

iASPP in the Balance Between Tolerance and Autoimmunity

by

Elliot H. Akama-Garren

Submitted in Fulfilment of the Requirements for the Degree of
Master of Science by Research in Clinical Medicine

St. John's College

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Nuffield Department of Clinical Medicine

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ABSTRACT

Maintenance of immunological homeostasis between tolerance and autoimmunity is essential for the prevention of human diseases ranging from autoimmune disease to cancer. Adaptive immune responses require both antigenicity and adjuvanticity, and cell death can critically influence immunological homeostasis by modulating the adjuvanticity it elicits. Accumulating evidence suggests that in addition to inducing apoptosis, p53 mitigates phagocytosis-induced adjuvanticity thereby promoting tolerance following physiologic tissue turnover. Here I identify Inhibitor of Apoptosis Stimulating p53 Protein (iASPP), a negative regulator of p53 transcriptional activity widely considered to be oncogenic, as a novel master regulator of immunological tolerance. Surprisingly, iASPP deficiency promoted oncogenic K-ras driven lung adenocarcinoma and pancreatic cancer tumorigenesis, while iASPP deficient mice were less susceptible to autoimmune disease. Furthermore, immune responses to iASPP-deficient tumors exhibited hallmarks of immunosuppression, including activated regulatory T cells and exhausted CD8⁺ T cells, and iASPP-deficient CD4⁺ T cells were less responsive to TCR engagement *in vitro*. Interestingly, iASPP-deficient tumor cells, myeloid cells, CD4⁺, and $\gamma\delta$ T cells expressed elevated levels of PD-1H, a recently identified transcriptional target of p53 that promotes tolerogenic phagocytosis. These findings suggest that iASPP can inhibit p53-mediated immunosuppressive PD-1H cross-talk between malignant cells, phagocytes, and responding immune cells, thereby promoting adjuvanticity following phagocytosis. Identification of a novel iASPP/p53 axis of immune homeostasis provides an exciting therapeutic opportunity for both autoimmune disease and cancer.

Thesis Advisor: Xin Lu

Title: Professor of Cancer Biology

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CHAPTER 1: Introduction

The balance between tolerance and autoimmunity

A hallmark of adaptive immunity is the ability to recognize an incredible diversity of foreign pathogens while simultaneously avoiding responsiveness to endogenous antigens. This delicate balance between immunity and tolerance is critical not only for proper immune homeostasis, but also for more general human health given the myriad implications of the immune system in the pathogenesis of an increasing number of human diseases. Excessive immune activation in combination with loss of tolerance is the basis for the development of autoimmune disease, while excessive tolerance with impaired immunity leads to immunodeficiency and can contribute to cancer development.

Both immunity and tolerance are established in central lymphoid organs, where lymphocytes are educated to respond to foreign antigens presented by host MHC (positive selection), while avoiding the dissemination of autoreactive lymphocyte clones (negative selection). Central T cell tolerance is maintained in the thymus, where T cell clones with strongly self-reactive T cell receptors (TCR) either undergo apoptosis or develop into CD4⁺CD25⁺FoxP3⁺ natural regulatory T cells (nT_{reg}). Importantly, the immune system has also evolved mechanisms of peripheral tolerance that restrain autoreactive lymphocytes that escape central tolerance, including induction of anergy following TCR engagement without co-stimulation, negative selection in secondary lymphoid organs, conversion of autoreactive CD4⁺ T cells to induced T_{reg} cells (iT_{reg}), or T_{reg}-mediated immunosuppression.

Maintenance of immune homeostasis between immunity and tolerance is essential in the combat of human disease, particularly autoimmune disease and cancer.

Functionally robust immune responses are necessary for tumor immunosurveillance, as malignant lesions only develop if neoplastic cells can overcome immune elimination (Dunn et al., 2002; Mittal et al., 2014). Recent advances in cancer immunotherapy rely on bolstering endogenous anti-tumor responses or inhibiting inherent immunosuppressive mechanisms upregulated during extended immune activation, such as checkpoint molecules CTLA-4, PD-1, and TIM-3 (Blank and Mackensen, 2007; Hodi et al., 2010; Ngiew et al., 2011; Topalian et al., 2012; Waterhouse et al., 1995). Although these therapies are curative for a subpopulation of patients, most patients do not experience clinical benefit and overall mortality rates remains high (Hodi et al., 2010), calling for a better understanding of additional mechanisms of tumor immune escape. In contrast, autoimmune disease is axiomatically due to the breakdown of immune tolerance, for example by dysfunction or abrogation of T_{reg} cells (Kim et al., 2007; Oh et al., 2017; Sakaguchi et al., 1995; Sather et al., 2007; Takahashi et al., 1998). Therefore, a better understanding of the fundamental mechanisms underlying the maintenance of immune homeostasis has implications for both cancer and autoimmune disease therapy.

One of the most fundamental functions of the immune system is the clearance of dead cells following normal cell turnover, malignancy, injury, or infection. In this capacity, the immune system exhibits remarkable capability to discriminate whether to respond in an immunogenic or tolerogenic manner depending on the nature of cell death (Galluzzi et al., 2017; Garg et al., 2016; Green et al., 2009a). This decision

represents a fundamental component of endogenous immune responses, and therefore has significant influence on the maintenance of organism-wide immune homeostasis. The daily homeostatic turnover of billions of cells rarely invokes an immune response (Fuchs and Steller, 2011; Galluzzi et al., 2015; Garg et al., 2014, 2015a; van Kempen et al., 2015), while apoptosis of relatively few infected or malignant cells can elicit robust antigen-specific immune responses and immunological memory (van Kempen et al., 2015). Defects in this decision can lead to human disease, for example mistaken immunogenic phagocytosis in combination with epitope spreading contributes to autoimmune disease (Ni et al., 2013; Sánchez et al., 2000; Zhou et al., 2013), while tumors can evade the immune system by promoting tolerogenic phagocytosis (Fucikova et al., 2015; Gabrilovich et al., 2012; Li and Rudensky, 2016). Fundamentally, adaptive immunogenicity requires antigenicity and adjuvanticity, and although apoptosis and subsequent phagocytosis almost universally promotes antigen presentation, it can have varying adjuvanticity (Janeway, 1992; Matzinger, 1994). The effect of cell death on either immunity or tolerance is therefore largely determined by the adjuvanticity elicited following phagocytosis.

Historically, apoptosis was thought to be immunologically silent while necrosis was thought to stimulate inflammation (Galluzzi et al., 2015; Kerr et al., 1972; Thompson, 1995). Dying cells trigger phagocytosis through expression or secretion of ‘find-me signals’ (Elliott et al., 2009), ‘keep-out signals’ (Garg et al., 2010; Ravichandran, 2011; Yang et al., 2009; Zitvogel et al., 2010), and ‘eat-me signals’ (Garg et al., 2010; Hochreiter-Hufford and Ravichandran, 2013; Ravichandran, 2011; Yang et al., 2009; Zitvogel et al., 2010) and downregulation of ‘don’t eat-me signals’ (Chao et al.,

2010; Gregory and Brown, 2005). Phagocytosis of apoptotic cells, particularly by cross-presenting dendritic cells (DCs) (Albert et al., 2001; Belz et al., 2002; Ferguson et al., 2002; Heath and Carbone, 2001; Heath et al., 1998; Steinman et al., 2000), can establish immune-privileged niches (Griffith et al., 1996), promote allograft tolerance (Pradhan et al., 2008; Sun et al., 2004), lead to the development of short-lived 'helpless' cytotoxic T cells (Carlin et al., 2009; Green et al., 2009b; Griffith et al., 1996; Janssen et al., 2003, 2005), generate iT_{reg} cells and anergic cytotoxic T lymphocytes due to insufficient co-stimulation (Dudek et al., 2013; Green et al., 2009a; Kleinclauss et al., 2006; Lutz and Schuler, 2002; Maeda et al., 2005; Roux et al., 2008; Twyman-Saint Victor et al., 2015), stimulate immune checkpoint pathways (Arandjelovic and Ravichandran, 2015; Elliott and Ravichandran, 2010; Fuchs and Steller, 2011; Nagata, 2010; Nagata et al., 2010), promote immunosuppressive cytokine such as TGF- β and IL-10 release (Garg et al., 2010; Ravichandran, 2011; Vaught and Cook, 2015; Voll et al., 1997; Yang et al., 2009; Zitvogel et al., 2010), and induce systemic antigenic tolerance (Garza et al., 2000; Miyake et al., 2007). Conversely, non-physiologic cell death or necrosis can induce inflammation and occasionally autoimmune responses (GaipI et al., 2007) via recognition of pathogen-associated molecular patterns (PAMPs) (Barber, 2014; Bettigole and Glimcher, 2015; Broz and Monack, 2013; Cao, 2016; Gay et al., 2014; Kanneganti, 2010; McNab et al., 2015; Medzhitov and Janeway, 2002; Sica et al., 2015; Yoneyama et al., 2015), inflammasome activation and cytokine release (Latz et al., 2013), autophagy and eIF2A phosphorylation (Liang et al., 1998; Tallóczy et al., 2002; Travassos et al., 2010), damage-associated molecular patterns (DAMPs) (Fuchs and Steller, 2015; Kaczmarek et al., 2013; Vénéreau et al., 2015) in combination with antigenicity (van Kempen et al., 2015), NK cell ligands (Gasser et al., 2005), and

licensing CD40L-mediated CD4⁺ T cell help of cytotoxic T cell development (Garg et al., 2015b; Griffith et al., 2007; Kroemer et al., 2013; Schoenberger et al., 1998).

Notably, later studies challenged the initial model that apoptosis is necessarily tolerogenic and necrosis is immunogenic. Apoptotic tumor cells (Casares et al., 2005; Zitvogel et al., 2004) and cross-presentation of antigens from engulfed apoptotic cells (Galluzzi et al., 2015; Steinman et al., 2003) can prime functional antigen-specific immune responses, bacterial infection-induced apoptosis can induce a Th17 rather than T_{reg} polarized immune response (Torchinsky et al., 2009), activated peripheral blood mononuclear cells that undergo apoptosis can induce pro-inflammatory cytokine secretion (Johansson et al., 2007), and DNA damage-induced senescence can lead to the secretion of pro-inflammatory cytokines (Acosta et al., 2008; Kuilman et al., 2008; Wajapeyee et al., 2008) and functional anti-tumor immune responses (Xue et al., 2007). Conversely, necrotic cells can express tolerogenic 'eat-me signals' such as phosphatidylserine (Birge and Ucker, 2008; Bondanza et al., 2004; Brouckaert et al., 2004; Lauber et al., 2013; Petrovski et al., 2007; Segawa and Nagata, 2015), PAMPs that mediate immune tolerance to symbiotic microorganisms (Chu and Mazmanian, 2013), and DAMPs such as adenosine, S1P, and prostaglandin E2 that can also have immunosuppressive properties (Galluzzi et al., 2012; Hangai et al., 2016; Krysko et al., 2012; Luo et al., 2016). In addition to apoptosis and necrosis, other cell death pathways such as necroptosis (Krysko and Vandenabeele, 2010; Krysko et al., 2006; Newton et al., 2014; Poon et al., 2010), autophagic cell death (Chen et al., 2008; Garg et al., 2013b, 2013a; Petrovski et al., 2011; Verfaillie et al., 2013), parthanatos, and ferroptosis occur although their influence on immune responses remain poorly

characterized (Galluzzi et al., 2015). Despite these intricacies, the generalizable model holds that cell death can influence immune homeostasis between immunity and tolerance in conditions of infection and malignancy or physiologic cell death, respectively.

Clearly the immune system integrates multifaceted cellular and molecular inputs beyond simply the acting cell death pathway to determine whether cell death is immunogenic or tolerogenic. Dying cells can express or secrete a myriad of molecules that constitutionally or secondarily stimulate immune responses, such as heat shock proteins (Obeid et al., 2007a; Spisek et al., 2007; Srivastava, 2006), calreticulin (Obeid et al., 2007b; Panaretakis et al., 2008), lysophosphatidylcholine (Lauber et al., 2003; Le et al., 2001), ATP (Hanley et al., 2004; Koizumi et al., 2007), HMGB1 (Apetoh et al., 2007; Bell et al., 2006; Jiang et al., 2007; Kazama et al., 2008; Scaffidi et al., 2002), SAP130 (Yamasaki et al., 2008), and CLEC9A-binding actin filaments (Sancho et al., 2009; Zhang et al., 2012). In contrast, dying cells can also express receptors or secrete molecules that facilitate immune tolerance, such as MERTK, C1q, exposed phosphatidylserine (Fadok et al., 2000; Gaipl et al., 2006), MFGE8 (Hanayama et al., 2004) which binds TIM-4 on phagocytes (Kobayashi et al., 2007; Miyanishi et al., 2007), IL-10 (Gao et al., 1998), and TGF- β (Chen et al., 2001), and can also stimulate the engulfing cell to secrete immunosuppressive cytokines such as TGF- β (Fadok et al., 1998, 2000; Freire-de-Lima et al., 2000; Hoffmann et al., 2005; Huynh et al., 2002), IL-10, platelet-activating factor, prostaglandin E₂ (Chung et al., 2007; Fadok et al., 1998; Voll et al., 1997), 15-lipoxygenase, and 15-hydroxyeicosatetraenoic acid (Serhan and Savill, 2005). Caspase activation in dying cells can promote immunity by immunodominant epitope

proteolysis (Castiglioni et al., 2002; Tenev et al., 2007), facilitating cross-presentation by DCs (Rawson et al., 2007), and activating calreticulin expression (Obeid et al., 2007c) or DAMP secretion (Bianchi and Manfredi, 2007), or can promote tolerance by generating reactive oxygen species (ROS) (Ferguson et al., 2002; Griffith et al., 2007; Kazama et al., 2008) that inactivate HMGB1 via oxidation (Kazama et al., 2008). Autophagy in dying cells is necessary for the expression of 'eat me' signals (Jin et al., 2007; Qu et al., 2007), and can stimulate HMGB1 release (Thorburn et al., 2009) and CD8⁺ T cell cross-priming (Uhl et al., 2009).

The tissue context of the responding APC might also influence the immune consequences of cell death, as splenic CD8 α ⁺ DCs promote tolerance whereas splenic CD8 α ⁻ DCs promote immune response to injected apoptotic cells (Battisto and Bloom, 1966; Ferguson et al., 2002; Iyoda et al., 2002), while bone marrow-derived DCs induce tolerance (Ferguson et al., 2002; Griffith et al., 2007; Kazama et al., 2008), and cutaneous CD8 α ⁺ DCs promote anti-viral immunity (Allan et al., 2003). The balance between tolerance and immunity in response to cell death is also influenced by the developmental phenotype and activation status of the responding APC. M1 and M2 macrophages promote fundamentally different immune responses and their imbalance can promote development of various inflammatory diseases or cancer (Mantovani et al., 2002). Polarization of myeloid cells towards M1 or M2 phenotypes is influenced by numerous cytokines, chemokines, DAMPs, and PAMPs, many of which are also involved in cell death (Mantovani et al., 2004; Wang et al., 2014). A population of "tolerogenic" macrophages, distinguished by HSP27, CD163, GR-1, CD11c^{lo}, and CD40^{lo}, have demonstrated pro-tumorigenic potential and may be induced by Wnt5a,

dexamethasone, S1P, and GM-CSF (Banerjee et al., 2011; Bayne et al., 2012; Bergenfelz et al., 2012; Luo et al., 2016; Zheng et al., 2013). Interestingly, mature MHCII^{hi}CD80^{hi} DCs are necessary to mount effective immune responses (Albert et al., 2001), while immature DCs are tolerogenic (Steinman et al., 2000, 2003), although mature DCs can also elicit tolerance following phagocytosis of apoptotic cells (Ferguson et al., 2002; Griffith et al., 2007; Kazama et al., 2008). Therefore, the identity, location, activation status, and life history of the dying cell as well as those of the cell engulfing it, are all influential factors that dictate the nature of the immune response to cell death.

Interestingly, chemotherapy or radiation-induced cancer cell death represents a unique form of apoptosis that can stimulate either anti-tumor immunity or pro-tumor tolerance (Aderem, 2003; Dudek et al., 2013; Garg et al., 2015c; Maderna and Godson, 2003; Ravichandran, 2011; Vénéreau et al., 2015). This bilateral disruption of immune homeostasis is likely due in part to tumoral expression of both tumor-associated antigens (ectopically expressed self antigens) and tumor-specific antigens (neoantigens acquired by mutagenesis). Therefore, although phagocytosis of apoptotic cancer cells might be intrinsically tolerizing, engulfed cancer cells can be considered in part foreign and resultant immunity might be biased towards tumor-specific antigens (Apetoh et al., 2007; Segal et al., 2008; Zitvogel et al., 2008). Despite the improved response of tumors with high mutational burdens such as melanoma, non-small cell lung cancer, and colorectal cancer to checkpoint blockade (Gubin et al., 2014; Le et al., 2015; Van Allen et al., 2015), all cancers regardless of mutational burden can elicit effective adaptive immune responses when stimulated to release DAMPs (Garg et al., 2015a; Kroemer et al., 2013). Cancers with low

mutational loads (Alexandrov et al., 2013) such as pancreatic ductal adenocarcinoma (PDAC) might have sufficient antigenicity to elicit immunity (Garg et al., 2013c, 2014; Kroemer et al., 2013; Schumacher and Schreiber, 2015), but lack the adjuvanticity mediated by immunogenic cell death and phagocytosis necessary to mount an anti-tumor immune response, impairing the effectiveness of immunotherapy (Hanahan and Weinberg, 2011; Mahoney et al., 2015; Pardoll, 2012; Sharma and Allison, 2015a; Zappasodi et al., 2015). Thus, the exciting recent preclinical observation of synergistically enhanced anti-tumor immunity and substantially improved prognosis following combinatorial radiotherapy and checkpoint blockade (Baksh and Weber, 2015; Baumeister et al., 2016; Chen et al., 2016; Deng et al., 2014a; Haymaker et al., 2012; Le Mercier et al., 2015; Pardoll, 2012; Sharma and Allison, 2015b; Zou et al., 2016) is likely due to improved tumor adjuvanticity rather than antigenicity. Identification of mediators of tolerogenic phagocytosis that decrease cancer adjuvanticity is therefore critical to develop novel therapies that may be used in concert with checkpoint blockade to exploit the already inherent antigenicity of malignant cells.

Chemotherapy-driven cancer cell death can stimulate anti-tumor immune responses (Fucikova et al., 2011; Kroemer et al., 2013; Krysko et al., 2012) via calreticulin (Gardai et al., 2005; Obeid et al., 2007b), PDIA3 (Panaretakis et al., 2008), HSP90 (Fucikova et al., 2011; Galluzzi et al., 2014; Garg et al., 2013c, 2015d), ATP (Michaud et al., 2011), type I IFN and downstream CXCL10 (Sistigu et al., 2014), HMGB1 (Apetoh et al., 2007), and ANXA1 (Vacchelli et al., 2015). Irradiation (Bloy et al., 2014; Gameiro et al., 2014; Kurokawa et al., 2001; Strome et al., 2002), hypericin-based photodynamic therapy (PDT), and high hydrostatic pressure

(Fucikova et al., 2014; Garg et al., 2012a; Obeid et al., 2007b), but not accidental necrosis (Aaes et al., 2016; Casares et al., 2005; Goldszmid et al., 2003), can also trigger immunogenic cancer cell death mediated by CALR (Fucikova et al., 2014, 2016a; Gameiro et al., 2014; Garg et al., 2012b, 2012a; Golden et al., 2014; Obeid et al., 2007c), ATP (Fucikova et al., 2014; Garg et al., 2012b; Golden et al., 2014; Ko et al., 2014), type I IFN (Deng et al., 2014b; Lim et al., 2014), HSP70 (Brusa et al., 2009), TLR3 (Bernard et al., 2012; Salaun et al., 2011), HMGB1 (Brusa et al., 2009; Fucikova et al., 2014; Ko et al., 2014; Korbelik et al., 2011; Werthmöller et al., 2015), and inflammasome activation and IL-1 β release (Chen et al., 2012). DCs responding to PDT-induced cancer cell death upregulate CD80, CD86, and MCH class II, and secrete IL-1 β , IL-12, and TNF (Fucikova et al., 2014; Garg et al., 2012b) resulting in anti-tumor immunity and immunological memory (Garg et al., 2012b; Weiss et al., 2010). Immunogenic cell death is a critical component of anti-tumor immunity, and loss of DAMP signaling (Apetoh et al., 2007; Bidwell et al., 2012; Cirone et al., 2012, 2012; Elliott et al., 2009; Fucikova et al., 2015, 2016b; Garg et al., 2012b, 2015d; Ghiringhelli et al., 2009; Gilardini Montani et al., 2015; Kroemer et al., 2013; Krysko et al., 2012; Ladoire et al., 2015; Ma et al., 2013; Michaud et al., 2011; Obeid et al., 2007b; Sistigu et al., 2014; Yamazaki et al., 2014), pattern recognition receptors (PRR) or their adaptor proteins (Apetoh et al., 2007; Bergmann et al., 2011; Gast et al., 2011; Tittarelli et al., 2012; Vacchelli et al., 2015), inflammasome components (Ghiringhelli et al., 2009; Zitvogel et al., 2015), autophagy (Ko et al., 2014), or necroptosis machinery (Aaes et al., 2016; Galluzzi et al., 2016; Werthmöller et al., 2015; Yang et al., 2016) from apoptotic cancer cells disrupts adaptive immune responses to cancer and is associated with worse prognosis. Indeed, apoptotic cancer cells are capable of preventing the development of functional immune

responses following phagocytosis by DAMP (Fucikova et al., 2015; Gabrilovich et al., 2012; Li and Rudensky, 2016) or DAMP pathway components such as IRF7 (Bidwell et al., 2012) or STAT1 (Chan et al., 2012) reduction, and are sometimes tolerogenic (Bidwell et al., 2012; Katlinski et al., 2017; Mohme et al., 2017). Paradoxically, some DAMPs expressed by apoptotic tumor cells have demonstrated immunosuppressive or pro-tumor effects, such as HMGB1 (Kang et al., 2013; Liu et al., 2015) and IL-1 α -dependent malignancy, angiogenesis, and metastasis (Di Paolo and Shayakhmetov, 2016; Grivennikov et al., 2010; Hong et al., 2014), and tumor necrosis correlates with worse prognosis (Coulie et al., 2014; Garg et al., 2014; Ravichandran, 2011; Richards et al., 2011, 2012; Schumacher and Schreiber, 2015), although this might be simply a result of overwhelming the phagocytic system. Other molecules released by apoptotic cancer cells are intrinsically immunosuppressive, such as S100 family members that promote metastasis (Coffelt et al., 2015; Hiratsuka et al., 2006), extracellular potassium that limits TCR signaling (Eil et al., 2016), CXCL1 that attracts immunosuppressive CD11b⁺GR-1⁺ myeloid cells (Acharyya et al., 2012; Sistigu et al., 2014), CD47 that acts as a 'don't eat-me signal' (Majeti et al., 2009; Suzuki et al., 2012; Wang et al., 2015), externalized cardiolipin (Balasubramanian et al., 2015), phosphatidylserine and MFG-E8 that promotes tolerogenic phagocytosis and T_{reg} recruitment (Jinushi et al., 2007), thrombospondin, complement C1q (Birge and Ucker, 2008; Gregory and Brown, 2005; Poon et al., 2014), PD-1H that promotes phagocytosis and inhibits T cell activation (Yoon et al., 2015), prostaglandin E2 that promotes M2 macrophage polarization (Hangai et al., 2016), S1P that promotes EPO signaling leading to generation of tolerogenic macrophages (Luo et al., 2016), and necrosomes that induce immunosuppression (Seifert et al., 2016). Chemotherapy and radiation therapy themselves can be immunosuppressive

when administered at high doses (Derer et al., 2015; Dewan et al., 2009; Pasquier et al., 2010; Shalapour et al., 2015; Vanpouille-Box et al., 2017), and therefore can even interfere with the immune response following phagocytosis of apoptotic cancer cells as well as immunosurveillance of cancer cells that avoid therapy (De Henau et al., 2016; Pallasch et al., 2014).

Physiologic apoptosis and unnatural cell death elicit fundamentally different immune responses, yet the fundamental pathways intrinsic to dying cells responsible for modulating immune homeostasis and phagocytic response remain poorly characterized. Clearly, different methods of cell death utilize unique signaling pathways, which might also have direct effects on expression of immunomodulatory molecules. As described above, APC influence on the cell death-responsive immune response is governed by a vast number of cell surface molecules and secreted factors, suggesting that the nature of cell death and phagocytosis results in profoundly distinct cell-intrinsic transcriptional programs. Here I propose that iASPP and p53 are two transcriptional regulators whose mutual equilibrium influences immunogenic versus tolerogenic cell death, respectively.

p53 and PD-1H as modulators of the immune system

The mounting of an immunogenic or tolerogenic immune response to phagocytosis is determined by the underlying cause of cell death, whether it is homeostatic tissue turnover, infection, malignancy, or injury. These various tissue insults elicit mechanistically distinct forms of cell death that ultimately result in expression and secretion of a unique milieu of signals that influence the responding phagocyte. Dysregulation of the immune response to cell death can have profound influence on

the development of autoimmune disease and cancer. Therefore, the identification of master regulators that govern whether a cell undergoes immunogenic or tolerogenic cell death has the potential to lead to improved therapeutics with multi-disease implications. One potential master regulator of tolerogenic cell death is the tumor-suppressor gene *TP53* because of its role in the initiation of physiologic apoptosis (Muñoz-Fontela et al., 2016).

Adaptive immunity fundamentally requires two concurrent signals: antigen presentation and co-stimulation. The nature of the immune response to cell death, whether immunogenic or tolerogenic, is most often relayed from the innate to adaptive immune system by manipulation of co-stimulation and cytokine-mediated adjuvanticity. Immunogenic phagocytosis of infected or malignant dying cells will promote CD80 engagement with CD28 and secretion of IL-4 and IL-6, while tolerogenic phagocytosis leads to anergy due to loss of pro-inflammatory co-stimulation. Here I propose that p53 induces tolerogenic phagocytosis by mitigating adjuvanticity, leading to the development of non-functional adaptive immune responses and promoting T_{reg} development and function (**Figure 1**).

Tumor-suppressor genes such as p53 respond to cellular stresses such as DNA damage, oncogene activation, and ribosomal and metabolic stress by governing cell cycle arrest, senescence, metabolism, and apoptosis (Berkers et al., 2013; Bursac et al., 2014; Golomb et al., 2014; Iurlaro et al., 2014; Jones et al., 2005; Kruiswijk et al., 2015; Lane and Levine, 2010; Maddocks et al., 2013; Maiuri et al., 2010; Vousden and Prives, 2009; Vousden and Ryan, 2009; Wang and Gu, 2014). In this manner, p53 acts as a safeguard against cancer development, and loss of function mutations

in p53 lead to genomic instability and enhanced malignancy (Chaffer and Weinberg, 2015; Hanahan and Weinberg, 2011; Weinberg, 2014). Cancer immunosurveillance represents an independent safeguard against cancer development, in which the host immune system recognizes and eliminates developing tumor cells (Iannello et al., 2016; Kroemer et al., 2015; Lesokhin et al., 2015; Miciak and Bunz, 2016; Schreiber et al., 2011; Zitvogel et al., 2006, 2015). Recent studies have identified a novel role of p53 in immune response with potential non-cell-autonomous influences on tumor immunology (Bi et al., 2014; Bueter et al., 2006; Cooks et al., 2014; Forys et al., 2014; Guo and Cui, 2015; He et al., 2015; Huang et al., 2014; Jung et al., 2010; Kawashima et al., 2013; Li et al., 2015; Lujambio et al., 2013; Menendez and Anderson, 2014; Menendez et al., 2009, 2013; Mori et al., 2002; Muñoz-Fontela et al., 2008; Rivas et al., 2010; Shatz et al., 2012; Singh et al., 2010; Takaoka et al., 2003; Takatori et al., 2014; Taura et al., 2008; Watanabe et al., 2014; Yanai et al., 2007).

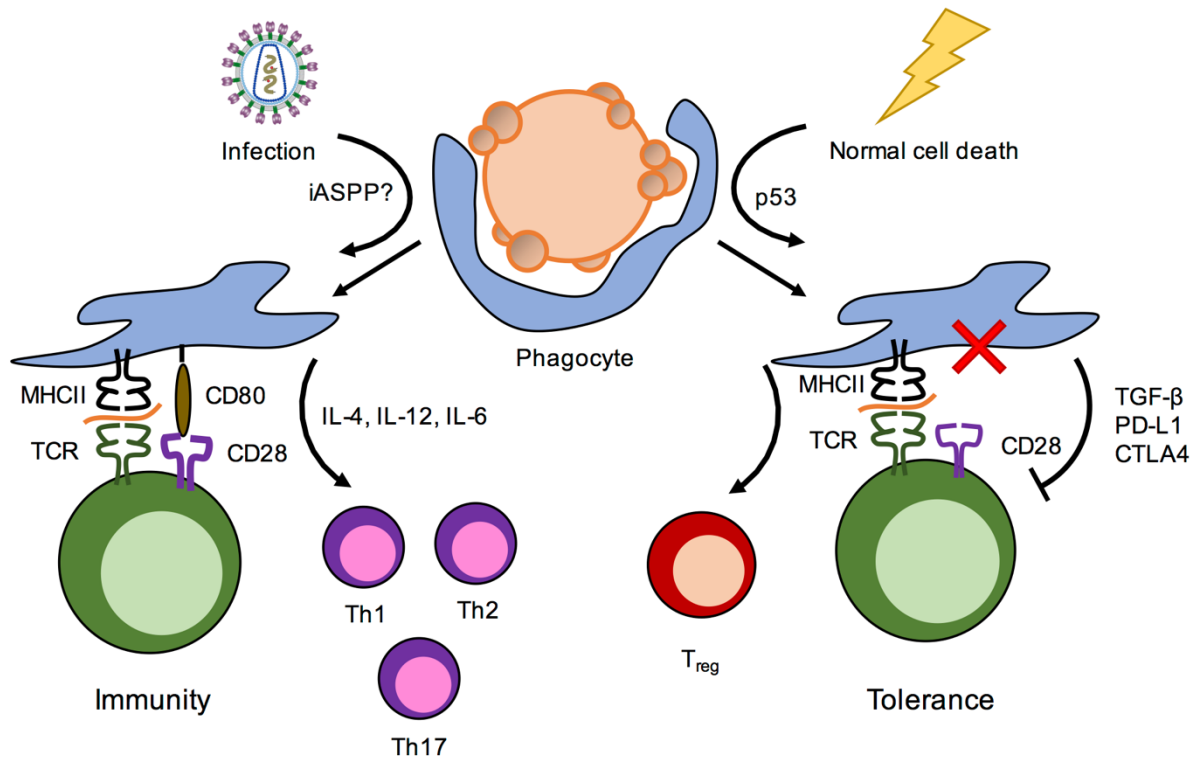


Figure 1: Immunogenic versus tolerogenic phagocytosis

The immunological consequences of phagocytosis and antigen presentation are determined by the underlying cause of cell death. Infection or necrosis and corresponding secretion of pro-inflammatory cytokines induces co-stimulation by antigen-presenting cells (APCs). Engagement of T cell receptor (TCR) with co-stimulation and pro-inflammatory cytokine release stimulates T cell activation, tipping the immunological balance towards immunity. During normal tissue turnover, p53-induced apoptosis and phagocytosis stimulates secretion of anti-inflammatory cytokines and antigen presentation without co-stimulation. Anergic TCR engagement and expression of checkpoint molecules tips the immunological balance towards tolerance. T_{reg}, regulatory T cell.

p53 directly transactivates expression of regulators of immune signaling pathways involved in pathogen recognition, cytokine secretion, and innate immunity (Bi et al., 2014; Menendez et al., 2013; Mori et al., 2002; Rivas et al., 2010; Watanabe et al., 2014; Yanai et al., 2007). TLRs, whose deregulation is associated with autoimmune disease such as systemic lupus erythematosus (SLE), inflammatory bowel disease, type 1 diabetes, multiple sclerosis, and rheumatoid arthritis (Mills, 2011; O'Neill et al., 2013), are also transcriptionally regulated by p53. Antiviral response genes such as CCL2, IRF5, IRF9, PKR, TLR3, and ISG15 (Iannello et al., 2013; Mori et al., 2002; Muñoz-Fontela et al., 2008; Taura et al., 2008; Yan et al., 2015) have been identified as p53 transcriptional targets, and p53-deficient mice are more susceptible to viral infection (Munoz-Fontela et al., 2005; Turpin et al., 2005). Type I IFN transcriptional activation of p53 following viral infection (Ding et al., 2006; Pampin et al., 2006; Takaoka et al., 2003; Townsend et al., 2004) suggests that p53 operates in a positive feedback loop to regulate antiviral intracellular immunity. In contrast, p53 negatively regulates STAT1 (Campbell et al., 2012; Cheon et al., 2014; McDermott et al., 2005; Youlyouz-Marfak et al., 2008; Zheng et al., 2005) and NF- κ B (Ak and Levine, 2010; Komarova et al., 2005; Murphy et al., 2011), preventing expression of pro-inflammatory cytokines (Komarova et al., 2005; Liu et al., 2009a; Lowe et al., 2014; Margulies and Sehgal, 1993; Santhanam et al., 1991), such as IL-6 (Okuda et al., 2003). Macrophages lacking p53 secrete elevated amounts of pro-inflammatory cytokines such as IL-1, IL-6, IL-12, and TNF- α (Gudkov et al., 2011; Komarova et al., 2005; Zheng et al., 2005). p53 can also promote anti-cancer NK cell immunity by transcriptionally activating NK cell ligands ULBP1 and ULBP2 (Textor et al., 2011), although it downregulates CD43, a regulator of immune cell proliferation (Kadaja-Saarepuu et al., 2008, 2012).

p53 also has multifaceted roles in the regulation of adaptive immunity. Dominant negative and missense mutations in *TP53* are associated with rheumatoid arthritis (Tak et al., 2000; Yamanishi et al., 2002a), SLE (Kovacs et al., 1997), ulcerative colitis (Hussain et al., 2000), and Sjögren's syndrome (Tapinos et al., 1999), demonstrating a necessary role of p53 in maintaining immune tolerance. Mice lacking p53 or its downstream mediators are more susceptible to systemic autoimmune disease and lupus (Balomenos et al., 2000; Campbell et al., 2012; Salvador et al., 2002; Santiago-Raber et al., 2001), arthritis (Yamanishi et al., 2002b), autoimmune diabetes (Yao et al., 2001; Zheng et al., 2005), and experimental autoimmune encephalomyelitis (Okuda et al., 2003). p53 is necessary for DNA damage-induced upregulation of PD-L1 on cancer cells (Yoon et al., 2015), and inhibits expression of the PD-L1 regulator miR-34 (Cortez et al., 2016). p53 might influence DC maturation and function (Gasparini et al., 2012; Muñoz-Fontela et al., 2011) via upregulation of MHC class I (Herzer et al., 2003; Wang et al., 2013a) and exosome production (Yu et al., 2006). p53 also promotes tolerance in CD4⁺ T cells by inhibiting IL-2 stimulated proliferation in the absence of antigen-specific TCR engagement (Watanabe et al., 2014), and CD4⁺ T cell activation and Th17 polarization via STAT3 (Kawashima et al., 2013; Zhang et al., 2011b). Intriguingly, p53 directly binds the *Foxp3* promoter in response to robust TCR engagement, promoting the development of T_{reg} cells (Jung et al., 2010; Kawashima et al., 2013; Park et al., 2013; Singh et al., 2010). Recently, c-MAF, a transcriptional activator of p53, was demonstrated to be necessary for RORγt⁺FoxP3⁺ iTreg maintenance of immunological tolerance in the intestine (Xu et al., 2018).

Although the role of p53 and its target genes in the induction and regulation of apoptosis is well established (Chipuk and Green, 2006; Green and Kroemer, 2009; Lane and Levine, 2010; Oren, 2003; Vousden and Prives, 2009), its effect on the immune response to apoptosis has only recently been appreciated (Yoon et al., 2015). p53 promotes phagocytosis of apoptotic cells by directly transactivating expression of PD-1H (also known as VISTA, Gi24, and DD1 α). PD-1H is an immunoglobulin superfamily member with sequence homology to the IgV domains of PD-1, PD-L1, and Tim-3 (Yoon et al., 2015). Interestingly, the cytoplasmic tail of PD-1H does not contain immunoreceptor tyrosine-based inhibitory or immunoreceptor tyrosine-based switch motifs, but rather contains conserved tyrosine and proline residues that are potential protein kinase C docking sites (Flies et al., 2011). Aged PD-1H deficient mice develop spontaneous immune infiltration of the skin, lung, and kidney (Yoon et al., 2015), suggesting that p53 promotes PD-1H mediated tolerogenic phagocytosis and in this manner prevents systemic autoimmune disease (Muñoz-Fontela et al., 2016). Interestingly, in contrast to the first identified immunosuppressive 'eat-me signals' and their scavenger receptors such as phosphatidylserine, BA11, TIM-4, and MERTK (Hochreiter-Hufford and Ravichandran, 2013; Kobayashi et al., 2007; Miyanishi et al., 2007; Oren, 2003), PD-1H is expressed on both dying cells and phagocytes and its homophilic interaction promotes engulfment (Yoon et al., 2015). The multifold expression of PD-1H on dying cells, phagocytes, and lymphocytes (Flies et al., 2011; Wang et al., 2011) suggests that PD-1H might promote immunosuppression via step-wise modulation of the immune response and fostering communication between dying cells and the immune system, providing the missing link between p53 and tolerogenic phagocytosis.

