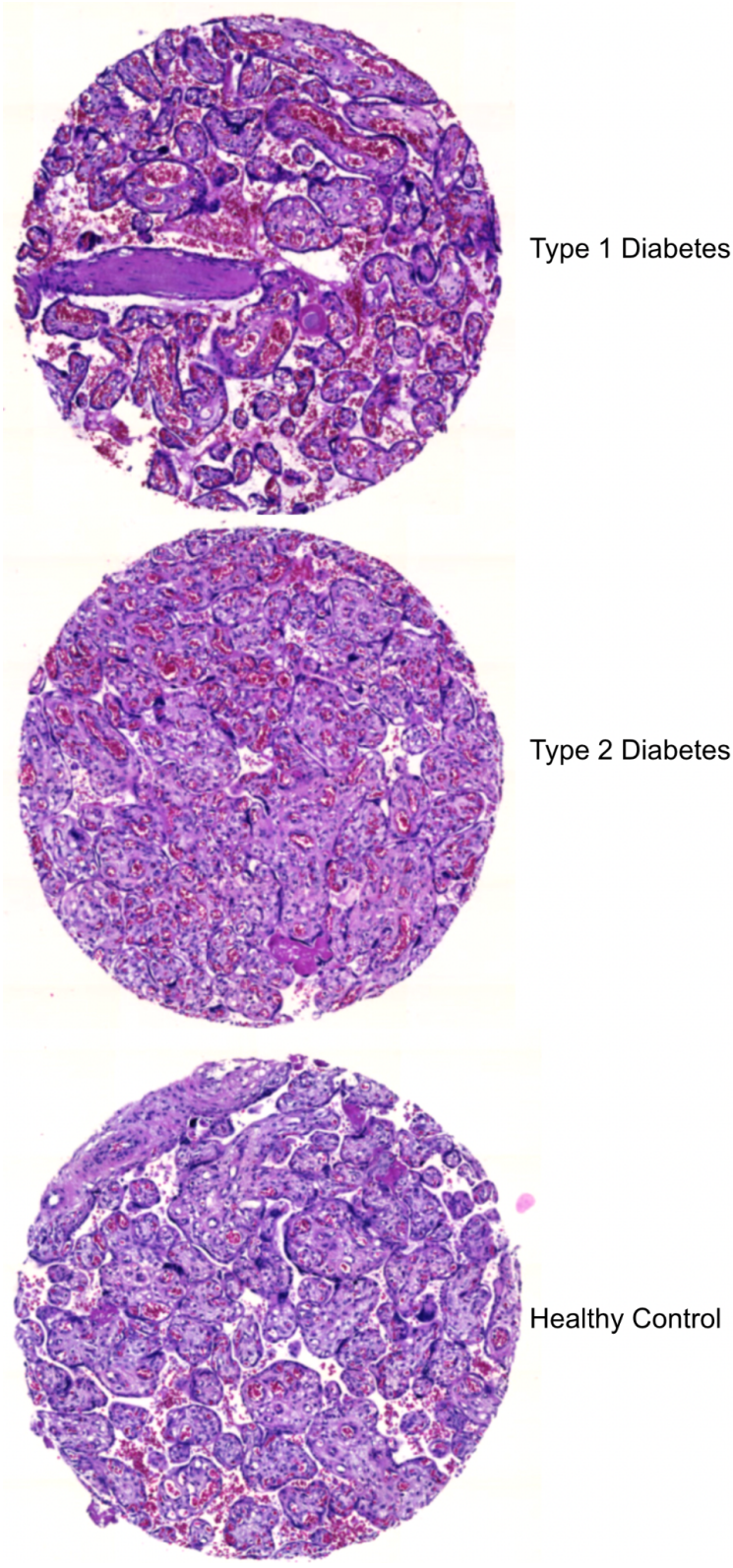


Figure 4.3. Selected H&E-stained cores from a patient with T1D, with T2D, and a healthy control.

Once constructed, the placenta TMA slides were sent to Ultivue Inc, a company that performs multiplex immunohistochemistry. A technician stained each slide for eight markers: CD4, CD8, Foxp3, CK, CD11c, CD20, CD63/CD163, and MHC Class II. CD4, CD8, and Foxp3 label subsets of T cells; CK (cytokeratin) labels epithelial cells; CD11c labels dendritic cells; CD20 labels B cells; CD63/CD163 labels macrophages; and MHC Class II is expressed by antigen-presenting cells. Stained slides were uploaded into HALO, and image processing identified the presence of each marker in relation to a DAPI-positive nucleus (Figure 4.4, 4.5).

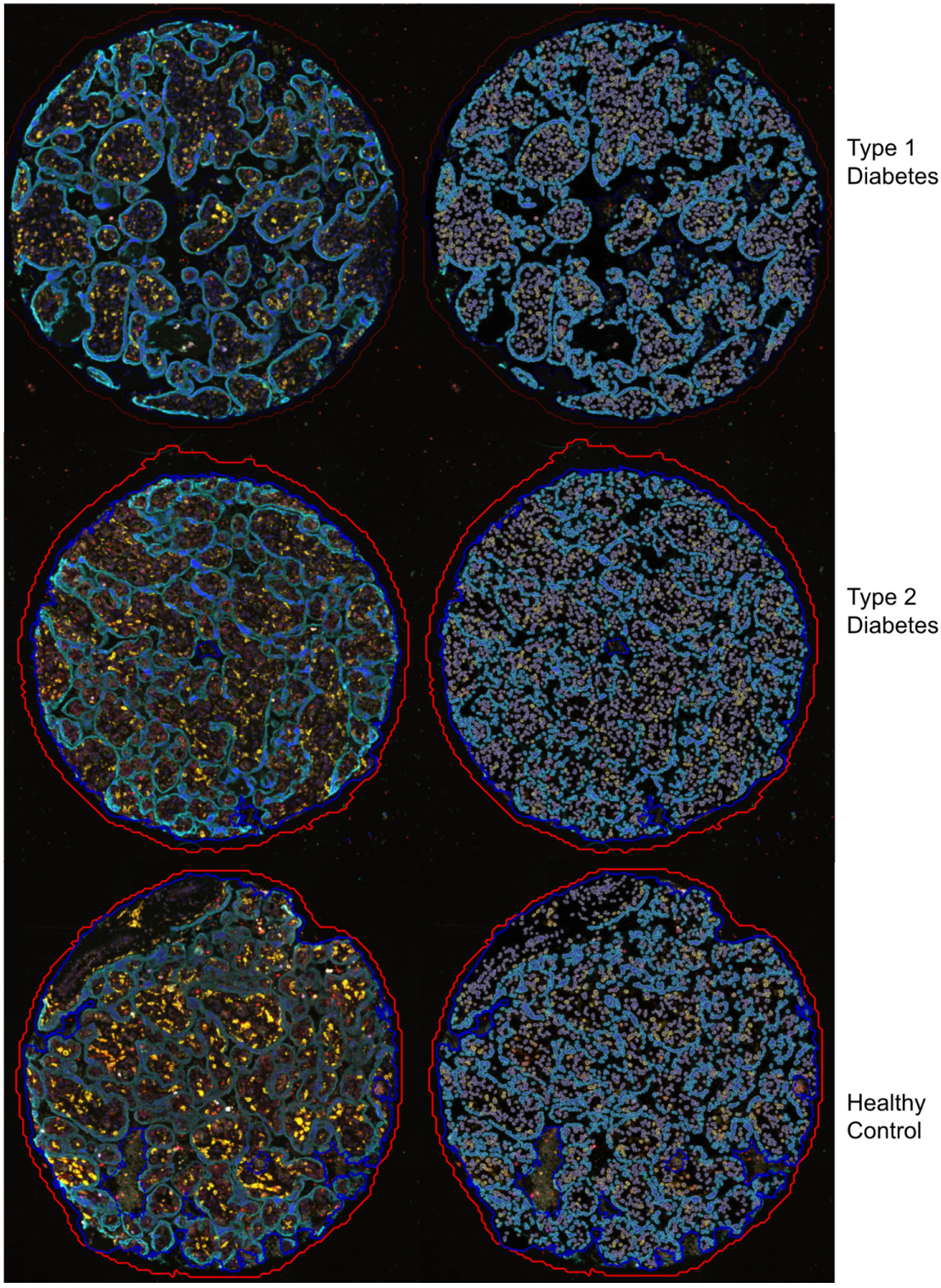


Figure 4.4 Selected cores from patients with T1D, T2D, and a healthy control, showing multiplex staining (left) and signals identified for analysis (right).

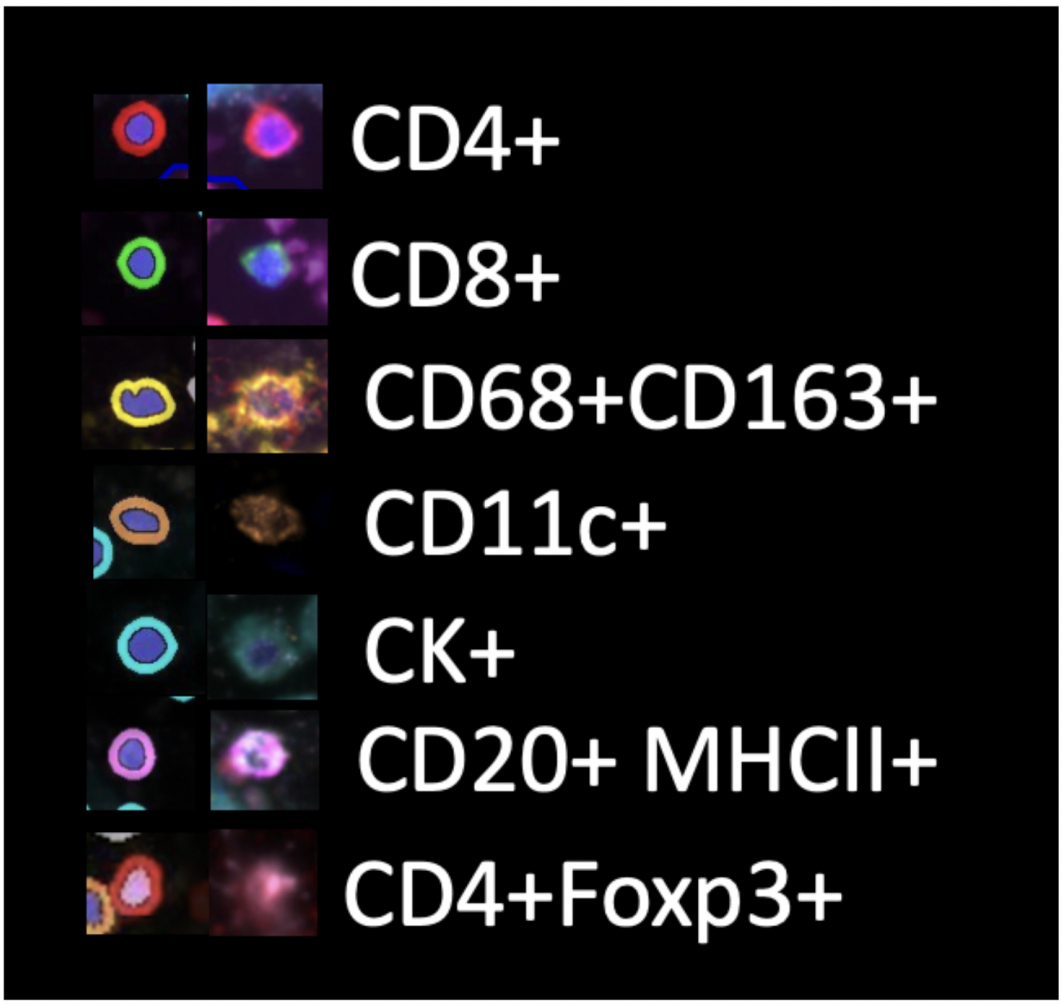


Figure 4.5. Examples of each marker in both stained cells and after being identified for analysis. CD63+CD163+ uses an antibody that identifies both CD markers. The example of a CD20+MHC Class II+ cell shows a cell positive for both CD20 (pink) and MHC Class II (white). The example of a CD4+Foxp3+ cell shows a cell positive for both Foxp3 (pink nuclear stain) and CD4 (red membrane stain).

As cores varied in their composition, readout of the total number of marked cells would be impossible to compare between groups. Data was reported as the percentage of cells positive for one of the 8 markers listed of DAPI+ nuclei. Each point represents an individual placental core, with 4 cores per patient; we analyzed 17 patients with T1D, 18 patients with T2D, and 17 healthy patients. Patients with T1D, T2D, and healthy controls all had a similar percentage of CD4+ cells (Figure 4.6). There was no trend in percentage of CD4+ cells with increasing A1c (Figure 4.7). Patients with T2D had a significantly higher percentage of CD8+ cells than healthy controls (Figure 4.8). The increased number of CD8+ cells may be found in patients with lower A1cs (Figure 4.9). Patients with T1D and T2D had significantly fewer CD11c+ cells than healthy controls (Figure 4.10). The decrease in CD11c+ cells does not seem to be correlated with A1c (Figure 4.11). Patients with T1D, T2D, and healthy controls all had a similar percentage of CD20+ cells (Figure 4.12). There was no trend in percentage of CD20+ cells with increasing A1c (Figure 4.13). Patients with T1D, T2D, and healthy controls all had a similar percentage of CD68/CD163+ cells (Figure 4.14). There was no trend in percentage of CD68/CD163+ cells with increasing A1c (Figure 4.15). Patients with T1D had significantly fewer Foxp3+ cells than healthy controls (Figure 4.16). The decrease in Foxp3+ cells appears to be attributable to patients with low A1cs (Figure 4.17). Patients with T1D had fewer MHC Class II+ cells than either patients with T2D or healthy controls; patients with T2D also had fewer MHC Class II+ cells than healthy controls (Figure 4.18). The decrease in MHC Class II+ cells appears dispersed across all A1cs (Figure 4.19).

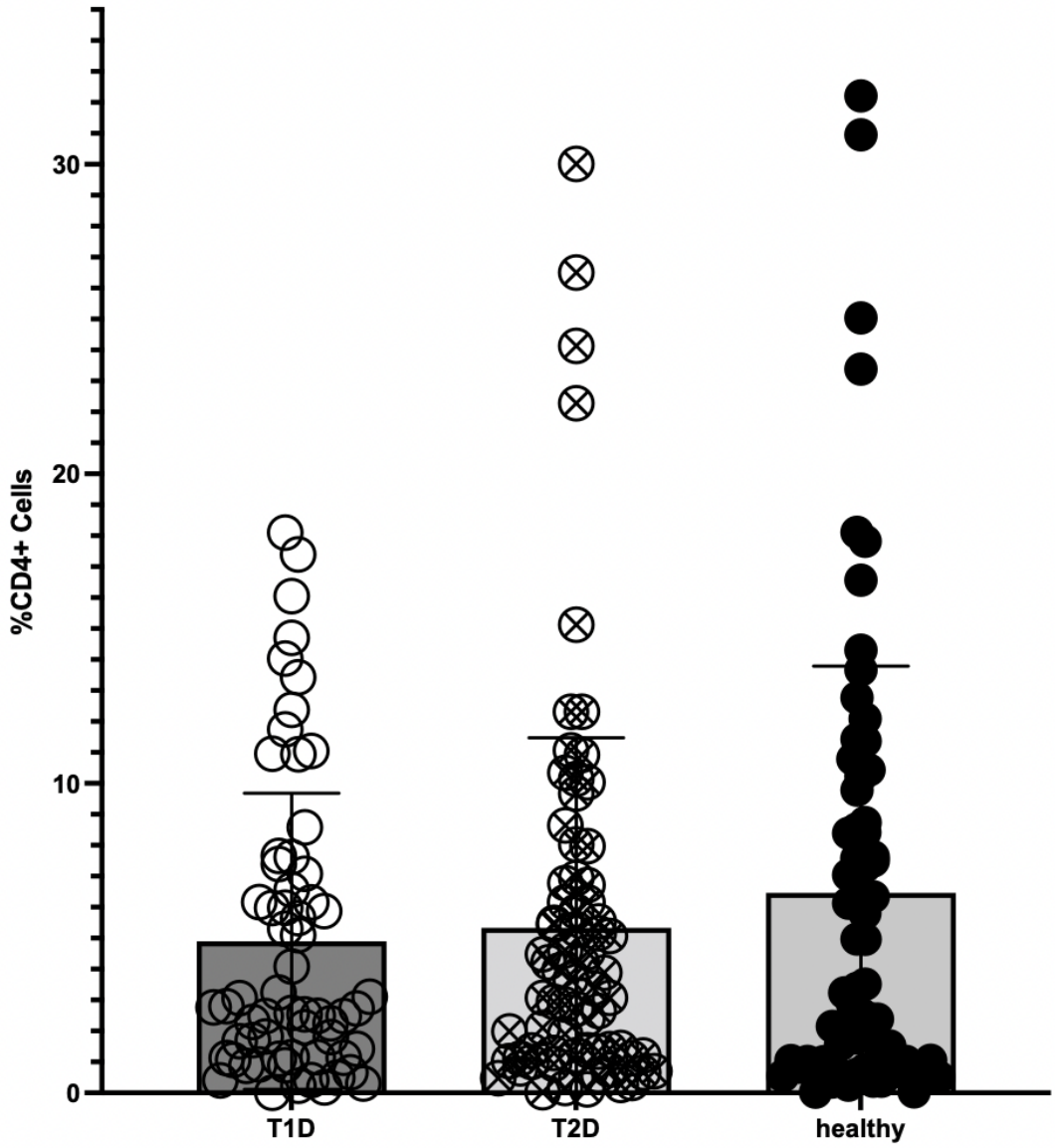


Figure 4.6. Percentages of CD4+ cells of total cells (identified with DAPI-positive nuclei) in patients with T1D, T2D, and healthy controls. Each dot represents an individual core.

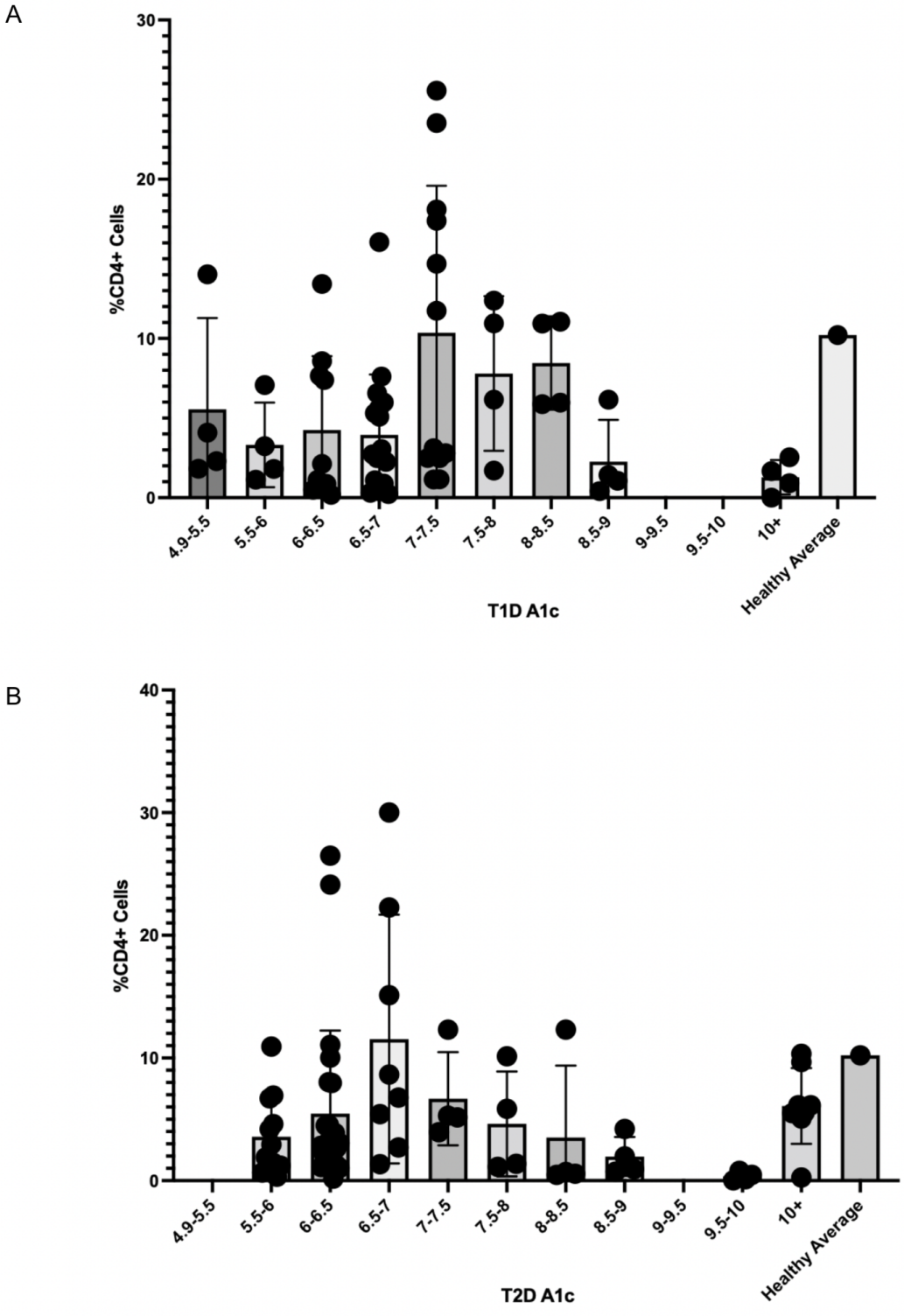


Figure 4.7. Percentages of CD4+ cells of total cells (identified with DAPI-positive nuclei) in patients with T1D, T2D, and healthy controls shown split by A1c. Each dot represents an individual core. An average of all percentages of CD4+ cells from healthy patients is shown for reference.