In addition to its recently discovered functions in phagocytes, PD-1H was initially characterized in CD4⁺ T cells (Wang et al., 2011). Engagement of cell surface PD-1H on CD4⁺ T cells can suppress their proliferation and secretion of IL-2 and IFN- γ , and this can be rescued by increased CD28 co-stimulation (Wang et al., 2011). Intriguingly, PD-1H is also necessary for the differentiation and maintenance of iT_{reg}, but not nT_{reg} pools *in vivo* (Wang et al., 2017). Mice deficient for PD-1H are more susceptible to experimental autoimmune encephalomyelitis (EAE) (Wang et al., 2011) and autoimmune hepatitis, have more robust antigen-specific immune responses, and are more resistant to a mouse model of glioma (Flies et al., 2014), suggesting that PD-1H is an essential mediator of immunological tolerance. In contrast, tumor cells overexpressing PD-1H can overcome protective antitumor immunity (Wang et al., 2011) and excessive PD-1H signaling using an engineered monoclonal antibody agonist prevents allogeneic graft-versus-host disease (Flies et al., 2011) and ameliorates CD4⁺ T cell mediated acute inflammation (Flies et al., 2014). Equivalently, PD-1H blockade attenuates tumor growth by promoting functional anti-tumor immunity via DC maturation, decreased tumor infiltration and function of MDSCs and T_{reg} cells, and increased CD4⁺ T cell proliferation, IFN- γ and GranzymeB secretion (Le Mercier et al., 2014). Interestingly, PD-1H mRNA was most prominently found in heart, brain, spleen, lung, and skeletal muscle tissues (Flies et al., 2011), identifying potential organs in which PD-1H maintains tolerance. The recent discovery and preliminary characterization of PD-1H suggests that PD-1H helps to orchestrate tolerogenic phagocytosis, serving as the molecular link between p53-induced apoptosis, phagocytosis, and adaptive immune homeostasis. Better understanding of the biological pathways influencing p53 regulation of PD-1H

and more general immune homeostasis is necessary, and might contribute to improved therapeutic approaches to autoimmune disease and cancer.

iASPP interactions with p53

Transcriptional regulation of cell death likely dictates not only how cells die, but also the immunological consequences of their death and ensuing phagocytosis. Master regulators of cell death, such as p53, might therefore be ultimately responsible for the establishment of an inherently immune activating or tolerizing transcriptional program. p53 is an evolutionarily ancient protein whose conserved function is the potent induction of apoptosis or cellular senescence following genotoxic stress by transactivating and transrepressing hundreds of target genes (Levine and Oren, 2009), including genes involved in immune regulation (Bi et al., 2014; Menendez et al., 2013; Mori et al., 2002; Rivas et al., 2010; Watanabe et al., 2014; Yanai et al., 2007). One of the most ancient regulators of p53 promoter selectivity and subsequent apoptosis is the Ankyrin repeat domain, SH3 domain and Proline rich sequence containing Protein (ASPP) family of proteins (Sullivan and Lu, 2007).

ASPP1 and ASPP2 interact with the DNA binding domains of p53, p63, and p73 to promote target gene transactivation (Bergamaschi et al., 2004; Kampa et al., 2009; Samuels-Lev et al., 2001; Vives et al., 2006), while inhibitor of Apoptosis Stimulating p53 Protein (iASPP) binds and inhibits the transcriptional activity but not expression of p53 (Bergamaschi et al., 2003), p63 in basal epithelial cells (Cai et al., 2012; Notari et al., 2011; Robinson et al., 2008), and NF- κ B subunit p65/RelA in cardiomyocytes and endothelium (Herron et al., 2005; Yang et al., 1999a, 1999b). This cell type-specific differential transcription factor binding of iASPP is likely due to

cellular localization of the iASPP protein (Lu et al., 2013, 2016; Slee et al., 2004), which can shuttle between the cytoplasm in differentiated epithelial cells (Chikh et al., 2011; Notari et al., 2011), the plasma membrane in differentiating keratinocytes and cardiomyocytes (Notari et al., 2015), and the nucleus in basal keratinocytes (Notari et al., 2011). iASPP can exist as a homo-oligomer in the cytoplasm, while phosphorylation of N-terminal serine residues (Lu et al., 2013; Slee et al., 2004) or caspase cleavage of the N-terminal region of iASPP (Hu et al., 2015) results in exposure of the C-terminal nuclear localization RanGDP and Ankyrin Repeats binding code as well as its p53 and RelA/p65 binding sites (Lu et al., 2014; Slee et al., 2004; Yang et al., 1999a). Nuclear iASPP inhibits p53-induced apoptosis by binding to the central core domain of p53 (Bergamaschi et al., 2003, 2006; Sullivan and Lu, 2007), and modulating p53 promoter selectivity and transcriptional activity (T. Carroll, unpublished data). Here I propose that regulation of p53 activity by iASPP is responsible for dictating whether cell death is immunogenic or tolerogenic.

iASPP was first described as an oncoprotein that can cooperate with Ras to transform rat embryonic fibroblasts *in vitro* by antagonizing p53-induced apoptosis (Bergamaschi et al., 2003). Indeed, overexpression of iASPP is associated with breast cancer (Bergamaschi et al., 2003), non-small cell lung cancer (Chen et al., 2010), bladder cancer (Wu et al., 2017b), glioma and glioblastoma (Li et al., 2011a; Liu et al., 2016b), hepatocellular carcinoma (Lin et al., 2011; Lu et al., 2010; Pang et al., 2010), acute leukemia (Jia et al., 2014; Zhang et al., 2005), oral cavity squamous cell carcinoma (Kim et al., 2015b), prostate cancer (Morris et al., 2014; Zhang et al., 2011a), colorectal cancer (Saebø et al., 2006), melanoma (Lu et al., 2013), head and neck squamous cell carcinoma (Liu et al., 2012), and chemoresistance in ovarian

cancer (Jiang et al., 2011) and cervical cancer (Xiong et al., 2017). Activation of iASPP is one mechanism tumors employ to overcome persistence of wild type p53 (Dong et al., 2015; Sullivan and Lu, 2007), and iASPP overexpression promotes mouse embryonic fibroblast cell migration and metastasis (Laska et al., 2009). Conversely, iASPP knock-down antagonizes human cancer cell proliferation *in vitro* (Cai et al., 2012; Chen et al., 2010, 2014; Lin et al., 2011; Liu et al., 2009b, 2011; Lu et al., 2010; Pang et al., 2010; Zhang et al., 2011a). Collectively, these data have led to the widespread acceptance that iASPP is an oncogene that functions by inhibiting p53-mediated apoptosis and potentially also EMT suppression (Bieging et al., 2014; Dong et al., 2013; Godar et al., 2008; Lin et al., 2005).

Notably, p53-independent functions of iASPP have also been characterized. The wavy hair phenotype observed in Wa3 mice, who harbor a mutation in *Ppp1r13l*, the gene encoding iASPP, is thought to be caused by NF- κ B defectiveness (Herron et al., 2005). Indeed, in addition to preventing p53-induced cell death and senescence, iASPP stabilizes desmosomes (Notari et al., 2015) and promotes wound healing (Z. Dedeić, unpublished). iASPP can even have pro-apoptotic functions, as RelA/p65 is pro-proliferative and associated with cancer development (Karin and Lin, 2002), and iASPP inhibition of RelA/p65 can promote DNA damage-induced apoptosis (Laska et al., 2007). NF- κ B pathway overactivation might have cardiological consequences, as iASPP-deficient mice have enhanced NF- κ B transcriptional activity (Herron et al., 2005), develop cardiocutaneous disorders (Notari et al., 2015) and impaired epithelial stratification (Notari et al., 2011), and human and bovine cardiocutaneous syndrome is associated with a nonsense mutation in *PPP1R13L* (Falik-Zaccai et al., 2017; Herron et al., 2005; Notari et al., 2015; Simpson et al., 2009; Whittington and

Cook, 1988). Human fibroblasts and murine cardiomyocytes with mutant, knocked-down, or lacking iASPP are hypersensitive to lipopolysaccharide stimulation of NF- κ B inflammatory cytokine release, and exhibit enhanced RelA/p65 binding to the *IL1B* and *TNFA* promoters (Falik-Zaccai et al., 2017). However, unraveling the consequences of iASPP inhibition of p53 and RelA/p65 is difficult, given the complex stimulus and cell type-specific cross-talk between the two pathways during apoptosis (Armstrong et al., 2006; Frank et al., 2011; Hu et al., 2015; Ryan et al., 2000) and immune response (Komarova et al., 2005; Liu et al., 2009a; Lowe et al., 2014; Margulies and Sehgal, 1993; Santhanam et al., 1991). How the biological cross-talk between iASPP, p53, and RelA/p65 influences apoptosis remains largely unknown.

Despite the widespread acceptance of an oncogenic function of iASPP in a plethora of cancer types (Dong et al., 2015), no study to date has demonstrated a definite causal link between iASPP activity and tumorigenesis. *In vitro* analyses of tumor cell proliferation and growth dynamics (Cai et al., 2012; Chen et al., 2010, 2014; Lin et al., 2011; Liu et al., 2009b, 2011; Lu et al., 2010; Pang et al., 2010; Zhang et al., 2011a) critically fail to represent the multitude of influences on carcinogenesis present *in vivo* (Hanahan and Weinberg, 2011), and have thus only demonstrated the cell-autonomous oncogenic properties of iASPP. In addition, clinical observation and association studies demonstrating iASPP expression in developed tumors (Bergamaschi et al., 2003; Cai et al., 2012; Chen et al., 2010; Jia et al., 2014; Lin et al., 2011; Liu et al., 2016b; Lu et al., 2010, 2013; Morris et al., 2014; Pang et al., 2010; Saebø et al., 2006; Zhang et al., 2005) fail to establish causal links between iASPP and tumorigenesis, or even make claims of necessity or sufficiency.

Elucidation of the temporal dynamics of iASPP expression during tumorigenesis from

neoplasia to metastasis is necessary, as iASPP might only be expressed in advanced lesions that have already escaped immunosurveillance or other negative pressures. To address these shortcomings of current iASPP cancer biology research, it is necessary to study the role of iASPP in autochthonous *in vivo* models of tumor development, which allows for bona fide immunoediting of the developing tumor (DuPage et al., 2011; Mittal et al., 2014; Schreiber et al., 2011). To date, no autochthonous mouse cancer model has been established to investigate the role of iASPP in tumorigenesis.

The consequences of iASPP inhibition of p53 on apoptosis and tumorigenesis remain relatively unclear. Given the recent implication of p53 in immune homeostasis, I hypothesized that iASPP regulates p53-mediated induction of tolerogenic apoptosis and phagocytosis by fundamentally altering the transcriptional profiles of dying cells. Alteration of the adjuvanticity of cell death, for example by expression of PD-1H, can significantly modulate the ensuing organism-wide and non-cell autonomous immune response. Under this model, iASPP and p53 represent novel master regulators of transcriptional signatures intrinsic to dying cells responsible for communicating the ultimate nature of cell death, whether physiologic or unnatural, to the responding immune system. The mounting of a correct immune response, whether immunogenic or tolerogenic, is critical for the prevention of autoimmune disease and cancer, and therefore would require proper iASPP and p53 cross-talk in this model. To this end, I subjected iASPP-deficient mice to models of autoimmune disease and autochthonous tumorigenesis to characterize the role of iASPP in the response to immunologic challenge.

CHAPTER 2: Materials and methods

Mice

iASPP^{-/-} (Notari et al., 2011, 2015), *iASPP*^{fl/fl} (Notari et al., 2011), *ASPP2*^{fl/fl} (Sottocornola et al., 2010), *K-ras*^{LSL-G12D} (Jackson et al., 2001), *Pdx1-Cre* (Hingorani et al., 2003), *Trp53*^{LSL-R127H/+} (Olive et al., 2004), *Rosa26*^{LSL-EYFP} (Srinivas et al., 2001), and *Rosa26*^{CreER} (Vooijs et al., 2001) mice have been previously described. *iASPP*^{fl/fl} mice were back-crossed into a C57BL/6 background strain by Z. Dedeić prior to being bred with *Pdx1-Cre* and *Rosa26*^{LSL-EYFP/EYFP} mice (Jackson Laboratory) by P. Miller to generate *iASPP*^{fl/fl};*Rosa26*^{LSL-EYFP/EYFP} (i) and *iASPP*^{fl/fl};*Pdx1-Cre*;*Rosa26*^{LSL-EYFP/EYFP} (iC) mice. *K-ras*^{LSL-G12D/+};*Rosa26*^{LSL-EYFP/EYFP} (K), *K-ras*^{LSL-G12D/+};*Pdx1-Cre*;*Rosa26*^{LSL-EYFP/EYFP} (KC), *K-ras*^{LSL-G12D/+};*iASPP*^{fl/fl};*Rosa26*^{LSL-EYFP/EYFP} (Ki), and *K-ras*^{LSL-G12D/+};*iASPP*^{fl/fl};*Pdx1-Cre*;*Rosa26*^{LSL-EYFP/EYFP} (KiC) mice were generated by P. Miller by crossing *K-ras*^{LSL-G12D} heterozygous mice with iC or *Pdx1-Cre* mice. *K-ras*^{LSL-G12D/+};*Trp53*^{LSL-R127H/+} (KP) and *K-ras*^{LSL-G12D/+};*Trp53*^{LSL-R127H/+};*Pdx1-Cre*;*Rosa26*^{LSL-EYFP/EYFP} (KPC) mice were generated by P. Miller by crossing KC mice with *Trp53*^{LSL-R127H} heterozygous mice. *ASPP2*^{fl/fl} mice were back-crossed into a C57BL/6 background strain by S. Zhong prior to being bred with *K-ras*^{LSL-G12D} heterozygous mice by P. Miller to generate *K-ras*^{LSL-G12D/+};*ASPP2*^{fl/fl};*Rosa26*^{LSL-EYFP/+} (KA) mice. Mice heterozygous for deletion of exon 8 of *Ppp1R13l* (*iASPP*^{Δ8/+}) were used to generate *iASPP*^{-/-} mice and littermate wild type controls. Aged (>30 wk old) *iASPP*^{-/-} mice were generously provided by E. Slee. The *iASPP*^{fl/fl};*Rosa26*^{CreER} colony was maintained by breeding homozygous *iASPP*^{fl/fl};*Rosa26*^{CreER} mice.

iASPP^{fl/fl};Rosa26^{CreER} mice were maintained in a mixed Sv129 x BALB/c background, while all other mice were maintained in a pure C57BL/6 background. The health of mice was monitored daily by veterinary staff at the Wellcome Trust Centre for Human Genetics at the University of Oxford. All mice were housed and all procedures were performed at the Wellcome Trust Centre for Human Genetics at the University of Oxford. All animal studies described in this study were performed in accordance with guidelines provided by the University of Oxford Institutional Animal Care and Use Committee. Procedures were performed under the Home Office Animal Scientific Procedures Act 1986 guidelines (license PPL 30/3451 and PPL 30/3096).

PCR genotyping

After mice were weaned, DNA was isolated from ear clips by incubation in TDB (50 mM KCl, 10 mM Tris-HCl, 0.1% Triton-X100 in H₂O) with 0.4 mg/mL Proteinase K (Qiagen) for 2 hr at 55 °C, then 10 min at 95 °C. *Pdx1-Cre* and *Rosa26^{CreER}* mice were genotyped using the following primers: Cre-F, 5'-CATTGTTGGGCCAGCTAAACAT-3'; Cre-B, 5'-ATTCTCCCACCGTCAGTACG-3'; Actin-F, 5'-GGTGTCATGGTAGGTATGGGT-3'; and Actin-B, 5'-CGCACAATCTCACGTTTCAG-3'. *ASPP2^{fl/fl}* mice were genotyped using the following primers: SDL1s, 5'-TGGACAGGCTGAGCTGTTAG-3'; SDL2s, 5'-ACACTTTCACCAACCCTAGGTC-3'. *iASPP2^{fl/fl}* mice were genotyped using the following primers: FRANT9, 5'-GGGTAGGAAAAAGGGCTGAG-3'; FLP2, 5'-CCGAATTGGAGAAGTGAAGC-3'. *K-ras^{LSL=G12D/+}* mice were genotyped using the following primers: Y116, 5'-TCCGAATTCAGTGAAGTACAGATG-3'; Y117, 5'-CTAGCCACCATGGCTTGAGT-3'; Y118, 5'-ATGTCTTTCCCCAGCACAGT-3'.

Rosa26^{LSL-EYFP/EYFP} mice were genotyped using the following primers: YFPc, 5'-AAAGTCGCTCTGAGTTGTTAT-3' and YFPwt, 5'-GGAGCGGGAGAAATGGATATG-3'; or YFPc and YFPmut, 5'-AAGACCGCGAAGAGTTTGTC-3'. *Trp53^{LSL-R172H/+}* mice were genotyped using the following primers: p53u, 5'-CTTGGAGACATAGCCACACTG-3' and p53wt, 5'-TTACACATCCAGCCTCTGTGG-3'; or p53u and p53mut, 5'-AGCTAGCCACCATGGCTTGAGTAAGTCTGCA-3'. *iASPP^{-/-}* mice were genotyped using the following primers: I8-2, 5'-ACAGGCAGCTACTGGTATTC-3' and E8-2, 5'-AGAGCAGCCTCAGAGCATGG-3'; or I8-2 and FLP2.

PCR reactions were performed on 0.5 µL of sample DNA using Green *Taq* DNA Polymerase (GenScript) according to manufacturer's protocols. The *Pdx1-Cre* and *Rosa26^{CreER}* allele yields a 308 bp product while the wild type allele yields a 753 bp product from primers Cre-F, Cre-B, Actin-F, and Actin-B. The *ASPP2^{fl/fl}* allele yields a 203 bp product while the wild type allele yields a 169 bp product from primers SDL1s and SDL2s. The *iASPP^{fl/fl}* allele yields a 400 bp product while the wild type allele yields a 265 bp product from primers FRANT9 and FLP2. The *K-ras^{LSL-G12D}* allele yields a 327 bp product while the wild type allele yields a 450 bp product from primers Y116, Y117, and Y118. The *Rosa26^{LSL-EYFP}* allele yields a 320 bp product from primers YFPc and YFPmut, while the wild type allele yields a 600 bp product from primers YFPc and YFPwt. The *Trp53^{LSL-R172H}* allele yields a 270 bp product from primers p53u and p53mut, while the wild type allele yields a 166 bp product from primers p53u and p53wt. The *iASPP^{-/-}* allele yields a 700 bp product from primers I8-2 and FLP2, while the wild type allele yields a 600 bp product from primers I8-2 and E8-2.

Preparation of mouse T cell populations

Spleens from 6-8-week-old mice were dissociated by mechanical dissociation using the plunger of a 2.5 mL syringe on a 70 μ m cell strainer (Falcon). Red blood cells were lysed with ACK Lysis buffer (150 mM NH_4Cl , 10 mM KHCO_3 , 0.1 mM EDTA in H_2O , pH 7.4) for 2 min at RT. Following centrifugation cells were resuspended in RPMI 1640 (Gibco) supplemented with 1% heat-inactivated fetal calf serum (HI-FCS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin and counted using a hemocytometer. Splenocytes were then washed and resuspended in 0.5% BSA and 2 mM EDTA in PBS at 1×10^7 cells/mL for magnetic-activated cell sorting (MACS). CD4^+ T cells were isolated by negative selection using a CD4^+ T Cell Isolation Kit and LS columns (Miltenyi Biotec), naive $\text{CD4}^+\text{CD44}^-\text{CD62L}^+$ T cells were isolated using a Naive CD4^+ T Cell Isolation Kit and LS columns (Miltenyi Biotec), and $\text{CD4}^+\text{CD25}^-$ conventional T cells (T_{conv}) and $\text{CD4}^+\text{CD25}^+$ regulatory T cells (T_{reg}) were isolated by sequential negative and positive selection using a $\text{CD4}^+\text{CD25}^+$ Regulatory T Cell Isolation Kit and LD and MS columns (Miltenyi Biotec) according to manufacturer's protocols.

In vitro T cell activation

CD4^+ T cells were washed and resuspended at 1×10^7 cells/mL in PBS and labeled with 5 μ M CellTrace carboxyfluorescein succinimidyl ester (CFSE) Cell Proliferation Dye (Life Technologies) for 10 min at 37 $^\circ\text{C}$. Staining was quenched with five volumes of HI-FCS and cells were resuspended at 5×10^5 cells/mL in RPMI 1640 supplemented with 10% HI-FCS, 10 mM HEPES (Life Technologies), 50 μ M β -mercaptoethanol (Sigma), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μ g/mL

streptomycin with or without 40 ng/mL murine IL-2 (PeproTech), Cells were stimulated with indicated concentrations of plate-bound anti-CD3 ϵ mAb (145-2C11, BioLegend) at 200 μ L/well in a 96-well plate, and CFSE dilution was measured by flow cytometry after 72 hr.

T_{reg} suppression assay

CD4⁺CD25⁻ T_{conv} cells were washed and resuspended at 1.5 x 10⁶ cells/mL in PBS and labeled with 5 μ M CFSE for 10 min at 37 °C. Staining was quenched with five volumes of HI-FCS and cells were resuspended at 5 x 10⁵ cells/mL in RPMI 1640 supplemented with 10% HI-FCS, 10 mM HEPES (Life Technologies), 50 μ M β -mercaptoethanol (Sigma), 1mM sodium pyruvate (Life Technologies), 100 mM non-essential amino acids (Life Technologies), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin. Control splenocytes were resuspended at 1 x 10⁷ cells/mL and exposed to 30 Gy gamma irradiation by J. Thompson and labeled with soluble 1 μ g/mL anti-CD3 ϵ mAb. CD4⁺CD25⁺ T_{reg} cells were co-cultured at indicated ratios with 5 x 10⁴ irradiated splenocytes and 2.5 x 10⁴ T_{conv} cells/well in round-bottom 96-well plates (Corning). After 72 hr cells were washed and CFSE dilution was measured by flow cytometry.

CD4 differentiation assay

Naive CD4⁺CD44⁻CD62L⁺ T cells were resuspended at 1 x 10⁶ cells/mL in RPMI 1640 supplemented with 10% HI-FCS, 50 μ M β -mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin and stimulated with plate-bound 1 μ g/mL anti-CD3 ϵ mAb and 1 μ g/mL anti-CD28 (37.51, BioLegend) at 200 μ L/well in a

96-well plate. Cells were polarized as indicated using the following culture conditions: Th0, 5 $\mu\text{g/mL}$ anti-IL-4 mAb (11B11, BioLegend) and 5 $\mu\text{g/mL}$ anti-IFN- γ mAb (XMG1.2, BioLegend); Th1, 10 ng/mL murine IL-12 (PeproTech) and 5 $\mu\text{g/mL}$ anti-IL-4 mAb; Th2, 10 ng/mL murine IL-4 (PeproTech) and 5 $\mu\text{g/mL}$ anti-IFN- γ mAb; Th17, 5 $\mu\text{g/mL}$ anti-IL-4 mAb, 5 $\mu\text{g/mL}$ anti-IFN- γ mAb, 10 ng/mL human IL-23 (PeproTech), 1 ng/mL human TGF- β (PeproTech), and 5 ng/mL murine IL-6 (PeproTech); T_{reg}, 1 ng/mL human TGF- β , 5 $\mu\text{g/mL}$ anti-IL-4 mAb, and 10 $\mu\text{g/mL}$ anti-IFN- γ mAb. After 72 hr cells were analyzed by intracellular flow cytometry for CD4⁺ Th subset polarization.

Preparation of mouse macrophages

Peritoneal macrophages were isolated by injecting and recovering 10 mL DMEM (Gibco) into the left peritoneum of 6-8-week-old mice through a 19 G needle immediately following cervical dislocation. Recovered cells were centrifuged at 400 g for 10 min at 4 °C and resuspended at 1×10^6 cells/mL in DMEM supplemented with 10% HI-FCS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin and plated at 700 $\mu\text{L/well}$ of a 24-well plate. After 2 hr, cells were washed twice with PBS, media was replaced, and replaced every 72 hr thereafter.

Bone marrow cells were isolated by flushing freshly-dissected femurs and tibiae from 6-8-week-old mice with DMEM through a 27 G needle. Bone marrow was dissociated by trituration and filtration through a 70 μm cell strainer with the plunger of a 2.5 mL syringe. Red blood cells were lysed with ACK Lysis buffer for 2 min at RT and cells were resuspended at 1×10^6 cells/mL in DMEM supplemented with

10% HI-FCS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin and plated at 700 µL/well of a 24-well plate. To promote bone marrow-derived macrophage (BMDM) differentiation cells were treated with 20 ng/mL murine M-CSF (PeproTech), while to promote bone marrow-derived dendritic cell (BMDC) differentiation cells were treated with 20 ng/mL murine GM-CSF (PeproTech). Media was replaced every 72 hr and experiments were performed once wells were 80% confluent.

BMDMs were stimulated with or without 10 ng/mL murine IFN- γ (PeproTech) for 4 hr, followed by overnight incubation with or without 100 ng/mL lipopolysaccharide (LPS, Sigma) and with or without 20 µg/mL polyinosinic:polycytidylic acid (poly(I:C), Sigma) and 2 µM GolgiStop™ (BD Biosciences). The following day macrophages were dissociated by mechanical dissociation in the presence of 5 mM EDTA and analyzed by flow cytometry.

Macrophage phagocytosis assay

BMDMs were harvested, washed, and resuspended at 5×10^6 cells/mL in PBS followed by labeling with 1 µM CellTrace Far Red Cell Proliferation Dye (Life Technologies) for 10 min at 37 °C. Staining was quenched with five volumes of HI-FCS and cells were plated at 2×10^5 cells/well in a 24-well plate (for time-lapse imaging) or 5×10^4 cells/well in a 96-well plate (for flow cytometry). After 24 hr, cells were starved with phenol red free DMEM/F12 (Life Technologies) supplemented with 2% FCS for 30 min at 37 °C. Thymuses were isolated from 6-week-old mice and mechanically dissociated using the plunger of a 2.5 mL syringe on a 70 µm cell

strainer. To induce apoptosis, thymocytes were resuspended at 1×10^6 cells/mL and exposed to 2 Gy gamma irradiation by J. Thompson. Apoptotic thymocytes were washed and resuspended at 5×10^6 cells/mL in PBS followed by labeling with 5 μ M CFSE for 10 min at 37 °C. Staining was quenched with five volumes of HI-FCS and 1×10^5 (for time-lapse imaging) or 5×10^4 (for flow cytometry) thymocytes were added per well to pre-equilibrated BMDMs. Individual BMDMs were monitored using time-lapse imaging on a TE 2000-E Eclipse Inverted Microscope (Nikon) every 1 min for a total of 90 min. For flow cytometric analysis of phagocytosis, BMDMs and thymocytes were co-cultured for 60 min, after which BMDMs were extensively washed and identified by flow cytometry via forward and side scatter parameters and CellTrace Fare Red positivity. Apoptotic thymocyte-engulfing phagocytes were identified via CFSE positivity.

Concanavalin A-induced autoimmune hepatitis

Autoimmune hepatitis was induced in 8-10-week-old male mice by intravenous (i.v.) injection of 15 mg/kg concanavalin A (ConA, Sigma) in PBS. Blood samples were collected from the tail vein 6 and 12 hr after i.v. injection of ConA, and serum was stored at -80 °C until further analysis. Any mice exhibiting adverse signs related to hepatitis such as hunched posture, piloerection, prolonged lethargy, or respiratory distress were humanely euthanized. All mice were sacrificed 24 hr after induction of autoimmune hepatitis and spleens, livers, blood, and inguinal lymph nodes (iLN) were collected for analysis.

Experimental autoimmune encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE) was induced in 8-10-week-old female mice by bilateral subcutaneous (s.c.) injection of a total of 200 µg MOG₃₅₋₅₅ (MEVGWYRSPFSRVVHLYRNGK, Severn Biotech) emulsified 1:1 in complete freund's adjuvant supplemented with 400 µg H37Ra mycobacterium tuberculosis (CFA, Chondrex) in incomplete freund's adjuvant (Sigma). Immunization was boosted immediately and 48 hr after s.c. injection with 300 ng pertussis toxin (PTx, Merck Chemicals) by i.v. injection. Mice were weighed and neurological impairment was measured daily in a genotype-blind fashion using the following clinical severity scoring system: 0, healthy; 0.5, partial tail limpness; 1, tail paralysis; 1.5, righting reflex weakness; 2, hind limb weakness; 2.5, paralysis of one hind limb; 3, paralysis of both hind limbs; 3.5 hind limb paralysis and paralysis of one fore limb; 4, quadriplegia; 5, moribund. When mice reached a score of 1, cages were changed to dry bedding and wet mash was placed on the cage floor. Under recommendation of the animal ethics committee, mice were humanely euthanized when they reached a clinical score greater than or equal to 3. Any mice exhibiting adverse signs related to EAE such as greater than 20% weight loss, labored breathing, or inability to access food were humanely euthanized. Mice remaining alive 30 days after immunization were sacrificed and spleens, brains, spinal cords, iLNs, and blood were collected for analysis.

MOG₃₅₋₅₅ stimulation assay

Splenocytes were washed and resuspended at 1×10^7 cells/mL in PBS and labeled with 5 µM CFSE for 10 min at 37 °C. Staining was quenched with five volumes of HI-FCS and cells were resuspended at 1×10^6 cells/mL in RPMI 1640 supplemented

with 40 ng/mL murine recombinant IL-2, 10% HI-FCS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. Splenocytes were then stimulated with indicated concentrations of MOG₃₅₋₅₅ peptide for 72 hr, and CFSE dilution was measured by flow cytometry.

Adenoviral infection

Autochthonous lung adenocarcinoma was initiated in 9-13 week old mice by intranasal infection with 2.5×10^7 PFU adenovirus as previously described (DuPage et al., 2009; Jackson et al., 2005). Briefly, Ad-CMV-iCre (Vector Biolabs) was resuspended in 50 µL/mouse Opti-MEM (Life Technologies) with 10 mM calcium chloride. Mice were anesthetized with isoflurane until respiration rate reached 2 respirations/sec and 25 µL of adenovirus suspension was administered dropwise to a single nostril. After 5 min of recovery, adenoviral administration was repeated on the same nostril. Mice were monitored daily and any mice exhibiting adverse signs related to tumor burden such as hunched posture, labored respiration, or greater than 15% weight loss were humanely euthanized. All mice were sacrificed 15 weeks after tumor initiation and spleens, lungs, livers, and mediastinal lymph nodes (mLN) were collected for analysis.

3D acinar cell culture

24-well plates were coated with 250 µL/well of 3 mg/mL rat tail collagen (Life Technologies) and 0.42% sodium bicarbonate in RPMI 1640 (Life Technologies) for 1 hr at 37 °C. Freshly-isolated pancreas from six-week-old mice were washed in ice cold HBSS and minced into small fragments using scissors. Acini were dissociated

by incubating in 200 µg/mL collagenase P (Sigma) in HBSS (Life Technologies) for 20 min at 37 °C with gentle agitation. Following three washes in 5% FCS in HBSS, acini were filtered through a 100 µm cell strainer then layered on top of 30% FCS in HBSS and acini were collected by centrifugation at 300 g for 3 min. Acini were resuspended at 8×10^3 acini/mL in 3D culture base medium (RPMI 1640 (Gibco) with 1% FCS, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.1 mg/mL soybean trypsin inhibitor (Sigma), 1 µg/mL dexamethasone (Sigma)) then mixed 1:1 with 3 mg/mL rat tail collagen and plated at 0.5 mL/well in pre-coated 24-well plates. After solidifying for 1 hr at 37 °C, the cell-collagen mixture was fed with 1 mL/well 3D culture base medium with or without 50 ng/mL human recombinant TGF- α (PeproTech) and with or without 1 µM 4-hydroxytamoxifen (4-OHT, Sigma). Media was replaced after 24 hr, and every 48 hr thereafter.

Daily images were acquired using a Primo Vert Inverted Phase Contrast Microscope (Zeiss). Number and diameter of acini that underwent ductal metaplasia was measured using Fiji (ImageJ, NIH). After 3 or 7 days acini were released from the collagen matrix by digestion with 200 µg/mL collagenase P in HBSS for 30 min at 37 °C with gentle agitation. Cells were centrifuged at 700 g for 3 min and stored at -80 °C until RNA isolation. RNA was isolated using the RNeasy Mini Kit (Qiagen) according to manufacturer's protocols without DNaseI treatment.

Caerulein-induced chronic pancreatitis

Chronic pancreatitis was induced in 6-8-week-old mice by six weekly intraperitoneal (i.p.) injections of 250 µg/kg of caerulein (Sigma) in PBS. Approximately equal

numbers of male and female mice were used in all experimental groups. Mice were monitored daily and any mice exhibiting adverse signs related to chronic pancreatitis or tumor burden such as hunched posture, piloerection, greater than 15% weight loss, palpable abdominal masses, lethargy, diarrhea, rectal bleeding, ulcerated tumors, tumors interfering with locomotion, feeding, breathing, or vision, genital prolapse, or dermatitis were humanely euthanized. Mice were sacrificed 48 hr following the final caerulein injection and spleens, pancreas, lungs, and livers were collected for analysis.

Tissue collection

Mice were sacrificed by cervical dislocation and blood was immediately collected via cardiac puncture. Blood was kept at RT for 1 hr to promote coagulation, then centrifuged at 1500 g for 15 min at 4 °C to isolate serum, which was stored at -80 °C. Mice were then transcardially perfused with 40 mL PBS and tissues were allocated for immunohistochemistry, immunofluorescence, flow cytometry, RNA isolation, *in vitro* assays, or tissue lysate preparation. In cases where tissues were only taken for immunohistochemistry or immunofluorescence, mice were subsequently perfused with paraformaldehyde-lysine-periodate fixative (PLP; 50 mM phosphate buffer with 1% paraformaldehyde (Sigma), 0.1 M L-lysine buffer (pH 7.4), 0.2% NaIO₄ (Sigma)). Tissue lysates were prepared by homogenizing organs in 50 µL/mg RPMI with 1% Triton X-100 (Sigma) with a rotor-stator tissue homogenizer for 15 sec, followed by centrifugation at 5000 g for 10 min. RNA was prepared from snap-frozen tissues by grinding with mortar and pestle in liquid nitrogen, followed by tissue homogenization in 600 µL Buffer RLT (Qiagen) with β-mercaptoethanol and RNA isolation using the RNeasy Mini Kit according to manufacturer's protocols.

Immunohistochemistry and immunofluorescence

Isolated tissues were individually perfused with PBS followed by PLP. Tissues were fixed overnight in PLP at 4 °C and either stored in 70% ethanol until processing, or cyroprotected with 30 % sucrose (Scientific Laboratory Supplies Ltd) in PBS for 8 hr at 4 °C, perfused with 30% optimum cutting temperature compound (OCT; Sakura), and embedded in 100 % OCT in Standard Cryomolds (Tissue-Tek) on dry ice for immunofluorescence.

PLP-fixed tissues were dehydrated overnight using a Shandon Excelsior Tissue Processor (Thermo), embedded in paraffin, sectioned by I. Ratnayaka at a thickness of 4 µm, and stained with haematoxylin and eosin using standard methods. For immunohistochemistry (IHC), slides were dewaxed and antigen retrieval was performed by boiling in Tris-EDTA (pH 9) and 0.5% Tween-20 (Sigma) for 3 min at 120 °C in a pressure cooker. Slides were then cooled, washed with dH₂O, and endogenous peroxidases were blocked with 3% hydrogen peroxide (Sigma) in methanol (Sigma) for 15 min at RT. Tissues were blocked with 5% normal goat (Vector Laboratories) or donkey serum (Sigma) in PBS, and stained with primary antibody overnight at 4 °C followed by biotinylated secondary anti-rabbit, anti-hamster, anti-rat, or anti-goat (Vector Laboratories) for 30 min at RT and Vectastain Elite ABC (Vector Laboratories) for 30 min at RT. Staining was visualized using ImmPACT DAB Peroxidase (Vector Laboratories) using manufacturer's protocols, and slides were counterstained with haematoxylin for 4 sec, dehydrated to Histo-Clear II (National Diagnostics), and coverslipped using VectaMount Permanent Mounting Medium (Vector Laboratories). The following primary antibodies were

used for IHC: rabbit anti-CD3 (ab5690; 1:200, 4 min develop), hamster anti-CD11c (HL3, BD Biosciences; 1:50, 30 min develop), rat anti-FoxP3 (FJK-16s, eBioscience; 1:50, 30 min develop), rabbit anti-GFAP (Dako Z0334; 1:500, 1 min develop), goat anti-Iba1 (ab5076; 1:300, 3 min develop). Images were obtained using a NanoZoomer S210 (Hamamatsu) and analyzed using NDP.view2 software, or using a Leica DM5500B and analyzed using Fiji.

Luxol fast blue staining was performed by incubating sections in 0.1% luxol fast blue (Fisher) in 95% ethanol with 0.5% glacial acetic acid overnight at 56 °C. Slides were then differentiated in 0.05% lithium carbonate (Scientific Laboratory Supplies Ltd) in dH₂O for 30 sec, then 70 % ethanol for 30 sec, and counterstained with 0.1 % cresyl violet acetate (MP Biomedicals) in dH₂O with glacial acetic acid.

PicroSirius staining was performed by counterstaining slides in Weigert's iron haematoxylin (Sigma) for 8 min, followed by 0.1% Sirius red (Sigma) in saturated picric acid (Fisher) for 60 min, and 0.5% glacial acetic acid for 2 min.

For immunofluorescence (IF), frozen sections were cut on a cryostat at a thickness of 25 µm and allowed to dry for 60 min at RT. Tissues were fixed in ice-cold acetone (Sigma) for 5 min at -20 °C, then permeabilized and blocked with 10% normal donkey serum in 0.2% BSA (Sigma), 0.05% sodium azide (Sigma), and 0.3% Triton X-100 in PBS for 1 hr at RT. Slides were stained with primary antibody overnight at 4 °C, washed with 0.3% Triton X-100 in PBS, and incubated with appropriate species-specific Alex Fluor-conjugated secondary antibody (Invitrogen; 1:400) overnight at 4 °C. Nuclei were counterstained with DAPI (5 µg/mL) and slides were

mounted using Vectashield Antifade Mounting Medium (Vector Laboratories). The following primary antibodies were used for IF: hamster anti-CD11c (HL3, BD Biosciences; 1:50), rat anti-B220 (RA3-6B2, BD Biosciences; 1:200), rabbit anti-CD3 (ab5690; 1:200), rat anti-FoxP3 (FJK-16s, eBioscience; 1:50). Images were acquired using a Zeiss LSM710 Multiphoton Confocal Microscope and analyzed using Fiji.

Histological quantification

Grading of pancreatic neoplasias was performed in a blind fashion by R. Goldin as described (Hruban et al., 2006). Briefly, pancreatic neoplasias were categorized as metaplasia when exhibiting non-invasive epithelial proliferation and cellular abnormalities, pancreatic intraepithelial neoplasia (PanIN) when glandular epithelial proliferation was confined to pancreatic ducts, and pancreatic ductal adenocarcinoma (PDAC) when exhibiting ductal differentiation and penetration of the basement membrane. Lung tumor grading was performed in a blind fashion as previously described (DuPage et al., 2012; Jackson et al., 2005; Nikitin et al., 2004). Briefly, lung adenomas were identified by prominent nucleoli, while lung adenocarcinomas were graded by extent of nuclear atypia, cellular pleomorphism, invasiveness, mitotic index, cellular heterogeneity, and desmoplasia.

IHC images were blinded using blindanalysis v1.0 (Jim Salter, 2016). T_{reg} infiltration was assessed in a blind fashion by counting the number of FoxP3⁺ nuclei present in IHC images of tumors. Tumor area was measured in Fiji by outlining the perimeter of tumors and calculating the enclosed area. For analysis, average T_{reg} infiltration was calculated by dividing the total number of infiltrating FoxP3⁺ cells by the total

tumor area per mouse. Tumor burden was measured per lung lobe in NDPview.2 by outlining the area of individual tumors and of individual lobes.

Flow cytometry

Lymph nodes were dissociated by mechanical dissociation between two frosted microscope slides (Fisher). Spleens, thymus, livers, and brains were dissociated by mechanical dissociation using the plunger of a 2.5 mL syringe on a 70 μ m cell strainer (Falcon). Livers and brains were further processed by centrifugation in a Percoll (VWR) gradient at 700 g for 25 min with no brake, and lymphocytes were isolated from the 40-70% Percoll interface.

Lungs, pancreas, kidneys, and hearts were placed in 5 mL collagenase buffer (125 U/mL collagenase IV (Worthington), 40 U/mL DNaseI (Roche), 25 mM HEPES, and 0.5% HI-FCS in 1X HBSS) in C tubes (Miltenyi Biotec). Tissues were dissociated using program “m_imp_tumor_01” for lungs, “m_imp_tumor_04” for pancreas, “multi_E_01” for kidneys, or “m_heart_01” for hearts on a gentleMACS dissociator (Miltenyi Biotec), incubated at 37 °C for 40 min with gentle agitation, then dissociation was repeated using program “m_imp_tumor_01” for lungs, “m_imp_tumor_04” for pancreas, “multi_E_02” for kidneys, or “m_heart_02” for hearts. Following dissociation cells were filtered through a 100 μ m cell strainer (Falcon).

Colons were cut longitudinally and washed in PBS, then cut into 5 mm pieces and incubated in 5 mM EDTA in PBS for 15 min at 37 °C. Washed tissues were then placed in 5 mL of RPMI 1640 supplemented with 5% HI-FCS, 20 mM HEPES, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 100 U/mL collagenase VIII (Sigma) in

C tubes. Tissues were dissociated using program “m_imp_intestine_01”, incubated for 60 min at 37 °C with gentle agitation, dissociated again using program “m_imp_intestine_01”, and filtered through a 100 µm cell strainer. Cells were then resuspended and centrifuged in a Percoll gradient at 700 g for 25 min with no brake, and lymphocytes were isolated from the 40-70% Percoll interface.

Red blood cells were lysed with ACK Lysis buffer for 2 min at RT and resuspended in RPMI 1640 supplemented with 1% HI-FCS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin, counted, and adjusted to 1×10^7 cells/mL. 3×10^3 to 1×10^7 cells were Fc receptor blocked with rat anti-CD16/CD32 (2.4G2, BD Biosciences; 1:50) for 5 min, and stained for 30 min on ice in round-bottom 96-well plates with Zombie Green Viability Dye (BioLegend; 1:1000) or Zombie NIR Viability Dye (BioLegend; 1:1000) and directly conjugated antibodies in 0.5% HI-FCS and 0.05% sodium azide in PBS. Following staining cells were fixed with Fixation/Permeabilization Buffer (eBioscience) overnight at 4 °C. The following surface antibodies were used: PerCP-Cy5.5 anti-CCR8 (SA214G2; 1:100), APC-Cy7 anti-CD3 (17A2; 1:100), PE-Cy5 anti-CD4 (RM4-5; 1:300), BV421 anti-CD4 (GK1.5; 1:100), FITC anti-CD4 (RM4-5, eBioscience; 1:100), APC anti-CD8 α (53-6.7; 1:200), BV510 anti-CD8 α (53-6.7; 1:100), BV785 anti-CD8 α (53-6.7; 1:200), BV510 anti-CD11b (M1/70; 1:100), PerCP-Cy5.5 anti-CD11b (M1/70; 1:50), BV421 anti-CD11c (N418; 1:100), PE-Cy7 anti-CD11c (N418; 1:250), BV650 anti-CD19 (6D5; 1:150), PE-Cy5 anti-CD24 (M1/69; 1:600), BV510 anti-CD25 (PC61; 1:50), BV785 anti-CD44 (IM7; 1:200), FITC anti-CD45 (30-F11; 1:100), PE/Dazzle 594 anti-CD49b (DX5; 1:50), Alexa Fluor 700 anti-CD62L (MEL-14; 1:150), PE-Cy7 anti-CD68 (FA-11; 1:250), BV605 anti-CD80 (16-10A1; 1:50), PE anti-CD80 (16-10A1; 1:100), PE

anti-CD86 (GL-1; 1:100), PE-Cy7 anti-CD103 (2E7; 1:100), PE/Dazzle 594 anti-CTLA-4 (UC10-4B9; 1:100), BV421 anti-F4/80 (BM8; 1:50), BV711 anti-F4/80 (BM8; 1:100), BV421 anti-KLRG1 (2F1/KLRG1; 1:100), BV785 anti-Ly6C (HK1.4; 1:500), PerCP-Cy5.5 anti-Ly6G (1A8; 1:250), Alexa Fluor 700 anti-MHCII (M5/114.15.2; 1:250), BV421 anti-OX40 (OX-86; 1:), BV711 anti-PD-1 (29F.1A12; 1:100), APC anti-PD-1H (MH5A; 1:100), PE-Cy7 anti-PD-L1 (10F.9G2; 1:250), PE anti-TCR $\gamma\delta$ (UC7-13D5; 1:100), and PE-Cy7 anti-Tim-3 (RMT3-23; 1:100). For intracellular staining, cells were washed with Permeabilization Buffer (eBioscience) following fixation, incubated with directly-conjugated antibodies in Permeabilization Buffer for 30 min on ice, washed, and acquired directly. The following intracellular antibodies were used: PE-Cy5 anti-FoxP3 (FJK-16s, eBioscience; 1:100), PE anti-GATA3 (16E10A23; 1:50), Pacific Blue anti-GranzymeB (GB11; 1:50), PE/Dazzle 594 anti-Helios (22F6; 1:100), PerCP-Cy5.5 anti-IFN- γ (XMG1.2; 1:50), BV421 anti-IL17a (TC11-18H10.1; 1:50), BV650 anti-ROR γ t (Q31-378, BD Biosciences; 1:50), BV605 anti-T-bet (4B10; 1:50), and BV510 anti-TNF- α (MP6-XT22; 1:50). All antibodies were purchased from BioLegend unless otherwise indicated.

Following fixation, cells were read using 3-11 fluorophore flow cytometry on a LSRT Fortessa X-20 (BD Biosciences) with 405, 488, 561, and 633 lasers.

Compensation matrices were determined for each individual acquisition date using unstained and single fluorophore-stained controls. Data was analyzed using FlowJo software (Tree Star) and cell numbers were calculated from tissue cell counts preceding staining.

Statistical analyses

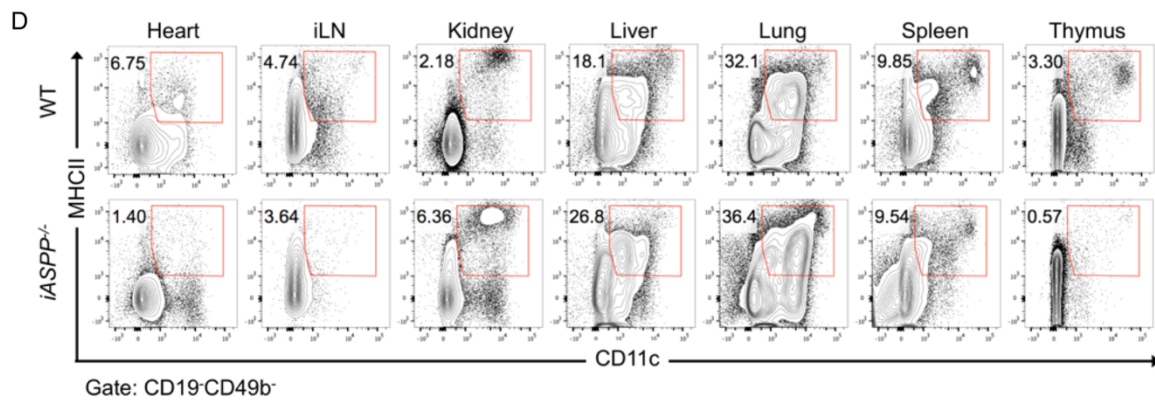
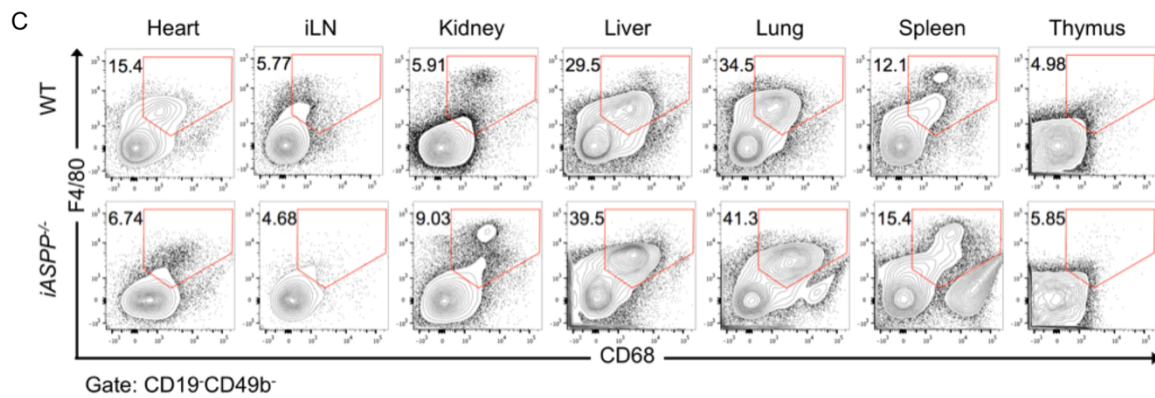
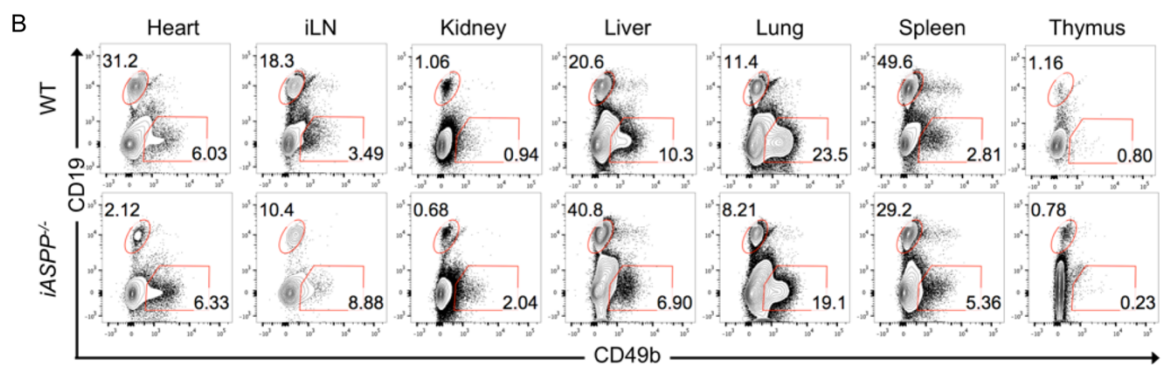
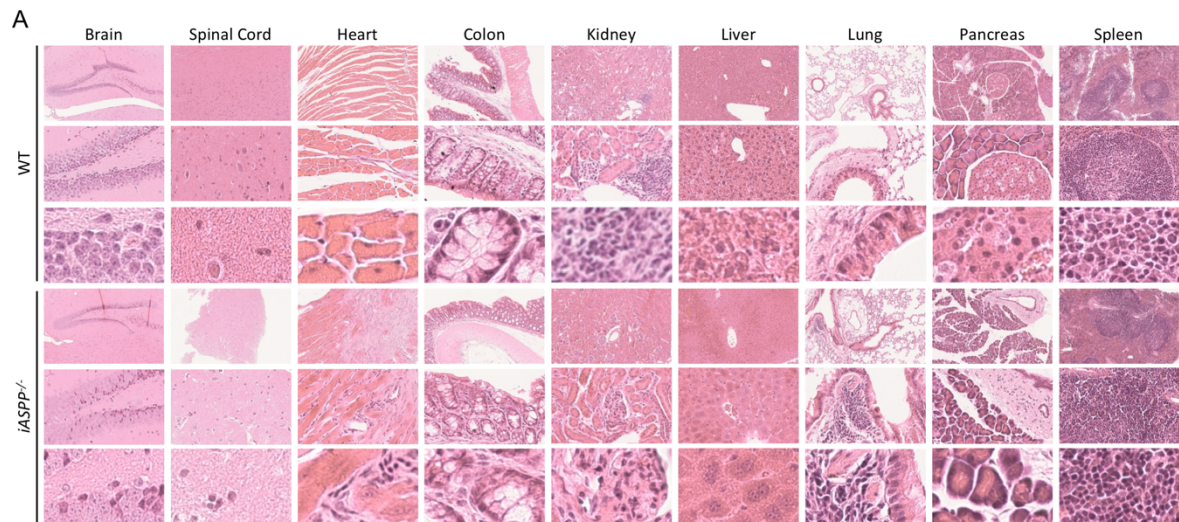
Statistical analysis was performed using unpaired two-tailed Student's *t*-tests where indicated. Where multiple comparisons were performed, statistical significance was determined using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli with a false-discovery rate (Q) = 5% without assuming consistent standard deviations (Benjamini et al., 2006). EAE clinical severity was analyzed using non-compartmental estimation of the area under the curve (AUC) using the log-trapezoidal rule. Median EAE duration was compared using the Mann-Whitney *U* test. Disease-free survival and overall survival Kaplan-Meier estimations were compared using the log-rank Mantel-Cox test with one degree of freedom, and hazard ratios were calculated using the Mantel-Haenszel method (Bernstein et al., 1981). Weight loss was compared using a two-way ANOVA with between-subject factors of genotype and within-subject factors of time point. MOG₃₅₋₅₅ stimulation-induced T cell proliferation was analyzed using two-way ANOVA with between-subject factors of genotype and within-subject factors of peptide concentration. Fisher's exact test was performed on contingency tables to determine statistical significance of differences in tumor grade between different genotypes. P-values less than 0.05 were considered significant, except in cases where adjusted q-values less than 0.05 were used to account for false discovery. For calculations of total cell counts, outliers were removed using robust nonlinear regression (Motulsky and Brown, 2006) with $Q = 1\%$. All analyses were performed in Prism 7 (GraphPad Software). Data are presented as mean $\pm$ standard error of the mean (s.e.m.).

CHAPTER 3: iASPP-deficient mice have a more tolerogenic peripheral immune response and decreased autoimmune disease severity

Aged iASPP-deficient mice have an immunosuppressed peripheral immune system

To investigate the role of iASPP in immune homeostasis, I characterized the peripheral immune response in aged *iASPP*^{-/-} mice. *iASPP*^{-/-} mice had normal litters, but exhibited features of arrhythmogenic right ventricular cardiomyopathy (ARVC) and die of sudden cardiac death after approximately nine months (Notari et al., 2015). Accordingly, to address the role of iASPP in development of autoimmune disease, I compared the immunological phenotypes of 33-week-old *iASPP*^{-/-} mice and age-matched littermate WT controls. Histological examination of brain, spinal cord, heart, colon, kidney, liver, lung, pancreas, and spleen revealed no major phenotypic alterations or inflammation (**Figure 2A**), besides previously described (Notari et al., 2015) cardiac right ventricular dilation and fibrotic deposits.

Flow cytometric analysis of hematopoietic cell subsets revealed that 7-week-old *iASPP*^{-/-} mice have normal numbers of cells in all tissues examined compared to their WT littermates (**Figure 2B-G**), suggesting that iASPP may not affect lymphocyte development. However, after 33 weeks, *iASPP*^{-/-} mice displayed elevated levels of polymorphonuclear neutrophil myeloid-derived suppressor cells (MDSC-P) and regulatory T cells (T_{reg}) in the liver (**Figure 3A**), while numbers of MDSC-P and T_{reg} cells remained similar in the spleen (**Figure 3C**). Additionally, CD4⁺ T cells in the inguinal lymph node (iLN), lamina propria of the colon (ColonLP),



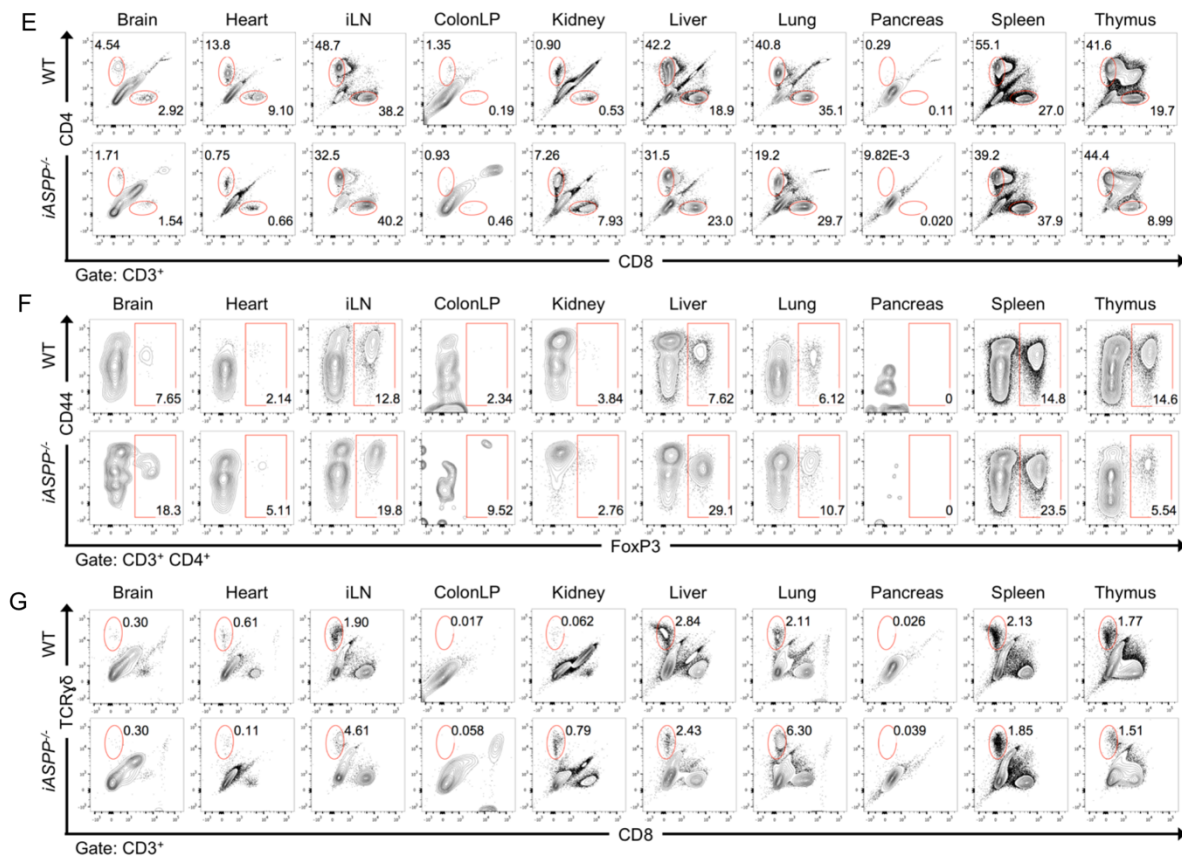
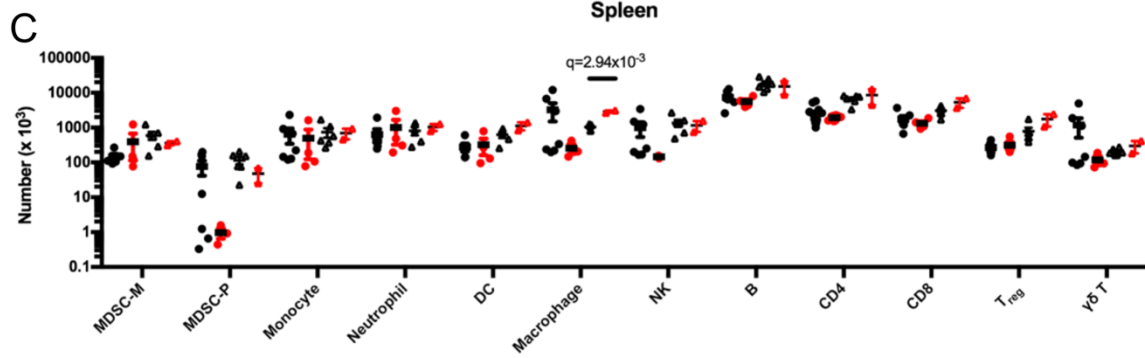
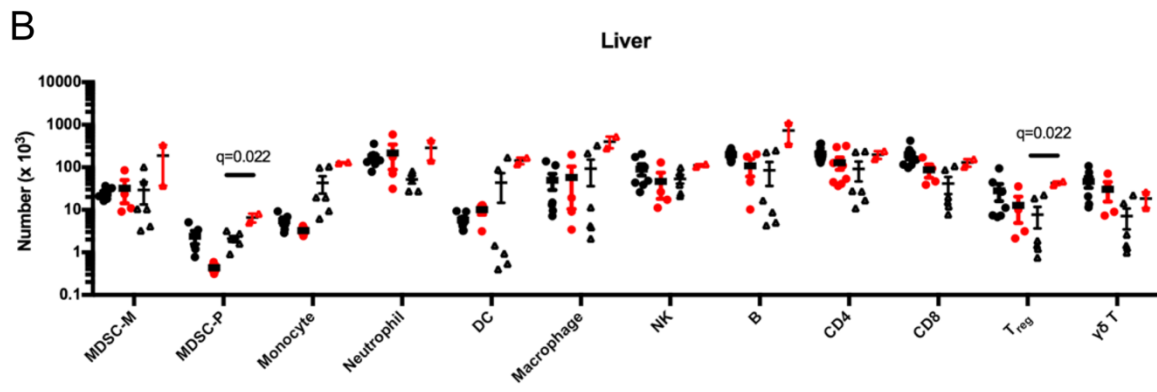
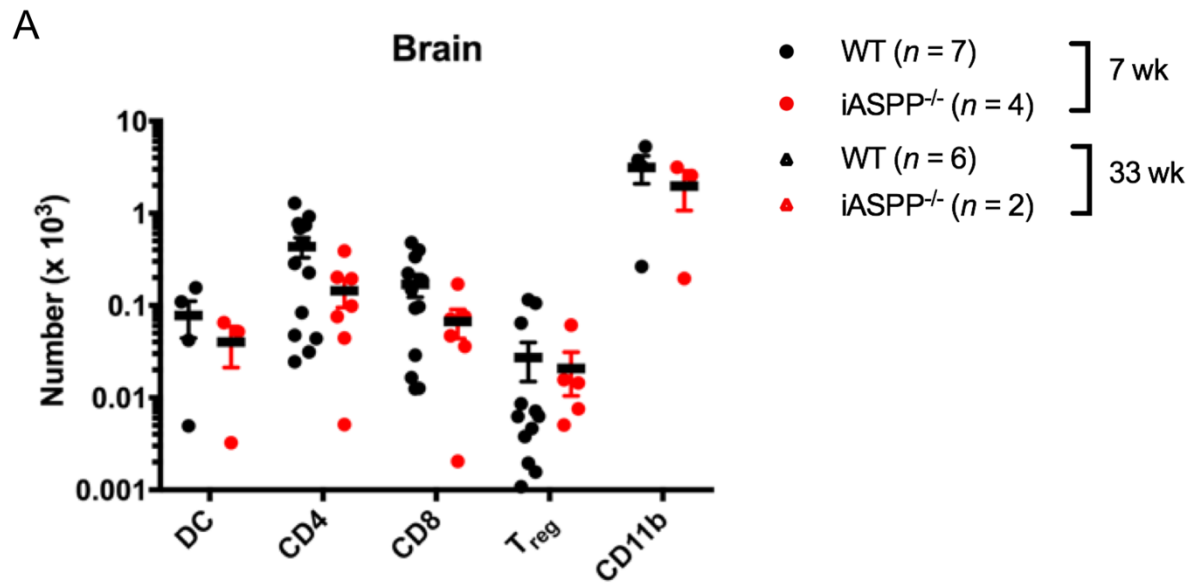


Figure 2: *iASPP*-deficient mice have unaltered total numbers of peripheral and central lymphocytes.

(A) Haematoxylin and eosin (HE) stains of brains, spinal cords, hearts, colons, kidneys, livers, lungs, pancreas, or spleens from 33-week-old WT ($n = 6$) mice or *iASPP*^{-/-} ($n = 2$) littermates. Higher magnification images are shown in lower rows.

(B-G) Flow cytometry contour plots of lymphocytes isolated from brains, hearts, inguinal lymph nodes (iLN), colon lamina propria (ColonLP), kidneys, livers, lungs, pancreas, spleens, or thymuses of 33-week-old WT or *iASPP*^{-/-} mice. Gating strategies to identify CD19⁺ B and CD49b⁺ NK cells (B), CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells (C), CD68⁺F4/80⁺ macrophages (D), CD3⁺CD4⁺FoxP3⁺ T_{reg} cells (E), CD11c⁺MHCII⁺ dendritic cells (DC) (F), and CD3⁺TCRγδ⁺ T cells (G) are indicated.



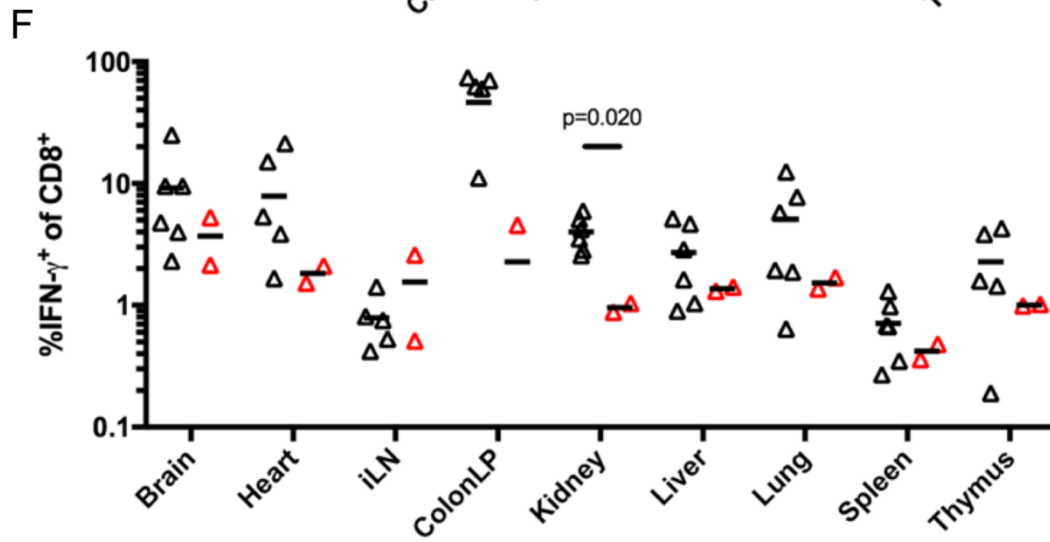
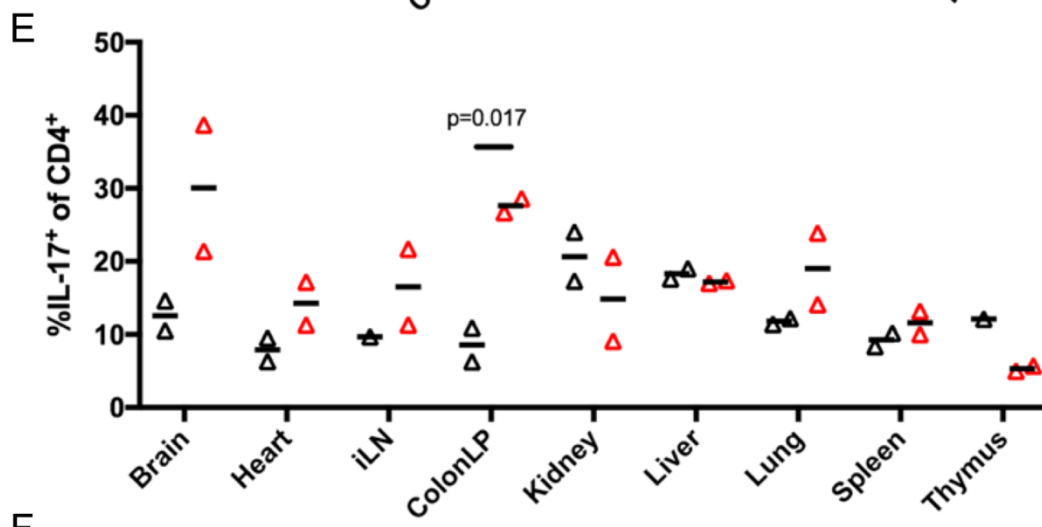
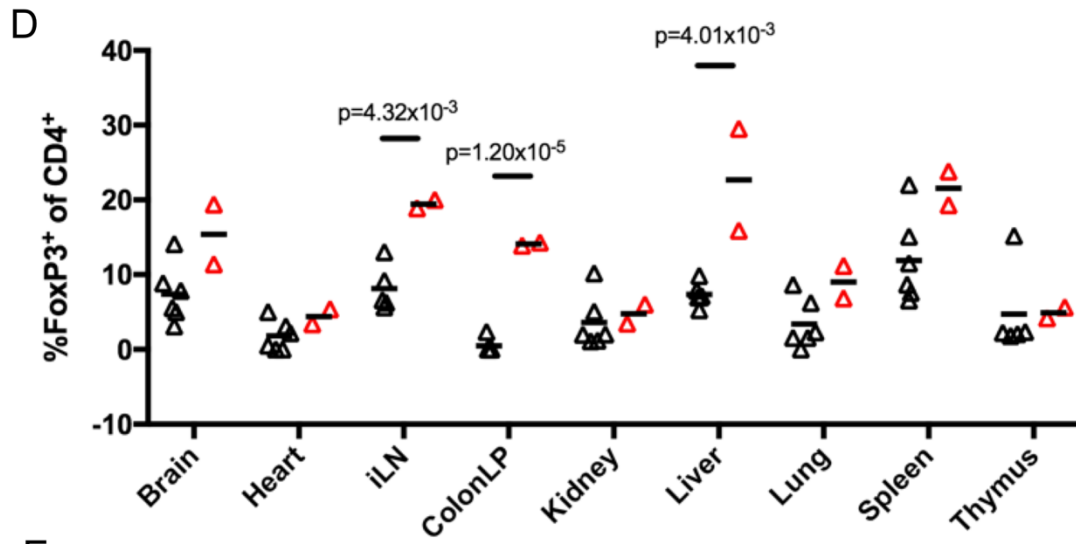
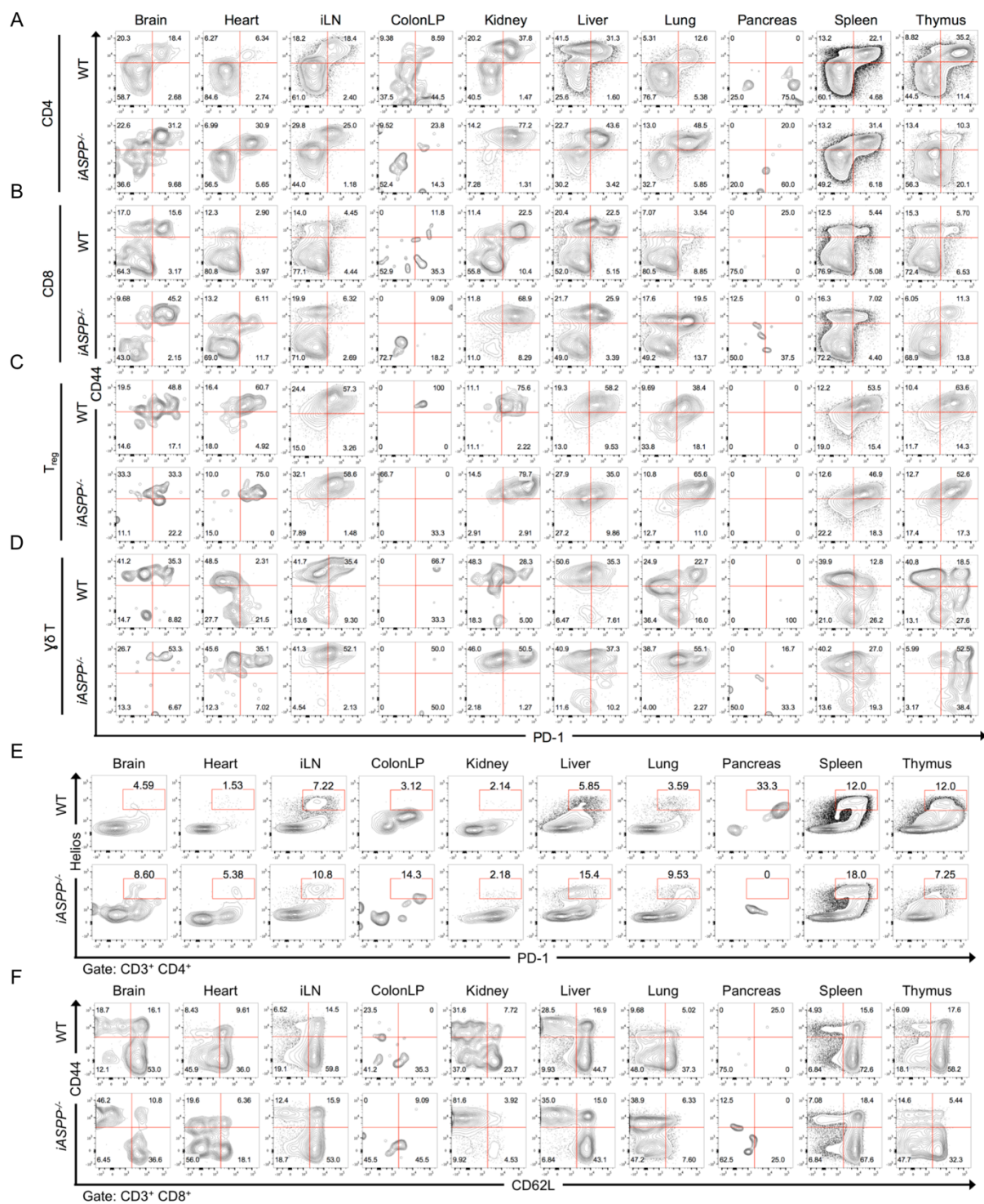