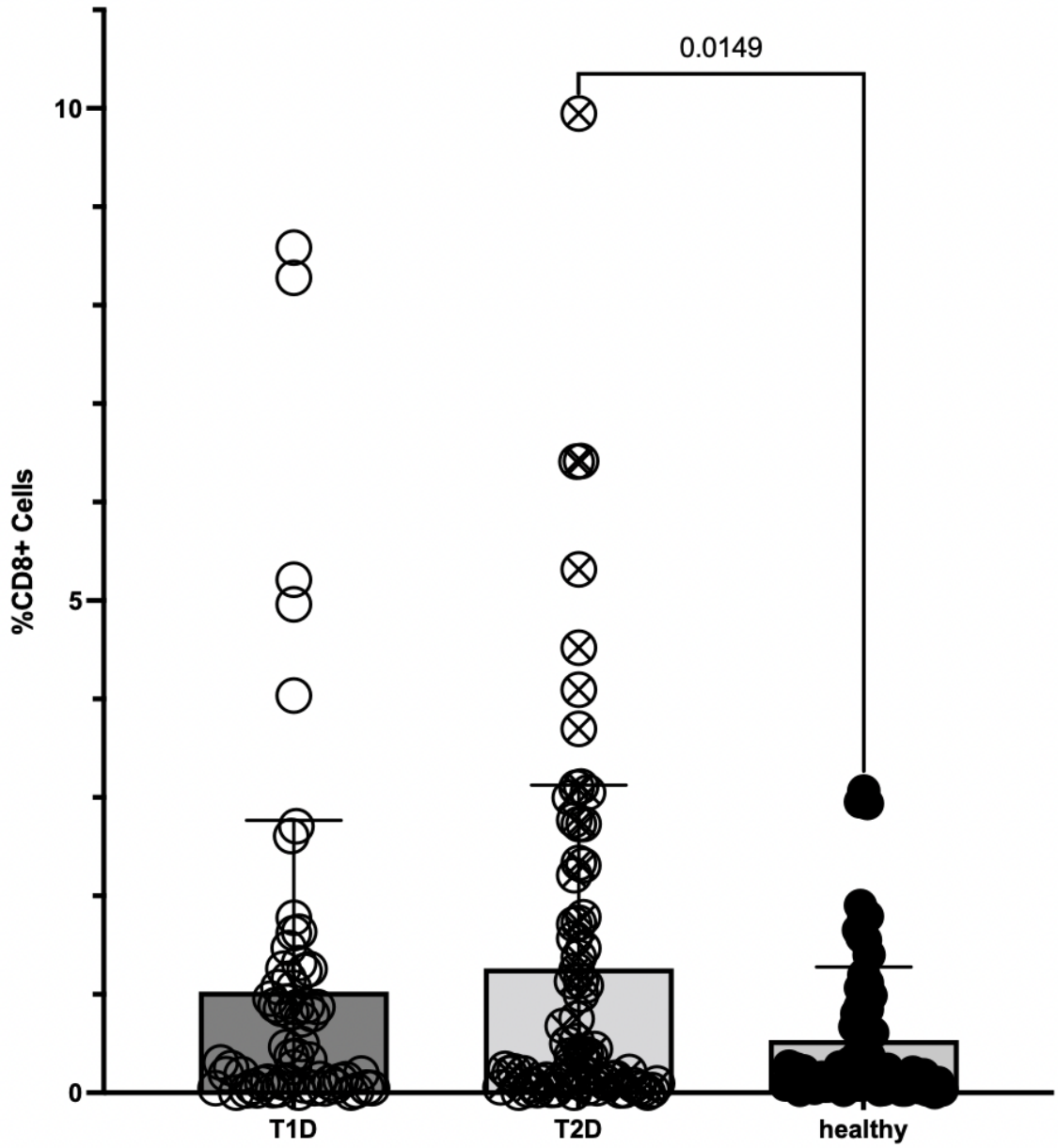


Figure 4.8. Percentages of CD8+ cells of total cells (identified with DAPI-positive nuclei) in patients with T1D, T2D, and healthy controls. Each dot represents an individual cores. Data was analyzed using a one-way ANOVA with multiple comparisons.

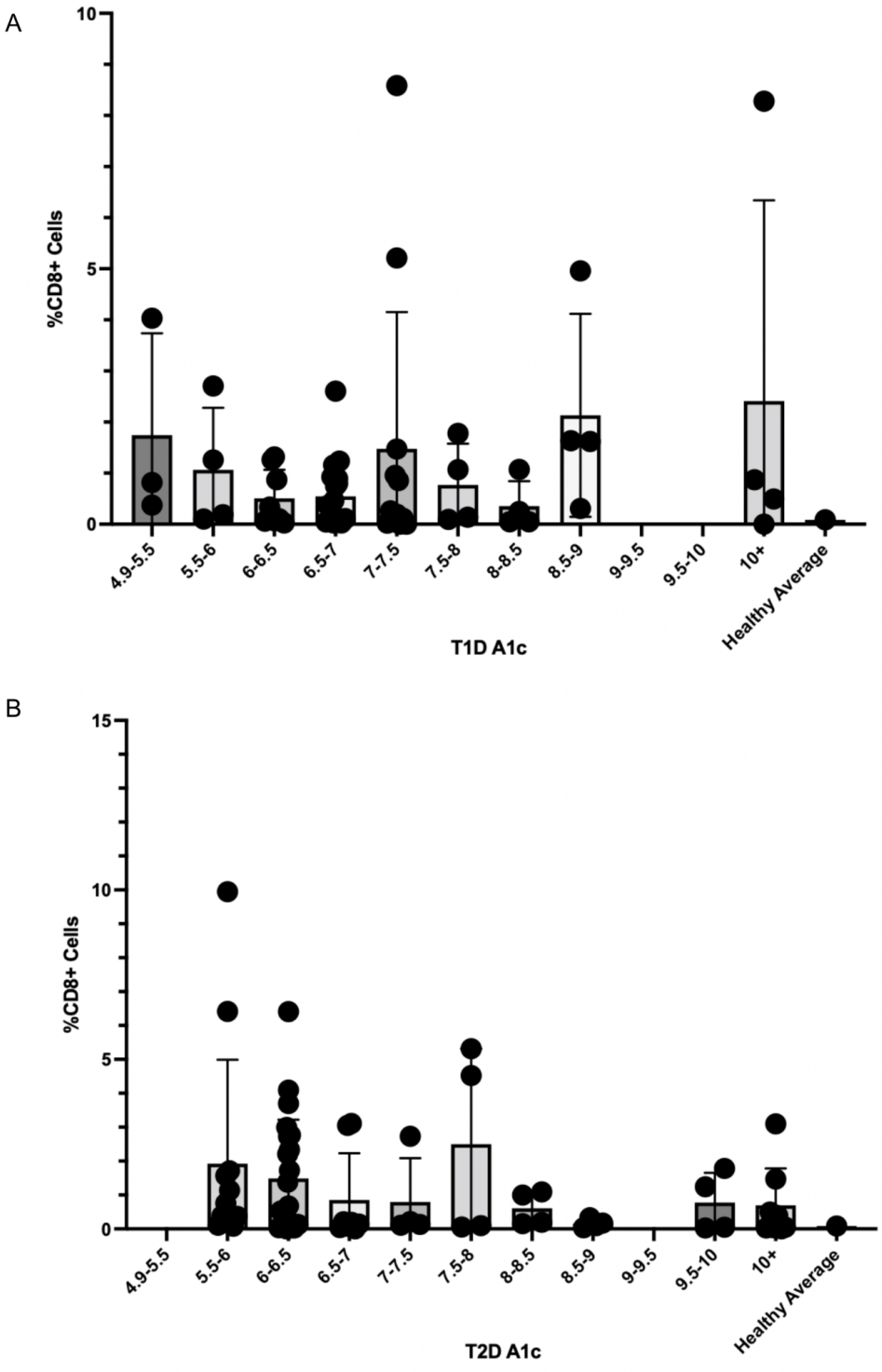


Figure 4.9. Percentages of CD8+ cells of total cells (identified with DAPI-positive nuclei) in patients with T1D, T2D, and healthy controls shown split by A1c. Each dot represents an individual core. An average of all percentages of CD8+ cells from healthy patients is shown for reference.

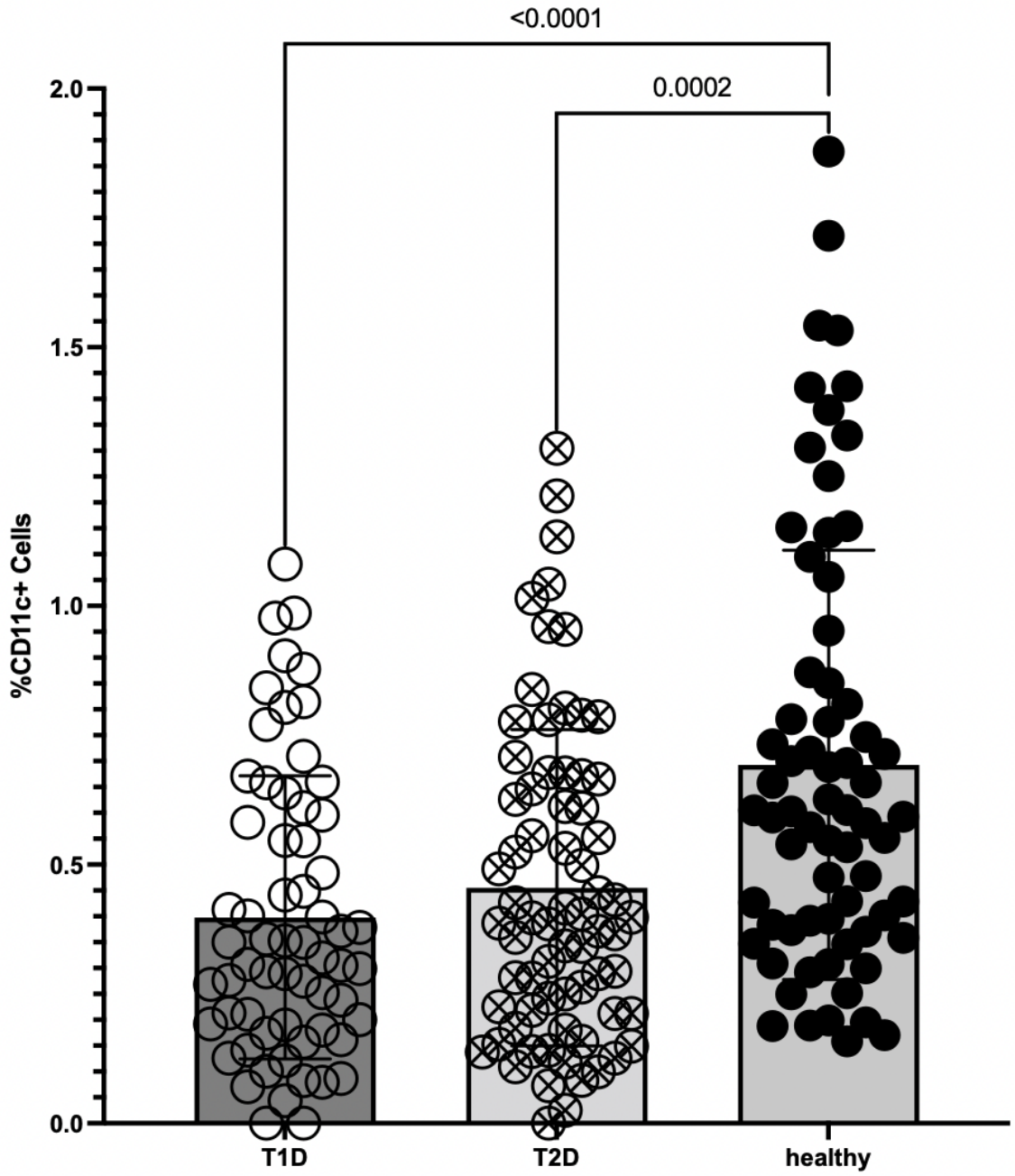


Figure 4.10. Percentages of CD11c+ cells of total cells (identified with DAPI-positive nuclei) in patients with T1D, T2D, and healthy controls. Each dot represents an individual core. Data was analyzed using a one-way ANOVA with multiple comparisons.

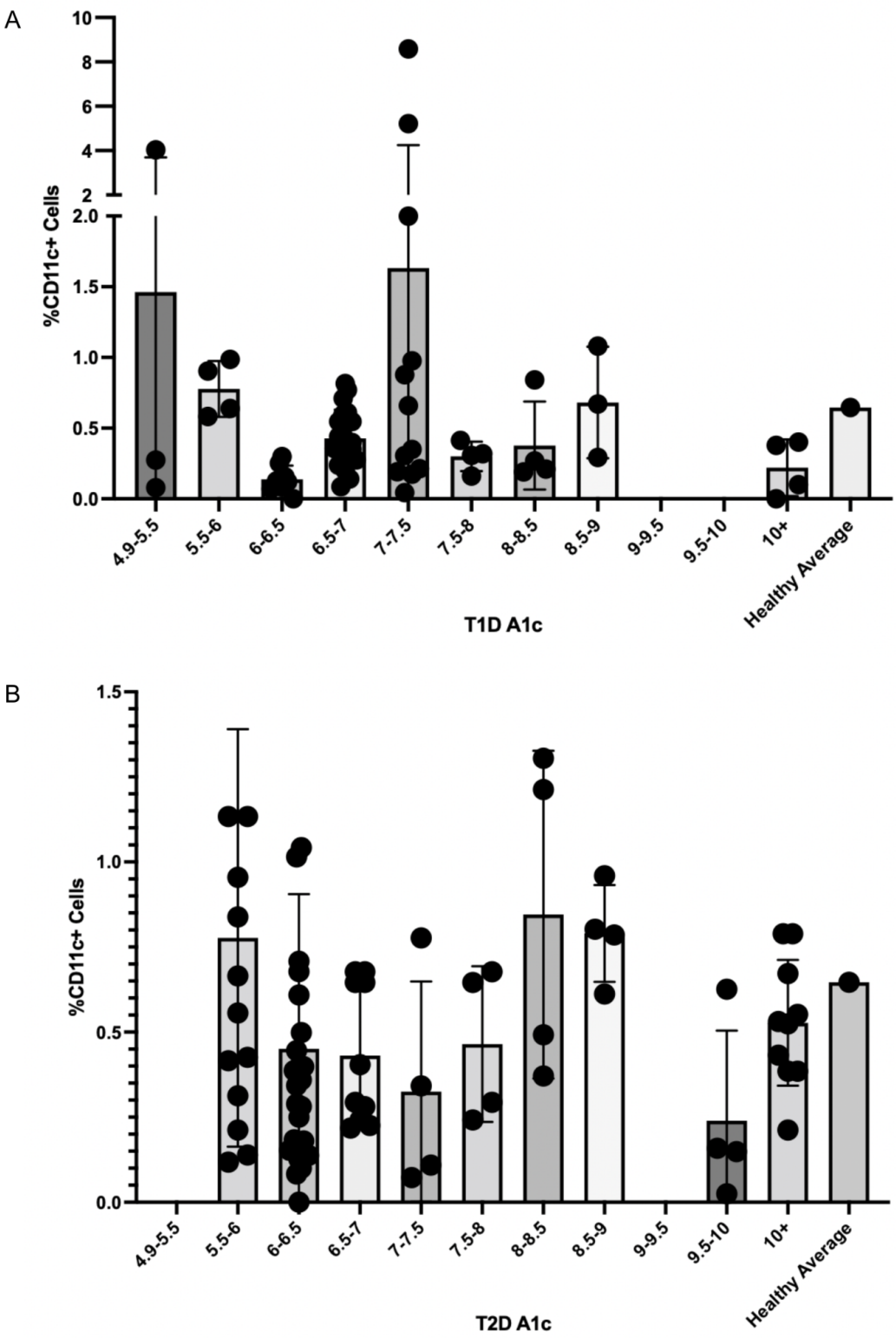


Figure 4.11. Percentages of CD11c+ cells of total cells (identified with DAPI-positive nuclei) in patients with T1D, T2D, and healthy controls shown split by A1c. Each dot represents an individual core. An average of all percentages of CD11c+ cells from healthy patients is shown for reference.

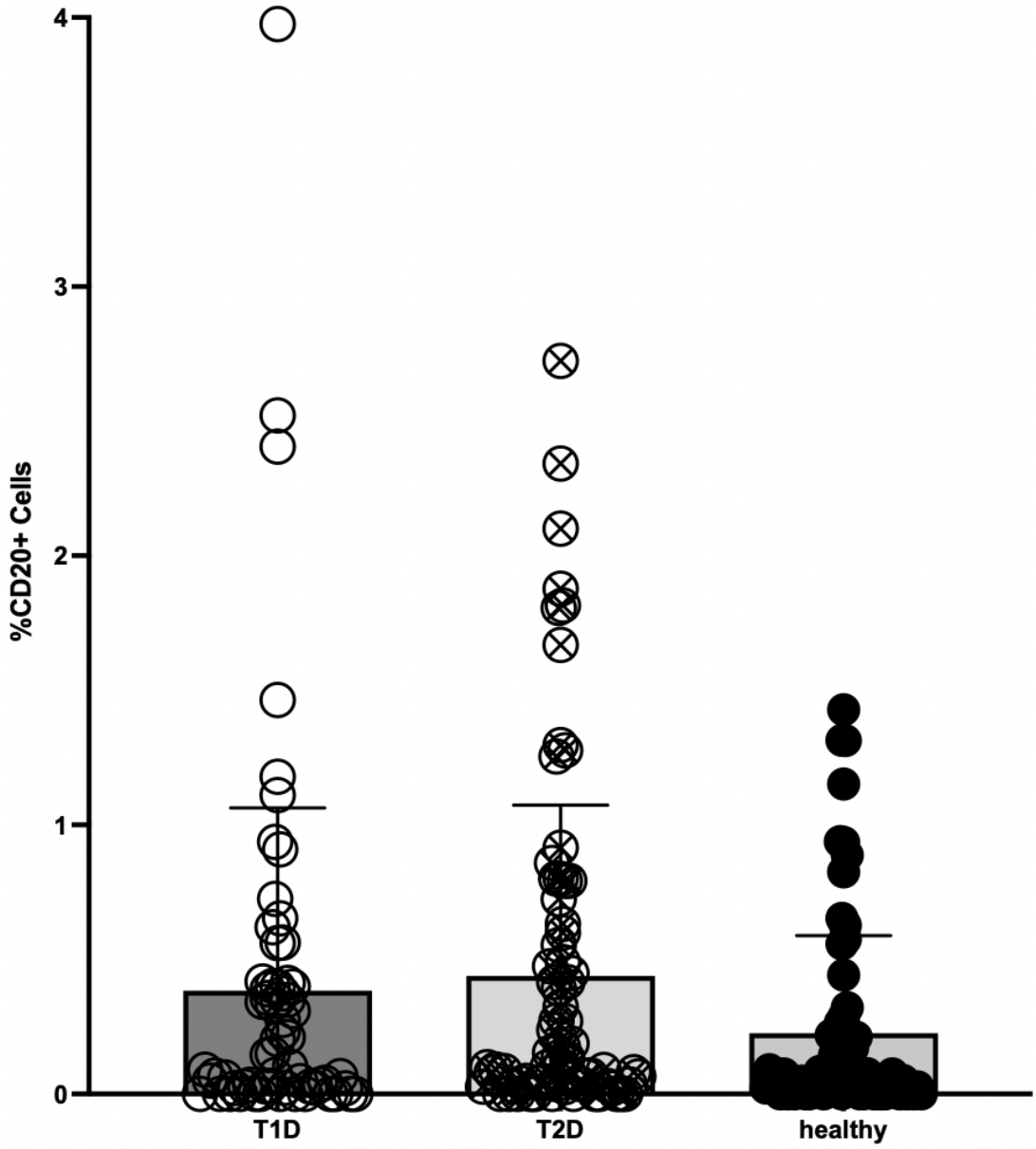


Figure 4.12. Percentages of CD20+ cells of total cells (identified with DAPI-positive nuclei) in patients with T1D, T2D, and healthy controls. Each dot represents an individual core.