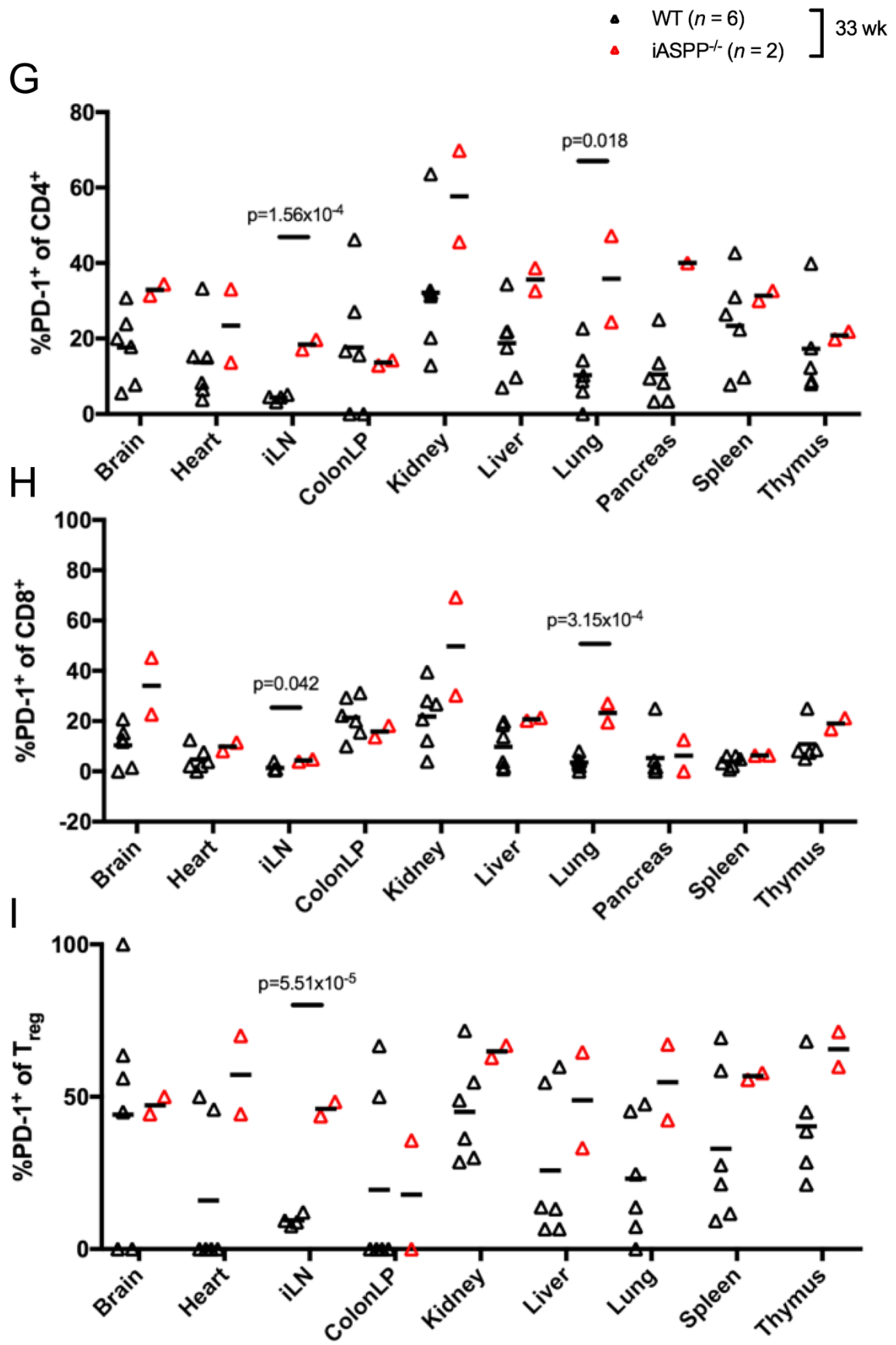
Figure 3: Peripheral lymphocytes in aged iASPP-deficient mice exhibit signs of immunosuppression

(A-C) Cell numbers in livers (A), brains (B), or spleens (C) of 7-week-old old WT ($n = 7$, black circles) mice or *iASPP*^{-/-} ($n = 4$, red circles) littermates, or 33-week-old WT ($n = 6$, black triangles) or *iASPP*^{-/-} ($n = 2$, red triangles) mice. Absolute numbers of lymphocytes were calculated by multiplying total cell count by percentage of cells determined by flow cytometry. Data are mean $\pm$ s.e.m.; significance determined using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli with $Q = 5\%$. (D-F) Quantification of T cell subpopulations from indicated organs of 33-week-old WT or *iASPP*^{-/-} mice as determined by flow cytometry. FoxP3⁺ T_{reg} cells (D) were identified as in **(Figure 2F)**, and IL-17 secreting CD4⁺ T cells (E) or IFN- γ secreting CD8⁺ cells (F) were identified from populations defined in **(Figure 2E)**. All data are representative of two independent experiments. Sample means indicated by horizontal bars; Student's *t*-test, $p < 0.05$ indicated on graph.

and liver had increased proportions of FoxP3⁺ cells (**Figure 3D**), while kidney CD8⁺ T cells had decreased IFN- γ secretion (**Figure 3F**) in iASPP-deficient mice. These results suggest that iASPP promotes age-related loss of peripheral immunosuppression by T_{reg} cells.

To specifically examine the activation status of peripheral T cells, I measured the levels of co-inhibitory checkpoint molecules on T cell subpopulations in 33-week-old *iASPP*^{-/-} mice. CD4⁺ and CD8⁺ T cells in the iLN and lung of *iASPP*^{-/-} mice expressed elevated levels of PD-1 (**Figure 4A-B**), suggesting that iASPP can prevent peripheral T cell exhaustion. Concertedly, iLN T_{reg} cells and $\gamma\delta$ T cells in *iASPP*^{-/-} iLN, kidney, and lung also increased PD-1 expression (**Figure 4C-D**), indicating that iASPP mediates inhibition of immunosuppressive T cell subpopulations. The proportion of CD4⁺ T cells co-expressing PD-1 and Helios, thought to represent a tolerized self-reactive population of T cells (Daley et al., 2013; Kim et al., 2015a), was increased in *iASPP*^{-/-} heart, ColonLP, liver, and lung (**Figure 4E**). CD8⁺ T cells in the brain, heart, iLN, liver, and thymus had increased levels of CD44⁺CD62L⁻ effector memory phenotype and decreased levels of CD44⁺CD62L⁺ central memory phenotype T cells in aged *iASPP*^{-/-} mice (**Figure 4F**). CD44 expression measured by mean-fluorescence intensity was increased in CD4⁺, CD8⁺, T_{reg}, and $\gamma\delta$ T cells in the periphery of aged *iASPP*^{-/-} mice (**Figure 5A-D**), suggesting that iASPP attenuates general activation of peripheral T cell populations. Interestingly, CD44 is transcriptionally repressed by p53 but transcriptionally activated by p63 (Boldrup et al., 2007; Carroll et al., 2006; Godar et al., 2008), and therefore the observed increase in CD44 expression in iASPP-deficient mice might be mediated by p63 rather than p53. This unique immune profile suggests that





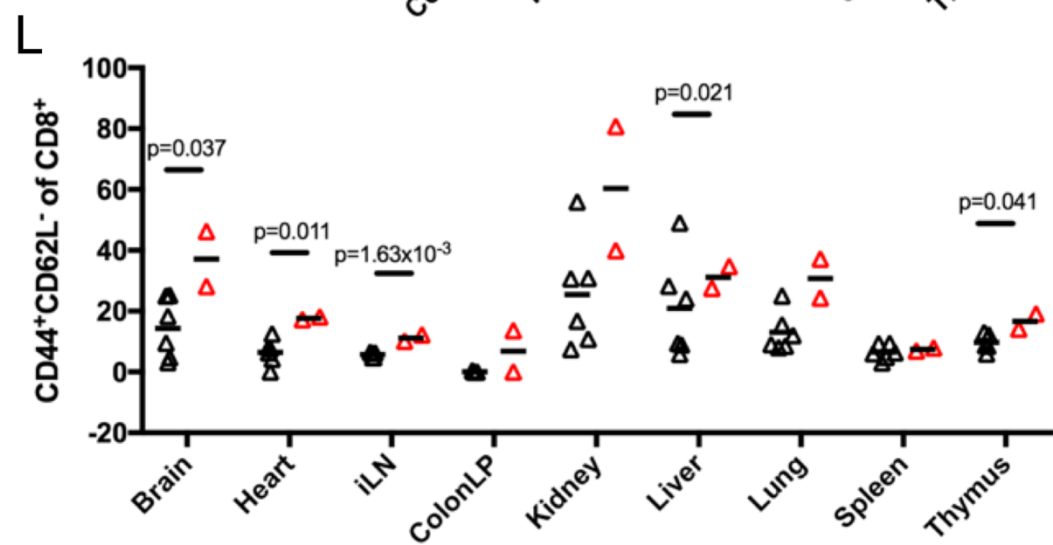
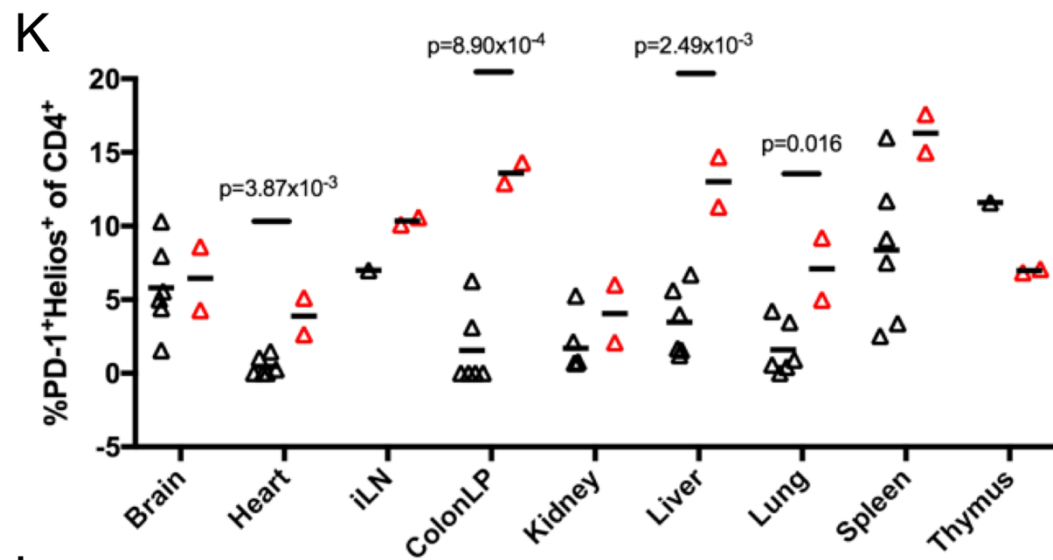
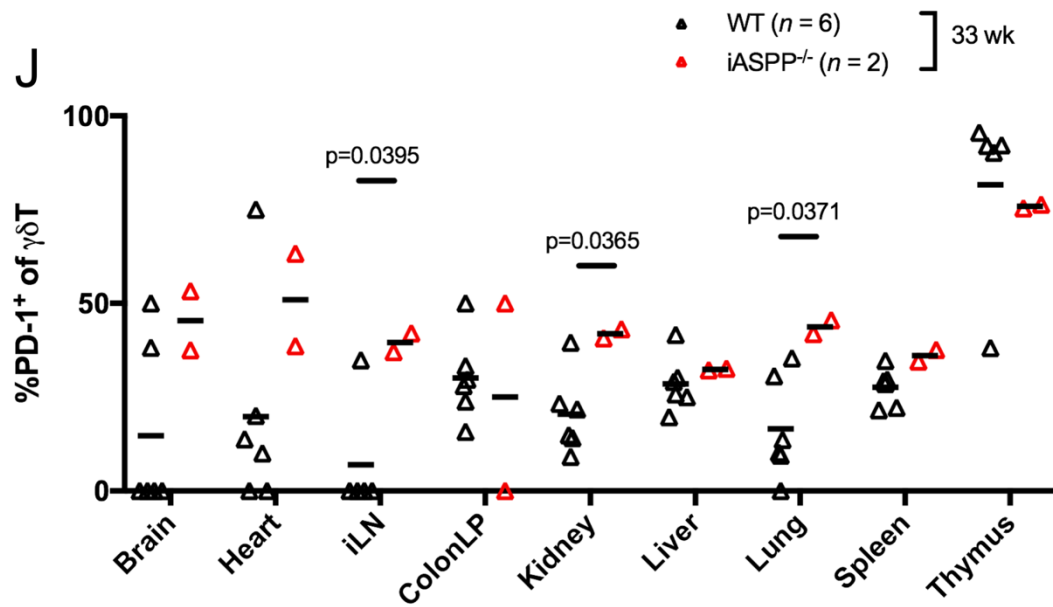


Figure 4: Peripheral CD4⁺ and CD8⁺ T cells in iASPP-deficient mice have an exhausted phenotype.

(A-D) Flow cytometry contour plots of CD44 and PD-1 expression by CD4⁺ (A), CD8⁺ (B), T_{reg} (C), or $\gamma\delta$ T (D) cells from indicated organs of 33-week-old WT ($n = 6$, black triangles) or *iASPP*^{-/-} ($n = 2$, red triangles) mice as gated in Figure 2B-G. (E-F) Flow cytometry contour plots of Helios and PD-1 expression by CD4⁺ T cells (E) or CD8⁺CD44⁺CD62L⁻ effector memory T cell (T_{em}) populations (F) from indicated organs of 33-week-old WT or *iASPP*^{-/-} mice. (G-L) Quantification of flow cytometry described above. All data are representative of two independent experiments. Data are mean $\pm$ s.e.m.; Student's *t*-test, $p < 0.05$ indicated on graph.

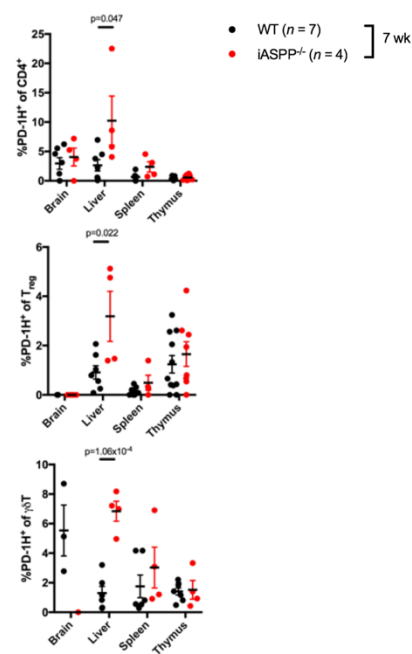
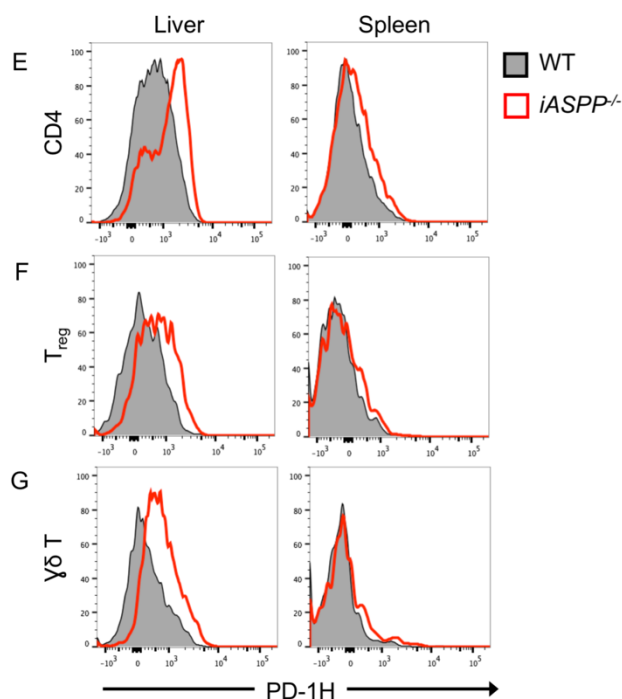
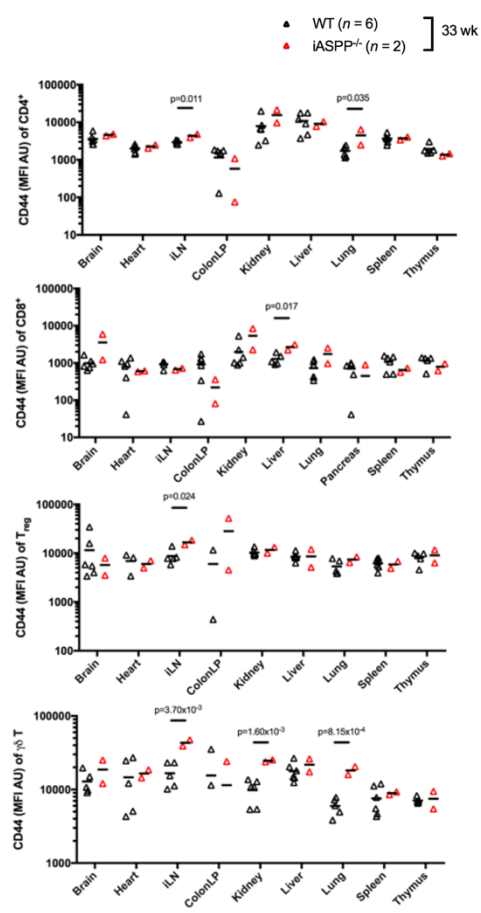
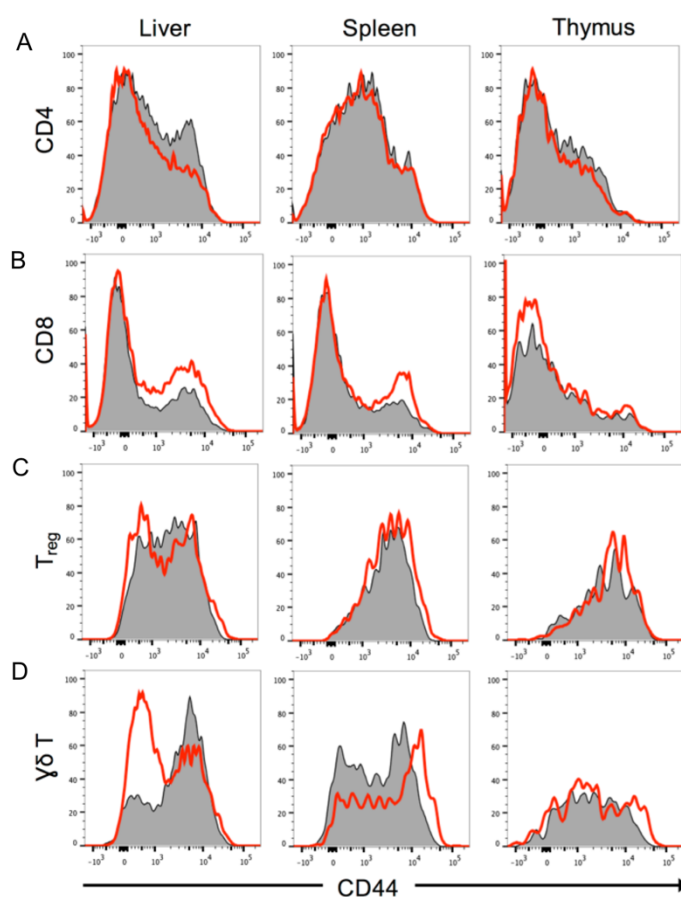


Figure 5: Peripheral CD4⁺, T_{reg}, and $\gamma\delta$ T cells in iASPP-deficient mice express elevated CD44 and PD-1H

(A-D) Flow cytometry histograms (left) and quantification (right) of CD44 expression by CD4⁺ (A), CD8⁺ (B), T_{reg} (C), or $\gamma\delta$ T (D) cells from livers, spleens, or thymuses of 33-week-old WT ($n = 6$; filled gray line, black triangles) or *iASPP*^{-/-} ($n = 2$; red line, red triangles) mice. (E-G) Flow cytometry histograms (left) and quantification (right) of PD-1H expression by CD4⁺ (E), T_{reg} (F), or $\gamma\delta$ T (G) cells from livers or spleens of 7-week-old WT ($n = 7$; filled gray line, black circles) or *iASPP*^{-/-} ($n = 4$; red line, red circles) mice. MFI AU, arbitrary units of median fluorescence intensity. All data are representative of two independent experiments. Data are mean $\pm$ s.e.m.; Student's *t*-test, $p < 0.05$ indicated on graph.

iASPP^{-/-} mice have a more immunosuppressive peripheral immune response to aging.

Interestingly, *p53*^{-/-} mice are more prone to autoimmune disease and have decreased numbers of peripheral T_{reg} cells (Kawashima et al., 2013). To explain the reciprocal relationship observed in the immune phenotypes of *p53*^{-/-} and *iASPP*^{-/-} mice, I hypothesized that iASPP inhibits expression of the p53 target gene *PD-1H* (Yoon et al., 2015). CD4⁺, T_{reg}, and γδ T cells in the livers but not spleens nor thymuses of aged *iASPP*^{-/-} mice expressed elevated levels of PD-1H (**Figure 5E-G**), suggesting that iASPP maintains immune homeostasis by inhibiting peripheral but not central expression of the immunosuppressive molecule PD-1H. Loss of iASPP leads to supraphysiological levels of PD-1H, likely through inhibition of p53 transcriptional activity. These results suggest that iASPP expression is necessary for peripheral immunity by attenuating the expression of PD-1H by T cells.

ConA-induced hepatitis is less severe in iASPP-deficient mice

Given the ability of iASPP to promote loss of immune tolerance in aged mice, I hypothesized that *iASPP*^{-/-} mice would develop less severe autoimmune disease. To test this possibility, I used a concanavalin A (ConA)-induced model of acute autoimmune hepatitis. ConA induces CD4⁺ and NKT cell-dependent acute inflammation of the liver (Tiegs et al., 1992), leading to hepatitis-related pathologies when used at sublethal doses (15 mg/kg). *iASPP*^{-/-} mice had markedly reduced liver fibrosis 24 hr following ConA administration (**Figure 6A**), supporting the hypothesis that iASPP is necessary for the hepatitis-associated autoimmune response. Interestingly, absolute numbers of immune cells were similar amongst WT and

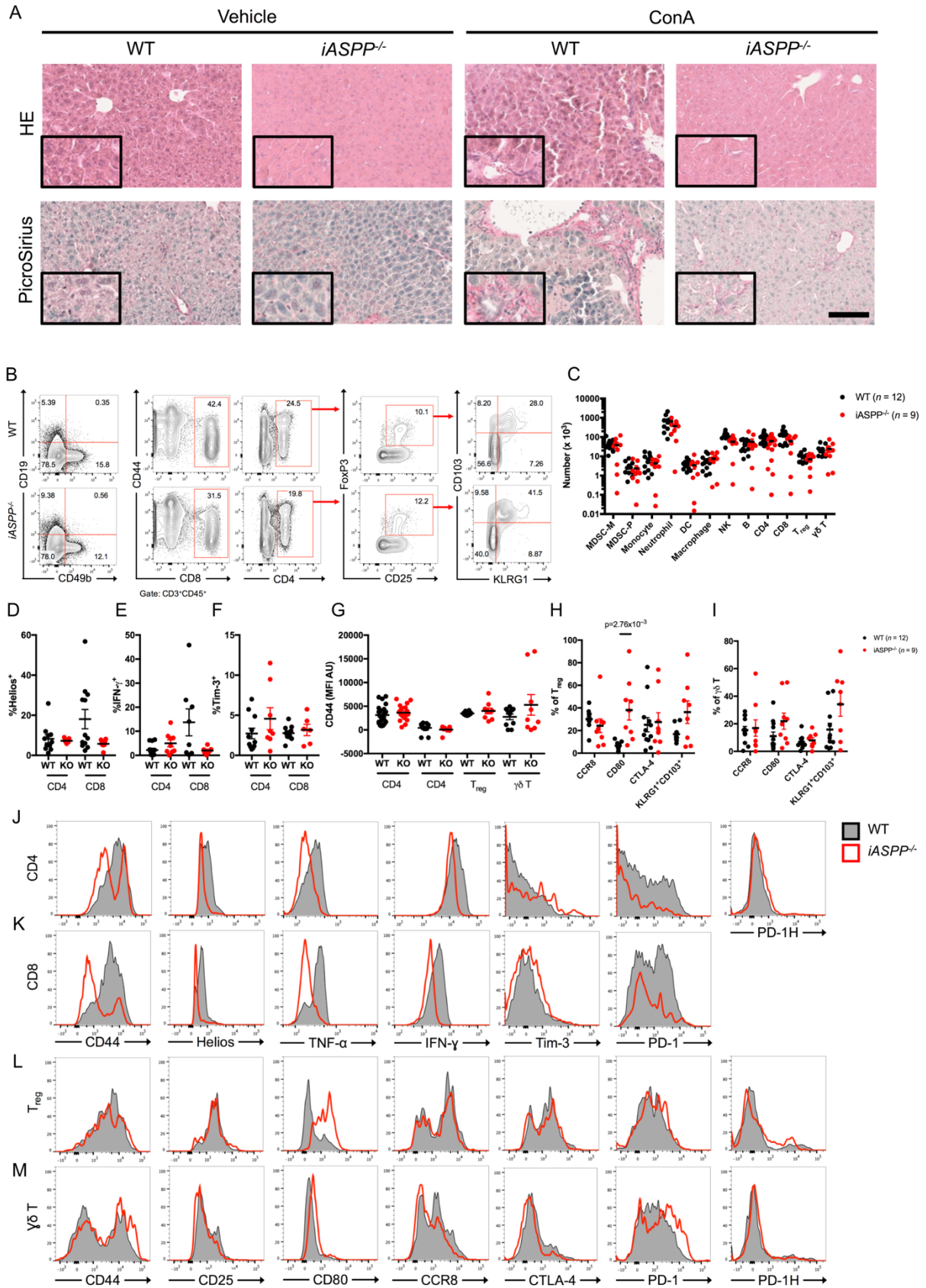


Figure 6: iASPP deficiency facilitates T_{reg} and $\gamma\delta$ T cell activation in autoimmune hepatitis.

(A) Representative HE (top) or PicroSirius (bottom) stains of livers isolated from WT or *iASPP*^{-/-} mice 24 hr after treatment with vehicle ($n = 7$ for WT, $n = 4$ for *iASPP*^{-/-}) or 15 mg/kg concanavalin A (ConA; $n = 12$ for WT, $n = 9$ for *iASPP*^{-/-}). Scale, 100 μ m. (B) Flow cytometry contour plots showing gating strategy used to identify CD19⁺ B cells, CD49b⁺ NK cells, CD3⁺CD8⁺ T cells, CD3⁺CD4⁺ T cells, CD3⁺CD4⁺CD25⁺FoxP3⁺ T_{reg} cells, and CD103⁺KLRG1⁺ T_{reg} cells from livers of WT or *iASPP*^{-/-} mice with acute autoimmune hepatitis. (C) Cell numbers in livers of WT (black circles) or *iASPP*^{-/-} (red circles) mice with acute autoimmune hepatitis determined by flow cytometry. (D-G) Quantification of Helios (D), IFN- γ (E), Tim-3 (F), and CD44 (G) expression in CD4⁺, CD8⁺, T_{reg}, and $\gamma\delta$ T cells from livers of WT or *iASPP*^{-/-} mice with acute autoimmune hepatitis measured by flow cytometry of gated population defined in (B). MFI AU, arbitrary units of median fluorescence intensity. (H-I) Quantification of CCR8, CD80, CTLA-4, and CD103⁺KLRG1⁺ double positivity in T_{reg}, (H) and $\gamma\delta$ T (I) cells from livers of WT or *iASPP*^{-/-} mice with acute autoimmune hepatitis measured by flow cytometry. (J-M) Flow cytometry histograms of CD44, Helios, TNF- α , IFN- γ , Tim-3, PD-1, PD-1H, CD25, CD80, CCR8, and CTLA-4 expression by CD4⁺ (J), CD8⁺ (K), T_{reg} (L), or $\gamma\delta$ T (M) cells from livers of WT (filled gray line) or *iASPP*^{-/-} (red line) mice with acute autoimmune hepatitis. All data are representative of five independent experiments. Data are mean $\pm$ s.e.m.; Student's *t*-test, $p < 0.05$ indicated on graph.

iASPP^{-/-} mice (**Figure 6B-C**), suggesting that the differences in liver pathology in *iASPP*^{-/-} mice are due to the activation status of resident lymphocytes rather than alteration of the cellular composition of liver infiltrates.

Immunophenotyping revealed that liver CD4⁺ and CD8⁺ T cells secreted less TNF- α and IFN- γ (**Figure 6E**) in *iASPP*^{-/-} mice with acute hepatitis, while liver T_{reg} and $\gamma\delta$ T cells had an activated phenotype, including upregulated expression of CD44, CD80, and PD-1 (**Figure 6H-M**). Therefore, iASPP likely contributes to autoimmune hepatitis by facilitating functional cytotoxic T cell responses and limiting activation of immunosuppressive T cell populations. A notable subpopulation of liver-infiltrating T_{reg} cells co-expressing CD103 and killer cell lectin-like receptor G1 (KLRG1) was increased in *iASPP*^{-/-} mice following acute hepatitis (**Figure 6B,H**). CD103 is a marker of activated tissue-resident T_{reg} cells (Sather et al., 2007; Suffia et al., 2005), KLRG1 is a marker of terminal differentiation (Beyersdorf et al., 2007; Cheng et al., 2012; Joshi et al., 2007), and double-positive CD103⁺KLRG1⁺ T_{reg} cells are thought to represent a homeostatically converted activated T_{reg} compartment (Feuerer et al., 2010). Together these data suggest that iASPP promotes CD4⁺ T cell-mediated autoimmune hepatitis severity by limiting T_{reg} activation following ConA insult.

Experimental autoimmune encephalomyelitis is less severe in iASPP-deficient mice

To examine the potential role of iASPP in more chronic autoimmune disease, I utilized the experimental autoimmune encephalomyelitis (EAE) model of T cell-mediated autoimmune demyelinating neuroinflammation (**Figure 7A**). Both WT and *iASPP*^{-/-} mice developed characteristic clinical features of EAE such as limp tail

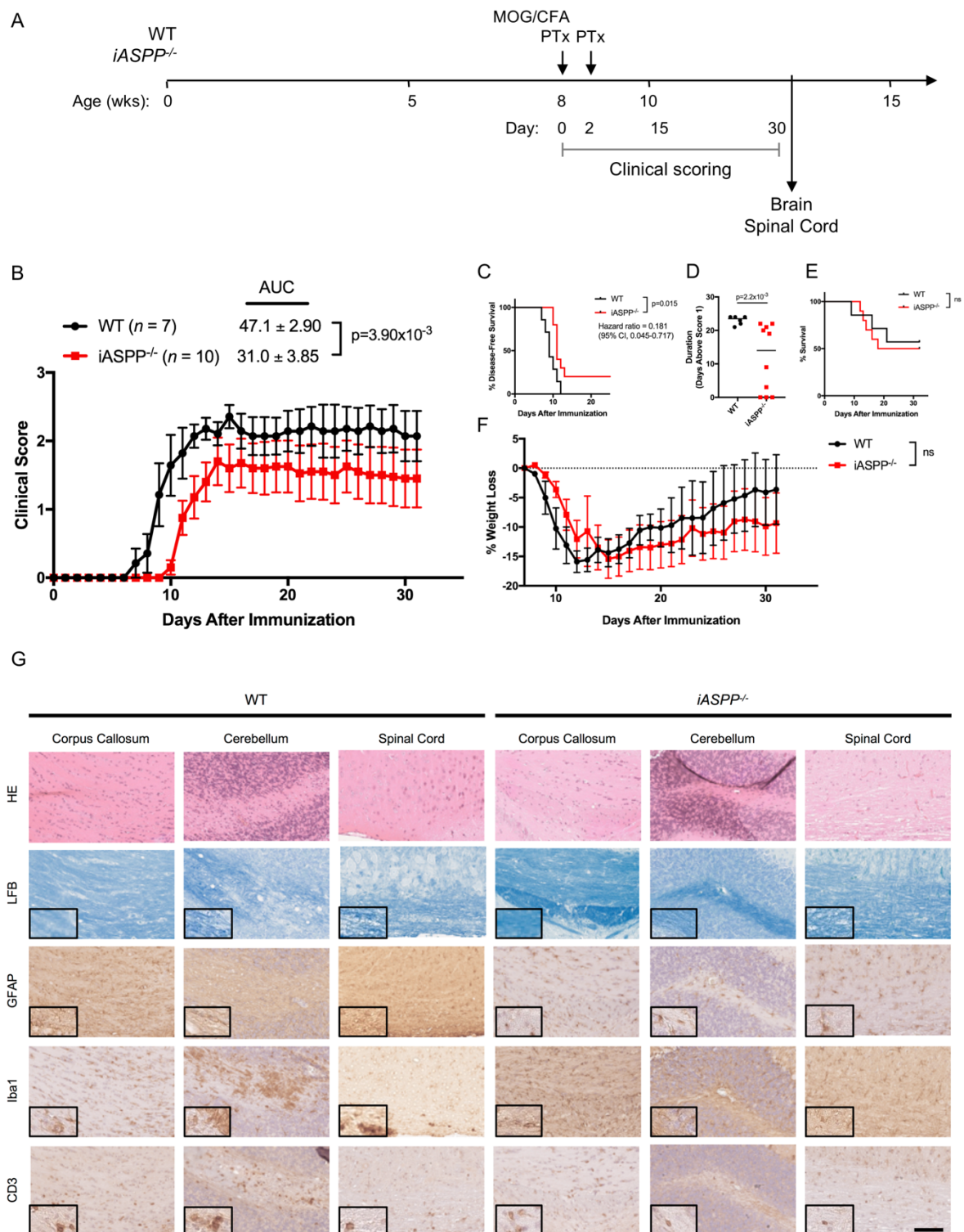


Figure 7: Experimental autoimmune encephalomyelitis severity is decreased in *iASPP*-deficient mice.