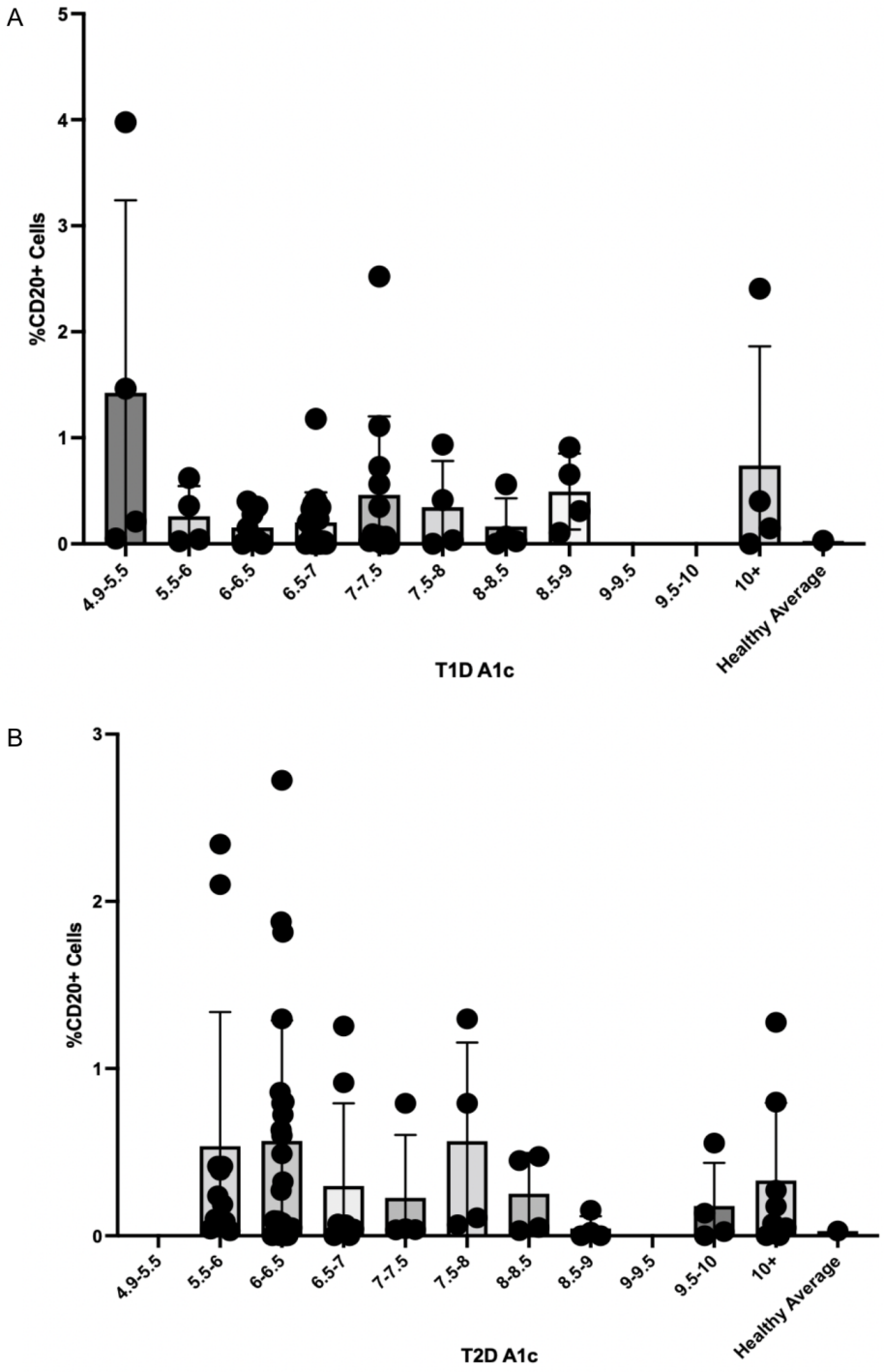


Figure 4.13. Percentages of CD20+ cells of total cells (identified with DAPI-positive nuclei) in patients with T1D, T2D, and healthy controls shown split by A1c. Each dot represents an individual core. An average of all percentages of CD20+ cells from healthy patients is shown for reference.

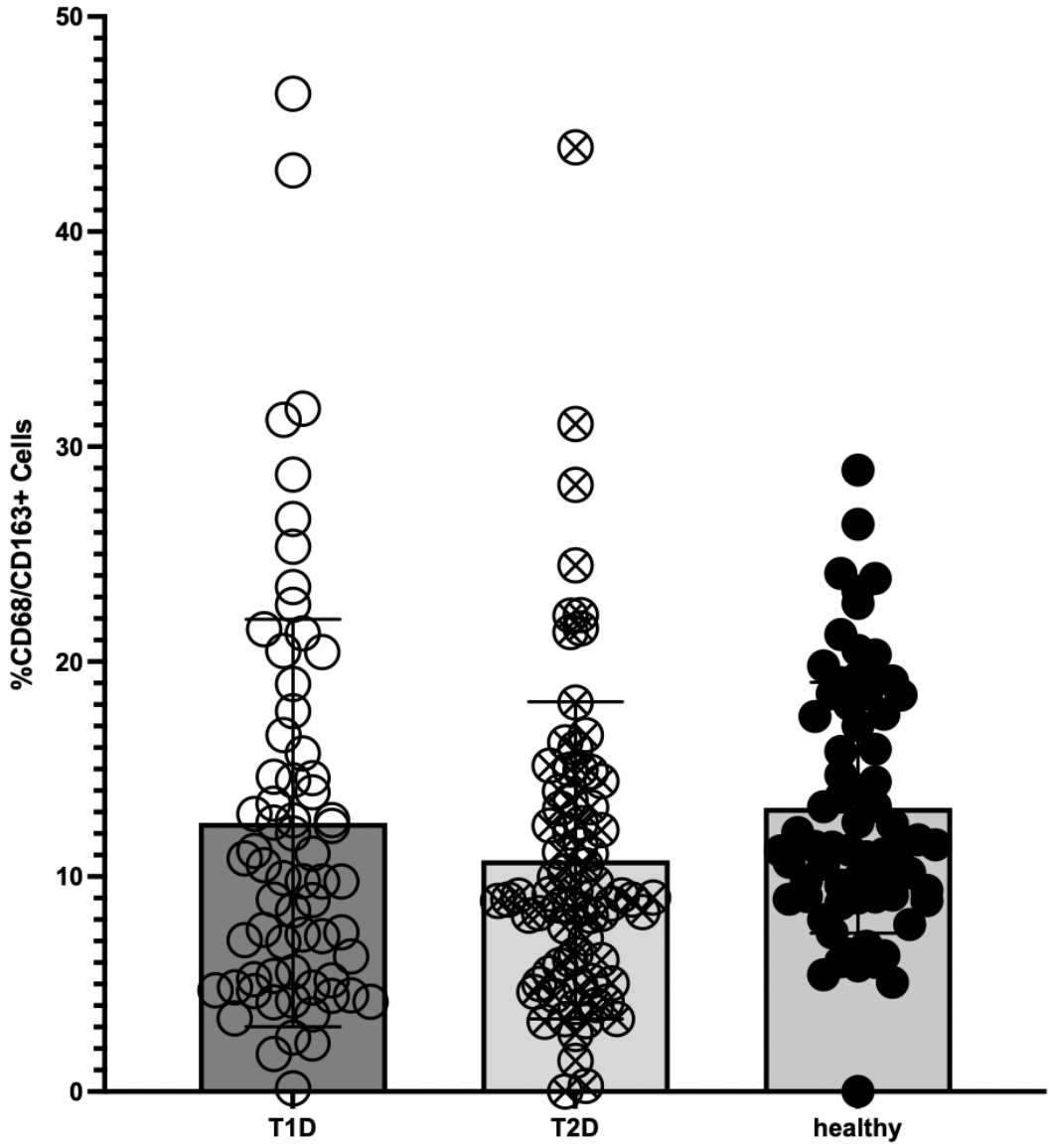


Figure 4.14. Percentages of CD68/CD163+ cells of total cells (identified with DAPI-positive nuclei) in patients with T1D, T2D, and healthy controls. Each dot represents an individual core.

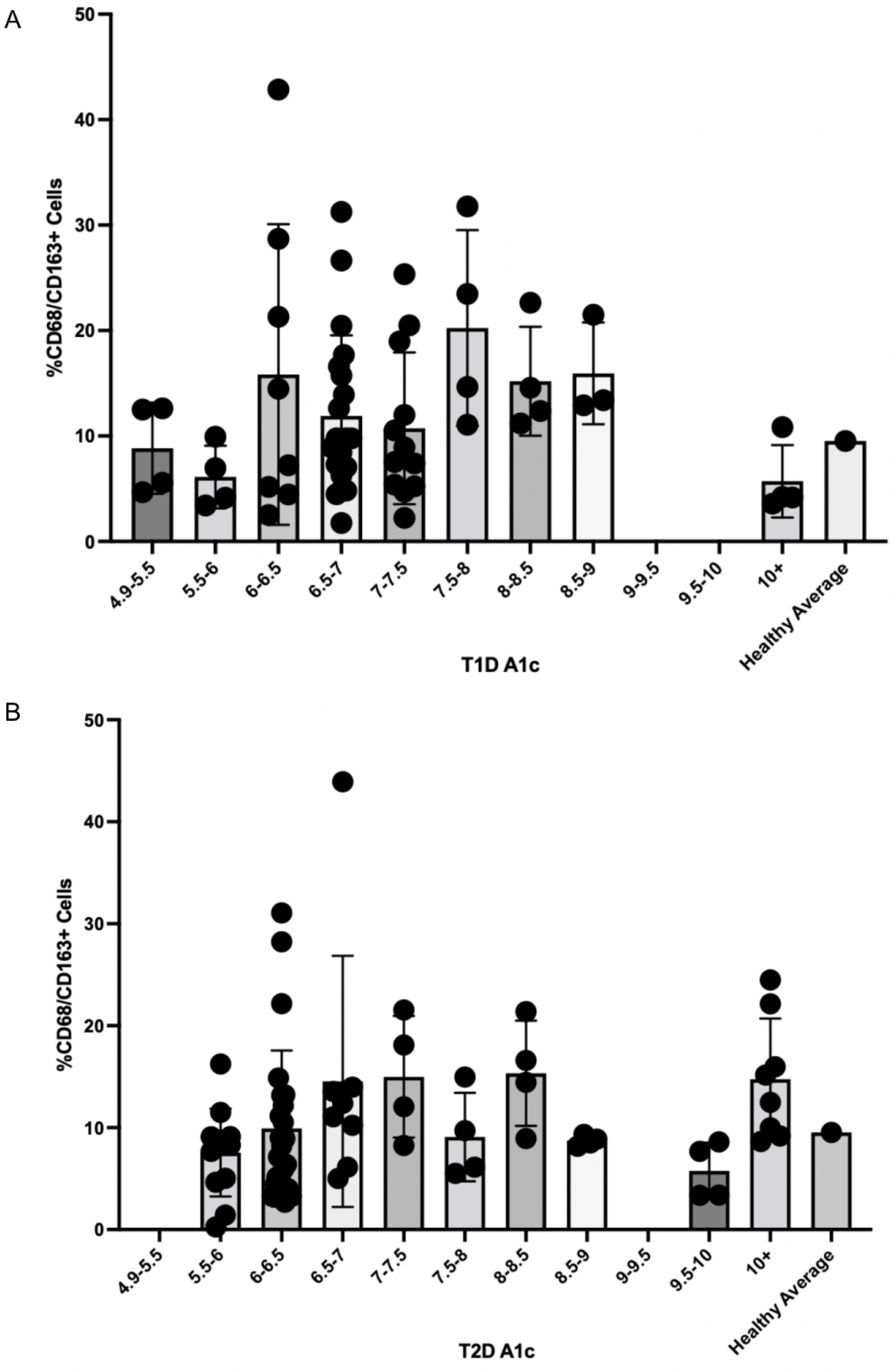


Figure 4.15. Percentages of CD68/CD163+ cells of total cells (identified with DAPI-positive nuclei) in patients with T1D, T2D, and healthy controls shown split by A1c. Each dot represents an individual core. An average of all percentages of CD68/CD163+ cells from healthy patients is shown for reference.

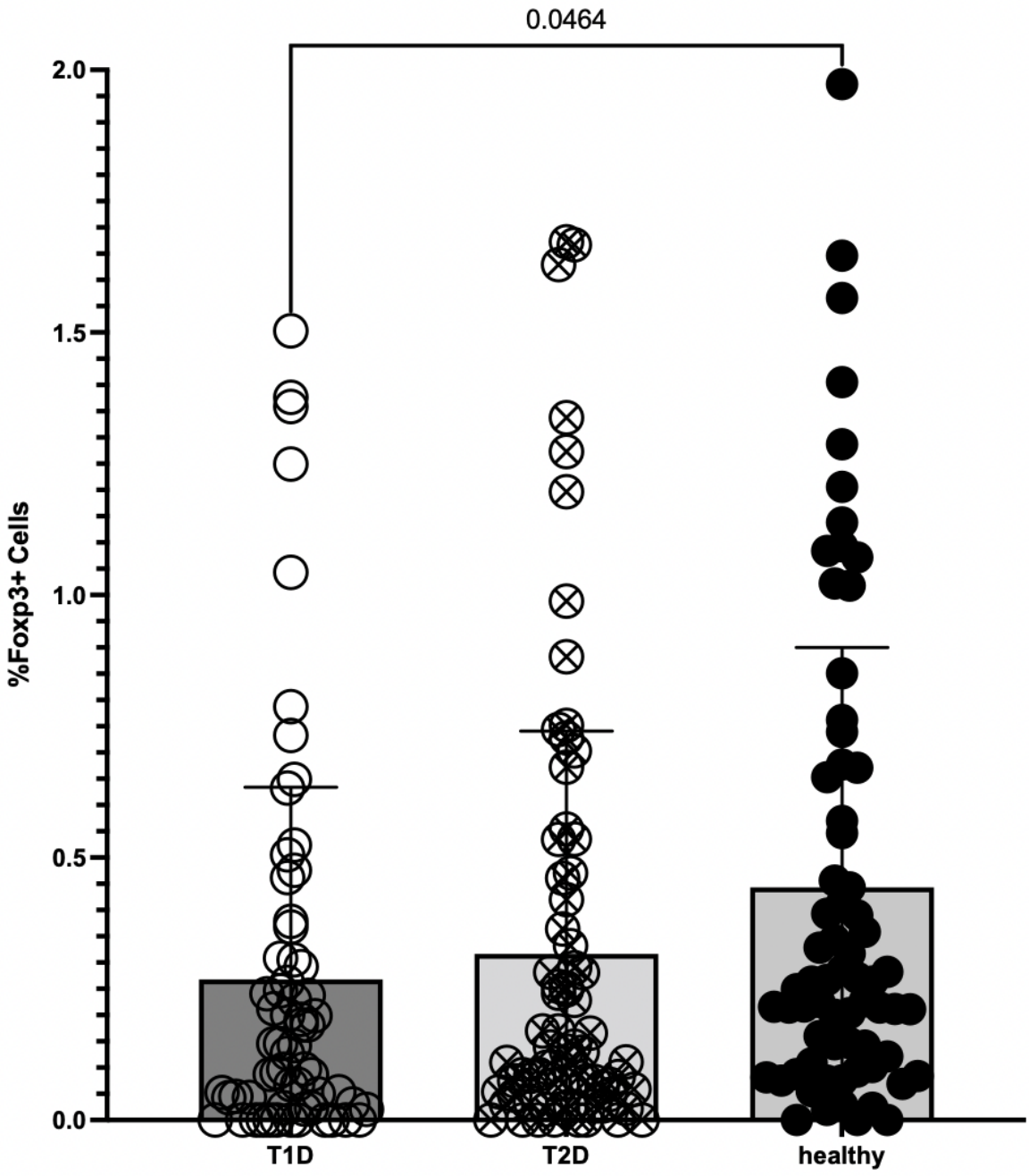


Figure 4.16. Percentages of Foxp3+ cells of total cells (identified with DAPI-positive nuclei) in patients with T1D, T2D, and healthy controls. Each dot represents an individual core. Data was analyzed using a one-way ANOVA with multiple comparisons.