(A) Schematic of experimental autoimmune encephalomyelitis (EAE) model and experimental design. Mice were monitored until 30 days after immunization with MOG/CFA or until reaching primary endpoint of bilateral hind limb paralysis (clinical severity score = 3). MOG, 200 μ g MOG₃₅₋₅₅ peptide (MEVGWYRSPFSRVVHLYRNGK); CFA, complete freund's adjuvant supplemented with 400 μ g H37Ra mycobacterium tuberculosis; PTx, 300 ng pertussis toxin. (B) Clinical course of MOG-induced EAE in WT ($n = 7$, black line) or *iASPP*^{-/-} ($n = 10$, red line) mice measured by daily assessment of clinical severity scores on a 1-5 scale. Data are mean $\pm$ s.e.m.; area under the curve (AUC) estimated using log-trapezoidal rule and compared using Student's *t*-test, $p < 0.05$ indicated on graph. (C) Kaplan-Meier estimate of disease-free survival of WT or *iASPP*^{-/-} mice following immunization with MOG/CFA. Median disease-free survival was 9 days for WT mice and 11 days for *iASPP*^{-/-} mice; hazard ratio calculated using Mantel-Haenszel method. Data compared using log-rank Mantel-Cox test; ns, $p > 0.05$; $p < 0.05$ indicated on graph. (D) Column scatter plot showing total number of days WT or *iASPP*^{-/-} mice with EAE spent above a clinical severity score of 1. Sample medians indicated by horizontal bars; Mann-Whitney *U* test, $p < 0.05$ indicated on graph. (E) Kaplan-Meier estimates as in (C) of overall survival. (F) Daily weights of WT or *iASPP*^{-/-} mice with EAE were compared to weight 7 days after immunization. Dashed line indicates no change from baseline weight. Data are mean $\pm$ s.e.m.; two-way ANOVA, ns, $p > 0.05$. (G) Representative HE, luxol fast blue (LFB), GFAP, Iba1, and CD3 immunohistochemistry stains of corpus callosum, cerebellum, and spinal cord isolated from WT ($n = 4$) or *iASPP*^{-/-} ($n = 6$) mice 30 days after immunization with MOG/CFA. Scale, 100 μ m. Inset shows higher magnification. All data are representative of two independent experiments.

followed by limb paralysis, and exhibited the expected relapsing-remitting disease course including initial attack and partial recovery after the peak phase of disease severity. However, *iASPP*^{-/-} mice had delayed disease onset and decreased duration of disease (**Figure 7B-D**). Although no significant difference was observed in overall survival (**Figure 7E**), weight loss (**Figure 7F**), or peak disease severity, the increased disease severity during the acute phase of EAE and earlier disease onset contributed to significantly decreased overall severity of EAE measured by area under the curve (**Figure 7B**) in *iASPP*^{-/-} mice. Histological examination of central nervous system (CNS) pathology 30 days after immunization revealed that *iASPP*^{-/-} mice had less extensive demyelination, particularly in the cerebellum (**Figure 7G**). *iASPP*^{-/-} mice also exhibited a consistent decrease in neuroinflammation, characterized by reduced astrocytosis, gliosis, and frequency of infiltrating T cells in the corpus callosum, cerebellum, and spinal cord (**Figure 7G**). Therefore, *iASPP* deficiency appears to decrease CNS autoimmune disease severity by attenuating neuroinflammation.

Given the decrease in autoimmune neuroinflammation observed in *iASPP*^{-/-} mice, I analyzed potential sources of immunosuppression by performing flow cytometry on brains isolated from EAE mice 30 days after immunization. *iASPP*^{-/-} mice had markedly decreased numbers of CNS-infiltrating CD4⁺ T cells, despite similar numbers of CD8⁺ and T_{reg} cells (**Figure 8A-B**). Tissue-infiltrating CD4⁺ and CD8⁺ T cells exhibited hallmark characteristics of exhaustion, including expression of checkpoint molecules PD-1 and Tim-3, and decreased secretion of pro-inflammatory cytokines TNF- α and IFN- γ (**Figure 8C-D**). These data suggest that *iASPP* deficiency ameliorates EAE severity by limiting CD4⁺ T cell activation. Notably,

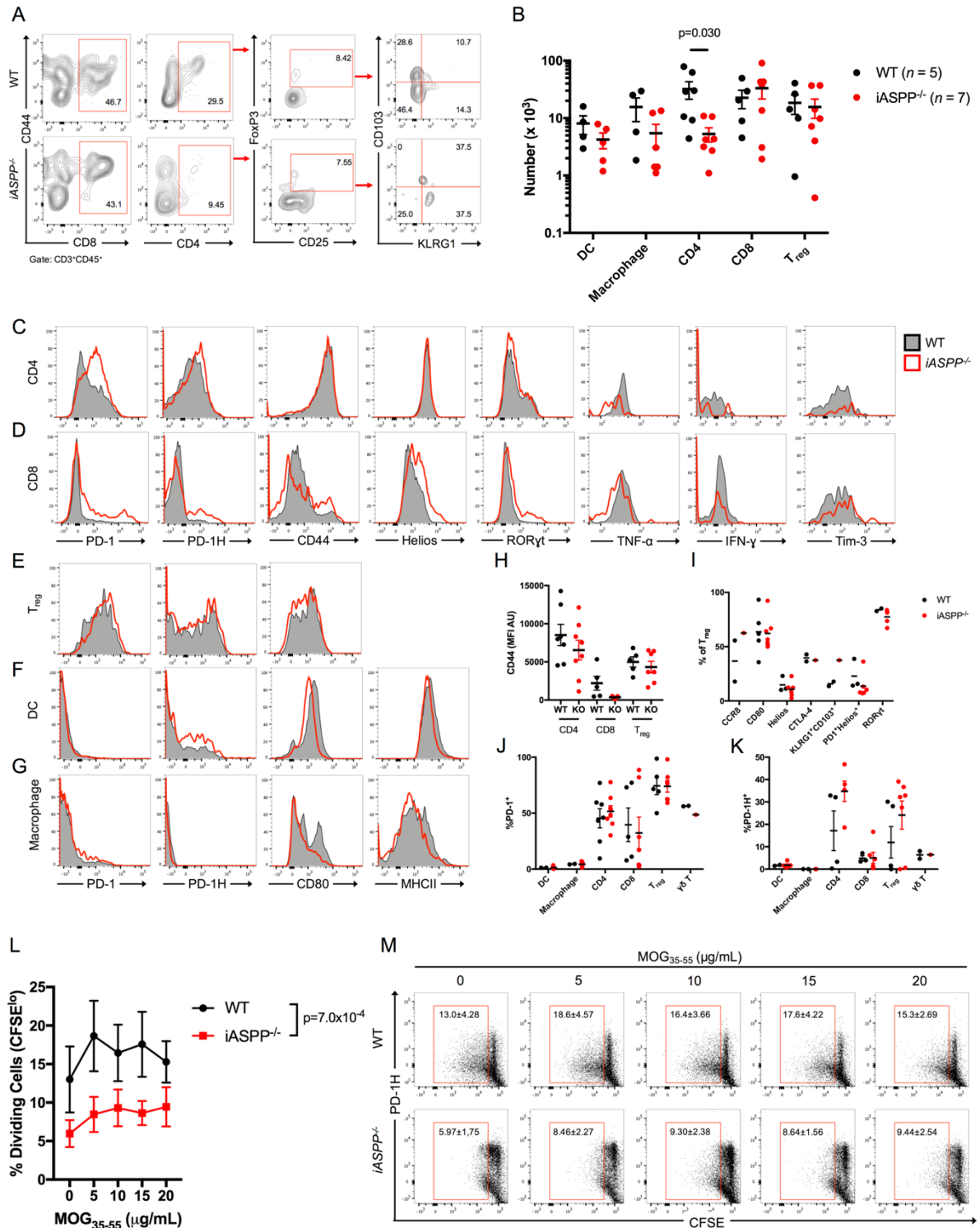


Figure 8: iASPP deficiency attenuates CD4⁺ T cell-mediated autoimmunity in experimental autoimmune encephalomyelitis.

(A) Flow cytometry contour plots showing gating strategy used to identify CD3⁺CD8⁺ T cells, CD3⁺CD4⁺ T cells, CD3⁺CD4⁺CD25⁺FoxP3⁺ T_{reg} cells, and CD103⁺KLRG1⁺

T_{reg} cells from brains of WT ($n = 5$) or *iASPP*^{-/-} ($n = 7$) mice with EAE. (B) Cell numbers in brains of WT (black circles) or *iASPP*^{-/-} (red circles) mice with EAE determined by flow cytometry. Data are mean $\pm$ s.e.m.; Student's *t*-test, $p < 0.05$ indicated on graph. (C-D) Flow cytometry histograms of PD-1, PD1-H, CD44, Helios, ROR γ t, TNF- α , IFN- γ , and Tim-3 expression by CD4⁺ (C) and CD8⁺ (D) T cells from brains of WT (filled gray line) or *iASPP*^{-/-} (red line) mice with EAE as gated in (A). (E-G) Flow cytometry histograms of PD-1, PD1-H, CD80, and MHCII expression by T_{reg} cells, DCs, and macrophages from brains of WT (filled gray line) or *iASPP*^{-/-} (red line) mice with EAE. (H) Quantification of CD44 expression in CD4⁺, CD8⁺, and T_{reg} cells from brains of WT or *iASPP*^{-/-} mice with EAE measured by flow cytometry of gated population defined in (A). MFI AU, arbitrary units of median fluorescence intensity. (I) Quantification of CCR8, CD80, Helios, CTLA-4, CD103⁺KLRG1⁺ double positivity, PD-1⁺Helios⁺ double positivity, and ROR γ t in T_{reg} cells from brains of WT or *iASPP*^{-/-} mice with EAE measured by flow cytometry. (J-K) Quantification of PD-1 (J) and PD-1H (K) expression in DCs, macrophages, CD4⁺, CD8⁺, T_{reg}, and $\gamma\delta$ T cells from brains of WT or *iASPP*^{-/-} mice with EAE measured by flow cytometry. (L) Splenocytes isolated from wild type (WT, $n = 4$) or *iASPP*^{-/-} ($n = 5$) mice were stained with CFSE and stimulated with indicated concentrations of MOG₃₅₋₅₅ and IL-2 for 72 hr. Cell division was analyzed using CFSE dilution by flow cytometry. Data are mean $\pm$ s.e.m.; two-way ANOVA, $p < 0.05$ indicated on graph. (M) Flow cytometry dot plots illustrating gating strategy used in (L). All data are representative of at least two independent experiments.

tissue-infiltrating T_{reg} cells expressed increased amounts of PD-1 (**Figure 8E**) and tissue-infiltrating dendritic cells (DCs) and macrophages decreased expression of CD80 and MHCII (**Figure 8F-G**) in *iASPP*^{-/-} mice, indicative of a more immunosuppressive tissue-resident lymphocyte population and attenuated response to EAE. CD4⁺, CD8⁺, T_{reg} cells, and DCs all expressed increased amounts of PD-1H in *iASPP*^{-/-} mice with EAE (**Figure 8C-G**), suggesting that iASPP deficiency ameliorates EAE pathology and autoimmunity by promoting expression of PD-1H on T cells and DCs.

Functionality of the endogenous autoimmune T cell response in EAE was examined by measuring splenocyte proliferation in response to challenge with immunogen. Splenocytes isolated from EAE mice at peak disease severity (clinical score = 3) were stimulated with increasing concentrations of MOG₃₅₋₅₅ peptide and extent of proliferation was measured using CFSE dilution. In concordance with the observed clinical disease course and flow cytometry immunophenotyping of CNS-infiltrating lymphocytes, splenocytes from *iASPP*^{-/-} mice with EAE were less responsive to *in vitro* immunogen challenge (**Figure 8L**) and expressed increased amounts of PD-1H (**Figure 8M**). Therefore, it appears that *iASPP*^{-/-} mice with EAE have a less potent autoimmune antigen-specific T cell response, suggesting that iASPP fosters T cell autoimmunity.

Together these data suggest that iASPP deficiency can decrease autoimmune disease by antagonizing loss of T_{reg}-mediated immune tolerance. Given the consequences of iASPP deletion on immune homeostasis observed *in vivo*, I next sought to understand the underlying cellular mechanisms by which iASPP deficiency

promotes tolerance. To this end, I performed various *in vitro* functional analyses of cell types implicated in these models of autoimmune disease.

CHAPTER 4: iASPP deficiency attenuates CD4⁺ T cell stimulation and potentiates T_{reg}-mediated suppression

Given the critical role of iASPP in maintaining immune homeostasis, I next asked whether the contribution of iASPP to autoimmune predisposition is CD4⁺ T cell intrinsic or a more generalizable immune phenotype implicating other cell populations. CD4⁺ T cells displayed the most marked phenotypes in aged and autoimmune-induced *iASPP*^{-/-} mice, and are also implicated in p53 (Watanabe et al., 2014) and PD-1H (Flies et al., 2014) regulated immunomodulation. To characterize the role of iASPP in T cell receptor (TCR)-driven T cell responses, CD4⁺ T cells were purified from *iASPP*^{-/-} mice and subject to polyclonal TCR stimulation using plate-bound anti-CD3 mAb. *iASPP*^{-/-} CD4⁺ T cells had a marked decrease in proliferation in response to anti-CD3, and the proliferation of both WT and *iASPP*^{-/-} CD4⁺ T cells was dependent on anti-CD3 dose (**Figure 9A**). Decreased *iASPP*^{-/-} CD4⁺ T cells proliferation was not due to increased apoptosis of iASPP-deficient cells, as *iASPP*^{-/-} CD4⁺ T cells surprisingly exhibited greater survival *in vitro* (**Figure 9B**). Therefore, iASPP expression in CD4⁺ T cells facilitates APC-independent polyclonal stimulation.

Effective immune responses and T cell proliferation is driven by the integration of signaling through the TCR and antigen-nonspecific inputs, such as cytokines and co-stimulatory molecules. Notably, p53 inhibits IL-2 dependent CD4⁺ T cell proliferation in the absence of antigenic stimuli (Watanabe et al., 2014). Surprisingly, iASPP deficiency did not affect CD4⁺ T cell proliferation in the presence of IL-2 alone (**Figure 9C**). This apparent inconsistency suggests that iASPP promotes CD4⁺ T

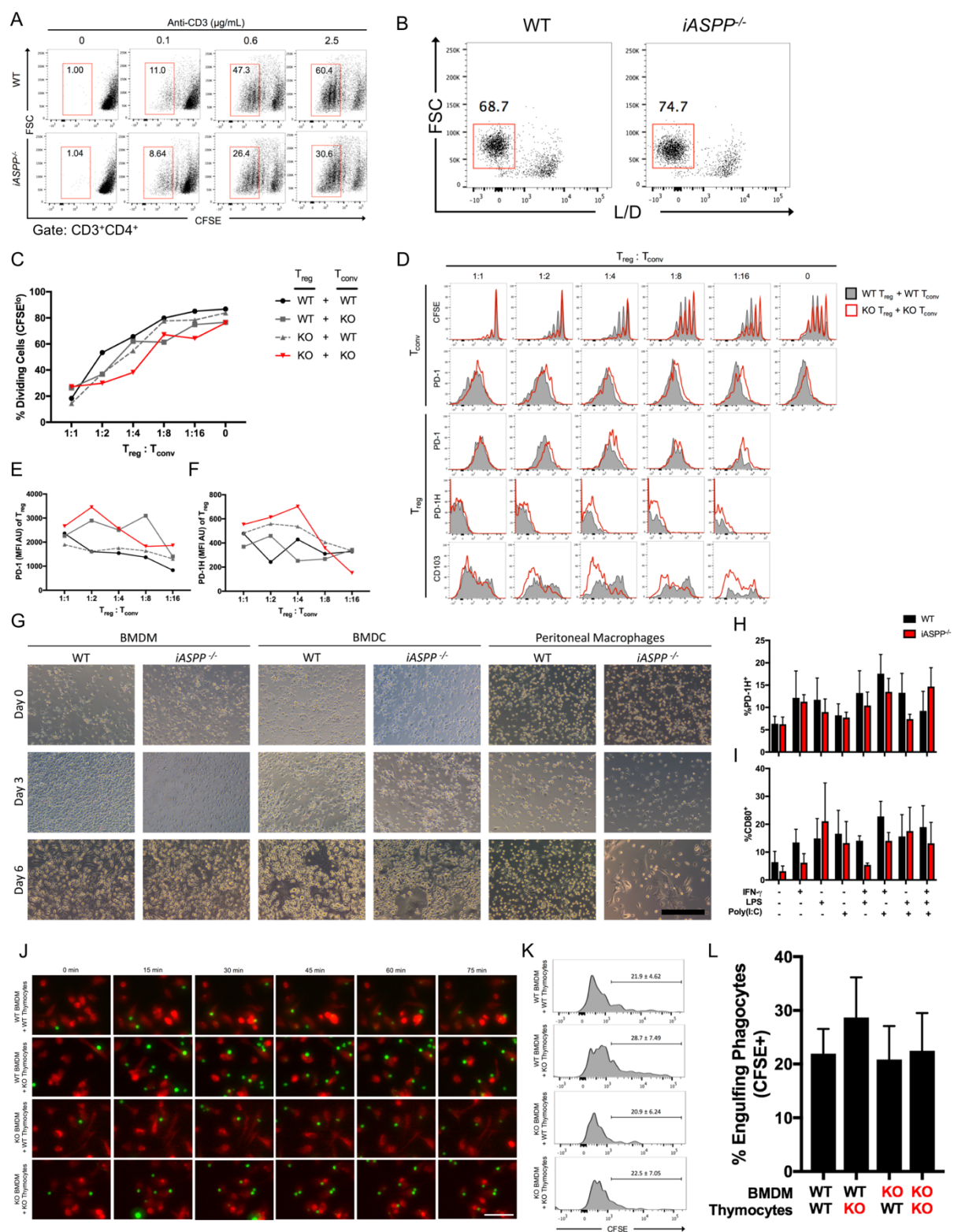


Figure 9: iASPP deficiency modulates CD4⁺ T cell responses but not macrophage function.

(A) Flow cytometry dot plots of CD4⁺ T cells isolated from wild type (WT, $n = 8$) or *iASPP*^{-/-} ($n = 4$) mice were pooled, stained with CFSE, and stimulated with indicated concentrations of anti-CD3 for 72 hr. Cell division was analyzed using CFSE dilution by flow cytometry. (B) Flow cytometry dot plot of live-dead stain of CD4⁺ T cells stimulated in (A). (C) WT or *iASPP*^{-/-} CD4⁺CD25⁺ T_{reg} and CD4⁺CD25⁻ T_{conv} cells were co-cultured at indicated ratios for 72 hr. The suppressive activity of T_{reg} cells was examined by cellular division of responder T_{conv} cells as measured by CFSE dilution. (D) Expression of CFSE, PD-1, PD-1H, and CD103 in T_{conv} (top) or T_{reg} (bottom) cells following co-culture described in (C). Histograms were gated for T_{conv} as CD4⁺CD25⁻FoxP3⁻ and for T_{reg} as CD4⁺CD25⁺FoxP3⁺. (E-F) Line graphs of PD-1 (E) or PD-1H (F) expression from (D). (G) Representative bright field images of bone marrow-derived macrophages (BMDM), bone marrow-derived dendritic cells (BMDC), or peritoneal macrophages isolated from WT or *iASPP*^{-/-} mice. Bone marrow cells were differentiated into BMDMs with M-CSF or into BMDCs with GM-CSF for six days. Scale, 500 μ m. (H-I) Induction of PD-1H and CD80 expression in BMDMs measured by flow cytometry following overnight stimulation with indicated conditions. (J) Time-lapse images of CFSE-labeled WT or *iASPP*^{-/-} gamma-irradiated apoptotic thymocytes (green) co-cultured with CellTrace Far Red-labeled WT or *iASPP*^{-/-} BMDMs (red). Images were acquired every 1 min, and representative images of engulfment are shown. Scale, 100 μ m. (K) Flow cytometry analysis of macrophage and apoptotic thymocyte co-culture in (L). Histograms are gated on CellTrace Far Red and statistic represents percentage of BMDMs engulfing CFSE-positive apoptotic thymocytes. (L) Bar graph representing quantification of flow cytometry results in (K). All data are representative of two independent experiments. Data are mean $\pm$ s.e.m.; Student's *t*-test, ns, $p > 0.05$.

cell expansion downstream of TCR signaling, and independently of p53 inhibition of antigen-independent CD4⁺ T cell activation.

Due to the apparent increased activation of T_{reg} cells in aged and autoimmune-induced *iASPP*^{-/-} mice, I tested whether *iASPP*^{-/-} T_{reg} cells are functionally distinct from WT T_{reg} cells. The suppressive activity of *iASPP*^{-/-} T_{reg} cells was examined *in vitro* by co-culturing CD4⁺CD25⁻ conventional T cells (T_{conv}) and CD4⁺CD25⁺ T_{reg} cells purified from healthy WT or *iASPP*^{-/-} mice. *iASPP*^{-/-} T_{reg} cells exhibited greater suppressive capacity than WT T_{reg} cells (**Figure 9C-D**), suggesting that iASPP expression in T_{reg} cells limits their suppressive potential. *iASPP*^{-/-} T_{conv} cells in general were more susceptible to T_{reg}-mediated suppression (**Figure 9C**), confirming that iASPP expression in CD4⁺ T cells facilitates their expansion. Interestingly, *iASPP*^{-/-} T_{reg} cells co-cultured with *iASPP*^{-/-} T_{conv} cells exhibited the greatest degree of suppression, implying a compounding immunomodulatory role of iASPP in both T_{reg} and T_{conv} cells. These differences were not observed at a T_{reg} to T_{conv} ratio of 1:1, likely due to a saturation limit of the assay.

Based on the markers of *iASPP*^{-/-} T_{reg} activation observed *in vivo*, I examined whether T_{reg} co-culture with T_{conv} cells could recapitulate these differences *in vitro*. Following co-culture, *iASPP*^{-/-} T_{reg} cells expressed increased amounts of PD-1 and PD-1H (**Figure 9D-F**), indicating that iASPP can regulate T_{reg} activation *ex vivo*. Notably, PD-1 expression was increased on both WT and *iASPP*^{-/-} T_{reg} cells only when cultured with *iASPP*^{-/-} T_{conv} cells (**Figure 9E**), suggesting that iASPP suppression of T_{reg} cell activation is non-cell autonomous. Conversely, PD-1H levels were increased only on *iASPP*^{-/-} T_{reg} cells regardless of the genotype of T_{conv} cells

(**Figure 9F**), suggesting that iASPP inhibition of PD-1H expression is T_{reg} cell-intrinsic. Together, these data suggest that iASPP deficiency promotes tolerance by both attenuating proliferation of TCR-stimulated T_{conv} cells, while also facilitating the suppressive potential of T_{reg} cells.

PD-1H is a cell surface molecule expressed by both T cells and antigen presenting cells (APCs) that is critical for tolerogenic phagocytosis (Yoon et al., 2015). Given the increased expression of PD-1H by both T cells and DCs observed in *iASPP*^{-/-} mice, I asked whether iASPP could also modulate APC activation and phagocytosis *in vitro*. Macrophages and dendritic cells differentiated at similar frequencies and with similar morphology from *iASPP*^{-/-} bone marrow (**Figure 9G**). *iASPP*^{-/-} peritoneal macrophages also exhibited similar development and characteristic processes following isolation and *in vitro* maintenance (**Figure 9G**). p53 inhibits autoimmune disease in part by downregulating phosphorylation of signal transducer and activator of transcription (STAT-1) and cytokine secretion by macrophages following lipopolysaccharides (LPS) or IFN- γ stimulation (Zheng et al., 2005). However, the expression of PD-1H and CD80 by *iASPP*^{-/-} bone-marrow derived macrophages (BMDMs) was comparable to that of WT BMDMs following stimulation with LPS, IFN- γ , or polyinosinic:polycytidylic acid (poly(I:C)) (**Figure 9H-I**), suggesting that iASPP does not regulate p53 interactions with STAT-1 in macrophages. Separately, p53-induced PD-1H expression by apoptotic cells is necessary for phagocytosis (Yoon et al., 2015), although *iASPP*^{-/-} apoptotic thymocytes were engulfed at similar frequencies to WT apoptotic thymocytes measured by both time lapse imaging (**Figure 9J**) and flow cytometry (**Figure 9K-L**). However, it is possible that apoptotic thymocytes upregulate PD-1H to saturated levels such that iASPP deficiency has a

negligible effect on PD-1H expression. Therefore, it appears that iASPP expression in macrophages does not regulate p53 pathways implicated in autoimmune disease.

Given the increased proportion of T_{reg} cells observed in the periphery but not spleens or thymus of *iASPP*^{-/-} mice, I examined whether iASPP deficiency protects mice from autoimmune disease by promoting T_{reg} cell differentiation. Indeed, p53 induces T_{reg} cell differentiation and *FoxP3* transcription by directly binding its promoter (Jung et al., 2010; Kawashima et al., 2013). Naive CD4⁺CD44⁻CD62L⁺ (T_{naive}) cells were purified and polarized toward T_{reg} or effector CD4⁺ subpopulations *in vitro* using Th0, Th1, Th2, Th17, or T_{reg} permissive conditions (**Figure 10A**). No significant difference was observed in the frequency of *iASPP*^{-/-} T_{naive} differentiation following *in vitro* polarization (**Figure 10B-F**), suggesting that iASPP does not modulate p53-regulated CD4⁺ polarization nor p53 binding to the *FoxP3* promoter. Therefore, the increased frequency and activation of peripheral T_{reg} cells observed in *iASPP*^{-/-} mice is likely due to non-cell-autonomous mechanisms of immune tolerance local to the site of immune insult rather than dysregulated CD4⁺ differentiation.

Together, these results suggest that iASPP deficiency decreases autoimmune disease severity by p53-regulated suppression of CD4⁺ T cell proliferation and potentiating T_{reg} cell functional suppression. Notably, iASPP does not appear to inhibit all mechanisms of p53-mediated immunosuppression, such as tolerogenic phagocytosis and transcriptional activation of *FoxP3*. Therefore, direct iASPP molecular interaction with p53 is likely immune cell-type specific, with greatest consequence of iASPP loss observed in CD4⁺ T cells.

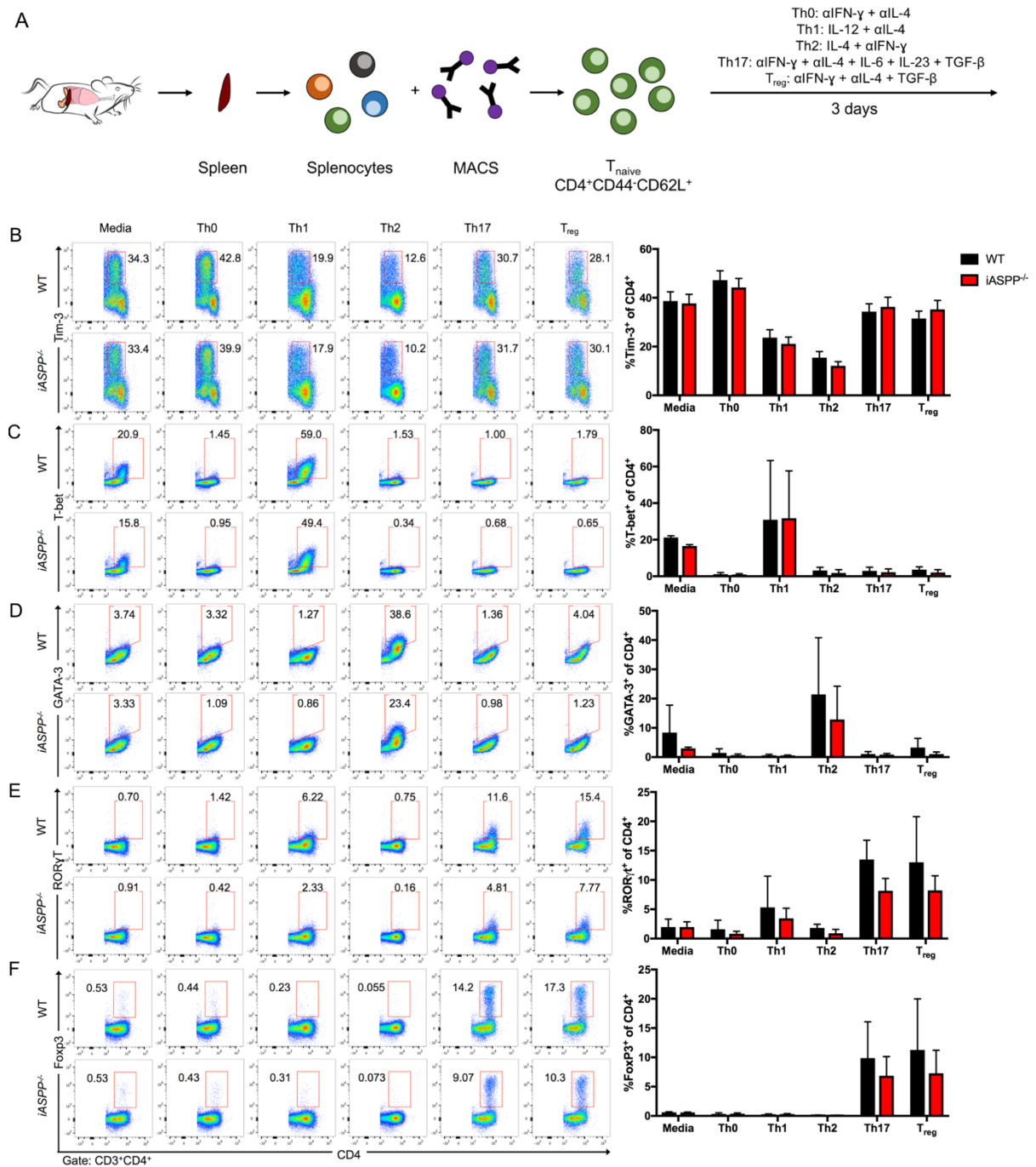


Figure 10: iASPP deficiency does not affect naive $CD4^+$ T cell differentiation.

(A) Schematic illustrating isolation of naive $CD4^+$ T cells (T_{naive}) using magnetic-activated cell sorting (MACS) and subsequent $CD4^+$ Th subset differentiation. T_{naive} cells were polarized towards Th0 with anti-IL-4 ($\alpha IL-4$) and anti-IFN- γ ($\alpha IFN-\gamma$); Th1 with IL-12 and $\alpha IL-4$; Th2 with IL-4 and $\alpha IFN-\gamma$; Th17 with $\alpha IL-4$, $\alpha IFN-\gamma$, IL-23, TGF- β , and IL-6; and T_{reg} with TGF- β , $\alpha IL-4$, and $\alpha IFN-\gamma$. (B-G) Flow cytometry dot plots (left) of WT ($n = 8$) or $iASPP^{-/-}$ ($n = 4$) T_{naive} cells cultured in Th0, Th1, Th2, Th17, or

T_{reg}-polarizing conditions for 72 hr. Tim-3 (B), T-bet (C), GATA-3 (D), ROR γ t (E), and FoxP3 (F) expression was examined by flow cytometry and quantified (right). Data are representative of two independent experiments.

CHAPTER 5: K-ras driven lung adenocarcinoma and pancreatic ductal adenocarcinoma development is accelerated in tumors lacking *iASPP*

iASPP deficiency accelerates K-ras driven lung adenocarcinoma

Given the profound stimulatory effect of *iASPP* on CD4⁺ autoimmunity, I hypothesized that *iASPP* might function reciprocally in cancer by facilitating immunosurveillance and functional anti-tumor immune responses. To test this hypothesis, I induced formation of autochthonous K-ras driven lung adenocarcinoma with tumor-specific deletion of *iASPP* via intranasal delivery of adenovirus expressing Cre recombinase (Ad-Cre) to *iASPP^{fl/fl};R26^{LSL-EYFP/EYFP}* (i), *K-Ras^{LSL-G12D/+};R26^{LSL-EYFP/EYFP}* (K), *K-Ras^{LSL-G12D/+};Trp53^{LSL-R127H/+}* (KP), *K-Ras^{LSL-G12D/+};iASPP^{fl/fl};R26^{LSL-EYFP/EYFP}* (Ki), or *K-Ras^{LSL-G12D/+};ASPP2^{fl/fl};R26^{LSL-EYFP/+}* (KA) mice (**Figure 11A**). Mutations leading to over-activation of the EGFR/Ras/ERK mitogen-activated protein kinase (MAPK) cascade have been associated with many cancer types and are drivers of cellular transformation and tumor development *in vivo* (Roberts and Der, 2007). Activating mutations in the proto-oncogene *K-RAS* are thought to be an early event in tumorigenesis, and are found in 10-30% of cases of human non-small cell lung cancer (NSCLC) (Herbst et al., 2008) and 70-90% of cases of pancreatic ductal adenocarcinoma (PDAC) (Pellegata et al., 1994). The EGFR/Ras/ERK MAPK cascade can be manipulated experimentally, for example by upstream pathway activation with exogenous EGFR ligand such as TGF- α (Yarden, 2001) or an activating glycine to aspartic acid mutation at codon 12 (G12D) in *K-ras* leading to constitutive pathway activation (Jackson et al., 2001; Johnson et al., 2001).

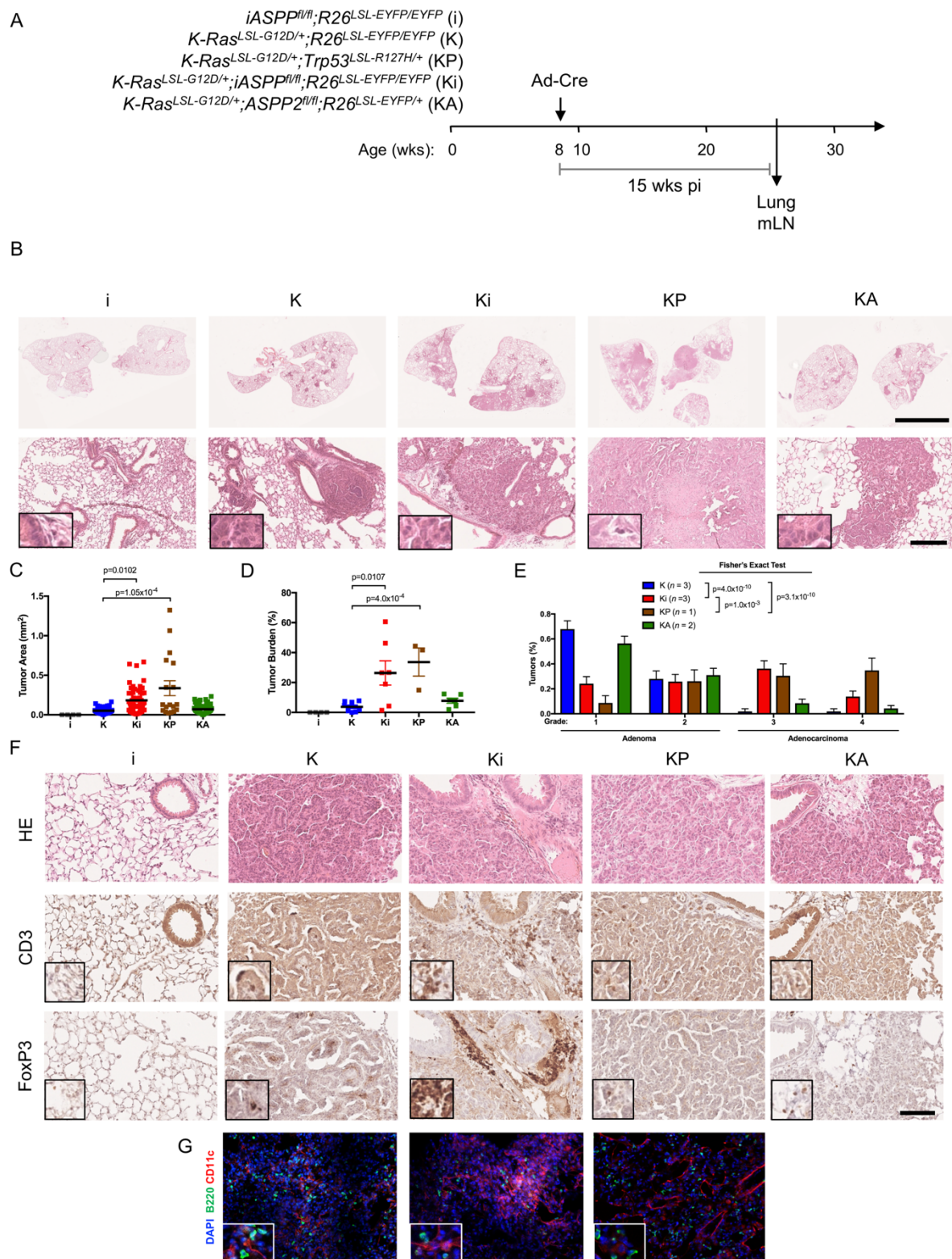


Figure 11: K-ras driven lung adenocarcinoma is accelerated in tumors lacking iASPP.

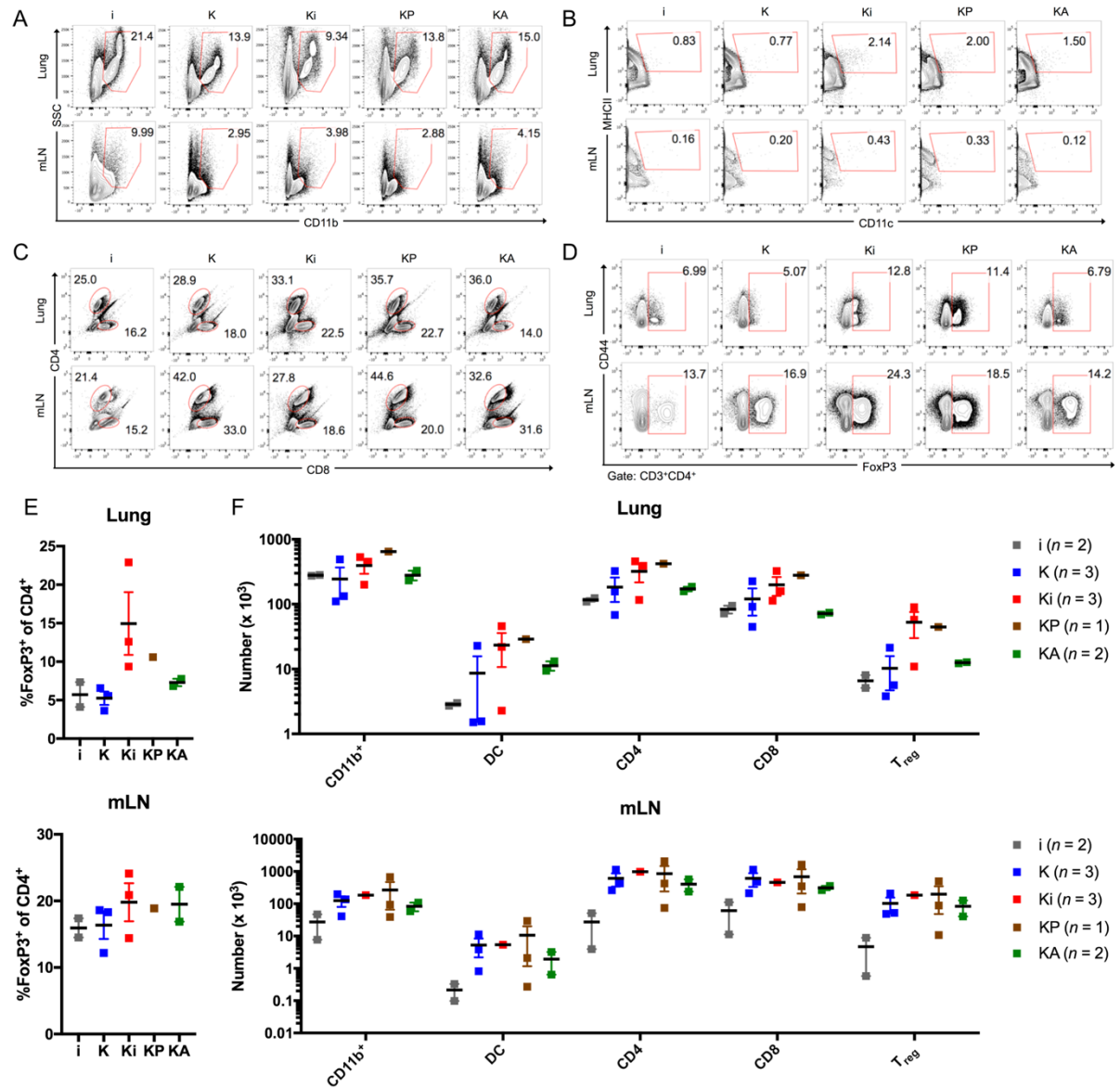
(A) Schematic of initiation of lung adenocarcinoma in *iASPP^{fl/fl};R26^{LSL-EYFP/EYFP}* (i), *K-Ras^{LSL-G12D/+};R26^{LSL-EYFP/EYFP}* (K), *K-Ras^{LSL-G12D/+};Trp53^{LSL-R127H/+}* (KP), *K-Ras^{LSL-G12D/+};iASPP^{fl/fl};R26^{LSL-EYFP/EYFP}* (Ki), and *K-Ras^{LSL-G12D/+};ASPP2^{fl/fl};R26^{LSL-EYFP/+}* (KA) mice with intranasal inhalation of adenoviral Cre (Ad-Cre). Lungs and mediastinal lymph node (mLN) were collected 15 weeks post-infection (pi). (B) Representative HE stains of lung lobes isolated from i ($n = 2$), K ($n = 3$), KP ($n = 1$), Ki ($n = 3$), and KA ($n = 2$) mice 15 weeks after tumor initiation. Higher magnification shown in lower row and insets. Scale, 5 mm (top) and 250 μ m (bottom). (C-D) Quantification of tumor area (C) and tumor burden (D) of lung tumors in (B). Tumor burden was calculated as total tumor area divided by total lung lobe area. Data are mean $\pm$ s.e.m.; Student's *t*-test, $p < 0.05$ indicated on graph. (E) Proportion of lung tumors classified by histological grade at 15 weeks after tumor initiation in K (blue), KP (brown), Ki (red), or KA (green) mice. Data are sample proportion $\pm$ standard error of the sample proportion; Fisher's exact test, $p < 0.05$ indicated on graph. (F) Representative HE, CD3, FoxP3, and CD11c immunohistochemistry stains of lung tumors 15 weeks after tumor initiation. Inset shows higher magnification. Scale, 100 μ m. (G) Confocal immunostaining for B220 (green) and CD11c (red) in lung tumors from K ($n = 2$), KP ($n = 1$), or Ki ($n = 3$) mice 15 weeks after tumor initiation. Inset shows higher magnification. All data are representative of two independent experiments.

After 15 weeks, KP mice had the greatest extent of lung cancer (**Figure 11B**), as expected given the tumor-suppressive role of p53 (DuPage et al., 2009; Iggo et al., 1990; Olive et al., 2004). Paradoxically, deletion of *iASPP*, an inhibitor of p53 (Bergamaschi et al., 2003, 2006; Lu et al., 2013), markedly accelerated lung adenocarcinoma compared to K mice. Ki mice had greater average tumor size (**Figure 11C**), tumor burden (**Figure 11D**), and histological grade (**Figure 11E**) of lung cancer compared to K mice. The majority (96%) of K tumors were graded as adenomas, whereas 50% of Ki tumors and 65% of KP tumors had progressed to lung adenocarcinoma 15 weeks after initiation. While K tumors were largely epithelial in nature with uniform nuclei, Ki and KP tumors had increased cellular heterogeneity with occasional stromal activation and greater nuclear atypia (**Figure 11B**). After 15 weeks no macroscopic lung cancer metastases were observed in the brain or liver. These histological observations indicate that loss of *iASPP* accelerates K-ras driven lung adenocarcinoma, despite being a canonical inhibitor of p53.

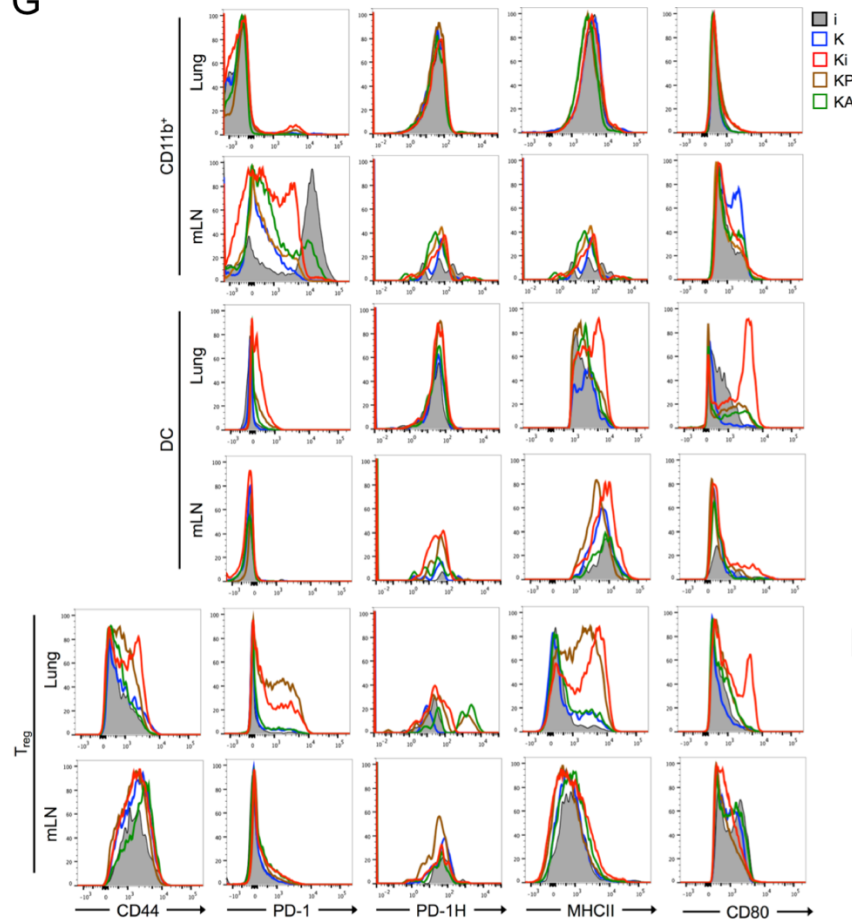
I hypothesized that an impaired anti-tumor immune response might explain the paradoxical finding that *iASPP* deletion accelerates lung adenocarcinoma development. Surprisingly, Ki tumors had a seemingly greater number of tumor-associated CD3⁺ T cells and B220⁺ B cells (**Figure 11F-G**). However, in Ki mice most tumor-associated T and B cells were retained in tertiary lymphoid structures (TLS) adjacent to lung tumors, while K and KP mice had a greater proportion of tumor-infiltrating T and B cells. TLSs are observed in human lung adenocarcinoma and correlate with positive prognosis (Dieu-Nosjean et al., 2008, 2014, 2016, 2016; Fridman et al., 2012; Germain et al., 2014, 2015; Hiraoka et al., 2016), but have only

been recently observed in mouse models of lung cancer that employ tumoral expression of strongly immunogenic antigens (Joshi et al., 2015). Therefore, Ki mice represent a novel model of immunogenic lung adenocarcinoma, in which TLS development surprisingly correlates with worse prognosis. Ki lung tumor-associated TLS contained vastly elevated amounts of FoxP3⁺ T_{regs} and harbored B220⁺ B cells and CD11c⁺ DCs (**Figure 11F-G**), which might indirectly facilitate T_{reg} activation and immunosuppression, suggesting that TLS formed in Ki mice might serve as sites of immunosuppression, rather than their typically accepted role of sites of anti-tumor T cell activation (de Chaisemartin et al., 2011; Neyt et al., 2012).

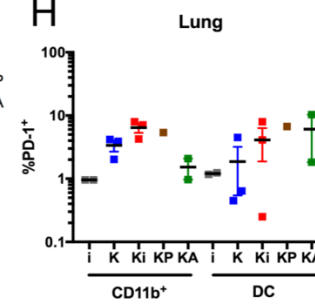
Immunophenotyping of Ki tumor-bearing lungs confirmed that loss of *iASPP* resulted in increased T_{reg}-infiltration into the lung, while no change was observed in the tumor-draining mediastinal lymph node (mLN) (**Figure 12A-F**). Notably, adenoviral infection of the lungs of Ki mice leads to selective activation of oncogenic *K-ras*^{G12D} and deletion of *iASPP* in lung epithelial cells, indicating that differential immune profiles observed in Ki mice are non-cell-autonomous and must be elicited by consequences of *iASPP* loss in tumor cells. Lung tumor-associated CD11b⁺ myeloid cells and DCs in Ki mice expressed increased CD80 although no difference was observed in the mLN (**Figure 12G-J**), suggesting that *iASPP* expression in lung adenocarcinoma prevents local activation of APCs. However, this activation is potentially immunosuppressive, as lung tumor-associated CD11b⁺ myeloid cells and DCs and mLN-resident CD11b⁺ myeloid cells in Ki mice expressed elevated levels of PD-1 (**Figure 12H**). Recently, PD-1 expression by tumor-associated macrophages (TAMs) was demonstrated to inhibit anti-tumor immunity by attenuating the phagocytic potency of TAMs (Gordon et al., 2017).



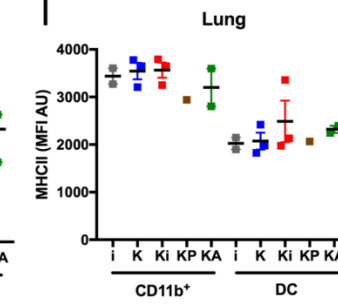
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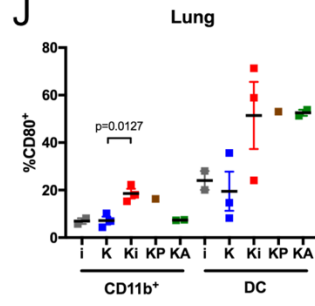
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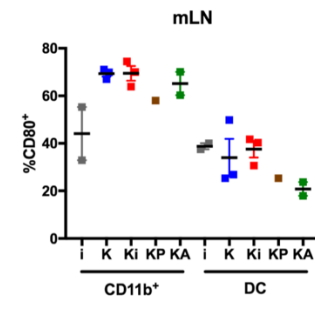
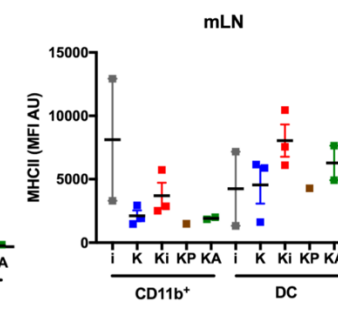
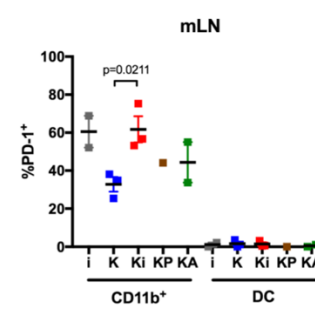
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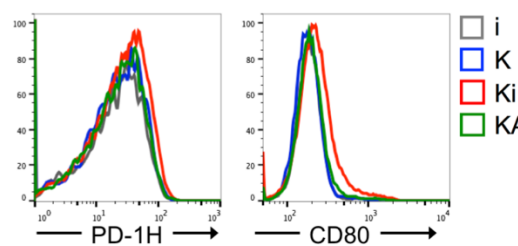
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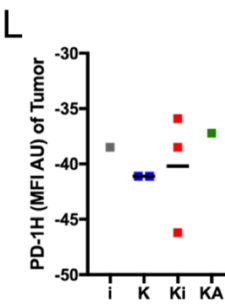
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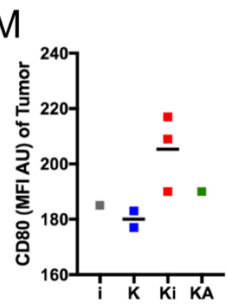
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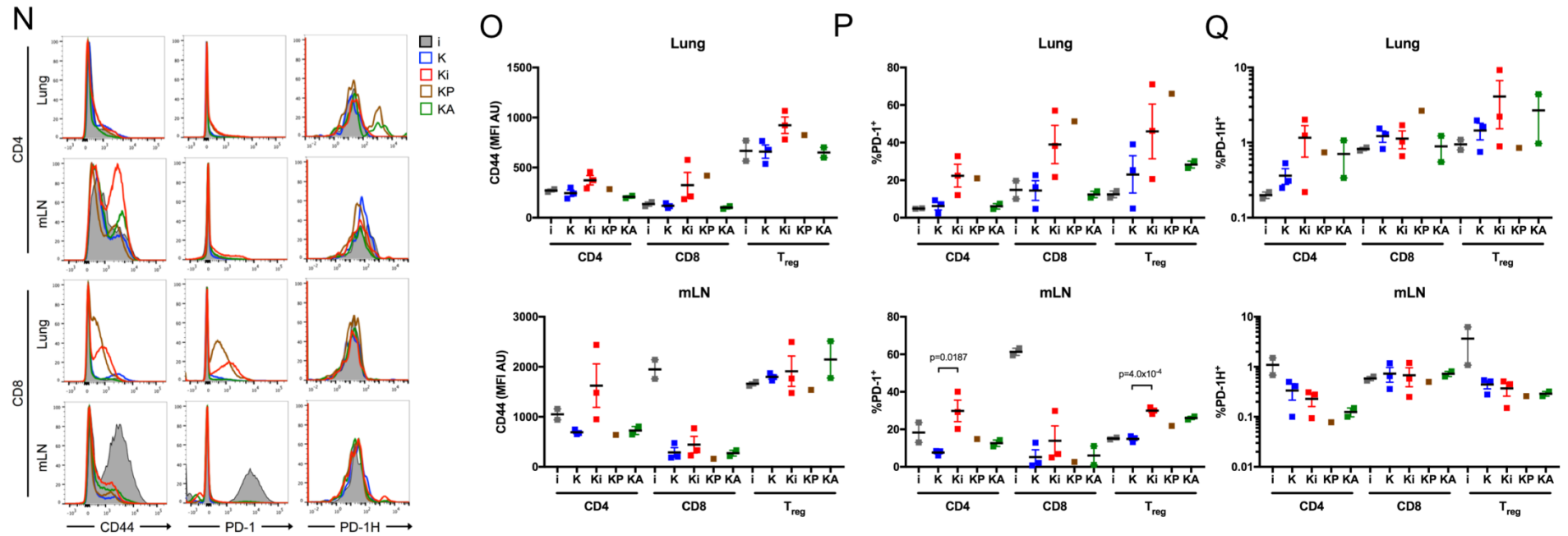


Figure 12: Lung tumor loss of iASPP expression promotes T cell exhaustion and Treg infiltration in lung adenocarcinoma.

(A-D) Flow cytometry contour plots of lymphocytes isolated from lungs of i ($n = 2$), K ($n = 3$), KP ($n = 1$), Ki ($n = 3$), and KA ($n = 2$) mice 15 weeks after tumor initiation. Gating strategies to identify CD11b⁺ myeloid cells (A), CD11c⁺MHCII⁺ DCs (B), CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells (C), and CD3⁺CD4⁺FoxP3⁺ T_{reg} cells (D) are indicated. (E) Quantification of FoxP3⁺ proportion of CD4⁺ cells from lungs (top) or tumor-draining mediastinal lymph nodes (mLN, bottom) of i (gray squares), K (blue squares), KP (brown squares), Ki (red squares), or KA (green squares) mice determined by flow cytometry gated as in (D). (F) Cell numbers in lungs (top) or mLNs (bottom) of mice as determined in (A-D). Absolute numbers of lymphocytes were calculated by multiplying total cell count by percentage of cells determined by flow cytometry. (G) Flow cytometry histograms of CD44, PD-1, PD-1H, MHCII, and

CD80 expression by myeloid, DCs, and T_{reg} cells from lungs or mLNs of i (filled gray line), K (blue line), KP (brown line), Ki (red line), or KA (green line) mice 15 weeks after tumor initiation as gated in (A-D). (H-J) Quantification of PD-1 (H), MHCII (I), and CD80 (J) expression by myeloid cells or DCs from lungs (top) or mLNs (bottom) of i (gray squares), K (blue squares), KP (brown squares), Ki (red squares), or KA (green squares) mice measured by flow cytometry of gated population defined in (A-B). (K) Flow cytometry histograms of PD-1H (left) and CD80 (right) expression by lung tumor cells from i ($n = 1$, gray line), K ($n = 2$, blue line), Ki ($n = 3$, red line), or KA ($n = 1$, green line) mice 15 weeks after tumor initiation. Lung tumor cells are gated from mice with the *R26^{LSL-EYFP}* allele as EYFP⁺CD3⁻CD4⁻CD8⁻CD11c⁻CD11b⁻F4/80⁻MHCII⁻. (L-M) Quantification of PD-1H (L) and CD80 (M) expression by lung tumor cells measured by flow cytometry as defined in (K). (N) Flow cytometry histograms of CD44, PD-1, and PD-1H expression by CD4⁺ or CD8⁺ cells from lungs or mLNs of mice 15 weeks after tumor initiation as gated in (C). (O-Q) Quantification of CD44 (O), PD-1 (P), and PD-1H (Q) expression by CD4⁺, CD8⁺, or T_{reg} cells from lungs (top) or mLNs (bottom) of mice measured by flow cytometry of gated population defined in (C-D). MFI AU, arbitrary units of median fluorescence intensity. All data are representative of two independent experiments. Data are mean $\pm$ s.e.m.; Student's *t*-test, $p < 0.05$ indicated on graph.

Given that loss of *iASPP* promoted an immunosuppressive lung tumor microenvironment that accelerated tumor development in a non-cell autonomous manner, I next asked what tumor cell-specific molecular mechanisms might be responsible for this immunosuppression. Flow cytometric analysis of transformed lung cancer cells revealed that Ki tumors express elevated levels of PD-1H and CD80 (**Figure 12K-M**) compared to K tumors, suggesting that PD-1H might be one of the factors responsible for mitigating anti-tumor immunity following *iASPP* loss. More detailed immunophenotyping of tumor-infiltrating lymphocytes revealed that CD4⁺, CD8⁺, and T_{reg} cells in both tumor-bearing lungs and the mLN express elevated PD-1, while only CD4⁺ and T_{reg} cells in the lung but not mLN upregulated PD-1H expression in Ki lung cancer (**Figure 12N-Q**). Together these data indicate that *iASPP* promotes anti-tumor immunity by dampening tumoral expression of the immunosuppressive molecule PD-1H, thereby allowing the development of functional CD4⁺ and CD8⁺ T cell responses while limiting T_{reg} infiltration and activation.

iASPP deficiency accelerates *K-ras* driven pancreatic cancer

To confirm whether *iASPP* paradoxically opposes cancer development by facilitating anti-tumor immunity in a different cancer type, I next examined the consequences of *iASPP* deletion in *K-ras* driven pancreatic ductal adenocarcinoma (PDAC). In concordance with lung adenocarcinoma findings, although *K-Ras*^{LSL-G12D/+};*Trp53*^{LSL-R127H/+};*Pdx1-Cre* (KPC) developed the most accelerated progression to PDAC, *K-Ras*^{LSL-G12D/+};*iASPP*^{fl/fl};*Pdx1-Cre* (KiC) mice had significantly accelerated pancreatic neoplasia development compared to control *iASPP*^{fl/fl};*Pdx1-Cre* (iC) and *K-Ras*^{LSL-G12D/+};*Pdx1-Cre* (KC) mice (**Figure 13A**). Rather than developing tumor-associated TLS, KiC pancreatic neoplasias had increased infiltration of FoxP3⁺ T_{reg} cells (**Figure**

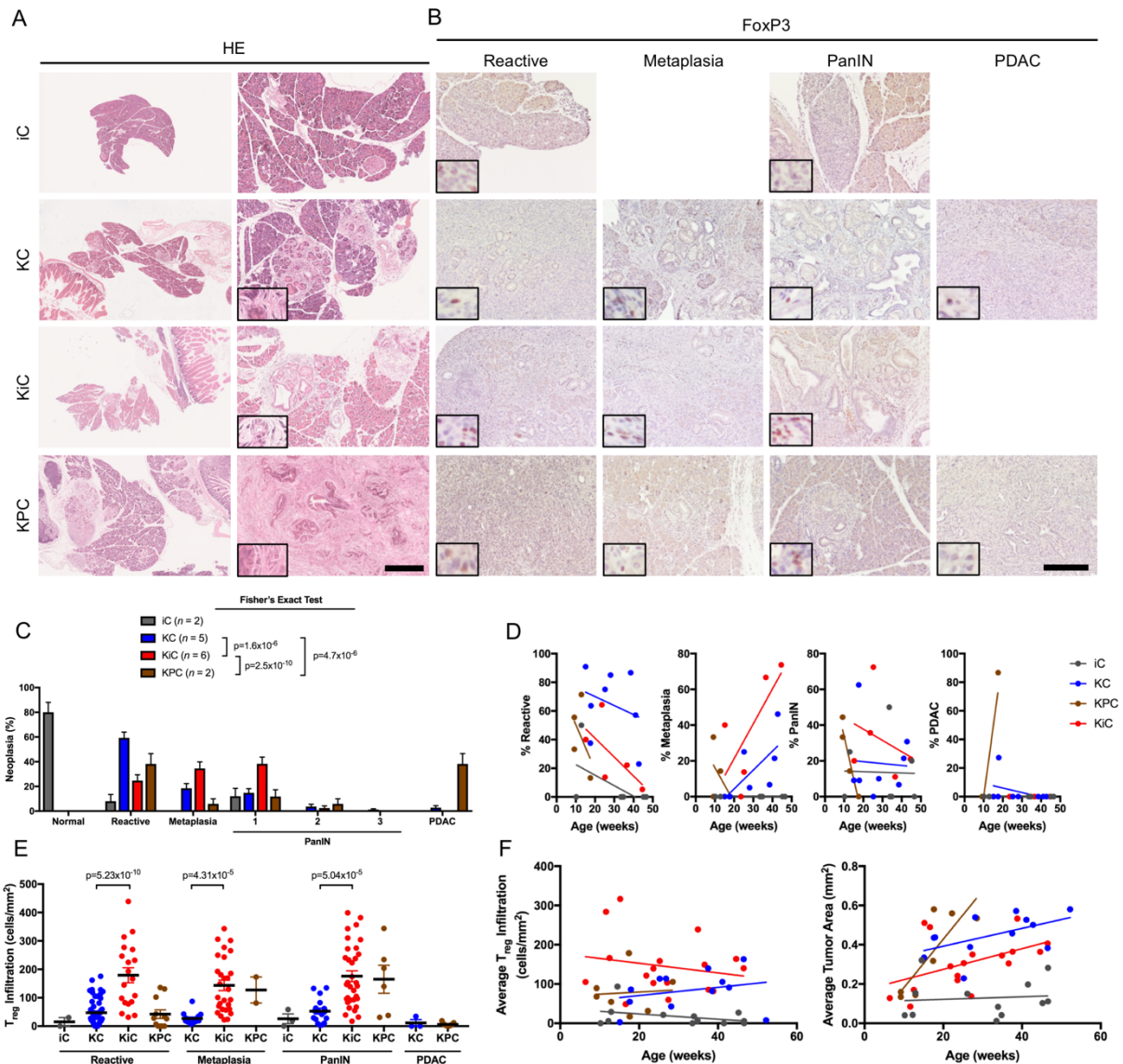


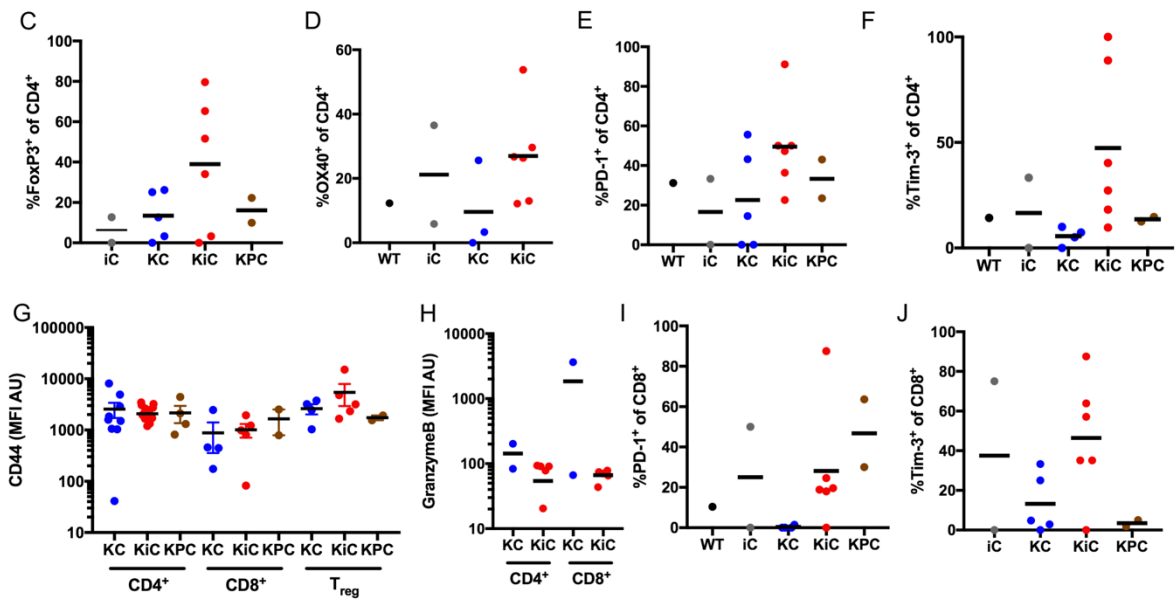
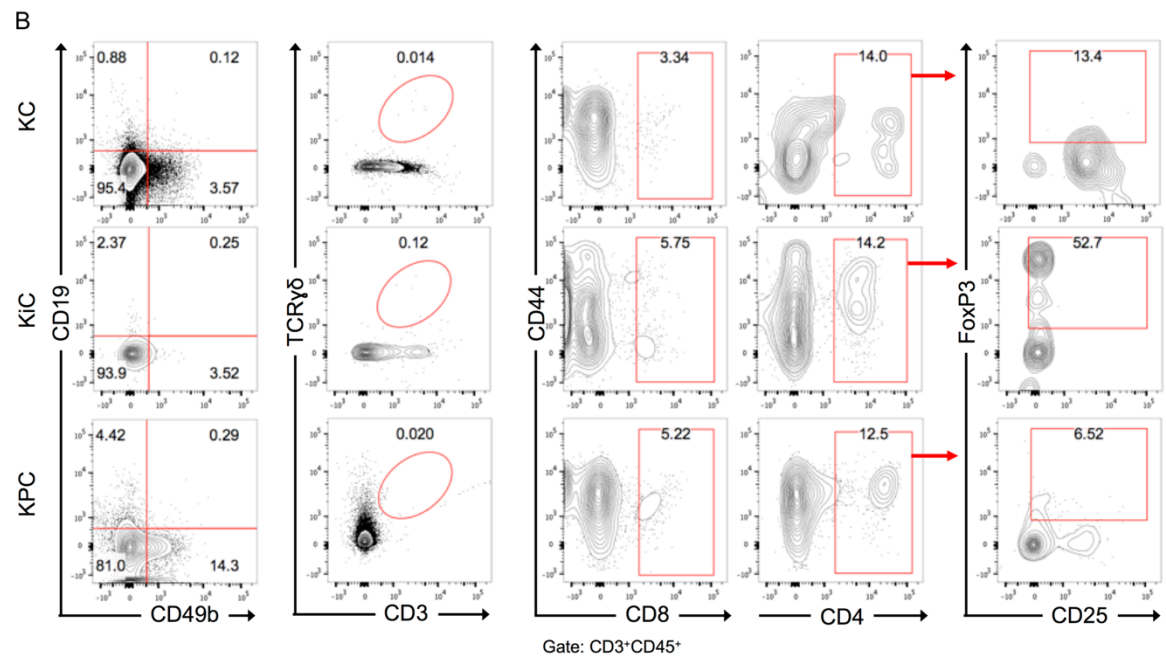
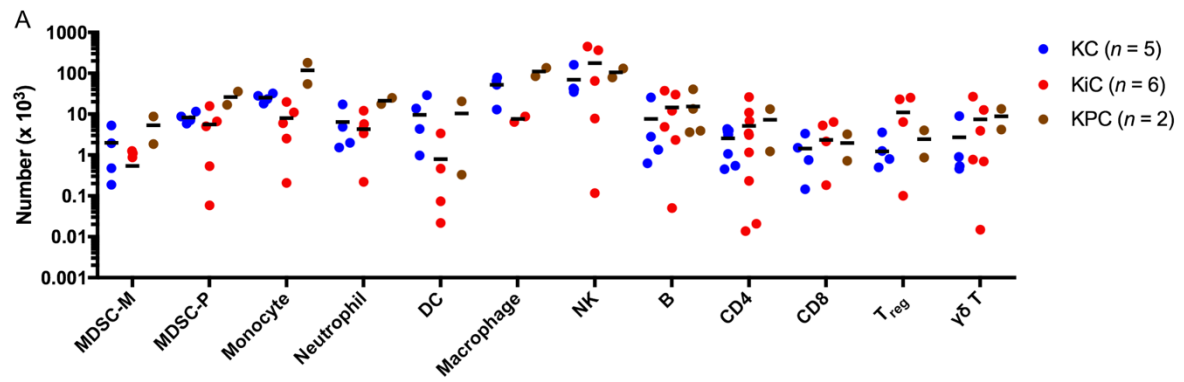
Figure 13: K-ras driven pancreatic ductal adenocarcinoma is accelerated in tumors lacking iASPP.

(A) Representative HE stains of pancreas isolated from 25-week-old *iASPP^{fl/fl};Pdx1-Cre* (iC, $n = 2$), *K-Ras^{LSL-G12D/+};Pdx1-Cre* (KC, $n = 5$), *K-Ras^{LSL-G12D/+};Trp53^{LSL-R127H/+};Pdx1-Cre* (KPC, $n = 2$), and *K-Ras^{LSL-G12D/+};iASPP^{fl/fl};Pdx1-Cre* (KiC, $n = 6$) mice. Higher magnification shown to right and in insets. Scale, 250 μ m. (B) Representative FoxP3 immunohistochemistry stains of pancreas with reactive neoplasia, metaplasia, pancreatic intraepithelial neoplasia (PanIN), or pancreatic ductal adenocarcinoma (PDAC) from iC, KC, KiC, or KPC mice. Higher magnification show in insets. Scale, 250 μ m. (C) Proportion of reactive neoplasia, metaplasia, PanIN, versus PDAC of all pancreatic neoplasia in iC ($n = 7$, gray), KC

($n = 8$, blue), KiC ($n = 5$, red), or KPC ($n = 4$, brown) mice at humane endpoint. Data are sample proportion $\pm$ standard error of the sample proportion; Fisher's exact test, $p < 0.05$ indicated on graph. (D) Scatter plots showing proportion reactive neoplasia, metaplasia, PanIN, versus PDAC of all pancreatic neoplasia in iC (gray circles), KC (blue circles), KiC (red circles), or KPC (brown circles) mice over time. Solid lines indicate linear regression estimates. (E) Column scatter plot shows quantification of pancreatic neoplasia-infiltrating FoxP3⁺ T_{reg} cells per area in reactive neoplasia, metaplasia, PanIN, versus PDAC from iC ($n = 5$ neoplasia, gray circles), KC ($n = 91$ neoplasia, blue circles), KiC ($n = 80$ neoplasia, red circles), or KPC ($n = 31$ neoplasia, brown circles) mice. Data are mean $\pm$ s.e.m.; Student's t -test, $p < 0.05$ indicated on graph. (F) Scatter plots of average infiltrating T_{reg} cells per area (left) or average tumor area (right) in iC ($n = 12$, gray circles), KC ($n = 12$, blue circles), KiC ($n = 17$, red circles), or KPC ($n = 7$, brown circles) mice over time. Solid lines indicate linear regression estimates. Data are representative of four independent experiments.

13B), suggesting that as in autoimmune disease and lung adenocarcinoma, iASPP suppresses T_{reg} cell infiltration in pancreatic ductal adenocarcinoma. While most KC neoplasias exhibited non-invasive epithelial proliferation and minimal cellular abnormalities after 25 weeks, most KiC pancreas harbored sites of acinar to ductal metaplasia (ADM) and pancreatic intraepithelial neoplasia (PanIN) (**Figure 13C-D**), indicative of more advanced disease. Importantly, KiC mice had greater extents of intratumoral T_{reg} cell infiltration than KC mice consistently across tumor histological grade (**Figure 13E**), demonstrating that increased presence of T_{reg} cells is dependent on loss of iASPP and not trivially on more advanced pancreatic cancer.

Immunophenotyping by flow cytometry confirmed the increase in infiltrating T_{reg} cells observed in KiC mice (**Figure 14A-C**), while absolute numbers of other lymphocyte populations remained relatively unchanged. Characterization of pancreas-infiltrating lymphocytes revealed that pancreatic CD4⁺ T cells exhibited a striking degree of exhaustion in KiC mice, including increased expression of OX-40, PD-1 and Tim-3 (**Figure 14D-F**). CD8⁺ T cells also increased expression of PD-1 and Tim-3 while decreasing expression of GranzymeB (**Figure 14H-J**), indicative of an exhausted non-functional cytotoxic T cell response in KiC mice. The pancreas infiltrating T_{reg} cell population was largely activated and immunosuppressive, expressing increased CD44, PD-1, and CD103 (**Figure 14K**), suggesting that iASPP also attenuates functional T_{reg} responses in pancreatic cancer. Together, these data suggest that accelerated PDAC development observed in KiC is likely due to the emergence of an immunosuppressive tumor microenvironment that suppresses CD8⁺ anti-tumor cytotoxicity.