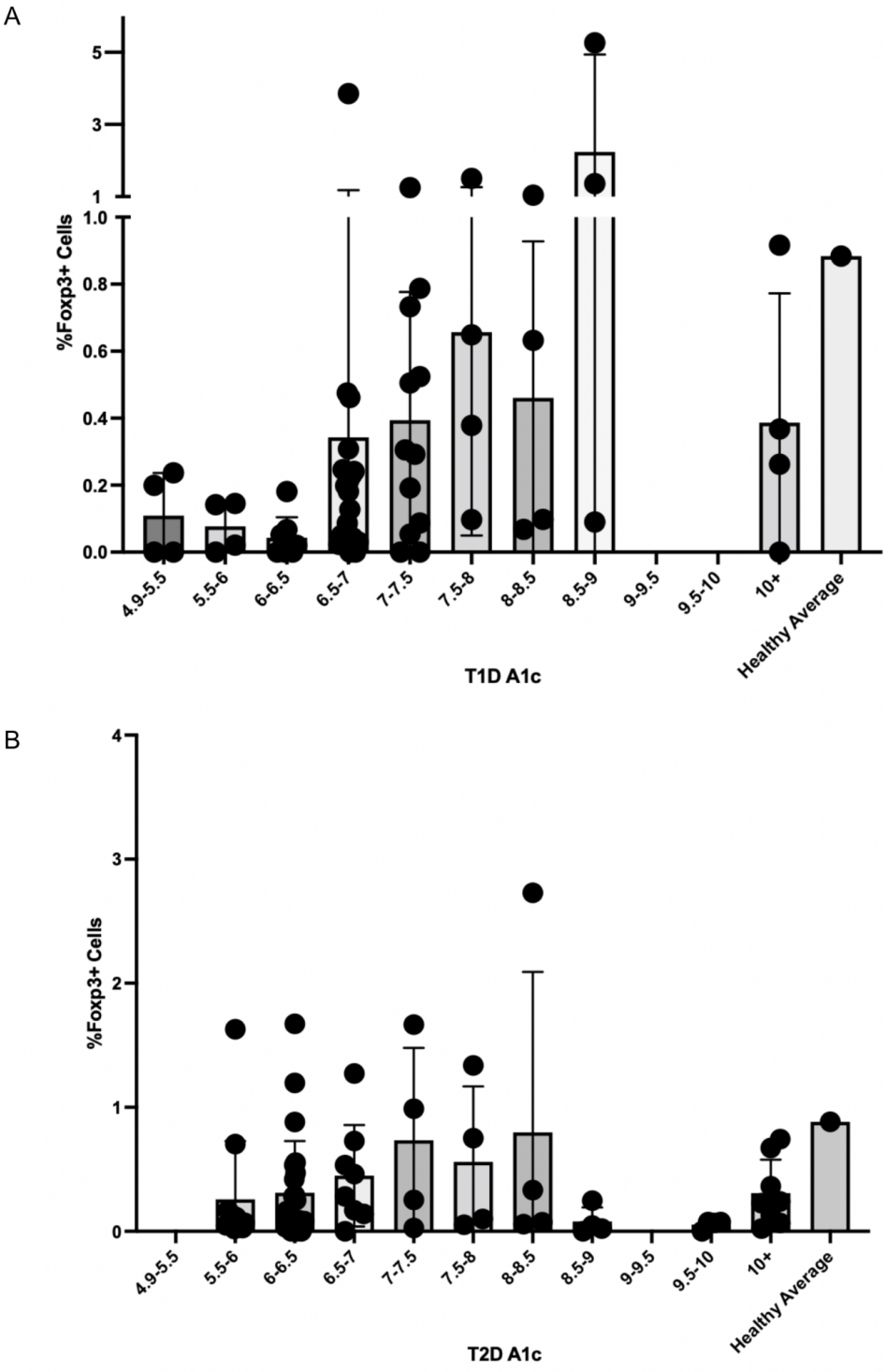


Figure 4.17. Percentages of Foxp3+ cells of total cells (identified with DAPI-positive nuclei) in patients with T1D, T2D, and healthy controls shown split by A1c. Each dot represents an individual core. An average of all percentages of Foxp3+ cells from healthy patients is shown for reference.

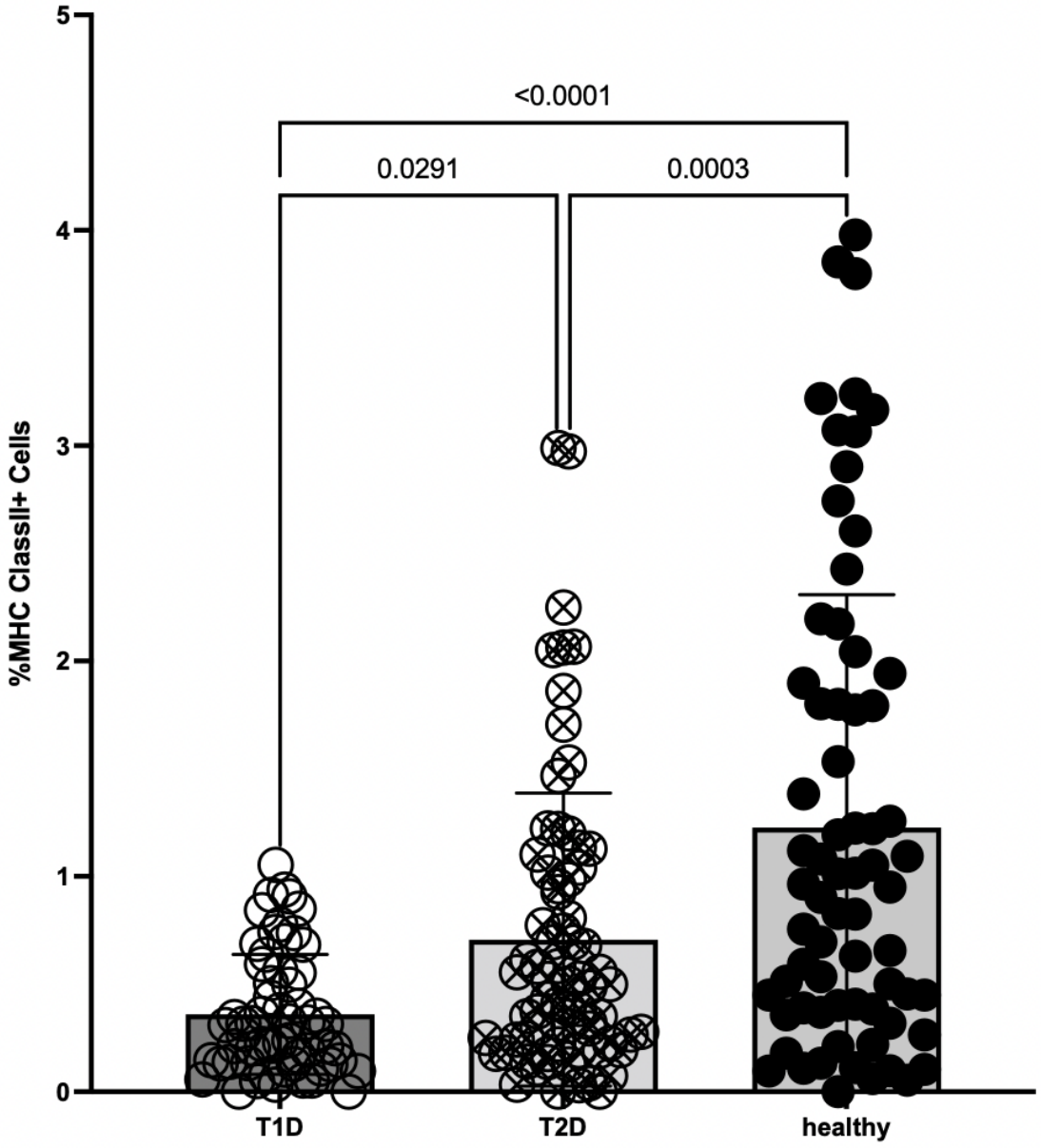


Figure 4.18. Percentages of MHC Class II+ cells of total cells (identified with DAPI-positive nuclei) in patients with T1D, T2D, and healthy controls. Each dot represents an individual core. Data was analyzed using a one-way ANOVA with multiple comparisons.

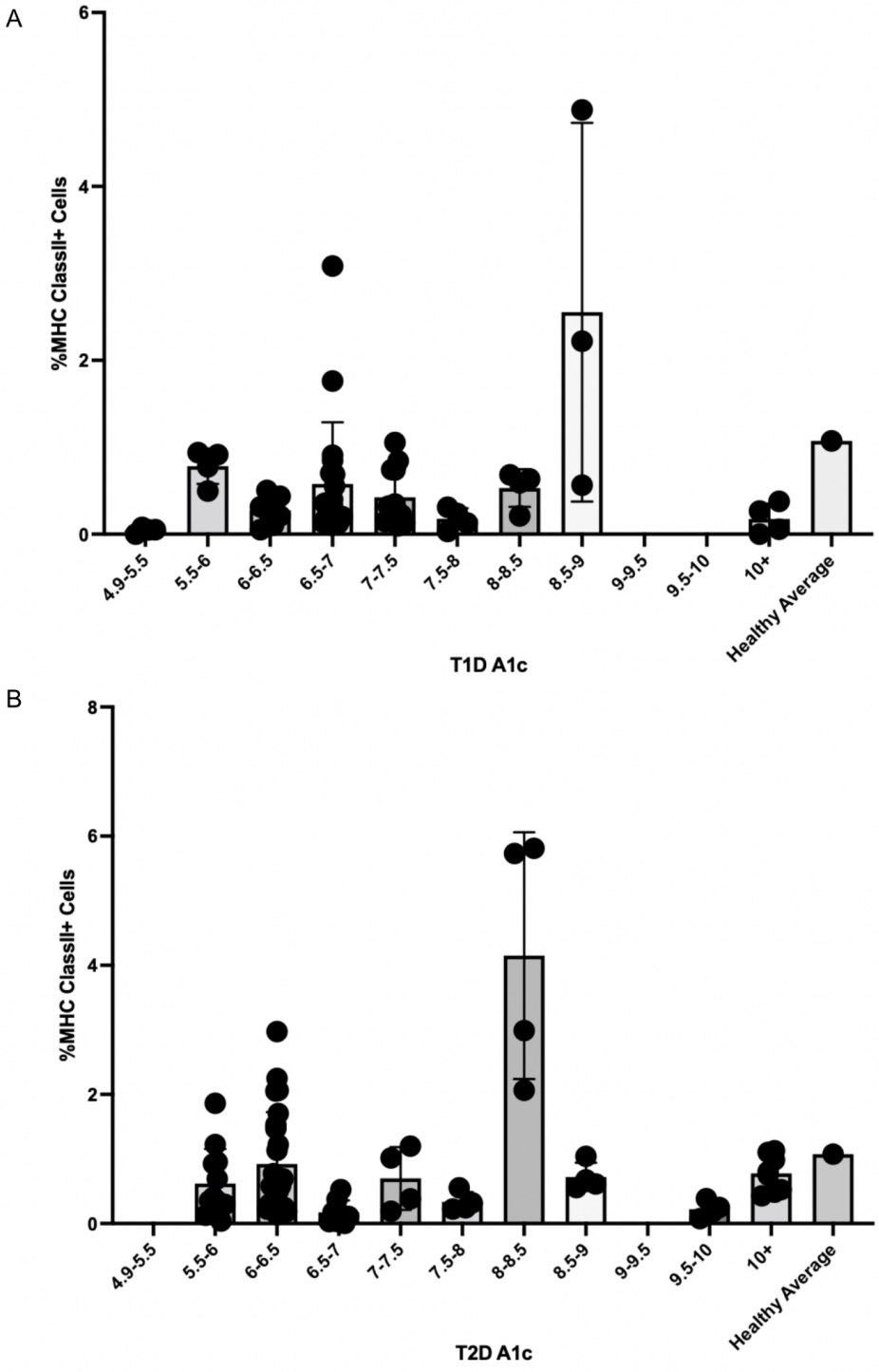


Figure 4.19. Percentages of MHC Class II+ cells of total cells (identified with DAPI-positive nuclei) in patients with T1D, T2D, and healthy controls shown split by A1c. Each dot represents an individual core. An average of all percentages of MHC Class II+ cells from healthy patients is shown for reference.

As most MHC Class II+ cells are antigen presenting cells, I examined the percentages of cells that were CD11c+MHC Class II+, CD68/CD163+MHC Class II+, and CD20+ MHC Class II+ (Figure 4.20A, B; Figure 4.21A). Additionally, some CD4+ cells can express MHC Class II when activated; I also examined the presence of CD4+ MHC Class II+ cells (Figure 4.21B). Patients with T1D had significantly fewer CD68/163+MHC Class II+ cells than healthy controls (Figure 4.20B). Both patients with T1D and T2D had significantly fewer CD11c+MHC Class II+ cells than healthy controls (Figure 4.21A). Patients with T1D also had significantly fewer CD4+MHC Class II+ cells than healthy patients (Figure 4.21B). Each dot represents an individual placental core. Together, these data indicate significant differences in the immunologic makeup of the human placenta in patients with both T1D and T2D. Notably, the specific changes in CD11c+ cells, Foxp3+ cells, and MHC Class II+ cells may be attributable to the additional burden of autoimmunity present for patients with T1D.

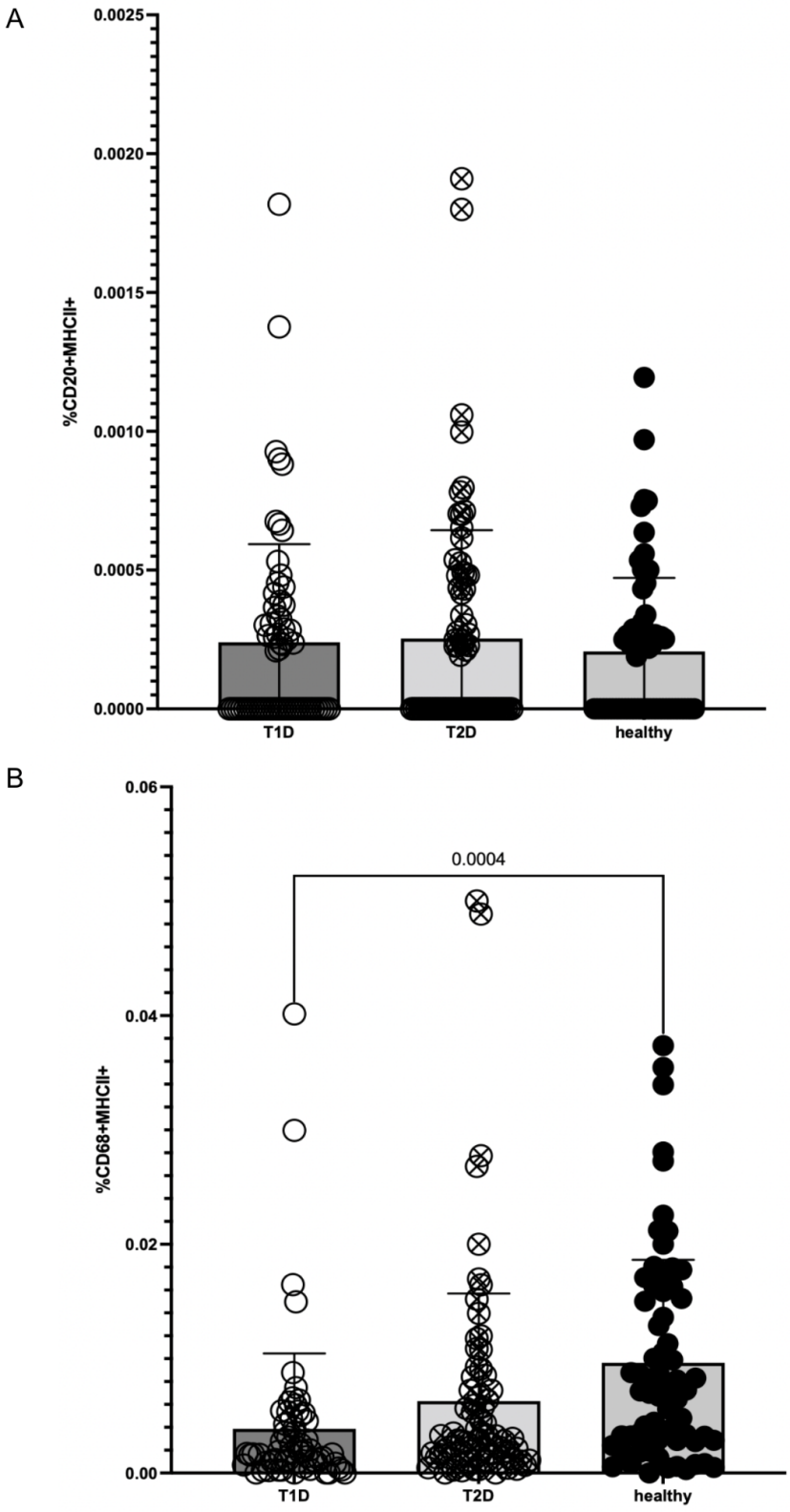


Figure 4.20. Percentage of cells that were CD11c+MHC ClassII+ (A) and CD68/163+MHC Class II+ (B) in patients with T1D, T2D, and healthy controls. Each dot represents an individual core. Data was analyzed using a one-way ANOVA with multiple comparisons.

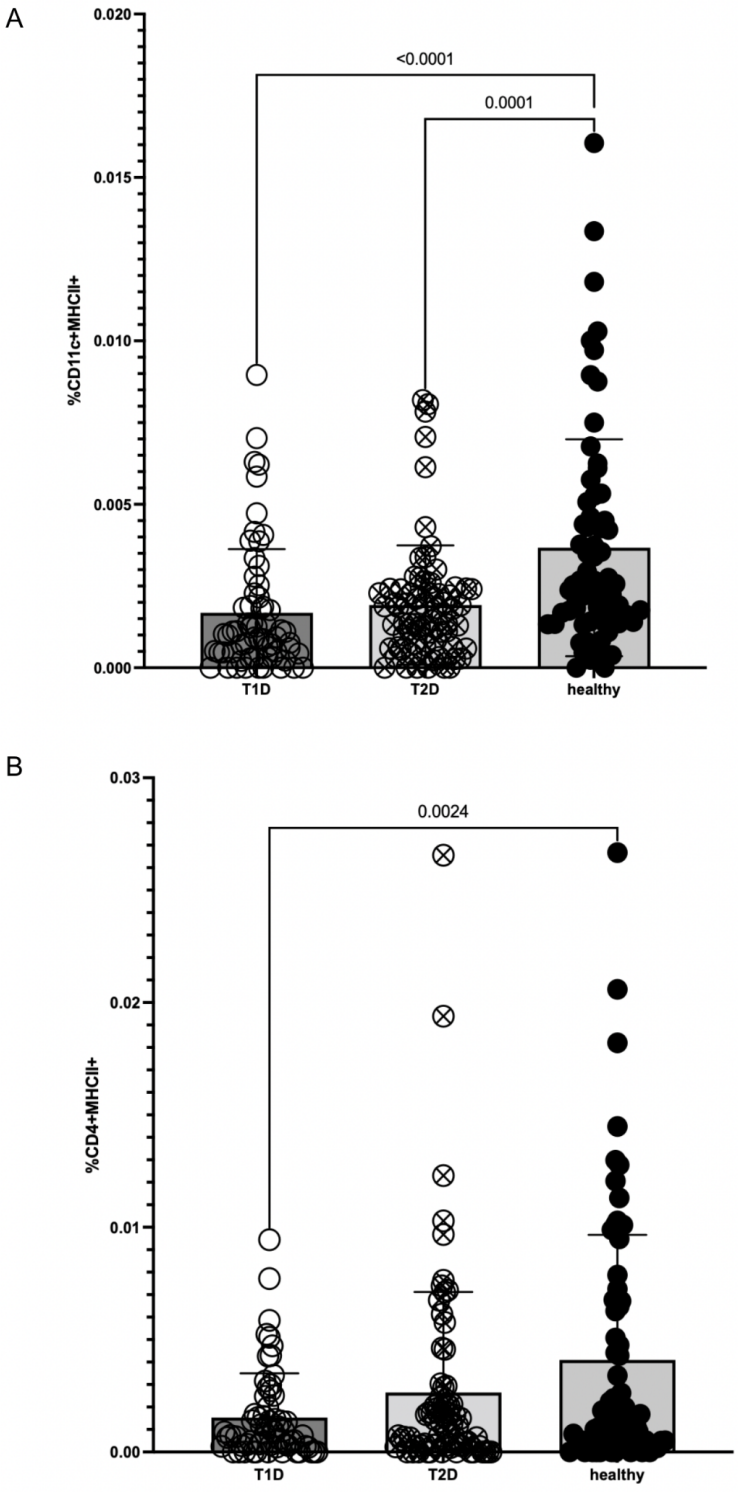


Figure 4.21. Percentage of cells that were CD20+MHC ClassII+ (A) and CD4+MHC Class II+ (B) in patients with T1D, T2D, and healthy controls. Each dot represents an individual core. Data was analyzed using a one-way ANOVA with multiple comparisons.

Vascular Markers in Placentas from Patients with Type 1 Diabetes Are Unchanged From Healthy Controls

In Chapter III, I identified vascular changes present in placentas from pre-diabetic NOD mice, including changes in CD31+ endothelial cells. I hypothesized similar changes may be present in the human placentas. Using immunohistochemistry, I measured CD31 on placenta TMAs (Figure 4.22). Once stained, slides were analyzed using HALO software, measuring CD31 signal in relation to DAPI+ nuclei (Figure 4.23). There is a large variation in the number of CD31+ cells within groups, but no significant difference between groups.

Several other proteins involved in vasculature were identified as being possibly dysregulated in diabetes. Angiopoietin 1 (ANGTP1) helps regulate angiogenesis and promote endothelial cell survival and the Thromboxane A2 Receptor (TBXA2R) interacts with Thromboxane A2 to promote platelet aggregation. When placenta TMA slides were stained for ANGTP1, I found no difference between conditions (Figure 4.24, 4.25).