K

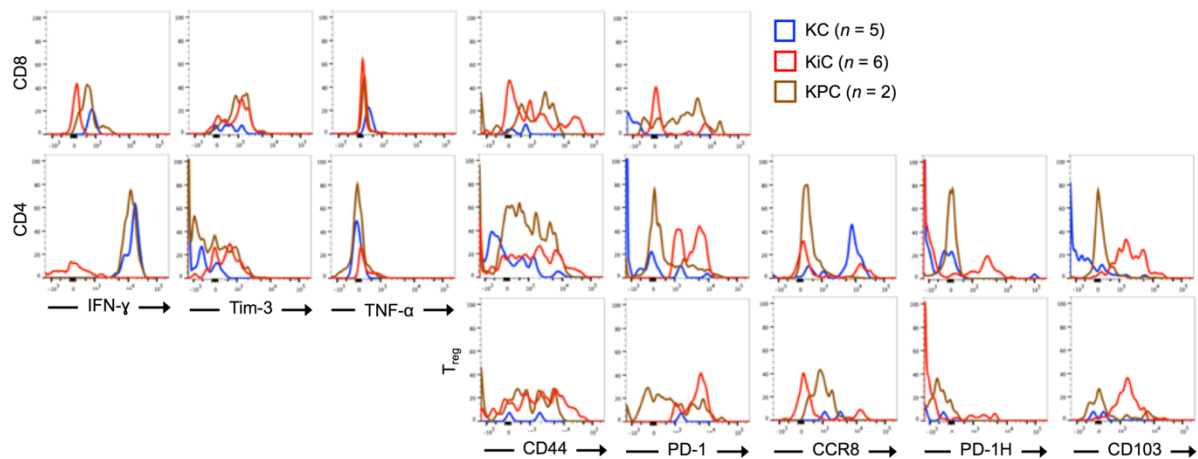


Figure 14: Pancreatic iASPP deficiency potentiates the development of an immunosuppressive tumor microenvironment

(A) Cell numbers in pancreata isolated from 25-week-old KC ($n = 5$, blue cricles), KiC ($n = 6$, red circles), or KPC ($n = 2$, brown circles) mice determined by flow cytometry. Horizontal line indicates sample means. (B) Flow cytometry contour plots showing gating strategy used to identify CD19⁺ B cells, CD49b⁺ NK cells, CD3⁺CD8⁺ T cells, CD3⁺CD4⁺ T cells, and CD3⁺CD4⁺CD25⁺FoxP3⁺ T_{reg} cells from pancreas of 25-week-old KC, KiC, or KPC mice. (C-F) Quantification of FoxP3 (C), OX40 (D), PD-1 (E), and Tim-3 (F) expression in CD4⁺ T cells from pancreas of 25-week-old WT ($n = 1$, black circles), iC ($n = 2$), KC ($n = 5$), KiC ($n = 6$), or KPC ($n = 2$) mice measured by flow cytometry of gated population defined in (B). Horizontal line indicates sample means. (G-H) Quantification of CD44 (G) and GranzymeB (H) in CD4⁺, CD8⁺, or T_{reg} cells from pancreas of 25-week-old KC ($n = 9$), KiC ($n = 6$), or KPC ($n = 4$) mice measured by flow cytometry of gated population defined in (B). MFI AU, arbitrary units of median fluorescence intensity. Data are mean $\pm$ s.e.m. (I-J) Quantification PD-1 (I) and Tim-3 (J) expression in CD8⁺ T cells from pancreas of 25-week-old WT, iC, KC, KiC, or KPC mice measured by flow cytometry of gated population defined in (B). Horizontal line indicates sample means. (K) Flow cytometry histograms of IFN- γ , Tim-3, TNF- α , CD44, PD-1, CCR8, PD-1H, and CD103 expression by CD8⁺ (top), CD4⁺ (middle), or T_{reg} (bottom) cells from pancreas of 25-week-old KC (blue line), KiC (red line), or KPC (brown line) mice. Data are representative of two independent experiments.

The immunosuppressive tumor microenvironment observed following iASPP deletion is particularly surprising given that KiC mice have pancreas-specific deletion of *iASPP* encoded by the *Pdx1-Cre* allele and otherwise wild type lymphocytes, suggesting that molecular signaling from transformed pancreatic tumor cells must be responsible for eliciting the observed immune phenotype. To investigate the identity of these signals, I isolated and characterized primary pancreatic acinar cells *in vitro* (**Figure 15A**). Three-dimensional culture of primary acinar cells can recapitulate acinar to ductal metaplasia (ADM), thereby modeling the initial cellular transformation characteristic of the progression from healthy pancreatic epithelium to PanIN to PDAC in K-ras driven pancreatic cancer (Means et al., 2005; Shi et al., 2013). Loss of function mutation of p53 significantly enhanced *K-ras*^{G12D}-induced ADM formation *in vitro* (**Figure 15B**), recapitulating the accelerated PanIN formation observed in KPC mice *in vivo*. Deletion of *iASPP* either with *Pdx1-Cre* (**Figure 15B**) or *in vitro* 4-OHT treatment of *Rosa26*^{CreER}-expressing cells (**Figure 15C**) markedly attenuated *K-ras*^{G12D}-induced ADM formation *in vitro* (**Figure 15D**), and was not rescued by orthogonal activation of the K-ras pathway with exogenous TGF- α , indicating that iASPP promotes ADM *in vitro* by acting downstream or at least independently of the K-ras/MEK/ERK pathway. These observations are seemingly contradictory to the accelerated PDAC development observed in KiC mice (**Figure 13**), suggesting that iASPP functions as an oncogene in a cell-autonomous manner *in vitro*, but as a tumor-suppressor in a non-cell autonomous manner *in vivo*. More generally, these findings highlight the necessity of taking care when extrapolating *in vitro* observations to mouse models or human pathophysiology.

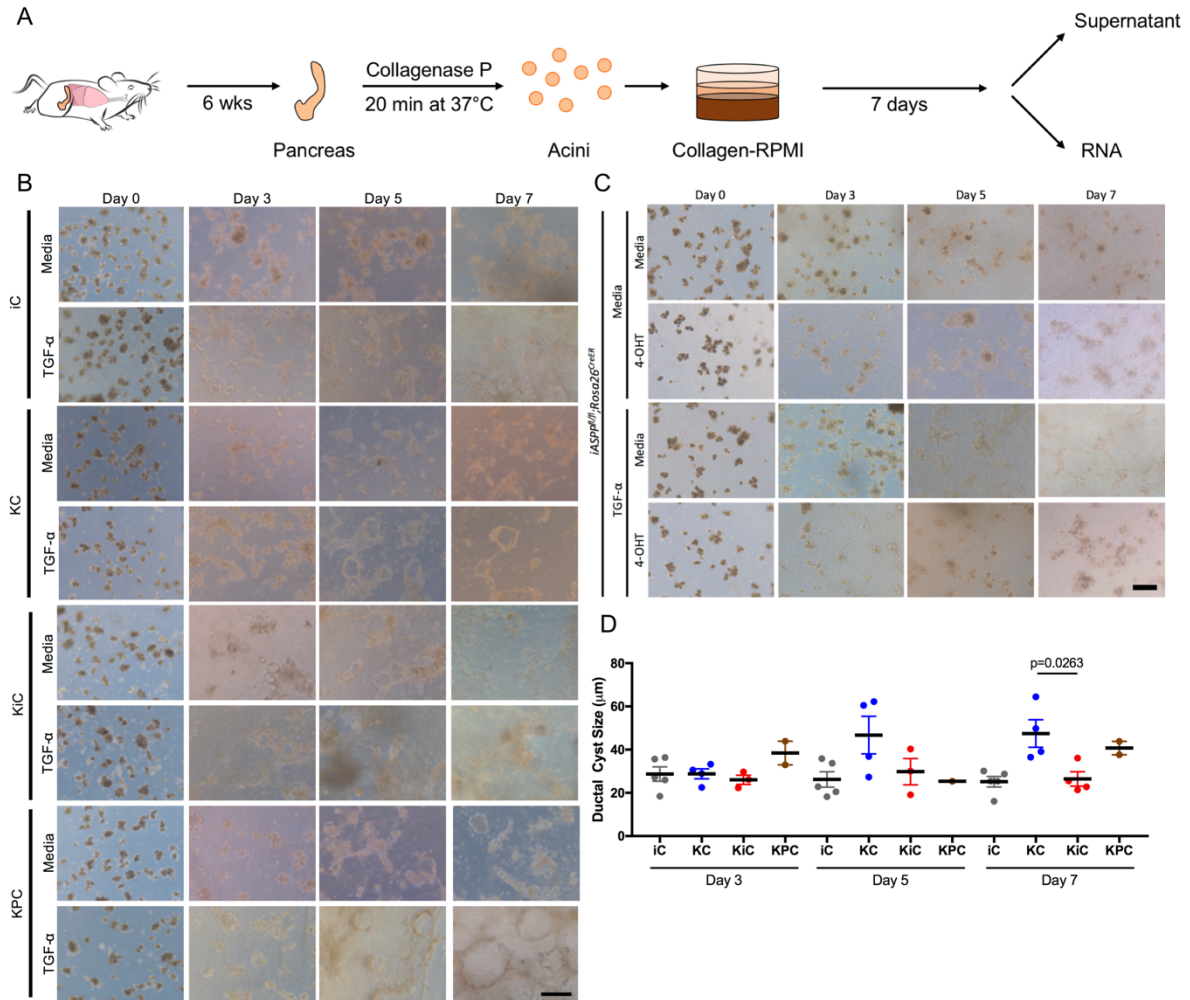


Figure 15: iASPP is necessary for efficient acinar to ductal metaplasia *in vitro*.

(A) Schematic illustrating protocol for 3D culture of primary pancreatic acinar cells. Pancreas was isolated from 6-week-old mice and dissociated acini were resuspended in a collagen-RPMI matrix. (B) Representative bright field images of acini isolated from iC ($n = 5$), KC ($n = 4$), KPC ($n = 2$), and KiC ($n = 4$) mice. Acinar to ductal metaplasia was assessed over 7 days with or without TGF- α . Scale, 250 μm . (C) Representative bright field images of acini isolated from *iASPP^{fl/fl}; Rosa26^{CreER}* ($n = 5$) mice and cultured with or without 4-hydroxytamoxifen (4-OHT) and with or without TGF- α . Scale, 250 μm . (D) Quantification of ductal cyst size over time from acini isolated from iC (black line), KC (blue line), KiC (red line), or KPC (brown line) mice treated with TGF- α . All data are representative of four independent experiments. Data are mean $\pm$ s.e.m.; Student's *t*-test, $p < 0.05$ indicated on graph.

Together these findings suggest that in reciprocity with its promotion of autoimmune disease, and despite inhibiting p53, iASPP can function as a tumor-suppressor in a non-cell autonomous manner by facilitating functional anti-tumor immunity. Both K-ras driven lung adenocarcinoma and pancreatic cancer exhibited accelerated development following iASPP deletion, which was associated with increased TLS presence or infiltration of T_{reg} cells. The immunosuppressive tumor microenvironment that develops in mice with iASPP-deficient tumors is likely due to loss of homeostatic control of the p53-regulated immunosuppressive molecule PD-1H, whose expression was increased on both lung and pancreatic tumors lacking iASPP. To further characterize this iASPP-mitigated immunosuppressive microenvironment in more detail, I next employed a mouse model of chronic pancreatitis to exacerbate the immunophenotype observed in KiC mice.

CHAPTER 6: Chronic pancreatitis promotes an immunosuppressive pancreatic tumor microenvironment in tumors lacking *iASPP*

iASPP-deficient pancreatic tumors exhibit greater chronic pancreatitis-induced lymphocyte infiltration

Epidemiological studies have demonstrated that chronic pancreatitis can predispose human patients to development of PDAC (Andrén-Sandberg et al., 1997; Lowenfels and Maisonneuve, 2006; Lowenfels et al., 1999), a finding that is recapitulated in mouse models of pancreatic cancer (Inman et al., 2014). Although there is controversy over whether chronic pancreatitis is necessary for PDAC development from mature pancreatic acini-specific expression of oncogenic *K-ras*^{G12D} (Liu et al., 2016a; Murtaugh and Leach, 2007), chronic pancreatitis certainly accelerates K-ras driven pancreatic cancer development (Gidekel Friedlander et al., 2009; Guerra et al., 2007, 2011; Habbe et al., 2008).

To both increase the population of pancreatic tumor-infiltrating lymphocytes for improved immunophenotyping and to examine if *iASPP* deficient K-ras driven pancreatic cancer remains responsive to chronic pancreatitis, I subjected KiC mice to caerulein-induced chronic pancreatitis (**Figure 16A**). Caerulein is a molecular analog of cholecystokinin that induces reversible pancreatitis by stimulating acinar cells to release amylase (Lampel and Kern, 1977), leading to digestive necrosis and responsive inflammation (Aghdassi et al., 2011; Lerch and Gorelick, 2013). Chronic pancreatitis resulted in substantial fibrosis and increased infiltration of CD3⁺ T cells into the pancreas (**Figure 16B**), and nearly all pancreatic neoplasias were reactive

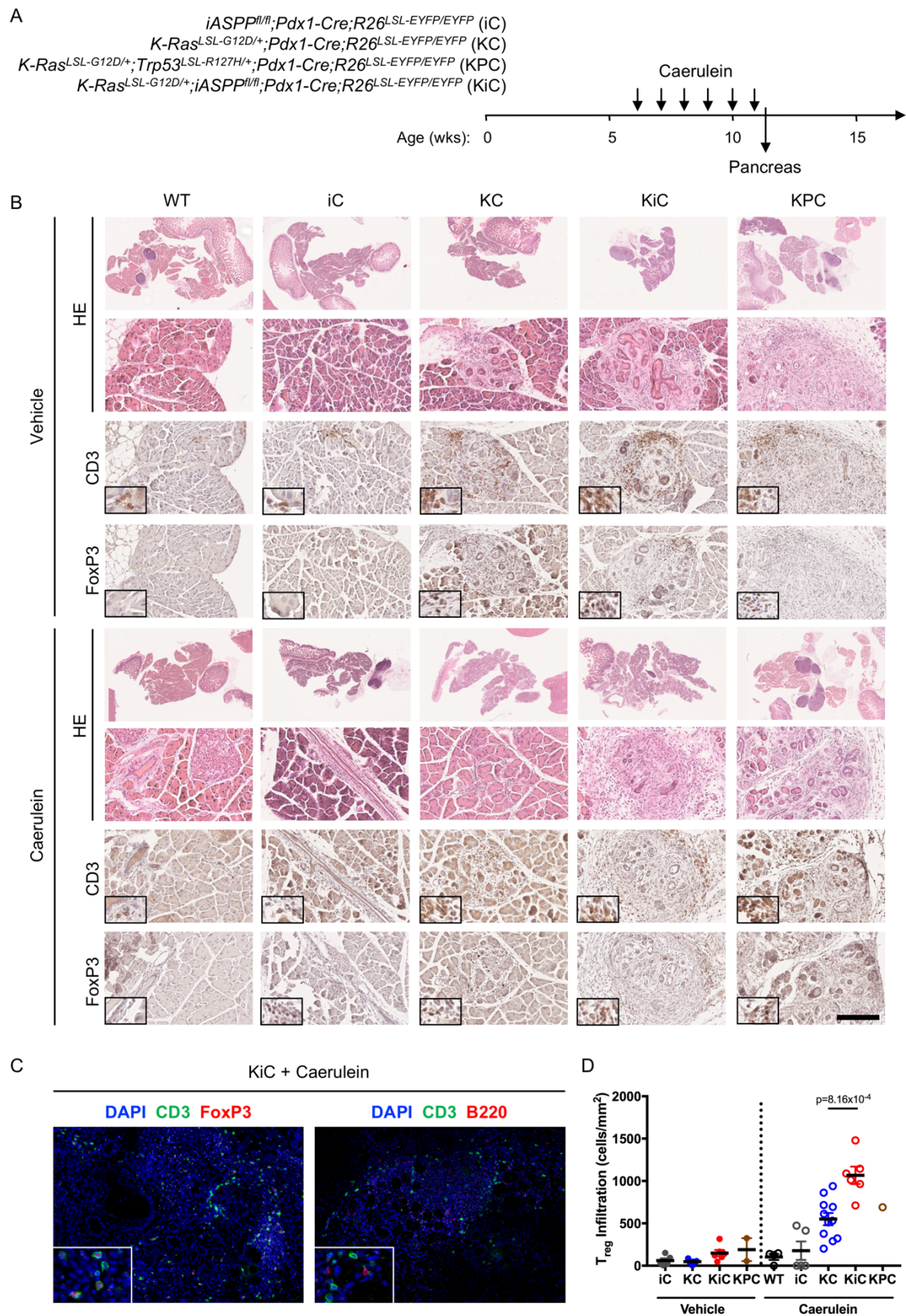


Figure 16: Chronic pancreatitis-induced lymphocyte infiltration is exacerbated in tumors lacking iASPP.

(A) Schematic of chronic pancreatitis model and experimental design. Six-week-old iC, KC, KPC, and KiC mice received six weekly doses of caerulein. Mice were sacrificed 48 hr after the final dose and pancreata were collected for analysis. (B) Representative HE, CD3, and FoxP3 immunohistochemistry stains of WT ($n = 6$ for vehicle, $n = 11$ for caerulein), iC ($n = 7$ for vehicle, $n = 13$ for caerulein), KC ($n = 3$ for vehicle, $n = 17$ for caerulein), KiC ($n = 6$ for vehicle, $n = 16$ for caerulein), or KPC ($n = 2$ for vehicle, $n = 4$ for caerulein) pancreata following chronic pancreatitis. Inset shows higher magnification. Scale, 250 μm . (C) Confocal immunostaining for CD3 (green) and FoxP3 (red, left) or B220 (red, right) in pancreata from KiC ($n = 5$) mice following chronic pancreatitis. Inset shows higher magnification. (D) Column scatter plot shows quantification of pancreatic neoplasia-infiltrating FoxP3⁺ T_{reg} cells per area in WT ($n = 4$ for caerulein, black hollow circles), iC ($n = 5$ for vehicle, gray circles; $n = 5$ for caerulein, gray hollow circles), KC ($n = 3$ for vehicle, blue circles; $n = 11$ for caerulein, blue hollow circles), KiC ($n = 6$ for vehicle, red circles; $n = 6$ for caerulein, red hollow circles), or KPC ($n = 2$ for vehicle, brown circles; $n = 1$ for caerulein, brown hollow circles) mice. All data are representative of five independent experiments.

as characterized by histologic grade. Pancreata expressing oncogenic *K-ras*^{G12D} exhibited relatively more severe acinar atrophy and fibrosis, and in combination with deletion of *iASPP* exhibited an even greater degree of reactive stroma following caerulein insult (**Figure 16B**). The increased FoxP3⁺ T_{reg} infiltration observed in KiC pancreata was exacerbated following chronic pancreatitis, although the presence of highly reactive intratumoral stroma appeared to constrain T_{reg} localization to more peripheral regions of PanIN and PDAC lesions (**Figure 16B-D**). Interestingly, loss of *iASPP* in K-ras driven pancreatic cancer with chronic pancreatitis seemed to elicit a T-cell dominant immune response (**Figure 16C**) in contrast to the substantial B cell response observed in K-ras driven lung adenocarcinoma (**Figure 11G**). These data validate caerulein-induced chronic pancreatitis as a model of accelerated PDAC development in KiC mice that displays similar histopathology to previously described chronically inflamed KC pancreatic cancer.

iASPP-deficient chronically-inflamed pancreatic tumors have greater myeloid-derived suppressor cell activation

Given the substantial increase in tumor-infiltrating T_{reg} cells observed in KiC mice with chronic pancreatitis, I sought to profile the infiltrating lymphocytes that comprise the KiC tumor microenvironment following chronic pancreatitis. *iASPP* deletion led to a substantial increase in the absolute numbers of pancreas-infiltrating B, CD4⁺, CD8⁺, and T_{reg} cells (**Figure 17A**). In contrast, *iASPP* deletion did not affect numbers of any of the examined immune cell populations in the spleens of tumor-bearing mice, demonstrating that *iASPP* mitigates T and B cell infiltration of pancreatic tumors following chronic pancreatitis without altering systemic immune profiles. Although absolute numbers of tumor-infiltrating myeloid populations

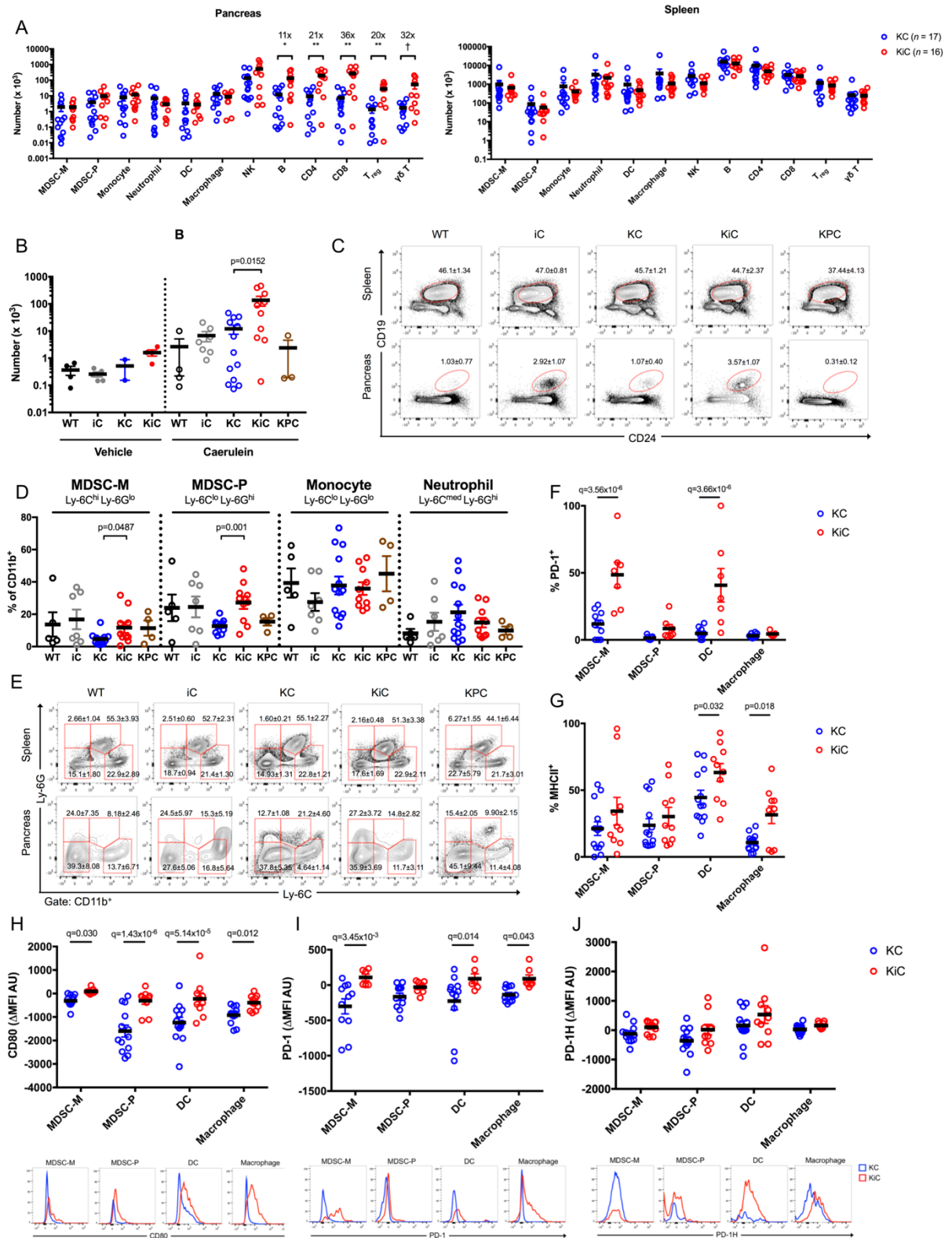


Figure 17: Pancreatic iASPP deficiency promotes myeloid-derived suppressor cell activation in the tumor microenvironment.

(A) Cell numbers in pancreas (left) or spleens (right) isolated from KC ($n = 17$, blue hollow circles) or KiC ($n = 16$, red hollow circles) mice determined by flow cytometry. Data are mean $\pm$ s.e.m.; significance determined using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli with $Q = 5\%$, $*q=0.0320$, $**q=7.32 \times 10^{-3}$. (B) Number of CD19⁺ B cells in pancreas from WT ($n = 6$ for vehicle, black circles; $n = 11$ for caerulein, black hollow circles), iC ($n = 7$ for vehicle, gray circles; $n = 13$ for caerulein, gray hollow circles), KC ($n = 3$ for vehicle, blue circles; $n = 17$ for caerulein, blue hollow circles), KiC ($n = 6$ for vehicle, red circles; $n = 16$ for caerulein, red hollow circles), or KPC ($n = 4$ for caerulein, brown hollow circles) mice following chronic pancreatitis. Data are mean $\pm$ s.e.m.; Student's t -test, $p < 0.05$ indicated on graph. (C) Flow cytometry contour plots showing gating strategy used to identify CD19⁺ B cells from spleens (top) or pancreata (bottom) following chronic pancreatitis. (D) Proportion Ly-6C^{hi}Ly-6G^{lo} monocytic myeloid-derived suppressor cells (MDSC-M), Ly-6C^{lo}Ly-6G^{hi} polymorphonuclear neutrophil MDSCs (MDSC-P), Ly-6C^{lo}Ly-6G^{lo} monocytes, and Ly-6C^{med}Ly-6G^{hi} neutrophils of CD11b⁺ myeloid cells in WT, iC, KC, KiC, or KPC pancreata following chronic pancreatitis as determined by flow cytometry. Data are mean $\pm$ s.e.m.; Student's t -test, $p < 0.05$ indicated on graph. (E) Flow cytometry contour plots of Ly-6G and Ly-6C expression in CD11b⁺ myeloid cells showing gating strategy used to identify myeloid subpopulations in (D) from spleens (top) or pancreas (bottom) following chronic pancreatitis. Statistic represents mean $\pm$ s.e.m. (F-J) Quantification (top) or flow cytometry histograms (H-J, bottom) of PD-1 (F), MHCII (G), CD80 (H), PD-1 (I), or PD-1H (J) expression by MDSC-M, MDSC-P, DCs, and macrophages from KC (blue line) or KiC (red line) pancreas. Myeloid populations gated as in (E). Data are mean $\pm$ s.e.m.; significance determined using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli with $Q = 5\%$, $q < 0.05$ indicated on graph, or p -values listed when $q > 0.05$ but $p < 0.05$. All data are representative of five independent experiments.

remained unchanged in KiC mice (**Figure 17A**), the proportions of the immunosuppressive Ly-6C^{hi}Ly-6G^{lo} monocytic myeloid-derived suppressor cells (MDSC-M) and Ly-6C^{lo}Ly-6G^{hi} polymorphonuclear neutrophil MDSCs (MDSC-P) CD11b⁺ subpopulations were significantly increased in KiC mice (**Figure 17D-E**). KiC pancreas-infiltrating DCs and macrophages expressed substantially increased amounts of MHCII, CD80, and PD-1 (**Figure 17H**), indicative of the emergence of a more activated albeit potentially immunosuppressive population of APCs following *iASPP* deletion, analogous to the PD-1⁺ myeloid and DC populations observed in Ki lung adenocarcinoma (**Figure 12G**). In support of the possibility of myeloid immunosuppression following *iASPP* deletion, KiC pancreas-infiltrating MDSC-Ms expressed elevated PD-1 and CD80 (**Figure 17F,H,I**). Notably, KiC pancreatic MDSC-M, MDSC-P, DCs, and macrophages exhibited slightly elevated PD-1H (**Figure 17J**), suggesting that pancreatic expression of *iASPP* attenuates immune tolerance by suppressing expression of PD-1H by MDSCs and APCs. These data indicate that MDSCs and PD-1⁺ DCs and macrophages contribute to an immunosuppressive tumor microenvironment in KiC mice following chronic pancreatitis.

iASPP-deficient chronically-inflamed pancreatic tumors have immunosuppressed CD4⁺ and CD8⁺ T cells

Surprisingly, *iASPP* deletion led to significantly increased CD4⁺ and CD8⁺ T cell infiltration into the pancreas following chronic pancreatitis (**Figure 18A**). Although KiC pancreas-infiltrating CD4⁺ T cells expressed increased CD44, they also increased expression of PD-1H (**Figure 18D**), suggesting that they might be subject to immunosuppression. Indeed, while CD4⁺ and CD8⁺ T cells secreted substantially

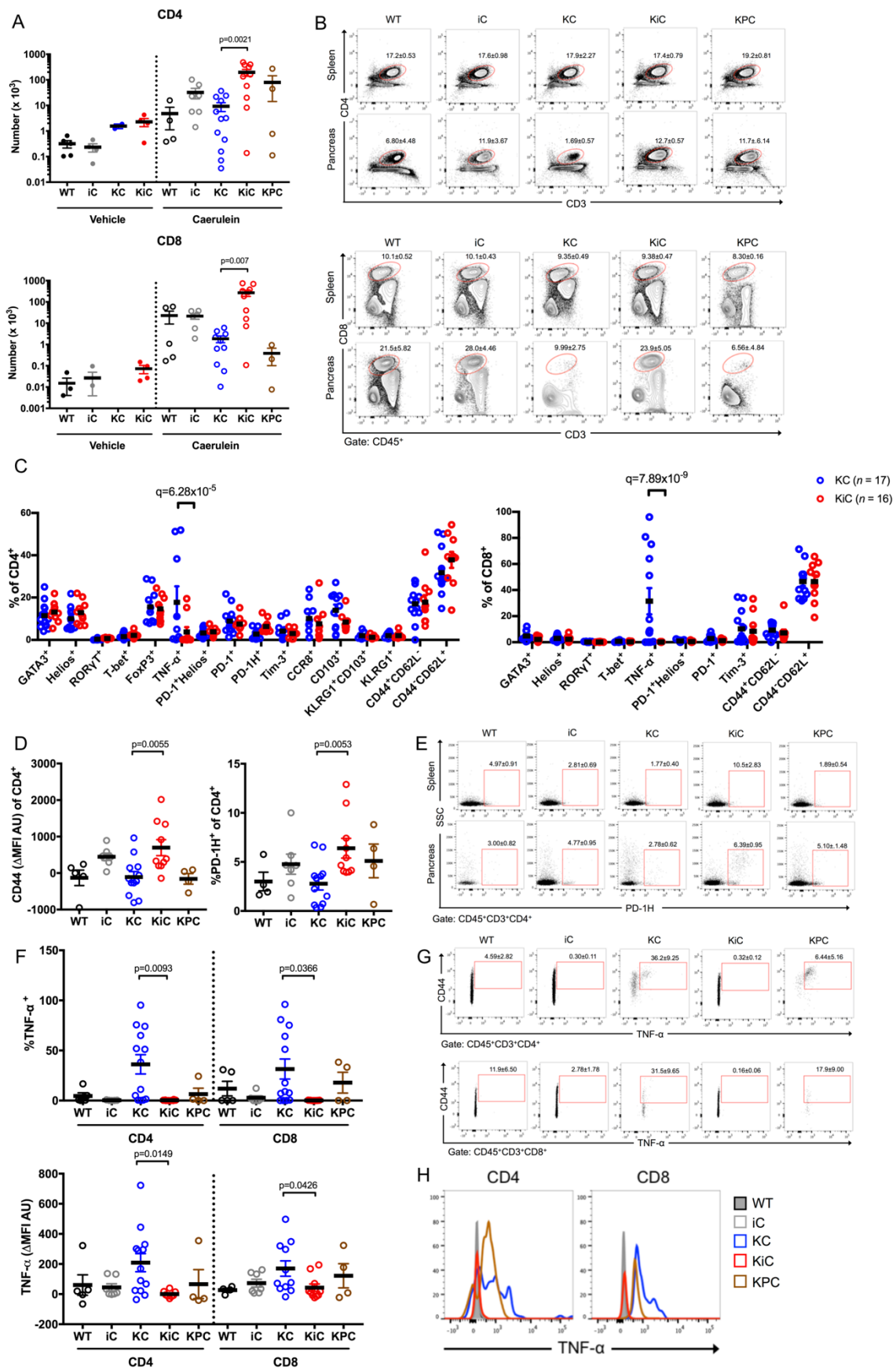


Figure 18: Pancreatic iASPP is necessary for TNF- α secretion by CD4⁺ and CD8⁺ T cells in the tumor microenvironment.