Placenta slides stained for TBXA2R could not be interpreted using HALO because the staining intensity made it difficult to identify cell membranes (Figure 4.26). After consultation with a pathologist, a 1-3 grading scale was developed based on intensity of staining; values for the four cores per patient were added together to give a lowest possible score of 4 and highest possible score of 12 (Figure 4.27). There may be a slight increase in TBXA2R staining in patients with T1D and T2D, but no significant difference (Figure 4.28). While vascular complications are a known sequelae of pregnancy in both NOD mice and patients with T1D, they appear to not be attributable to alterations in CD31, ANGPT1, or TBXA2R found in the placenta at delivery.

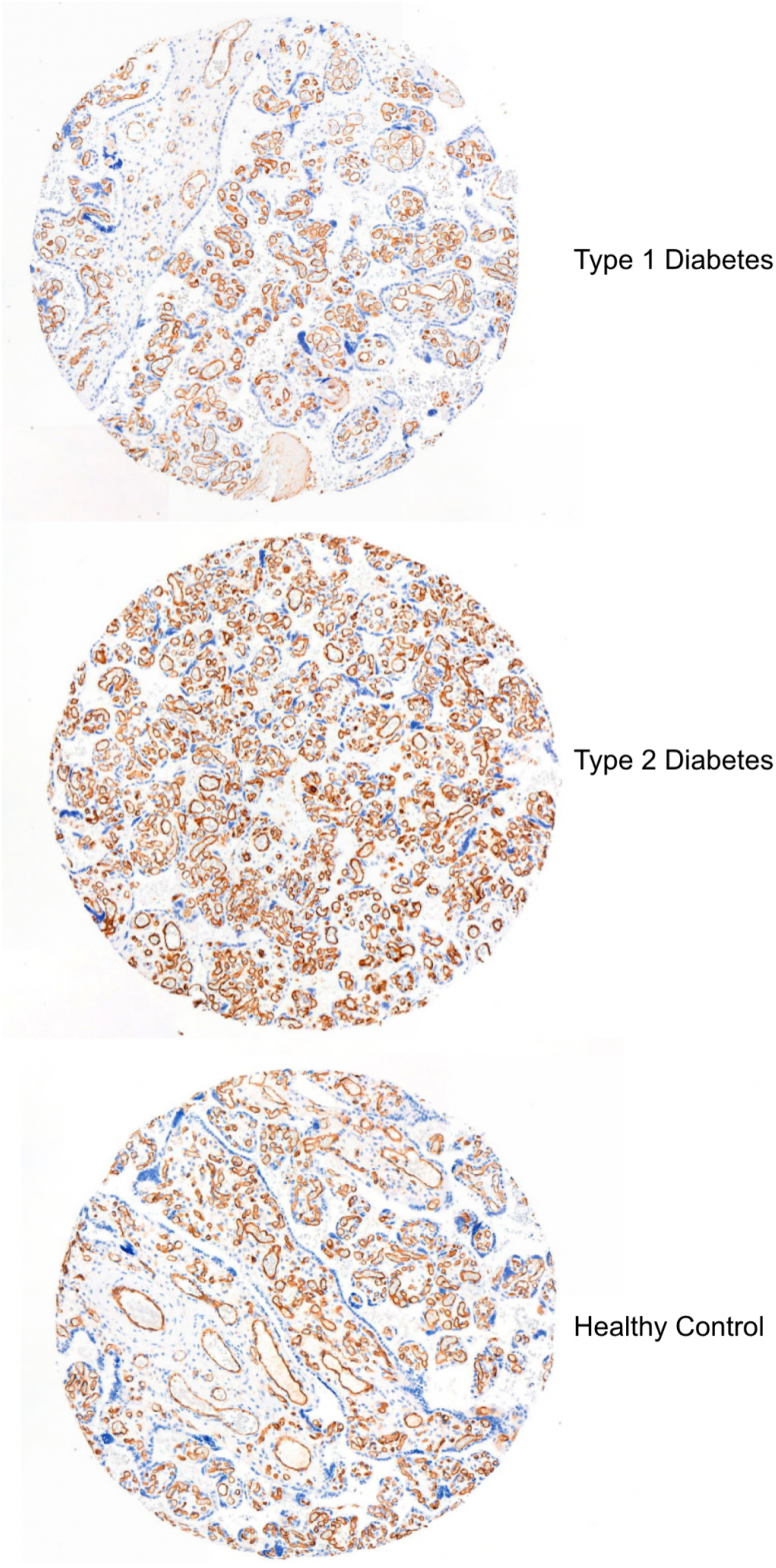


Figure 4.22. Selected CD31-stained cores from a patient with T1D, with T2D, and a healthy control.

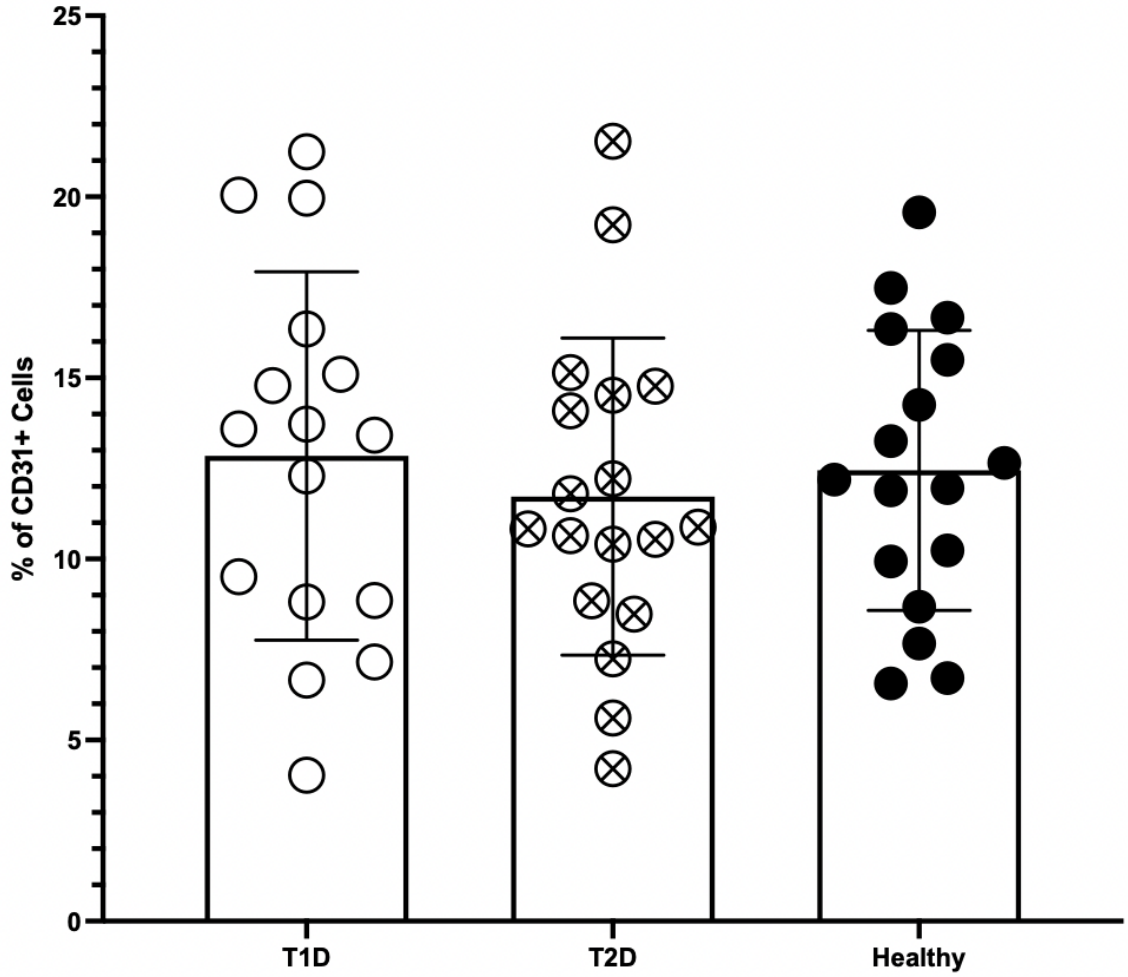


Figure 4.23. Percentage of cells that were CD31+ in placenta cores from patients with T1D, T2D, and healthy controls. Each dot represents the average of four cores per patient.

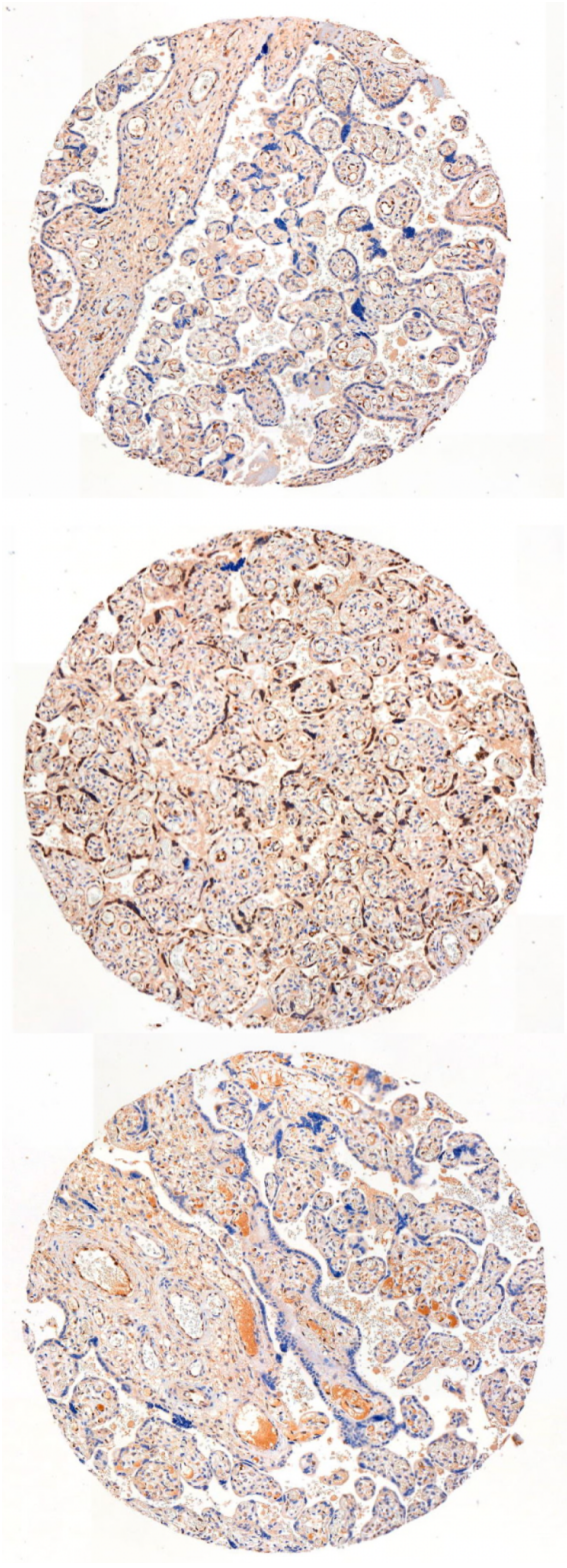


Figure 4.24. Selected ANGPT1-stained cores from a patient with T1D, with T2D, and a healthy control.

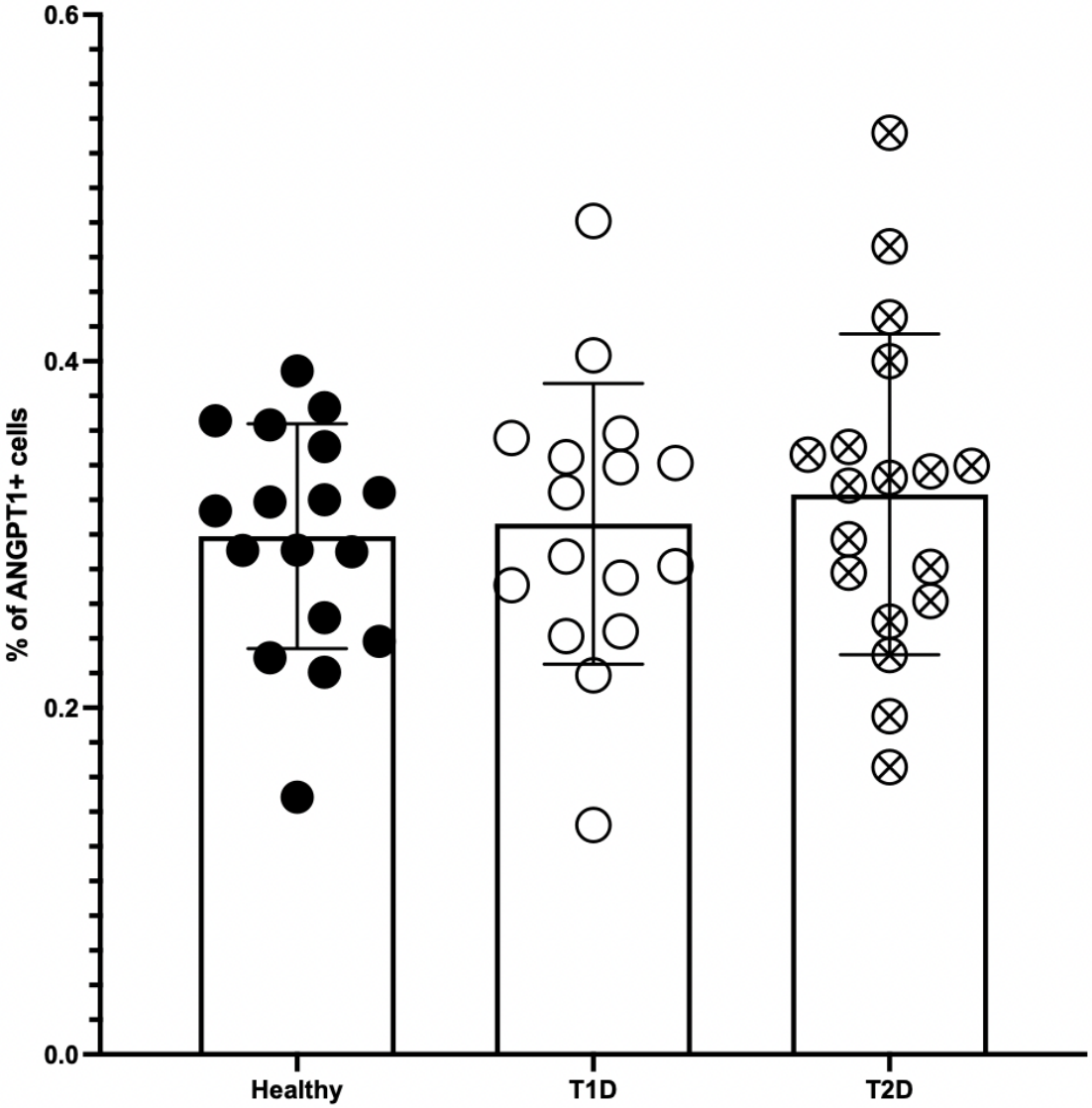


Figure 4.25. Percentage of cells that were ANGPT1+ in placenta cores from patients with T1D, T2D, and healthy controls. Each dot represents the average of four cores per patient.

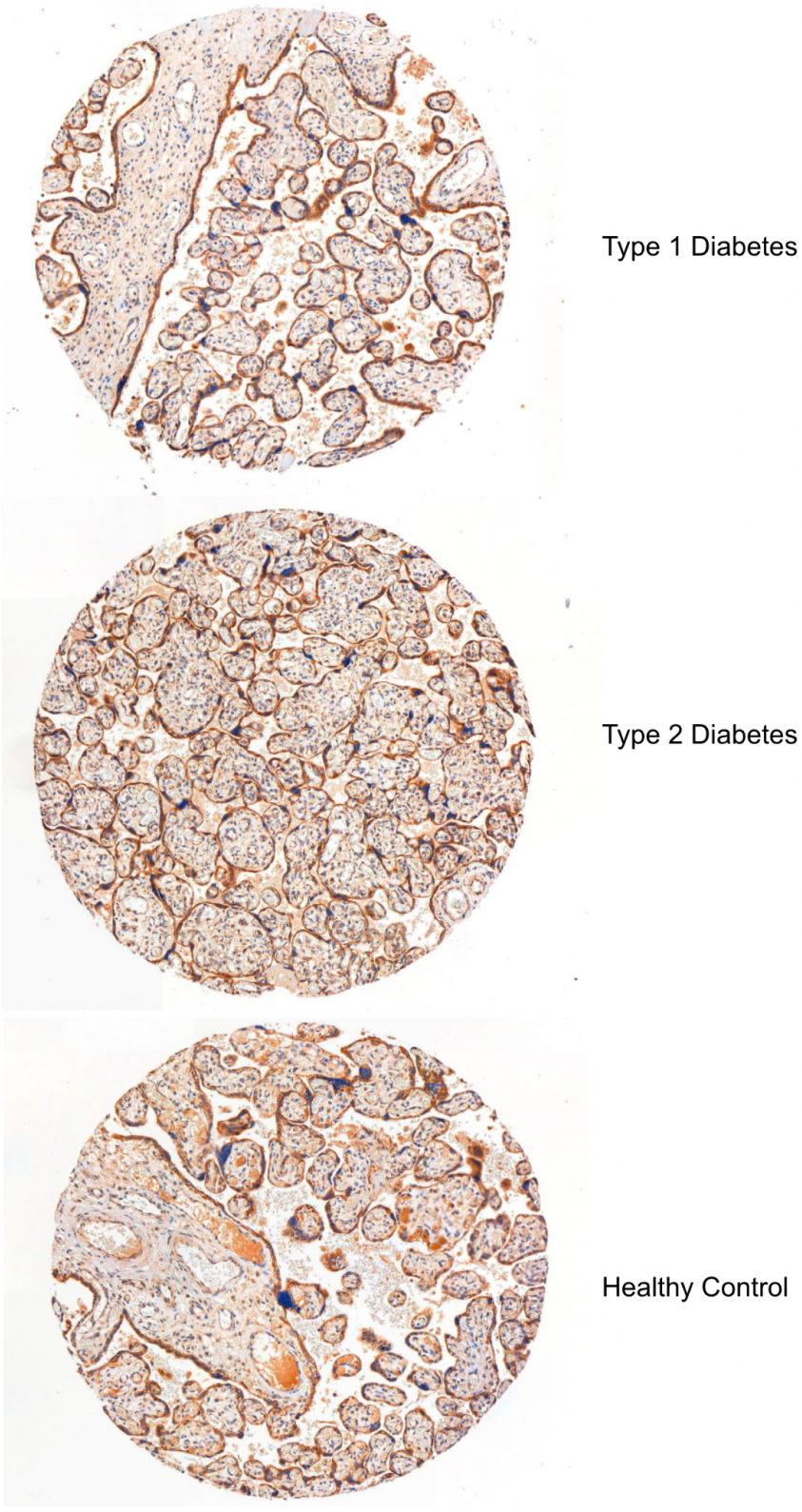
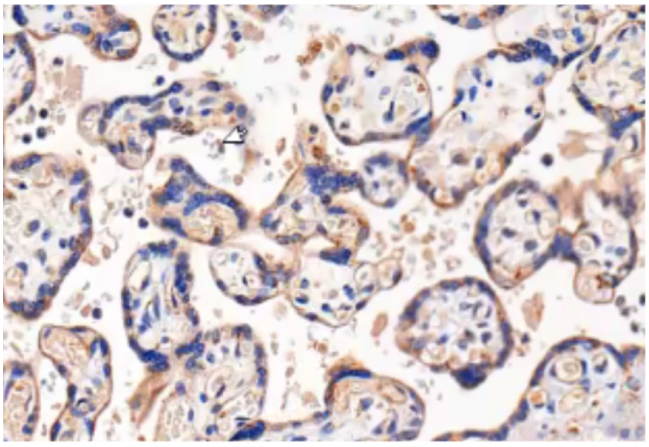
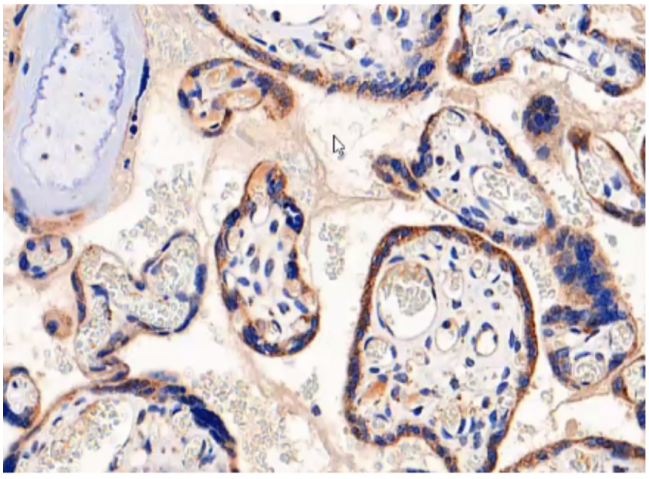


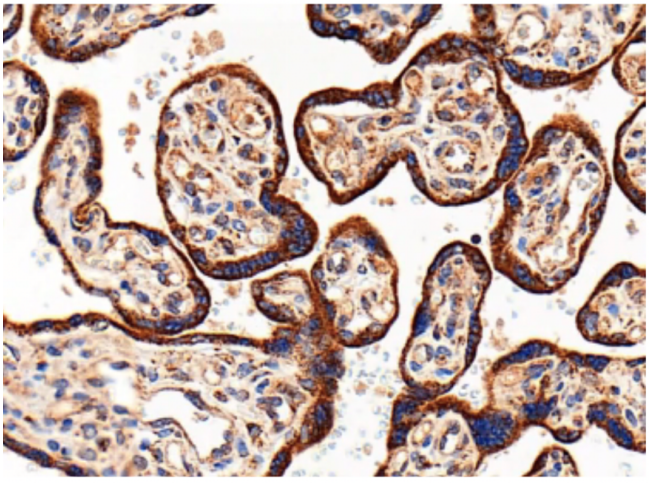
Figure 4.26. Selected TBXA2R-stained cores from a patient with T1D, with T2D, and a healthy control.



Score: 1
Staining in endothelium is the same color as platelets



Score: 2



Score: 3
Difficult to visualize nuclear detail or delineate cell membranes

Figure 4.27. Selected images representing numbers 1-3 on the grading scale; a value of 1 was assigned to cores with minimal staining that rendered endothelium similar in color to platelets. A value of 3 was assigned to cores with heavy staining that obscured nuclei and cell membranes.

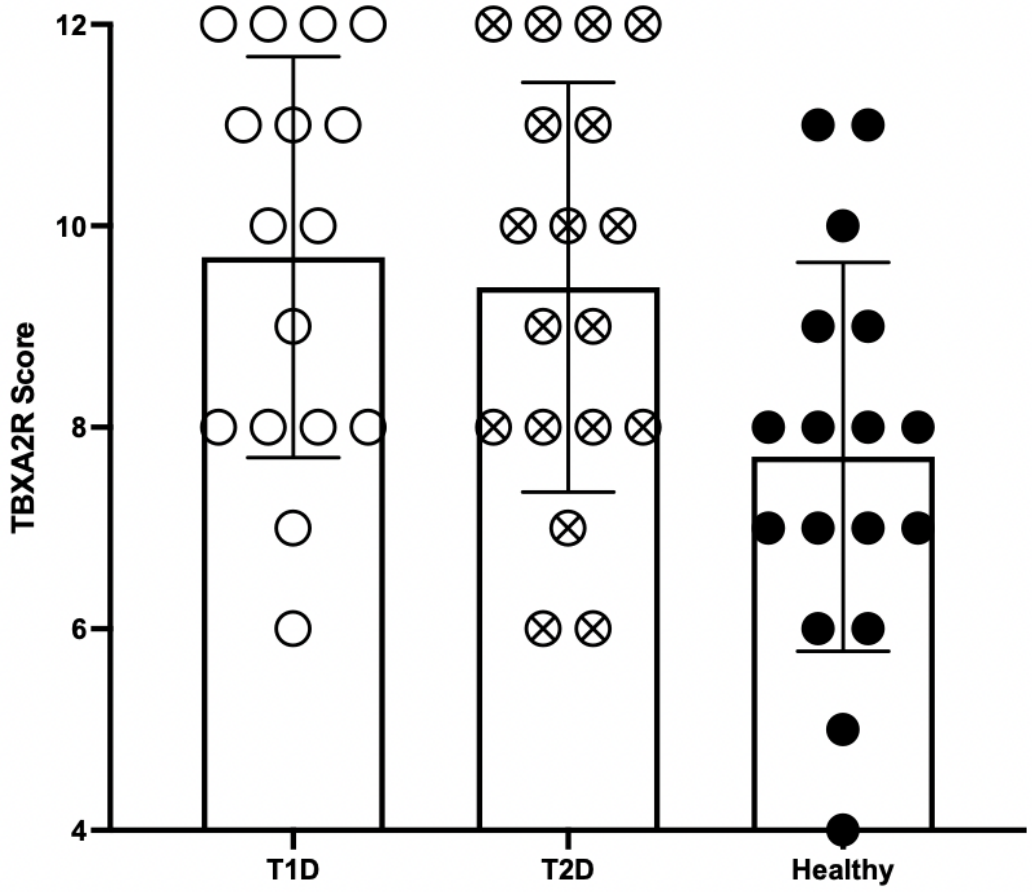


Figure 4.28. Percentage of cells that were TBXA2R+ in placenta cores from patients with T1D, T2D, and healthy controls. Each dot represents the average of four cores per patient.

Patients with T1D Have an Increased Risk of Adverse Vascular Outcomes Across All A1cs

Studies in patients with T1D have reported increased risks of vascular complications like preeclampsia, but the impact of normal or target A1C on vascular complications remains poorly understood relative to the general pregnant population. With the help of collaborators, I used the EHR at Vanderbilt University Medical Center to identify 354 pregnant people with and 45,467 without T1D. I identified specific adverse outcomes that could be attributable to vascular disease, which were grouped into Phecodes (Figure 4.29).

Among pregnant people with T1D, 55 (15.5%) experienced a vascular complication in pregnancy compared to 3,633 (8%) of those without T1D (Figure 4.30). Given the reported increased risk of adverse vascular outcomes with higher preconception A1c, I hypothesized that hemoglobin A1c early in pregnancy modifies the probability of vascular outcomes. I found that patients with T1D have an increased likelihood of experiencing vascular complications in pregnancy regardless of their A1c (Figure 4.31A). Even at a normal A1c of 5.5, pregnant individuals with T1D had increased odds (2.17, 95% Confidence Interval [CI]: 1.3, 3.62) of vascular pathology relative to patients without T1D (Figure 4.31B). Furthermore, I found that pregnant individuals with T1D were more likely to experience preeclampsia, eclampsia, or HELLP syndrome (conditions only found in pregnancy that can include symptoms of new-onset hypertension with headache and liver disease, seizures, and thrombocytopenia) relative to patients without T1D regardless of their A1c (Figure 4.32A). Notably, the odds of developing preeclampsia, eclampsia, or HELLP syndrome were increased (4.02, 95% CI: 2.2, 7.33) even at a normal A1c, 5.5 (Figure 4.32B). Figure 4.31A and 4.32A show the shape of the relationship between A1c and each outcome among people with T1D.

Phecode Labels and Outcome Assignment.			
Phecode	Label	Vascular Composite Outcome Inclusion	Preeclampsia, Eclampsia, HELLP Syndrome Inclusion
907.2	Placental infarction	Yes	No
908	Edema, proteinuria and hypertensive disorders in pregnancy, childbirth and the puerperium	Yes	No
908.1	Preeclampsia and eclampsia	Yes	Yes
908.11	Severe pre-eclampsia or HELLP syndrome	Yes	Yes
908.12	Eclampsia	Yes	Yes
908.3	Gestational [pregnancy-induced] hypertension without significant proteinuria	Yes	No
908.4	Gestational edema and proteinuria	Yes	No
https://github.com/abraham-abin13/a1c_pregnancy_outcomes.git			

Figure 4.29. List of Phecodes used to compile a list of vascular composite outcomes and preeclampsia, eclampsia, and HELLP syndrome outcomes.

	No Type 1 Diabetes (N=45467)	Type 1 Diabetes (N=354)	Overall (N=45821)
Hemoglobin A1c Close to Delivery			
Median [Q1, Q3]	5.30 [5.23, 5.37]	7.50 [6.50, 8.70]	5.30 [5.23, 5.37]
Min, Max	4.89, 5.72	4.70, 14.0	4.70, 14.0
Missing	0 (0%)	127 (35.9%)	127 (0.3%)
Hemoglobin A1c Close to Conception			
Median [Q1, Q3]	5.30 [5.23, 5.37]	6.90 [6.10, 7.90]	5.30 [5.23, 5.37]
Min, Max	4.86, 5.72	4.70, 12.8	4.70, 12.8
Missing	0 (0%)	127 (35.9%)	127 (0.3%)
Age (years)			
Median [Q1, Q3]	27.6 [23.2, 31.8]	26.2 [21.7, 30.6]	27.6 [23.2, 31.8]
Min, Max	8.37, 48.9	15.2, 41.5	8.37, 48.9
Race			
White	29734 (65.4%)	279 (78.8%)	30013 (65.5%)
Black	7744 (17.0%)	58 (16.4%)	7802 (17.0%)
Unknown	5546 (12.2%)	13 (3.7%)	5559 (12.1%)
Asian	2256 (5.0%)	4 (1.1%)	2260 (4.9%)
Native American	187 (0.4%)	0 (0%)	187 (0.4%)
Deprivation Index			
Median [Q1, Q3]	0.340 [0.254, 0.435]	0.350 [0.275, 0.420]	0.340 [0.254, 0.435]
Min, Max	0.0210, 0.827	0.0980, 0.827	0.0210, 0.827
Missing	15533 (34.2%)	126 (35.6%)	15659 (34.2%)
Any Vascular Pathology			
No	41834 (92.0%)	299 (84.5%)	42133 (92.0%)
Yes	3633 (8.0%)	55 (15.5%)	3688 (8.0%)
Pre-Eclampsia/Eclampsia/HELLP Syndrome			
No	43889 (96.5%)	315 (89.0%)	44204 (96.5%)
Yes	1578 (3.5%)	39 (11.0%)	1617 (3.5%)

Figure 4.30. Characteristics of patients pulled from the EHR at Vanderbilt University Medical Center. Individuals without T1D hemoglobin A1c values were missing by design and were imputed randomly from a normal distribution with mean 5.3 and standard deviation of 0.1 informed based on published literature to avoid singularity problems in the regression models.(Harrell , 2015; Selvin et al., 2009)

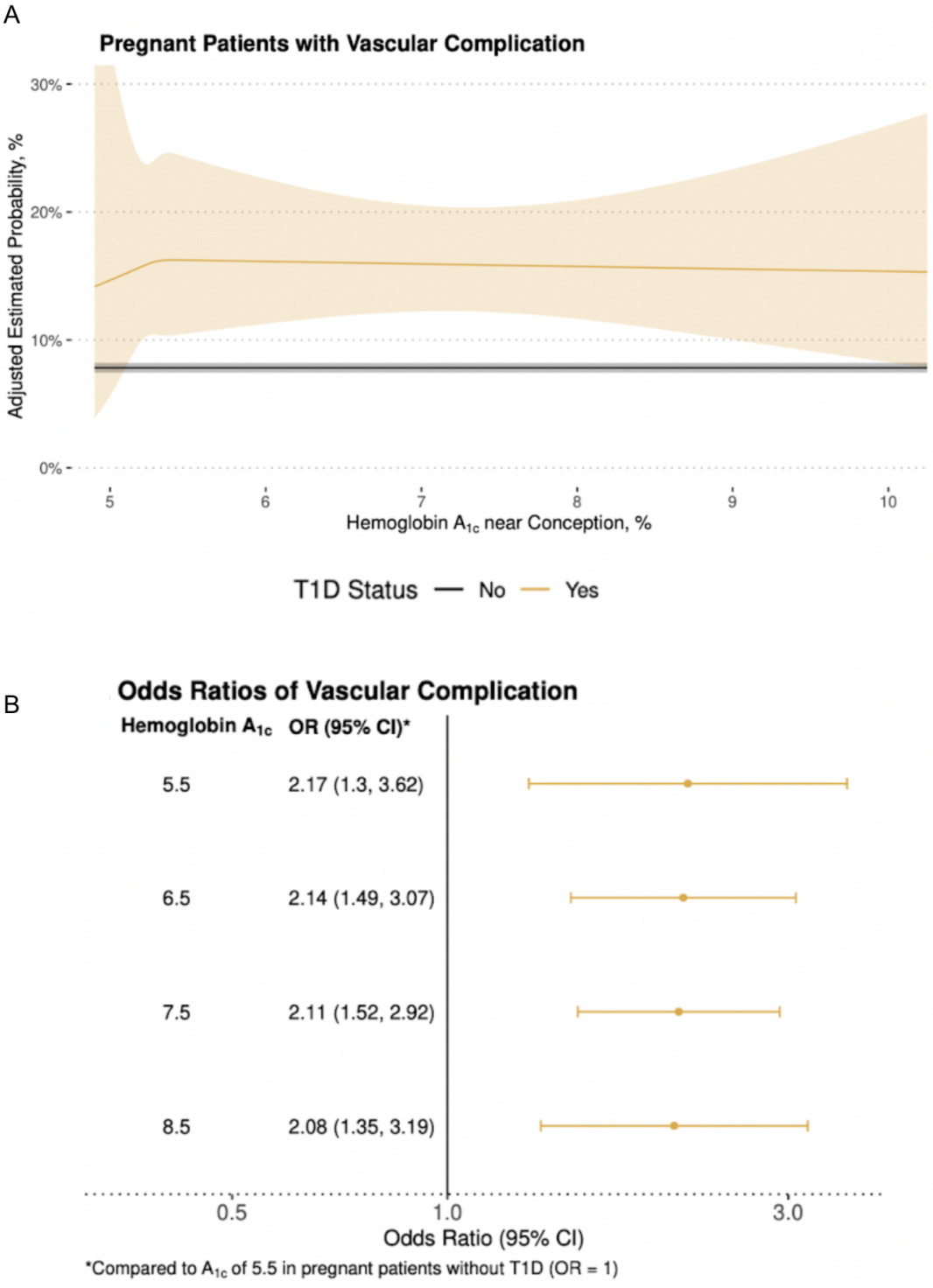


Figure 4.31. EHR data illustrating differences in vascular outcomes in patients with T1D compared to the general population. Proportion of pregnant patients with a vascular complication in the general population (black line) and with T1D (gold line with 95% CI) (A). When stratified by Hemoglobin A1c at the first recorded antenatal visit, patients with T1D had an increased odds ratio of a vascular complication across all A1c values (B).

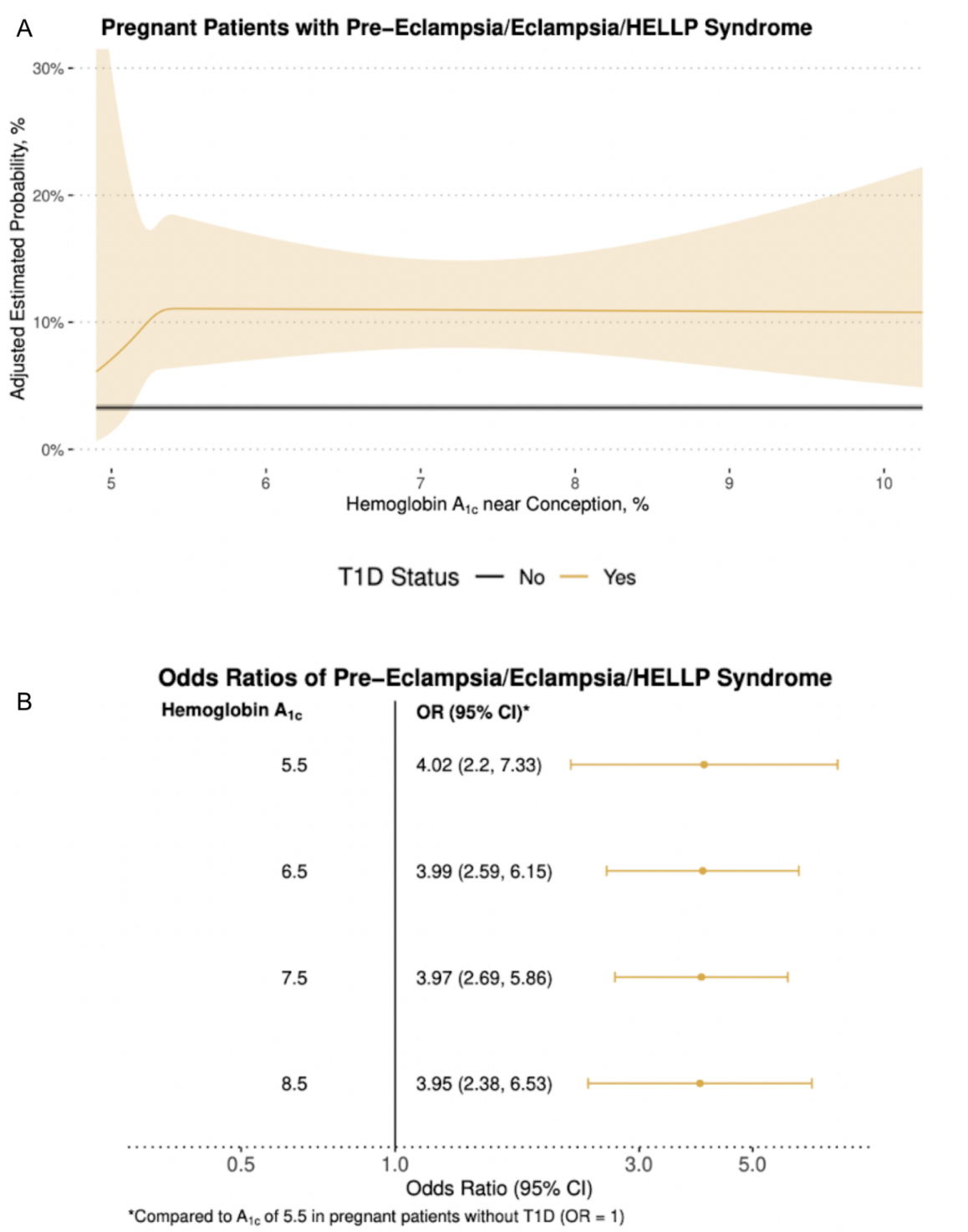


Figure 4.32. Proportion of patients with Phecodes for preeclampsia, eclampsia, or HELLP syndrome in the general population (black line) and with T1D (gold line with 95% CI) (A). When stratified by Hemoglobin A1c near conception, patients with T1D had an increased odds ratio of preeclampsia, eclampsia, or HELLP across all A1c values (B).

While I detected high probabilities of adverse vascular outcomes regardless of A1c, the small cohort size resulted in wide confidence intervals at higher A1cs. The probability of adverse vascular outcomes may increase more dramatically at higher A1C values than shown in Figures 31A and 32A. Even so, the increase in vascular complications at normal A1c compared to the general population shows an effect independent of glycemia on pregnancy outcome (Figure 4.33).

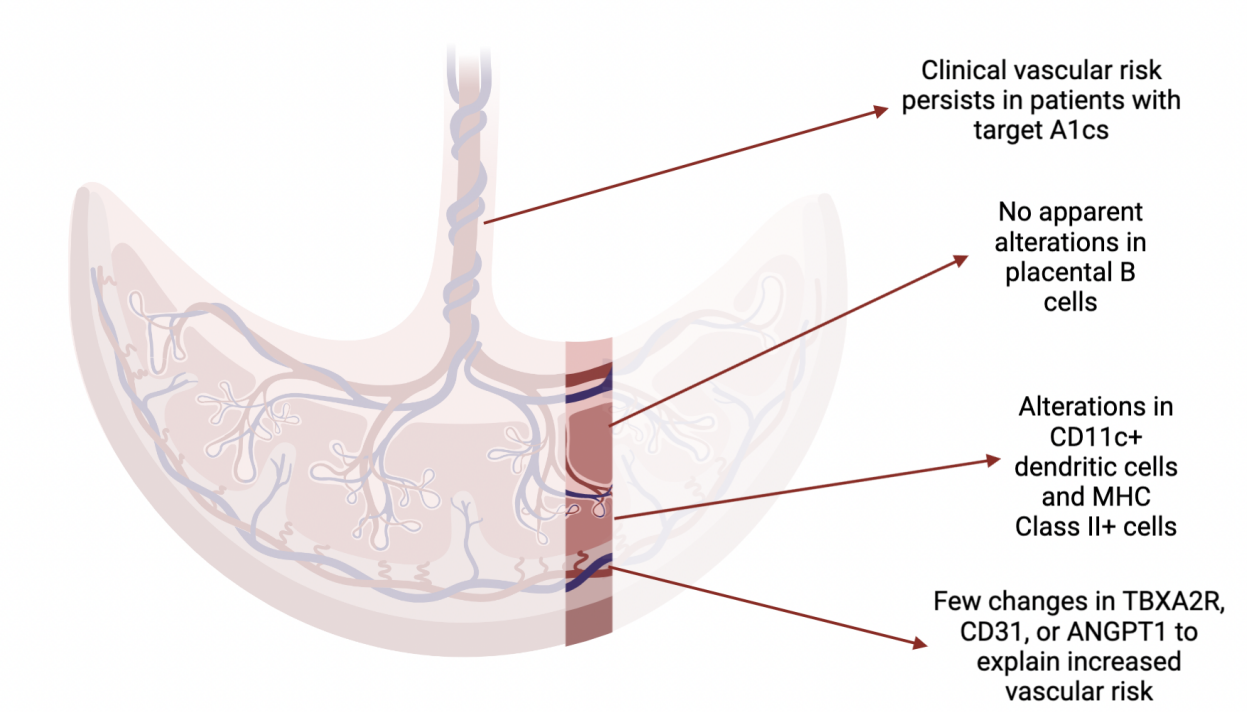


Figure 4.33. Overview of discoveries from Chapter IV.

Conclusions

The work done in this chapter has, for the first time, investigated the impact of autoimmunity in T1D on human pregnancy. By examining human placentas for immunologic changes, I found that several cell types in placentas from patients with T1D differ from those of healthy controls. Both patients with T1D and T2D have fewer CD11c+ dendritic cells than healthy controls. In late pregnancy, when all the placenta samples were acquired for this study, dendritic cells are thought to shift from a tolerogenic role to a pro-inflammatory one to promote parturition (Shah et al., 2017). The decrease in dendritic cells in patients with both T1D and T2D may reflect an effect of hyperglycemia on inflammation; perhaps fewer dendritic cells are needed to achieve the appropriate inflammatory state.

Placentas from patients with T1D were found to have fewer Foxp3+ cells than those from healthy controls. Stratification by A1c revealed that the T1D placentas with fewest Foxp3+ cells were at the lowest A1cs (5-6.5). By the late third trimester of pregnancy, the T cell landscape of the placenta should have shifted from one containing more Foxp3+ cells promoting tolerance to one containing more CD8+ cells encouraging parturition. However, this shift should also occur in healthy patients; a deficit in Tregs relative to healthy patients may indicate a deeper disturbance.

Patients with T1D also had a deficit in MHC Class II+ cells. Although I examined the antigen-presenting cells known to express MHC Class II, there is still a sizable deficit of cells that express MHC Class II that appear to be missing from the placentas of patients with T1D. There was also a significant decrease in CD4+MHC Class II+ cells in T1D. Unlike T cells from mice, activated human T cells can express MHC Class II and even present autoantigen without assistance from classical antigen-presenting cells (Gerrard et al., 1986; Holling et al., 2004; LaSalle et al., 1991). This decrease in CD4+MHC Class II+ cells may be protective against the effects of autoimmunity in gestation.

Future studies should trace the trafficking of placental immune cells in T1D over the course of gestation. Examination of fresh human placental tissue via methods like flow cytometry would provide more information on cell functions like cytokine secretion or proliferation. In particular, examination of the development of CD4+ MHC Class II+ cells may reveal a method by which T1D placentas avoid effects of autoreactive cells.

Examination of vascular proteins like CD31, ANGPT1, and TBXA2R did not yield significant differences. Changes in CD31 found in the NOD mouse were noted at day 17.5 of gestation, which is preterm. By 34-40 weeks, when these placentas were delivered, it is likely that any changes in proteins involved in vascular remodeling or angiogenesis may have already resolved. Future studies should examine vascular changes over time to identify early changes that may indicate later vascular comorbidities. Furthermore, a study was just published that promoted early identification of preeclampsia risk by detection of circulating RNA; they identify several preeclampsia-related genes that could be examined in patients with T1D (Rasmussen et al., 2022).

Examination of the prevalence of vascular complications in human pregnancy revealed an increased proportion of patients with T1D and vascular complications across all A1cs. These data indicate that adverse events in T1D pregnancy cannot purely be tied to glycemic control and may suggest a role for abnormal or autoimmunity in pregnancy. Furthermore, the control group also likely included individuals with other autoimmune conditions or metabolic conditions, which would have biased the effect estimates towards non-significance. While the low number of included pregnant people with T1D and adverse vascular outcomes makes it difficult to accurately assess risk at higher A1cs, the findings at lower A1cs directly relate to the hypothesis of increased risk of vascular complications at lower A1cs. The clinical information gathered and analyzed reveals a strong signal of an effect that builds on previous knowledge of complications in humans with T1D; combined with immunologic alterations seen in the placenta, this promotes an independent effect of autoimmunity on pregnancy. Further studies may allow for a

mechanistic understanding of how vascular and other complications arise in euglycemic patients with T1D.

CHAPTER V

Discussion and Future Directions

The work presented in this dissertation has identified several challenges to inducible immune tolerance in NOD mice and people with T1D. The presence of autoimmunity creates an increased barrier to tolerance; inflammation and autoreactivity must coexist with a tolerogenic, anti-inflammatory immune response. In both transplantation and pregnancy, I have discovered components of the immunologic hurdle that must be overcome to promote true tolerance.

Dual Roles for B and T Cells in Immune Tolerance

During development, multiple mechanisms are meant to stop the development of self-reactive adaptive immune cells (Goodnow et al., 2005). In T1D, both autoreactive T and B cells escape these numerous checkpoints to create destruction of the pancreatic beta cells. Clinical trials meant to overcome autoreactivity have used broad strokes to wipe out entire lymphocytic populations, like using anti-CD3 to eliminate T cells (Herold et al., 2002) or anti-CD20 to eliminate B cells (Pescovitz et al., 2009). These therapies are effective while patients are actively taking them, but fail to provide long term tolerance; additionally, they require chronic immunosuppression for patients.

The tolerizing therapy I discuss in Chapter II, αCD45RB, stands in stark contrast; not only does it not result in broad immunosuppression, but the effects persist past a seven-day course without additional treatment. I discovered that NOD mice at 20 weeks of age had large clumps of B cells in the thymus that resembled germinal centers (Figure 2.7). These germinal centers may be promoting the refinement of autoreactive B cells, which then could present self-antigen to allow for the persistence of autoreactive T cells. Furthermore, autoreactive B cells produce autoantibodies. While no role has been found for autoantibodies in direct

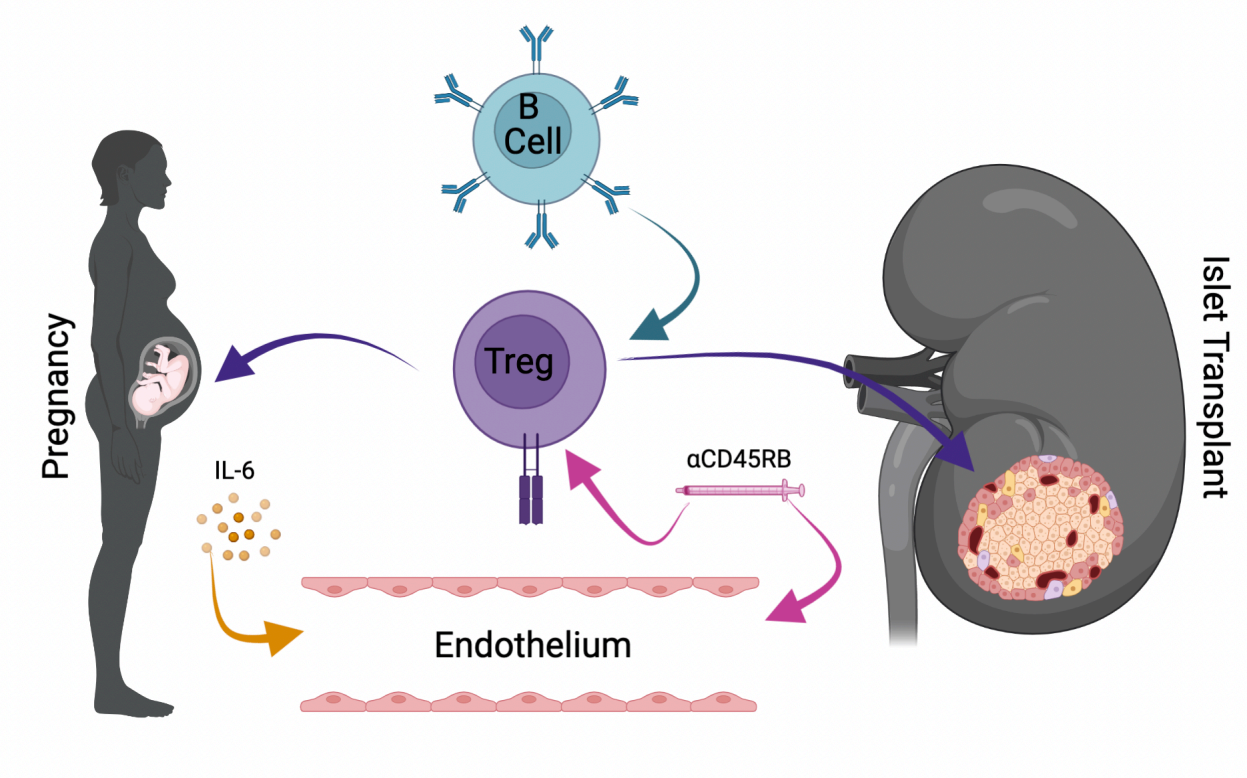


Figure 5.1. Overview of discoveries in this dissertation.

pathogenesis of islet destruction, autoantibody production in the thymus has been thought to eliminate mTECs that usually function to promote tolerance. I found an increase in Follicular and Marginal Zone B cells from ages 3 weeks to 6 weeks in both B6 and NOD mice; however, αCD45RB rescues some of the increase in Marginal Zone B cells (Figure 2.9). These data indicate a window in which reform of autoreactive B lymphocytes could lead to an easier state of tolerance induction.

It appears that pregnant NOD mice do not require therapy to reduce the presence of B cells in the placenta (Figures 3.4, 3.6). In both syngeneic and allogeneic NOD pregnancies, B cells are significantly decreased in the placenta. The decreases are not attributable to one particular B cell subset (Figure 3.8); rather, this broad decrease indicates that the presence of B cells may perturb a fragile tolerance established during gestation. While human patients with T1D did not have fewer B cells in the placenta than healthy patients (Figure 4.12), I was unable to obtain additional phenotypic information about the nature of the placental B. Human placental B cells have been thought to predominantly secrete anti-inflammatory IL-10 (Benner et al., 2020), but I was unable to find a notable cohort of IL-10 secreting B cells in mouse placenta (Figure 3.15). At most, in my data, the percentage of IL-10 secreting B cells in mouse placenta is similar to IL-10 secreting T cells and macrophages. If these data reflect differences between the role of B cells in mouse and human pregnancies, then the B cells present at appropriate numbers in the placentas of human patients with T1D may also be appropriately secreting IL-10. No functional analysis has been done on cytokine secretion into amniotic fluid in T1D; indeed, the transition to pro-inflammatory cytokines at parturition makes detection of secreted IL-10 difficult. Other researchers have noted the role of IL-10 in promoting Treg development in mouse pregnancies, so further research into IL-10 levels in pregnancy may contribute to an understanding of tolerance induced in pregnancy (Busse et al., 2019). Additionally, if B cells in the thymus are thought to be secreting autoantibodies, B cells in the placenta may be doing the same thing; perhaps a relative decrease in B cells is protective against autoantibody secretion.

In addition to decreasing Marginal Zone B cells, αCD45RB increases thymically-derived Tregs in 4-week-old mice, with a smaller but present expansion at 6 weeks (Figure 2.8). This Treg expansion may have contributed to some of the loss of infiltrative lymphocytes seen around the αCD45RB-treated islet graft in the kidney (Figure 2.15). Notably, I did not find any deficit in percentage of Tregs present in the NOD placenta (Figure 3.6), as is true for other organs in the NOD mouse (D'Alise et al., 2008). Simply possessing the same percentage of Tregs in the placenta as non-autoimmune B6 mice appears to be insufficient to prevent the adverse pregnancy outcomes present in NOD mice.

NOD Tregs may be similar in number to B6 mice, but disturbing Treg number is immediately more deleterious to NOD pregnancies than B6 pregnancies (Figure 3.9). Treatment with αCD25 severely impacted NOD pregnancies, resulting in only 19% normal pups. The mild peripheral Treg depletion induced by αCD25 did not impact B6 pregnancies, possibly because B6 placentas actually had increased Tregs after αCD25 treatment. The non-autoimmune B6 mouse may have an innate mechanism to increase Treg trafficking to the placenta to protect the developing fetus that the NOD mouse lacks. In contrast, however, treatment with αIL-2 had a greater impact on B6 pregnancies than NOD pregnancies (Figure 3.11). Although B6 mice start with a higher level of IL-2 signaling, the NOD mouse may be adapted to persisting even with decreased IL-2 signal (Figure 2.10). I show that additional IL-2 does not significantly impact downstream phosphorylation in NOD mice with αCD45RB treatment, whereas it increases phosphorylation of STAT5 in B6 mice; it is possible that pregnancy acts as a similar agent of induction of tolerance as αCD45RB treatment in B6 mice, and that removing the extra IL-2 signal can be deleterious to pregnancy.

People with T1D had a lower percentage of Foxp3+ Tregs in the placenta compared to healthy patients or people with T2D (Figure 4.16). Notably, this decrease seems to be attributable to patients with the lowest A1cs (Figure 4.17); patients with an A1c of 4.9-6 seemed to have only 20% of the Tregs of healthy controls. Pregnancy complications in patients with T1D

increase in prevalence with higher A1cs, but are present for all patients. It may be that the low number of Tregs in patients with T1D and normal A1cs is responsible for some of the adverse outcomes that they experience.

The complicated interplay between B and T cells in T1D pervades multiple organs and often escapes therapeutic intervention. I discovered roles for both B cells and Tregs in preserving tolerance within the challenge of autoimmunity. Both pregnancy and transplant rely on preferential activation of tolerogenic cells over their autoreactive counterparts; αCD45RB may be a mechanism through which tolerance is chosen.

Immunologic Interactions with Vasculature

Unexpectedly, my data have revealed how vascular complications impede the induction of immune tolerance. Both pregnancy and transplant rely on precise angiogenesis, the creation of new blood vessels to supply the foreign tissue with nutrients. The intersection between tolerance and appropriate angiogenesis has been implicated several times throughout the course of this work.

I found that αCD45RB was able to produce tolerance to foreign antigen in a young NOD mouse through an ex-vivo model of transplantation, the mixed lymphocyte reaction (Figure 2.12). This data represents a rare successful induction of tolerance in a B-cell sufficient NOD mouse; by treating younger mice, the Treg expansion and deficit of Marginal Zone B cells induced by αCD45RB is able to function to promote tolerance. However, when αCD45RB was used to treat young mice after islet transplant, the treated mice experienced loss of islet grafts faster than non-treated counterparts (Figure 2.14, 2.16). Even a different transplant location away from the highly vascular kidney was unable to facilitate successful engraftment and long-term acceptance (Figure 2.16). Transplanted islets require endothelial cells from the transplant recipient to promote revascularization (Brissova and Powers, 2008). It appears that αCD45RB may have hindered this migration; on histologic examination, the islet graft from a mouse

treated with αCD45RB showed active hemorrhage, complete with hemosiderin-laden macrophages indicating prior hemorrhage. Endothelial cells at any stage of development are not thought to express CD45RB, so it seems unlikely that αCD45RB would have a direct effect on revascularization. Other researchers have found hemorrhage surrounding grafts in mice treated with αCD45RB, but the hemorrhage was part of a larger rejection phenotype that also included lymphocytic infiltration (Luke et al., 2006). My discoveries in pregnancy, however, identified a role for NK cells in endothelial remodeling (Figure 3.20). αCD45RB treatment decreases the frequency of NK cells in the spleen (Stocks et al., 2017) –perhaps in younger mice as in pregnancy, NK cell presence is required for vascular remodeling and therefore graft revascularization. Regardless, αCD45RB is able to promote specific, durable tolerance to foreign antigen in older B6 mice; I may have identified a developmental stage at which αCD45RB is deleterious to endothelial cells.

In NOD pregnancies, endothelial cells are directly involved in creating adverse pregnancy outcomes. I found an increased number of IL-6 secreting CD31+ endothelial cells in NOD placentas as compared to B6 placentas (Figure 3.17). When secreted IL-6 was neutralized by administration of a monoclonal antibody, pregnancy outcomes for NOD mice improved (Figure 3.18). Additionally, IL-6 neutralization increased the percentage of NK cells present in the placenta, a cell type known to be involved in remodeling spiral arteries (Robson et al., 2012) (Fraser et al., 2015). CD31+ endothelial cells appear to be part of a destructive cycle in NOD pregnancy; CD31+ cells secrete IL-6, which prevents NK cells from appropriately aiding in spiral artery remodeling. As a result, there are increased CD31+ cells to continue secreting IL-6, which harms fetal outcomes. Additionally, spiral artery remodeling is known to be impacted in diseases like preeclampsia (Benyo et al., 2001; Fraser et al., 2015; Robson et al., 2012; Smith et al., 2009), a disease in which serum IL-6 is elevated (Lamarca et al., 2011; Prins et al., 2012). This data suggests that pathogenic findings in NOD mice mirror those of patients with T1D.

Patients with T1D have a well-established increased risk of preeclampsia, particularly at high A1cs (Hanson and Persson, 1998; Hiilesmaa et al., 2000; Holmes et al., 2011; Temple et al., 2006; Weissgerber and Mudd, 2015). This has been attributed to the hypothesized interaction between T1D and preeclampsia both as causes of vascular injury. Because of this prevailing theory, the impact of autoimmunity on pregnancy in T1D has been understudied. I performed a retrospective study of pregnant patients with T1D using EHR data. I found that patients with T1D, even those with target A1cs in pregnancy, still had a vastly increased risk of preeclampsia and other vascular complications in pregnancy (Figure 4.31, 4.32). While all patients with T1D have a risk of vascular injury from hyperglycemia, this risk is likely to be spread across the cohort of patients, or even clustered amongst the patients who entered pregnancy with higher A1cs. The findings of an increased risk of vascular disease in pregnancy with lower A1cs suggests autoimmunity as a primary driver of complications. Notably, presence of autoantibodies is also associated with an increased likelihood of developing HELLP syndrome during pregnancy (Weitgasser et al., 2000), again linking autoimmunity with vascular disease. While the patients we studied in the human placenta TMA did not have significant alterations in the three proteins we examined (CD31 [Figure 4.23], ANGPT1 [Figure 4.25], TBXA2R [Figure 4.28]), investigating protein levels at parturition may not have uncovered pathologic findings during gestation. Notably, we were unable to stain for IL-6 on the human placenta TMA because of the nature of sample collection; IL-6 levels present in human placentas may guide our understanding of how immunity can influence vasculature.

Intersection of Pregnancy, Heritability of Autoimmunity, and Transplant in T1D

Both pregnancy and transplant require adaptive immune changes in order to create tolerance to foreign DNA present in the host. While these immunologic transformations are very similar, they create a compounding system of tolerance. It is well established that children who

develop T1D are more likely to have a father with T1D than a mother with T1D (Turtinen et al., 2019). This data implies a protective effect of being exposed to T1D in utero. Accordingly, mice treated with proinsulin fused to Fc while in utero were protected from diabetes development, a technique the researchers call “transplacental antigen vaccination” (Culina et al., 2015). However, antibody transmission during pregnancy is thought to be involved in creating autoreactivity in offspring (Greeley et al., 2002). While autoantibodies passing through FcRN into the placenta may lead to the development of autoreactive fetal cells, it appears that antigen administration in pregnancy can lead to tolerance–similar to the discoveries of Billingham, Brent and Medawar in 1953 (Billingham et al., 1953).

Pregnancy can alter outcomes not just for the fetus, but for the gestating parent. Administration of pregnancy hormones prevents diabetes development and reduces lymphocytic infiltrate into the pancreas in NOD mice (Atwater et al., 2001); similarly, administration of h-CG reversed pancreatic infiltrate and prevented diabetes (Khan et al., 2001). As Bregs express the h-CG receptor, this may be a mechanism by which h-CG is helpful for slowing disease progression; it would be interesting to look at changes in the B cell population after h-CG administration. Finally, NOD mice given pancreatic autografts in early pregnancy had less immune infiltrate into transplanted tissue than non-pregnant mice (Chen et al., 1996).

These data suggest that pregnancy is intricately linked to the autoimmune pathogenesis of Type 1 Diabetes. Pregnancy, while an incredible stress on beta cells that can reveal a predilection towards later beta cell failure, appears to provide a slowing–and even reversal–of autoimmunity, with some protection passed on to offspring. So much is still unknown about the role of autoantibodies in pregnancy, about the necessary alterations in placental lymphocytes to promote tolerance, and about the resultant changes in cytokines.

Future Directions

Future studies will benefit from the relative knowledge deficit in this field and the close mirroring of human pregnancy by gestation in the NOD mouse. Researching pregnancy outcomes in NOD mice, in both dam and offspring, may reveal an impact of pregnancy timing on diabetes development or phenotypic changes in immune cell presence. The deficit of B lymphocytes in the NOD mouse, even if not precisely reflected in human pregnancy, deserves investigation; by understanding the functions of B cells in B6 placentas, we may uncover more information about what is lacking in NOD pregnancies. If B cells aren't secreting tolerogenic IL-10, are they functioning in an antigen-presentation capacity to T cells? Furthermore, if B cells in the NOD thymus are secreting autoantibodies that harm mTECs, are B cells in the NOD placenta secreting autoantibodies that contribute to disease pathogenesis in neonates or to pregnancy loss? Is the ability of younger mice to be tolerized to transplant with αCD45RB indicative of a phenotype induced during gestation that wains with age?

Additionally, a mechanistic understanding of how CD31+ endothelial cells interact with NK cells may reveal how spiral artery remodeling goes away in NOD mice–and in people with T1D. While an increase in secreted IL-6 is found in both people with preeclampsia and NOD mice, the mechanism of how IL-6 impacts NK cell function and prevents remodeling is still elusive. This understanding would impact not only pregnancy in T1D, but for all patients with vascular complications of pregnancy—and, possibly, transplant recipients experiencing hemorrhagic rejection of their graft.

If αCD45RB was unable to support graft acceptance in young NOD mice because of abnormal vascularization, other tolerizing therapies may be able to produce tolerance to islet transplant in young NOD mice. My data suggests that the failure of αCD45RB was not immunologic, opening the possibility that tolerance to transplant may be possible in the NOD mouse. My identification of a window before the major onslaught of autoimmunity in which NOD

mice may be able to be tolerized to foreign antigens tracks with discoveries in patients with T1D, where immunotherapy is found to be more effective before overt hyperglycemia (Herold et al., 2019); (Felton, 2021). Understanding this immunologic window would allow for appropriate targeting of immunotherapy to the patients who would most benefit. Alternately, investigation of how αCD45RB impacts revascularization may produce insights into vascular pathology in pregnancy as well.

So much is still to be uncovered about human pregnancy in patients with T1D, both immunologic and vascular. Further interrogation into the decreased presence of Tregs in people with lower A1cs may reveal how glycemia can interact with immunity in pregnancy. Broadly, expanding the sample to more patients may create a deeper understanding of immune cell function and presence throughout gestation. Collection of placentas at different gestational time points may also reveal information about the “switch” from anti-inflammatory in gestation to pro-inflammatory in parturition, and if this change impacts diseases like preeclampsia that are usually diagnosed late in gestation. Collection of fresh placental tissue would also allow for collection of RNA to measure cytokine transcripts, producing novel information about how IL-6 may be a cause or effect of vascular disease. Finally, comparing vascular development of placentas from patients with T1D may reveal early findings that predispose towards conditions like preeclampsia or other vascular concerns.

In this dissertation, I investigated induction of immune tolerance in transplant and pregnancy, and showed how labile the established tolerance can be. I discovered that young NOD mice are capable of responding to tolerizing immune therapy and may be able to accept islet transplants with αCD45RB. I uncovered IL-6 as a driver of poor pregnancy outcomes in NOD mice, and created a framework in which vascular and immunologic determinants of pregnancy collaborate to undermine healthy gestation. Finally, these findings are mirrored in pregnant patients with T1D, who have alterations in placental leukocytes and a predisposition to developing vascular complications of pregnancy across all A1cs. My work highlights the impact

and connections between autoimmunity, transplant, and pregnancy. Future studies, based on the research presented here, have the opportunity to improve outcomes for generations of patients with T1D.

CHAPTER VI

Methods

Animal Care

C57BL6/J (B6), C3H/HeJ (C3H), and NOD/ShiLtJ (NOD) mice were purchased from the Jackson Laboratory. Mice were housed and bred in a specific pathogen-free facility at Vanderbilt University according to the protocols approved in IACUC M1500016-02.

α-CD45RB Treatment

Mice treated with α-CD45RB (BioXCell #BE0019) received 100 μg in saline intraperitoneally on days 0, 1, 3, 5, and 7.

Splenic and Thymic Isolation

Mice were euthanized by isoflurane inhalation followed by cervical dislocation. For each mouse, spleen and thymus were removed and individually placed in a 70 micron filter suspended in phosphate-buffered saline (PBS). Organs were manually dissociated and centrifuged to wash. Splenocytes were incubated for 5 minutes in ACK Lysis Buffer to lyse erythrocytes, then quenched with PBS.

Flow Cytometry

Thymocytes or splenocytes were stained with the following antibodies: CD45 (30-F11), B220 (RA3-6B2), CD8 (53-6.7), $H2K^d$ (SF1-1.1), CD21 (7G6), CD49b (DX5), CD31 (MEC13.1) purchased from BD Bioscience; $H2K^b$ (AF6-88.5.5.3), Foxp3 (FJK-16S), IgM (II/41), CD23 (B3B4), CD3 (17A2), TCRβ (H57-597), CD19 (eBio1D3), (CD11B M1.70) purchased from

eBioscience; and CD4 (RM4-4), CD19 (6D5), CD268 (7H22-E16) from Biolegend. The eBioscience Foxp3 Transcription Factor Staining Kit (catalog 00-5523-00) was used for detection of Foxp3. Splenocytes were used as compensation controls.

Histopathology

Thymuses were placed into cassettes in 10% formalin, processed routinely, embedded in paraffin, and slides cut at 5 microns were stained with hematoxylin and eosin. Immunohistochemistry for B220 (BD Bioscience #553086) was performed. Slides were imaged at the Vanderbilt Cell Imaging Shared Resource.

Phospho-Flow Cytometry with IL-2 Stimulation

Splenocytes were isolated using protocols previously discussed and were kept on ice. Prior to each time point (60, 45, 30, 15 minutes), cells were plated into a 96-well V-bottom plate at a concentration of 1 million cells per well. The plate was centrifuged and the cells were resuspended in cell culture media (DMEM + 10% FBS + Penicillin/Streptomycin) with 20 ng/mL IL-2 (Gibco #PMC0024) and incubated at 37°C for the appropriate amount of time. Cells were washed in PBS, and immediately permeabilized with the eBioscience Foxp3 Transcription Factor Staining Kit (catalog 00-5523-00).

Mixed Lymphocyte Reaction

Splenocytes were isolated using protocols previously discussed and were kept on ice. Cells acting as the antigen (B6, NOD and C3H) were plated in cell culture media containing 10 µg/mL mitomycin C (Fisher #BP253110) in the incubator at 37°C for 2 hours. Following mitomycin treatment, cells were washed five times with PBS, then plated back in routine cell culture media at 37°C for two additional hours. Experimental responder splenocytes were stained with Cell Trace Violet (ThermoFisher #C34557) according to the standard protocol. Following staining, all

cells were counted. In cell culture media, “antigen” cells were plated at 500,000 cells/well and “responder” cells were plated at 1,000,000 cells/well into a 96-well round bottom plate. The plate was incubated at 37°C for five days. After five days, the cells were washed with PBS and analyzed using flow cytometry according to the protocols above.

Streptozotocin-Induced Diabetes

Mice were injected with 225 mg/kg Streptozotocin (Sigma #18883-66-4) dissolved in a Sodium Citrate Buffer at pH 4.5. Blood glucose values were measured for a week post injection; mice who had become hyperglycemic (BG >300 mg/dL) were eligible for transplant.

Islet Harvest

C3H mice were administered ketamine/xylazine as an anesthetic. An incision was made through skin and peritoneum and intestines were relocated to the mouse’s left flank. A pair of fine-tipped forceps were introduced under the Common Bile Duct and used to draw a length of suture (Fisher #14-516-128) underneath. The Common Bile Duct was tied off, and a bent 30 gauge needle was introduced into the Common Bile Duct. 5 mL of ice-cold 0.5 mg/mL Collagenase P (Sigma #11249002001) in HBSS. The solution was injected into the pancreas, resulting in inflation of the pancreas. The pancreas was dissected out of the abdomen and placed in additional HBSS + Collagenase P solution on ice. After all pancreases were collected, the pancreases were shaken in a 37°C water bath for 7 minutes. The digestion was quenched by ice-cold HBSS with 5% FBS. The cells were washed in additional HBSS and then plated in a 10 cm dish. Islets were sequentially hand-picked under a microscope and placed into RPMI media containing 10% FBS and 1% Penicillin/Streptomycin overnight.

Islet Transplant

Islets were hand-picked out of culture media into a 1.6 mL eppendorf tube containing warm RPMI + 10% FBS + 1% Penicillin/Streptomycin and allowed to rest at the bottom. Mice with Streptozotocin-induced diabetes were administered ketamine/xylazine as an anesthetic. For mice receiving grafts under the kidney capsule, a small incision was made in the left flank to allow the kidney to rest on top of the body. A needle was used to create a small channel under the kidney capsule, and islets were relayed into this channel using a syringe fitted with a PE50 tube. The channel was sealed using surgical glue, and the kidney relocated into the body cavity. Suture was used to close the muscular layer and staples were used to close the skin layer. For mice receiving islet grafts into the ear pinna, a needle was used to create a small channel in the left ear. Islets were injected into this channel using a pipette-loading tip. The channel was closed using surgical glue. For both transplant locations, >300 MHC-mismatched islets were delivered at the time of transplant. Mice treated with anti-CD45RB received 100 μg on the day of transplant (day 0), and then at days 1, 3, 5, and 7 after transplantation. All mice received ketorolac on days 0, 1, and 2 for pain relief. Blood glucose was monitored at least every third day after transplant.

Mating Protocol

Male mice were housed individually. 3 days prior to mating, bedding from male cages was transferred into female cages to induce estrus. In the evening of day 0, one female mouse was placed into each individual male cage. Mice were separated the next morning at day 0.5 and mating was confirmed by presence of a mucous plug. Experiments were performed on day 17.5 of gestation in mice confirmed to be non-diabetic. The gravid uterus was examined for evidence of reabsorbed fetuses, as well as for pups that appeared normal or abnormal (small, aberrant in color, or with obvious fetal anomalies).

IL-6 Neutralization

Beginning at gestational day 5.5, pregnant dams were treated every two days with 200 μg of an IL-6 neutralizing antibody (αIL-6) injected intraperitoneally (clone MP5-20F3, BioXCell #BE0046).

IL-2 Neutralization

Beginning at gestational day 5.5, pregnant dams were treated every two days with 200 μg of an IL-2 neutralizing antibody (αIL-2) injected intraperitoneally (clone JES6-1A12, BioXCell #BE0043).

Treg Depletion

Pregnant dams were treated on gestational days 5 and 10 with 200 μg of a monoclonal antibody that depletes Tregs by reacting with IL-2Rα (αCD25) injected intraperitoneally (clone PC.61.5.3, BioXCell #BE0012).

Cytokine Secretion Assay

Pregnant dams were euthanized and individual placentas were dissected from the uterus with removal of the decidua. Manually dissociated placentas were plated overnight in 5% DMEM cell culture media (with 10% FBS, penicillin/streptomycin, and β-mercaptoethanol) with 1 μM R848 (Stemcell Technologies #73782) and 8 μg/mL αCD3/αCD28 (BD #553057, 553294). After 12 hours of stimulation, GolgiStop (BD #554724) was added at a concentration of 0.66 μL/mL cell culture media. 6 hours after addition of GolgiStop, cells were harvested and stained using a Cytofix/Cytoperm Plus Fixation/Permeabilization Kit (BD #554715). Cells were stained for extracellular markers as listed above with IL-6 (MP5-20F3) from eBioscience.

Cytokine Array and ELISA

Pregnant dams were euthanized and amniotic fluid was collected from healthy-appearing fetuses by inserting a heparinized tube (Fisher #02-668-10) into the amniotic sac. Amniotic fluid was collected in microcentrifuge tubes and centrifuged for 8 minutes at 6800 rcf to pellet RBCs. Amniotic fluid was stored at -80℃, prior to analysis for cytokines and IL-6. Samples were sent to Eve Technologies (Mouse Cytokine 32-Plex #MD31) for a cytokine array. IL-6 was measured using an IL-6 ELISA Kit (BD #555240).

Human Tissue Microarray (TMA) Construction

Patients with a diagnosis of Type 1 or Type 2 Diabetes (T2D) who delivered at Vanderbilt University Medical Center and had a placenta collected for histopathological examination were identified by a collaborator. With approval from IRB #201307, chart review was performed to verify diagnosis of Type 1 or Type 2 Diabetes. Healthy controls were identified by performing chart review on patients with a placenta collected for histopathological examination. The inclusion criteria were: pre-existing T1D, T2D, or healthy controls without diabetes; delivery by Cesarean Section; and gestational age between 33-40 weeks at delivery. The exclusion criteria were: gestational diabetes; any infection at the time of delivery, including chorioamnionitis and HIV; any fever at time of delivery; labor, induction of labor, or any contractions; multiple gestation; other autoimmune disease (with the exception of autoimmune thyroid disease); placenta accreta spectrum disorder; and major fetal anomalies, including hydrocephalus and myelomeningocele.

We identified 17 patients with T1D, 18 patients with T2D, and 17 healthy patients that fit both inclusion and exclusion criteria. Paraffin-embedded placenta blocks and corresponding slides were pulled from storage. Slides with a cross-section of each placenta were scanned into

CaseViewer, and four 1 mm cores identified per placenta. Two cores from each patient were placed on one of two TMAs, creating two TMAs with identical patient composition but different cores.

Human Tissue Microarray Multiplex Immunohistochemistry

Slides from both TMAs were sent to Ultivue, Inc for multiplex immunohistochemistry staining. Two Ultimapper kits were used simultaneously: the Treg kit, staining for Foxp3, CD8, CD4, and panCK; and the APC kit, staining for CD11c, CD20, CD63/CD163, and MHC Class II. The stained slides were analyzed by Ultivue technicians using Halo Software.

Human Tissue Microarray Immunohistochemistry

Immunohistochemistry on both TMA slides was performed by Vanderbilt TPSR. Slides were stained for CD31, ANGPT1, and TBXA2R. Slides were analyzed using Halo Software. For slides stained with TBXA2R, an intensity score was calculated by rating each core on a 0-3 scale, where 0 was no staining and 3 was staining so intense that nuclei were obscured and cell membrane definition was lost.

EHRs data and phenotyping

With approval from Vanderbilt University Medical Center (VUMC) IRB #212013, we accessed VUMC's de-identified database of electronic health records (EHRs, >3.1 million patients) and assembled a pregnancy cohort with T1D status and vascular complications during pregnancy. For each patient, we used billing codes that included International Classification of Diseases, 9th/10th Revision, Clinical Modification (ICD-9/10-CM) and Current Procedural Terminology (CPT) to demarcate the first pregnancy and delivery, ascertain T1D status, and identify vascular complications. EHRs of this cohort ranged from 1988-09-10 to 2020-12-30.

To demarcate the first pregnancy within an EHR, we required at least one billing code for delivery no more than 45 weeks after a billing code indicative of prenatal care. To avoid correlation among outcomes derived from the same individual with multiple pregnancies, we considered only the first pregnancy recorded in the EHR. Next, we used a validated and accurate phenotyping algorithm (Stanford University, 2020) that incorporated billing codes, clinical labs, and diabetes-related medications to ascertain women with T1D diagnosed before prenatal care as determined by first prenatal billing code. We assigned a A1C value measured closest to and within three months of the billing code indicative of prenatal care (Dennis et al., 2021). To identify pregnancies with vascular complications, we required patients to have at least one Phecode v2 (Wei et al., 2017; Wu et al., 2019), a condensed and expert curated set of diagnoses mapped from ICD-9 and ICD-10 codes, for vascular phenotypes as specified in Figure 4.29 occurring between nine months before delivery and three months after delivery.

Association of A1C level with vascular outcomes

Our exposure of interest was T1D status before the first recorded pregnancy's delivery date. Our outcomes included a composite of vascular phenotypes (Figure 4.29) and pre-eclampsia, eclampsia, or HELLP syndrome. We assessed for effect modification between T1D and hemoglobin A1c at each participant's first recorded prenatal visit in the EHR using an interaction term. We adjusted for covariates that may have confounded the relationship between T1D and the outcome. These included EHR-collected race (White, Black, Unknown, Asian, or Native American), age at the first pregnancy encounter, and socioeconomic status (Greenland et al., 1999),(Brokamp et al., 2019).

Individuals without T1D were missing hemoglobin A1c values by design. We therefore imputed them randomly from a normal distribution that reflected the population mean A1c among people

without T1D (mean of 5.3, standard deviation of 0.1) (Harrell , 2015; Selvin et al., 2009). Imputing from a normal distribution enabled computational convergence for regression models. We then performed 35 multiple imputations with chained equations with the exposures, covariates, and outcomes to account for missing covariate data (Harrell , 2015). Finally, we used logistic regression to assess the probability of each outcome by T1D status (with A1c fixed to 5.3 in people without T1D), modified by A1c, controlled for the above-listed covariates pooled across the imputed datasets (Harrell , 2015) . A1c was modeled as a restricted cubic spline with three knots given existing evidence that A1c has a non-linear relationship with vascular outcomes (Temple et al., 2006). Other continuous covariates were modeled as a linear relationship given the small number of outcomes. Analyses with EHR data were performed using Python v3.8 with Pandas v1.3.4 (McKinney, 2010) and NumPy v1.19 (Harris et al., 2020), and *R Statistical Software* (Version 4.1.0). Python and R code is available at https://github.com/abraham-abin13/a1c_pregnancy_outcomes.git.

Mouse Statistics

Mouse data were analyzed with GraphPad Prism version 9.2.0. An unpaired t-test with Welch's correction was used to compare differences between two groups. For multiple groups, a one- or two-way ANOVA with Šidák's multiple-comparisons post hoc test was used. Statistical values with $p \leqq 0.05$ were deemed significant.

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