(A) Number of CD4⁺ (top) and CD8⁺ (bottom) T cells in pancreas from WT ($n = 6$ for vehicle, black circles; $n = 11$ for caerulein, black hollow circles), iC ($n = 7$ for vehicle, gray circles; $n = 13$ for caerulein, gray hollow circles), KC ($n = 3$ for vehicle, blue circles; $n = 17$ for caerulein, blue hollow circles), KiC ($n = 6$ for vehicle, red circles; $n = 16$ for caerulein, red hollow circles), or KPC ($n = 4$ for caerulein, brown hollow circles) mice following chronic pancreatitis. Data are mean $\pm$ s.e.m.; Student's *t*-test, $p < 0.05$ indicated on graph. (B) Flow cytometry contour plots showing gating strategy used to identify CD4⁺ (top) or CD8⁺ (bottom) T cells from spleens or pancreas following chronic pancreatitis. (C) Quantification of expression of indicated markers of activation, exhaustion, and transcription factors in CD4⁺ (left) and CD8⁺ (right) T cells from KC (blue hollow circles) or KiC (red hollow circles) pancreas determined by flow cytometry. Data are mean $\pm$ s.e.m.; significance determined using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli with $Q = 5\%$, $q < 0.05$ indicated on graph. (D) CD44 (left) and PD-1H (right) expression in CD4⁺ T cells in WT, iC, KC, KiC, or KPC pancreata following chronic pancreatitis as determined by flow cytometry. Data are mean $\pm$ s.e.m.; Student's *t*-test, $p < 0.05$ indicated on graph. (E) Flow cytometry dot plot showing gating strategy used to identify PD-1H⁺CD4⁺ T cells in (D). (F) Proportion (top) and intensity (bottom) of TNF- α expression in CD4⁺ and CD8⁺ T cells from WT, iC, KC, KiC, or KPC pancreata following chronic pancreatitis as determined by flow cytometry. Data are mean $\pm$ s.e.m.; Student's *t*-test, $p < 0.05$ indicated on graph. (G) Flow cytometry dot plot showing gating strategy used to quantify TNF- α expression in CD4⁺ (top) and CD8⁺ (bottom) T cells in (F). (H) Flow cytometry histograms of TNF- α expression in CD4⁺ (left) and CD8⁺ (right) T cells from WT (filled black line), iC (grey line), KC (blue line), KPC (brown line), and KiC (red line) pancreas following chronic pancreatitis. MFI AU, arbitrary units of median fluorescence intensity. All data are representative of five independent experiments.

increased amounts of TNF- α in KC and to a lesser extent KPC mice with chronic pancreatitis, KiC pancreas-infiltrating CD8⁺ T cells secreted less TNF- α than any other genotype of mouse following chronic pancreatitis (**Figure 18F-H**). These data suggest that iASPP promotes functional CD4⁺ and CD8⁺ T cell responses via mitigation of PD-1H expression. Notably, pancreas-infiltrating CD4⁺ T cells did not upregulate PD-1 or Tim-3 in KiC mice relative to KC mice with chronic pancreatitis (**Figure 18C**) as they did in untreated 25-week-old KiC mice, indicating that caerulein-induced chronic pancreatitis does not precisely immunophenocopy normal K-ras driven pancreatic cancer. The persistence of chronic inflammation likely promotes local T cell exhaustion to such an extent that it overcomes the inhibitory effect of iASPP on CD4⁺ and CD8⁺ T cell exhaustion.

iASPP-deficient chronically-inflamed pancreatic tumors have increased activated T_{reg} and $\gamma\delta$ T cells

As observed by immunohistochemistry, KiC mice had a markedly increased amount of pancreas-infiltrating T_{reg} cells following chronic pancreatitis (**Figure 19A-B**). Unexpectedly, I also observed a significant increase in the number of pancreas-infiltrating $\gamma\delta$ T cells in KiC mice with chronic pancreatitis (**Figure 19A-B**). PDAC-infiltrating $\gamma\delta$ T cells have recently been implicated as mediators of pancreatic cancer oncogenesis that inhibit adaptive $\alpha\beta$ T cell anti-tumor immunity via PD-L1 (Daley et al., 2016). The populations of pancreas-infiltrating T_{reg} and $\gamma\delta$ T cells observed in KiC mice were distinctly activated, expressing higher amounts of CD44, CD103 and PD-1 (**Figure 19C-D**). Interestingly, a subpopulation of pancreas-infiltrating CD44⁺CD62L⁻ effector memory T_{reg} cells emerged in KiC mice, with a corresponding decrease in the naive CD44⁻CD62L⁺ compartment (**Figure 20A**). Notably, the

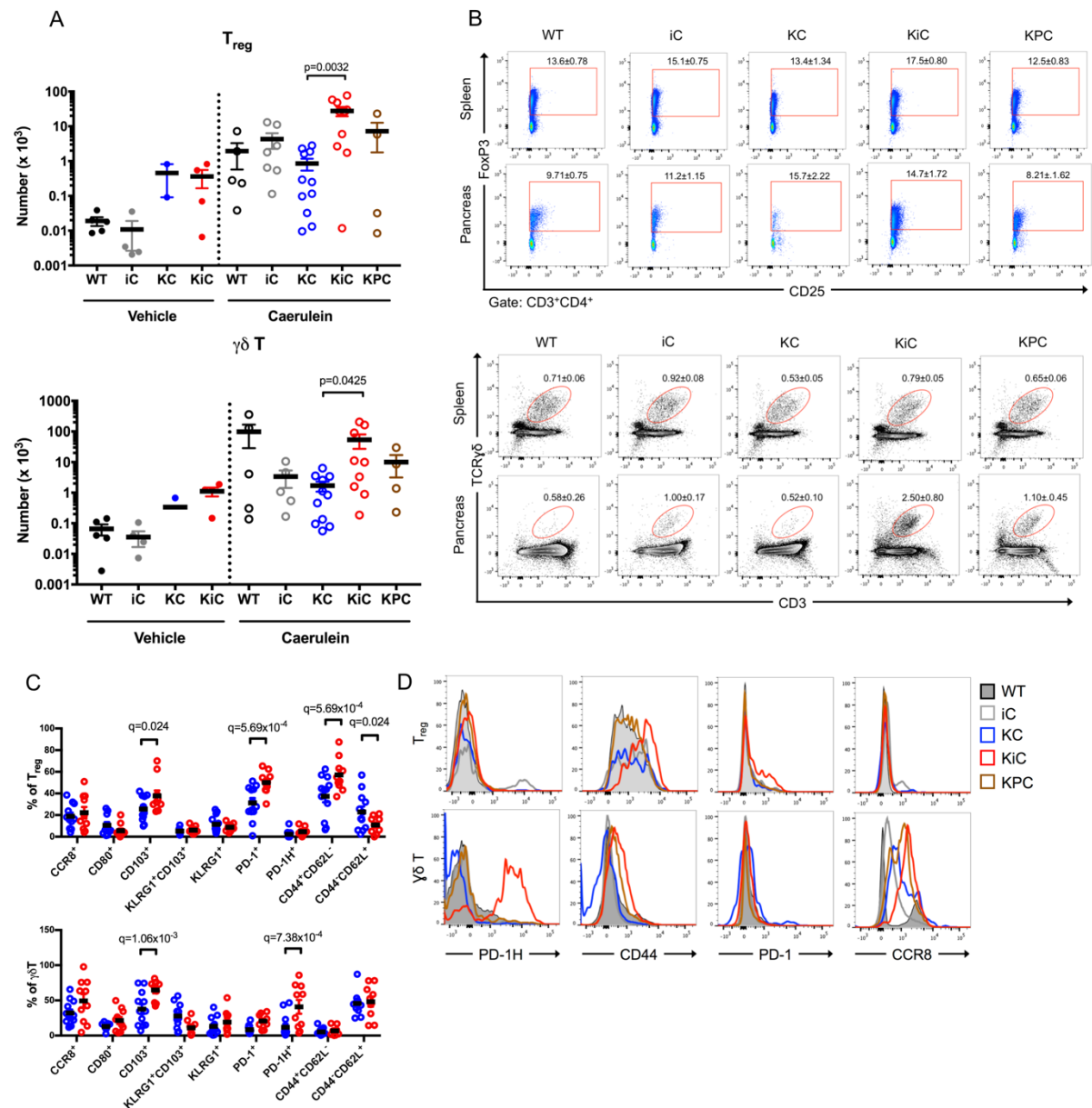


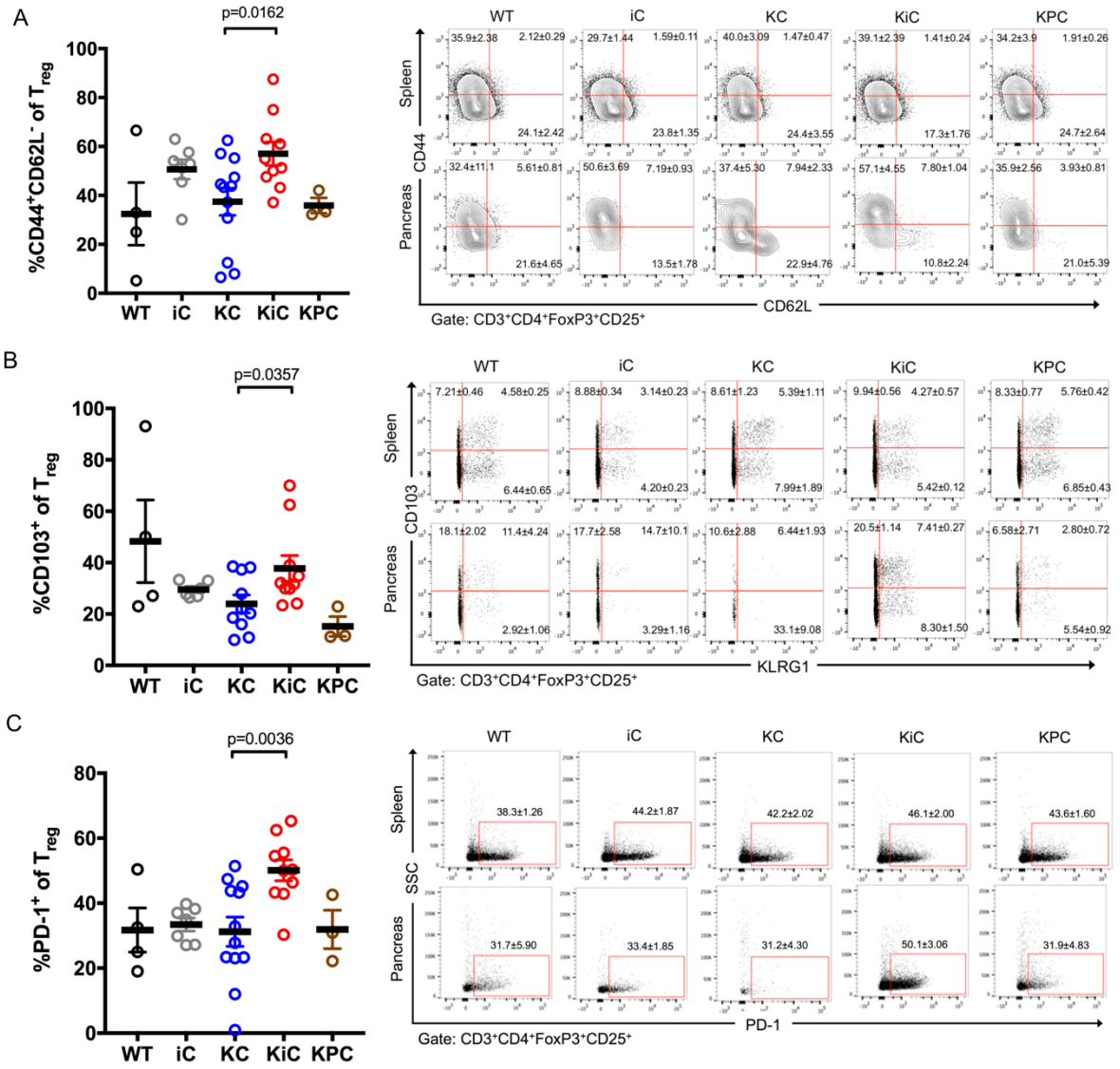
Figure 19: Pancreatic iASPP deficiency promotes T_{reg} and $\gamma\delta$ T cell infiltration and activation in the tumor microenvironment.

(A) Number of CD4⁺CD25⁺FoxP3⁺ T_{reg} (top) and CD3⁺TCR $\gamma\delta$ ⁺ (bottom) T cells in pancreas from WT ($n = 6$ for vehicle, black circles; $n = 11$ for caerulein, black hollow circles), iC ($n = 7$ for vehicle, gray circles; $n = 13$ for caerulein, gray hollow circles), KC ($n = 3$ for vehicle, blue circles; $n = 17$ for caerulein, blue hollow circles), KiC ($n = 6$ for vehicle, red circles; $n = 16$ for caerulein, red hollow circles), or KPC ($n = 4$ for caerulein, brown hollow circles) mice following chronic pancreatitis. Data are mean $\pm$ s.e.m.; Student's t -test, $p < 0.05$ indicated on graph. (B) Flow cytometry pseudocolor (top) or dot (bottom) plots showing gating strategy used to identify T_{reg}

(top) or $\gamma\delta$ T (bottom) cells from spleens or pancreata following chronic pancreatitis. (C) Quantification of expression of indicated markers of activation T_{reg} (top) and $\gamma\delta$ T (bottom) cells from KC (blue hollow circles) or KiC (red hollow circles) pancreas determined by flow cytometry. Data are mean $\pm$ s.e.m.; significance determined using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli with $Q = 5\%$, $q < 0.05$ indicated on graph. (D) Flow cytometry histograms of PD-1H, CD44, PD-1, and CCR8 expression in T_{reg} (top) and $\gamma\delta$ T (bottom) cells from WT (filled black line), iC (grey line), KC (blue line), KPC (brown line), and KiC (red line) pancreas following chronic pancreatitis. All data are representative of five independent experiments.

changes in T_{reg} phenotype in KiC mice were not recapitulated in KPC mice (**Figure 20A-C**), suggesting that *iASPP* deletion itself rather than the secondary effect of more advanced tumor stage is responsible for activating a distinct population of tumor-infiltrating T_{reg} cells. KiC pancreas-infiltrating $\gamma\delta$ T cells also expressed higher amounts of CCR8 (**Figure 20D**), a chemokine receptor recently identified as a marker of tumor-infiltrating T_{reg} cells of unknown function whose expression strongly correlates with worse breast cancer (Plitas et al., 2016), non-small cell lung cancer and colorectal cancer prognosis (De Simone et al., 2016). Together these observations indicate that *iASPP* prevents formation of an immunosuppressive tumor microenvironment by mitigating local activation of T_{reg} and $\gamma\delta$ T cells due to chronic pancreatitis, and is therefore necessary for establishment of a functional anti-tumor adaptive immune response.

Given the elevated expression of PD-1H on CD4⁺ and T_{reg} cells observed in aged mice (**Figure 5E-G**), EAE (**Figure 8K**), and lung adenocarcinoma (**Figure 14K**) following *iASPP* loss, I examined whether *iASPP* affects T_{reg} or $\gamma\delta$ T cell expression of PD-1H in KiC pancreatic cancer. Although PD-1H expression was unaltered on KiC pancreas-infiltrating T_{reg} cells, $\gamma\delta$ T cells expressed considerably higher amounts of PD-1H (**Figure 20G-H**). Furthermore, flow cytometric analysis of transformed cancerous pancreatic cells revealed that loss of *iASPP* in combination with expression of oncogenic *K-ras*^{G12D} led to significantly higher levels of PD-1H, but not PD-L1, on tumor cells themselves (**Figure 20I-J**). Because *Pdx1-Cre* limits deletion of *iASPP* to pancreatic cells, these data suggest that transformed KiC tumor cells modulate the surrounding tumor immune microenvironment via expression of PD-1H which might promote PD-1H expression by tumor-infiltrating lymphocytes via



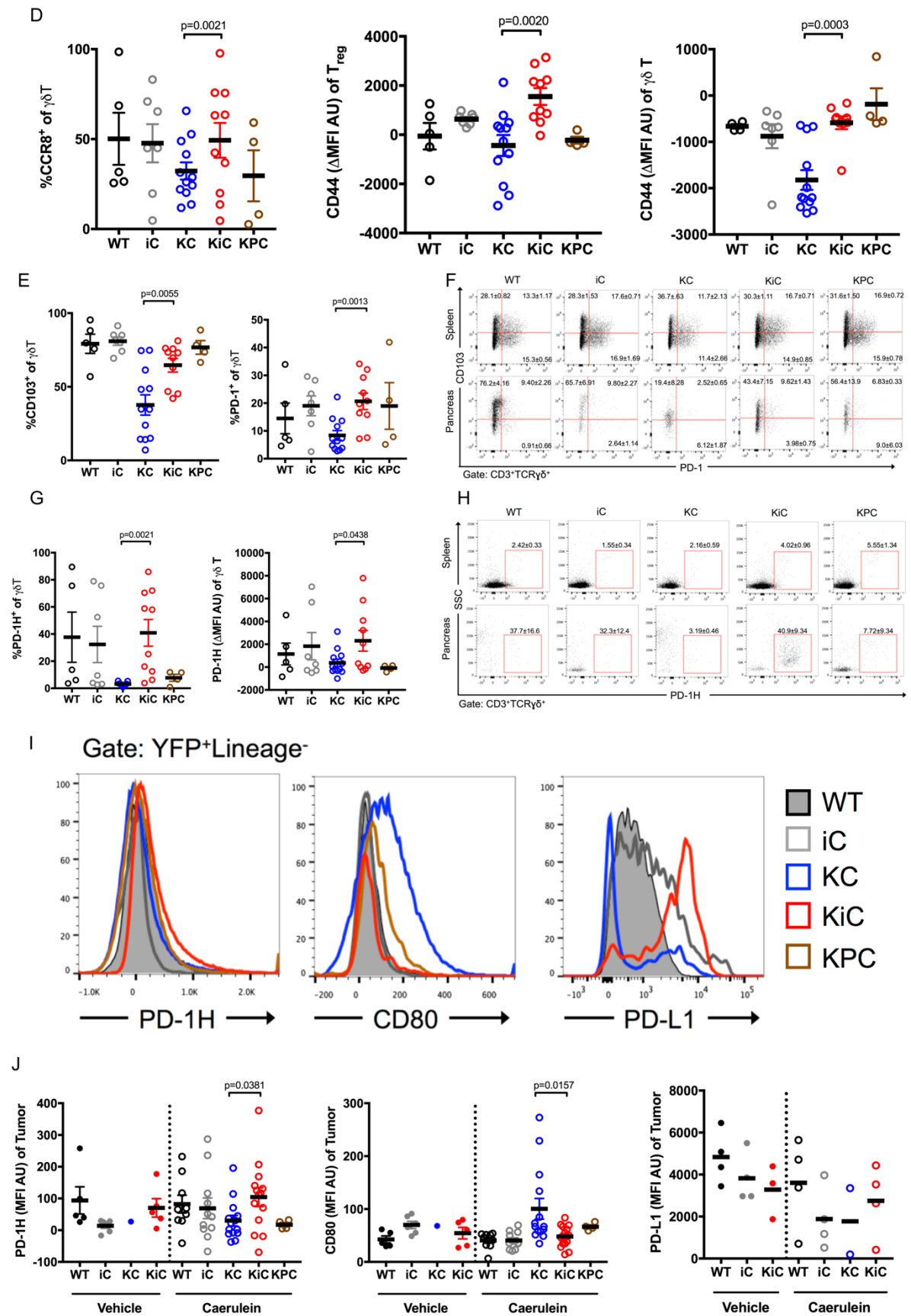


Figure 20: T_{reg} and $\gamma\delta$ T cells infiltrating iASPP-deficient pancreata have a distinctly activated phenotype

(A-C) Quantification (left) and flow cytometry contour plot (right) of CD44 and CD62L (A), CD103 and KLRG1 (B), and PD-1 (C) expression by pancreatic T_{reg} cells following chronic pancreatitis. (D) Quantification of CCR8 expression by $\gamma\delta$ T cells (left), CD44 expression by T_{reg} cells (middle), and CD44 expression by $\gamma\delta$ T cells (right) from pancreas following chronic pancreatitis. (E) Quantification of CD103 (left) and PD-1 (right) expression by pancreatic $\gamma\delta$ T cells following chronic pancreatitis. (F) Flow cytometry dot plot of CD103 and PD-1 expression by pancreatic $\gamma\delta$ T cells following chronic pancreatitis. (G) Proportion (left) and intensity (right) of PD-1H expression by pancreatic $\gamma\delta$ T cells following chronic pancreatitis. (H) Flow cytometry dot plot showing gating strategy used to identify PD-1H⁺ $\gamma\delta$ T cells in (G). (A-G) Data are mean $\pm$ s.e.m.; Student's *t*-test, *p*<0.05 indicated on graph. (I) Flow cytometry histograms of PD-1H (left), CD80 (middle), and PD-L1 (right) expression by transformed pancreatic tumor cells from WT (*n* = 9, filled black line), iC (*n* = 11, gray line), KC (*n* = 15, blue line), KiC (*n* = 14, red line), or KPC (*n* = 4, brown line) mice following chronic pancreatitis. Transformed pancreatic cells are gated from mice with the *R26^{LSL-EYFP}* allele as EYFP⁺CD3⁻CD4⁻CD8⁻CD11c⁻CD11b⁻F4/80⁻MHCII⁻. (J) Quantification of PD-1H (left), CD80 (middle), and PD-L1 (right) expression by pancreatic tumor cells measured by flow cytometry as defined in (I). Data are mean $\pm$ s.e.m.; Student's *t*-test, *p*<0.05 indicated on graph. For PD-L1 measurement, horizontal bars represent sample means and WT (*n* = 4 for vehicle, *n* = 4 for caerulein), iC (*n* = 4 for vehicle, *n* = 4 for caerulein), KC (*n* = 2 for caerulein), and KiC (*n* = 3 for vehicle, *n* = 4 for caerulein). MFI AU, arbitrary units of median fluorescence intensity. All data are representative of five independent experiments.

juxtacrine signaling. PD-1H⁺ DCs, MDSCs, and $\gamma\delta$ T cells may then orchestrate the establishment of an immunosuppressive microenvironment that hinders functional anti-tumor immune responses.

These data demonstrate that chronic pancreatitis recapitulates many but not all of the immune phenotypes observed in KiC pancreatic cancer. With chronic pancreatitis KiC mice consistently developed a strongly immunosuppressive tumor microenvironment characterized by activated MDSCs, T_{reg}, and $\gamma\delta$ T cells and functionally inept CD4⁺ and CD8⁺ T cells. Together, these findings suggest that tumoral expression of iASPP can hinder chronic pancreatitis-driven PDAC development by facilitating anti-tumor immune responses and inhibiting PD-1H mediated immunosuppression.

CHAPTER 7: Discussion and future directions

iASPP and p53 as master regulators of immune tolerance

Here I identify iASPP as a novel regulator of immune tolerance. These data suggest that iASPP diametrically opposes p53-mediated immunosuppression by inhibiting its transactivation of genes such as PD-1H, thereby promoting adaptive immune responses while antagonizing mechanisms of immune tolerance. iASPP can promote autoimmune responses *in vivo* by limiting T_{reg} and $\gamma\delta$ T cell activation (**Figure 6**), while it can also enhance cancer immunosurveillance by inhibiting T cell exhaustion and antagonizing T_{reg} and $\gamma\delta$ T cell-mediated immune tolerance (**Figure 20**). These mechanisms of immunity are maintained by distinct cell types in which iASPP has complementary functions, namely oncogenically transformed cancer cells and CD4⁺ T cells. These observations can be integrated into a model in which iASPP inhibits p53-mediated PD-1H cross-talk between tumor cells, APCs, and CD4⁺ T cells, ultimately shifting the balance of the adaptive immune response from tolerance to immunity (**Figure 21**).

Under this model, iASPP and p53 function as master regulators of transcriptional profiles intrinsic to dying cells that influence their adjuvanticity, thereby communicating to the immune system whether physiologic or unnatural cell death has occurred. During homeostatic tissue turnover, p53 induces tolerogenic apoptosis via transcriptional transactivation of the immunosuppressive cell surface molecule PD-1H (Yoon et al., 2015) and suppression of NF- κ B-mediated pro-

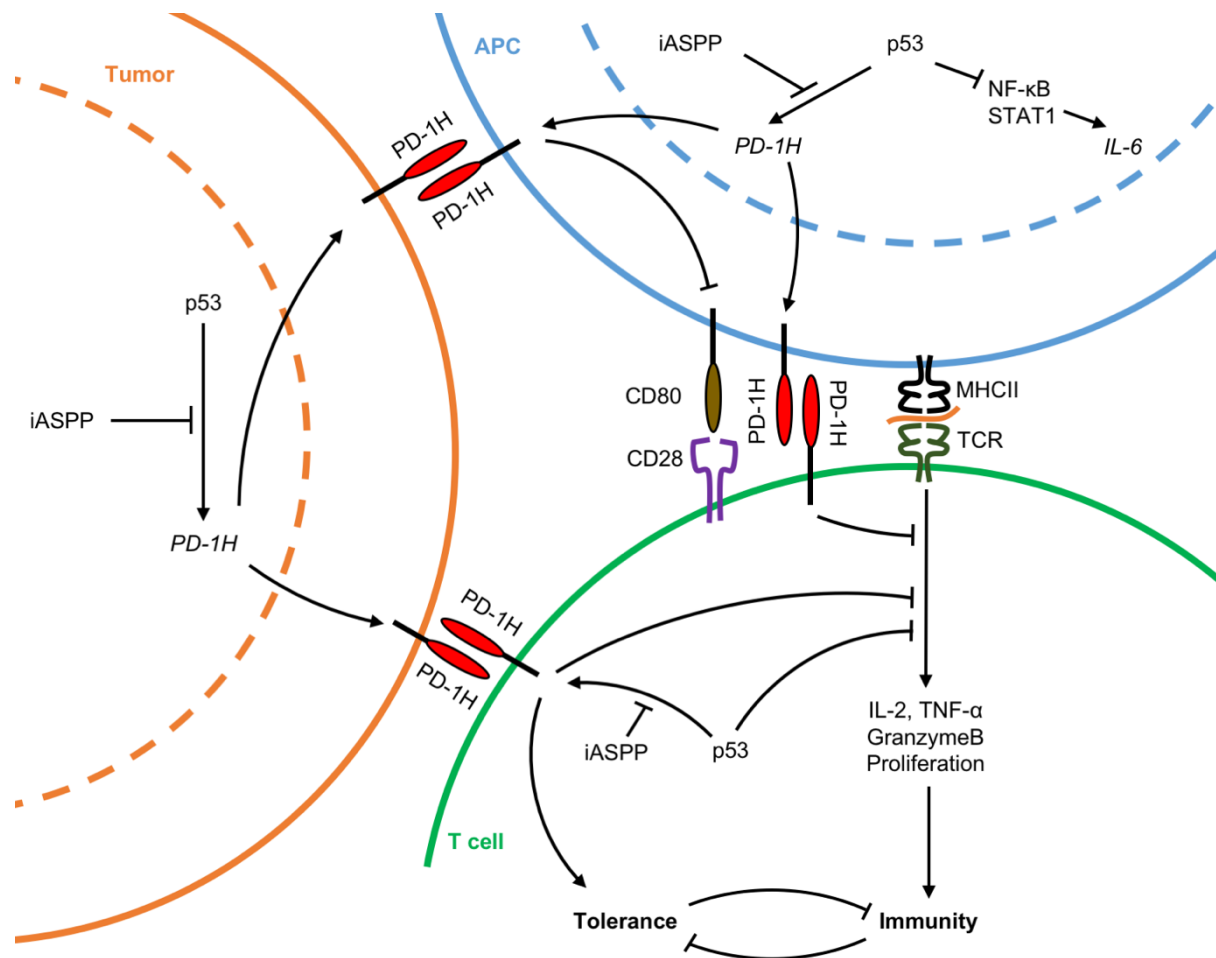


Figure 21: Proposed model of iASPP-mediated loss of immune tolerance.

iASPP promotes autoimmunity by inhibiting p53-mediated mechanisms of immune tolerance. p53 promotes immunosuppressive consequences of tolerogenic apoptosis and phagocytosis by activating transcription of PD-1H and expression of anti-inflammatory cytokines and checkpoint molecules. Surface expression and engagement of PD-1H amongst tumor, antigen presenting cells (APCs), and T cells attenuates endogenous TCR-dependent T cell activation and APC pro-inflammatory cytokine secretion and co-stimulation. iASPP-deficient mice lack regulation of p53-mediated immune tolerance, leading to supraphysiological expression of PD-1H and aberrant p53 immunosuppression. Loss of iASPP therefore tips the immunological balance toward tolerance, making iASPP-deficient mice more resistant to autoimmune disease but more susceptible to cancer development.

inflammatory cytokine release (Komarova et al., 2005; Liu et al., 2009a; Lowe et al., 2014; Margulies and Sehgal, 1993; Santhanam et al., 1991). Apoptotic cell surface expression of PD-1H might then physically promote tolerogenic phagocytosis by macrophages and DCs (Le Mercier et al., 2014; Yoon et al., 2015) while also directly mediating immunosuppression of TCR-driven CD4⁺ T cell activation (Flies et al., 2014; Le Mercier et al., 2014; Wang et al., 2011). Under this model, iASPP antagonizes p53-mediated tolerogenic apoptosis following infection or malignancy, leading to the expression of a fundamentally altered transcriptional program in dying cells. Downregulation of PD-1H allows for sufficient adjuvanticity in combination with inherent antigenicity for the development of a functional adaptive immune response, promoting loss of tolerance.

In addition to the autoimmune disease and autochthonous tumor models described here, other immunological disease models might be similarly influenced by iASPP inhibition of p53-mediated immunosuppression. For example, under the model described above, I hypothesize that iASPP deficient mice would be more susceptible to infection with influenza or hepatitis C virus. Analogous to the accelerated tumor development observed *in vivo*, chronic infection of iASPP deficient mice with LCMV clone 13 might lead to a less robust antigen-specific T cell response, accelerated onset of cytotoxic T cell exhaustion, and overall greater viral load. Potential future experiments also include generation of bone marrow chimeric mice to investigate whether the T cell-intrinsic immunosuppressive role of iASPP deficiency is sufficient to recapitulate the multi-disease implications of iASPP loss observed *in vivo*. If these predictions hold true, these findings would reinforce the potential of iASPP as an

immunological target with functions in myriad diseases, in addition to autoimmune disease and cancer.

A novel tumor-suppressive role of iASPP

The consequences of iASPP influence on immunogenic apoptosis were most striking in genetically-engineered mouse models (GEMMs) of cancer deficient for iASPP. While iASPP was necessary for efficient metaplastic transformation *in vitro* (**Figure 15**), its presence in neoplastic cells paradoxically hindered tumor growth and development *in vivo* (**Figure 13**), supporting a novel non-cell autonomous role of iASPP in preventing tumor development. These findings directly contradict the widely-accepted presumption that iASPP is a constitutive oncogene (Dong et al., 2015). However, this presumption was formed based on *in vitro* studies (Cai et al., 2012; Chen et al., 2010, 2014; Lin et al., 2011; Liu et al., 2009b, 2011; Lu et al., 2010; Pang et al., 2010; Zhang et al., 2011a) that fail to appreciate confounding non-cell autonomous influences present *in vivo*, and based on observational studies of iASPP expression in advanced human tumors or xenograft transplants (Bergamaschi et al., 2003; Cai et al., 2012; Chen et al., 2010; Jia et al., 2014; Lin et al., 2011; Liu et al., 2016b; Lu et al., 2010, 2013; Morris et al., 2014; Pang et al., 2010; Saebø et al., 2006; Zhang et al., 2005), which perhaps fail to appreciate the role of iASPP in neoplastic lesions. While iASPP might function as an oncogene once tumors have escaped immunosurveillance, its expression in developing tumors is critical for the establishment a functional anti-tumor immune response that hinders tumor growth and development. More generally, these findings support the importance of genetically-engineered autochthonous mouse models of tumor development that

recapitulate the entire process of immunoediting (Mittal et al., 2014), rather than *in vitro* growth characterization or xenograft models (Le Magnen et al., 2016).

Here I demonstrate that iASPP serves a non-cell autonomous tumor suppressive role by promoting immunosurveillance of neoplastic lesions. iASPP deletion in K-ras driven lung and pancreatic neoplasias elicited a more immunosuppressive tumor microenvironment, characterized by increased tumor-infiltrating PD-1⁺CD11b⁺, T_{reg}, and $\gamma\delta$ T cells, MDSC activation, and increased expression of PD-1 and Tim-3 but decreased TNF- α secretion by CD4⁺ and CD8⁺ T cells (**Chapter 5**). Although T cell exhaustion was assessed by PD-1 expression and impaired cytokine secretion, additional markers of T cell exhaustion such as CD160, Lag-3, TIGIT, CD73, and CD39 (Antonioli et al., 2013; Fang et al., 2016; Wherry and Kurachi, 2015) are appealing future candidates for genes modulated by iASPP expression. Increased presence of T_{reg} cells in lung tumor-associated tertiary lymphoid structures (Boissonnas et al., 2010; Fridman et al., 2012; Joshi et al., 2015; Teng et al., 2010), MDSC expansion (Gabrilovich and Nagaraj, 2009; Gabrilovich et al., 2012; Pylayeva-Gupta et al., 2012), and T cell exhaustion assessed by PD-1 and Tim-3 expression (Sakuishi et al., 2010; Wherry, 2011) all correlate with increased tumor development.

Notably, iASPP-deficient tumors also displayed novel mechanisms of immune escape not previously associated with lung or pancreatic cancer. CD11b⁺ myeloid cells associated with iASPP-deficient lung tumors (**Figure 12H**) and MDSC-Ms, DCs, and macrophages associated with iASPP-deficient pancreatic cancer (**Figure 17I**) expressed increased PD-1, whose expression on tumor-associated macrophages

was recently identified to limit phagocytosis and anti-tumor immunity in syngeneic tumor models (Gordon et al., 2017). These results suggest that the striking clinical efficacy of PD-1/PD-L1 blockade in various cancers (Hamid et al., 2013; Pardoll, 2012; Topalian et al., 2012, 2014) might be due in part to T cell-independent effects on tumor-associated macrophages and DCs. $\gamma\delta$ T cells were not only increased in chronically inflamed iASPP-deficient pancreatic cancer, but also expressed increased amounts of CCR8 and CD103 (**Figure 20**). CD103 is a well-characterized hallmark of activated tumor-infiltrating T_{reg} cells (Anz et al., 2011; Sather et al., 2007), while CCR8 is a recently identified marker of tumor-infiltrating T_{reg} cells that correlates with negative prognosis (De Simone et al., 2016; Plitas et al., 2016). $\gamma\delta$ T cells have recently been implicated in PDAC development via a novel immunosuppressive phenotype that specifically inhibits adaptive $\alpha\beta$ T cell-mediated anti-tumor immunity (Daley et al., 2016), although $\gamma\delta$ T cell expression of CCR8 or CD103 has not been previously described. Therefore, CCR8 and CD103 are potential markers of activated pancreatic tumor-infiltrating immunosuppressive $\gamma\delta$ T cells. More detailed phenotyping of the expanded $\gamma\delta$ T cell population observed in iASPP-deficient mice might allow for identification of distinct immunosuppressive subpopulations, such as V δ 1⁺CD27⁺CD25^{hi}, V δ 2⁺, or CD39⁺ $\gamma\delta$ T cells (Bhatnagar et al., 2017; Li et al., 2011b; Wu et al., 2017a). Chronic pancreatitis also resulted in significantly increased B cell infiltration in iASPP-deficient pancreatic cancer (**Figure 17B**), which might represent regulatory B cells (Fremd et al., 2013; Rosser and Mauri, 2015; Yoshizaki et al., 2012) or a recently identified immunosuppressive pro-tumorigenic IgA⁺IL-10⁺PD-L1⁺ B cell population (Shalapour et al., 2015). Indeed, IgA⁺IL-10⁺PD-L1⁺ B cells critically require PD-1/PD-L1 pathway activation from CD4⁺PD-1⁺CXCR5⁺BCL6⁺ T follicular helper cells (M. Karin, unpublished), and

CD4⁺PD-1⁺ T cells were increased in iASPP-deficient pancreatic cancer (**Figure 14E**). However, definitive immunophenotyping of the expanded B cell population, as well as the CD4⁺PD-1⁺ T cells, observed in iASPP-deficient pancreatic cancer will require further experimentation. The immunosuppressive tumor microenvironment observed in iASPP-deficient tumors might also be reflective of an expanded M2 relative to M1 macrophage population, although macrophage phenotypic markers such as IL-12, IL-10, CD163, CD38, and Egr2 (Jablonski et al., 2015; Röszer, 2015) were not examined here.

Although iASPP deletion promoted a more tolerogenic tumor microenvironment in autochthonous mouse models of lung and pancreatic cancer, the cell-intrinsic oncogenic properties of iASPP might dominate in other cancer types with less significant immunosurveillance. Indeed, not only does iASPP exhibit differential localization depending on cell type (Lu et al., 2013; Notari et al., 2011, 2015; Slee et al., 2004), but neoplastic cells also employ vastly diverse mechanisms of immune escape depending on the tissue context (DuPage and Jacks, 2013; Prehn, 1968). Future experiments in different mouse models of cancer are necessary to assess the universality of iASPP non-cell autonomous tumor-suppression. It also remains unclear whether iASPP promotes anti-tumor immune responses in an antigen-specific or non-specific manner. This question may easily be addressed in future experiments by delivering known immunogenic cDNA in the tumor-initiating virus encoding Cre recombinase. For example, intratracheal delivery of lentivirus encoding both ovalbumin and Cre recombinase followed by monitoring of SIINFEKL-specific immune responses either with tetramer or adoptive transfer of OT-I transgenic T cells (DuPage et al., 2011; Joshi et al., 2015) would allow for detailed

dissection of the differential effects of tumoral iASPP deletion on tumor antigen-specific and non-specific T cells.

Another advantage of GEMMs is the precise manipulation of combinatorial genetic insults in a tissue-specific manner. Many of the immune phenotypes observed following loss of iASPP in tumors expressing oncogenic *K-ras*^{G12D} were not observed following loss of iASPP in cells with wildtype *K-ras*, suggesting that oncogenic *K-ras*^{G12D} cooperates with iASPP deficiency to promote immunosuppression. In addition to the greater immunological insult provided by a more advanced neoplasia, oncogenic *K-ras*^{G12D} likely contributes to iASPP-regulated immunosuppression by activating endogenous p53. Upregulation and increased function of p53 in response to oncogenic stress is a well-documented homeostatic response to malignancy (Halazonetis et al., 2008; Junttila et al., 2010; Kruiswijk et al., 2015). Therefore, tumors expressing oncogenic *K-ras*^{G12D} and lacking iASPP functionally receive two independent stimuli to the p53 pathway, likely contributing to the greater immunophenotypic consequences observed compared to those in tumors bearing iASPP mutations only, supporting the signaling pathway proposed in **Figure 21**.

Despite similarly accelerated growth dynamics, tumors expressing oncogenic *K-ras*^{G12D} and lacking p53 were phenotypically distinct from tumors expressing oncogenic *K-ras*^{G12D} and lacking iASPP, suggesting that the mechanism underlying p53 tumor suppression is orthogonal to the mechanism underlying iASPP tumor suppression. The tumor suppressive function of p53 is well characterized; p53 responds to oncogenic stress by inducing cell cycle arrest and eventual apoptosis (Evan and Vousden, 2001; Kruiswijk et al., 2015). Here I describe a novel non-cell

autonomous tumor suppressive function of iASPP via inhibition of p53-mediated immunosuppression. Surprisingly, opposing manipulations of the same signaling pathway – deletion of p53 or deletion of its inhibitor iASPP – resulted in similar acceleration of tumor growth. This confusing result is likely due to etiological dominance of p53-mediated cell cycle arrest over p53-mediated immunosuppression, as greater regulatory redundancy and compensation exists in immunosuppression, in which p53 has a relatively minor role (Muñoz-Fontela et al., 2016), than cell cycle arrest and apoptosis, in which p53 has a pivotal role (Livingstone et al., 1992). Thus, p53-deficient tumors overcome cell cycle arrest while likely still experiencing sufficient immunosuppression, while iASPP-deficient tumors experience supraphysiological immunosuppression that pathogenically outweighs any concurrent increase in cell cycle arrest. Better understanding of these two contradictory roles of p53 and their influence by iASPP will require epistasis experiments in which mice bear tumors lacking both p53 and iASPP.

The precise mechanism of iASPP mediated adjuvanticity remains unclear. Mechanistically, as iASPP was specifically deleted in neoplastic cells in mouse models of both lung adenocarcinoma and pancreatic cancer using either intranasal adenoviral delivery or tissue-specific Cre recombinase, transformed cells themselves must modulate the activation of an otherwise wild type immune system, likely through juxtacrine or paracrine signaling. Immune tolerance following iASPP loss might result from tumor co-opting of regulatory pathways evolved to prevent the development of autoimmune disease. I suggest here that iASPP functions by inhibiting p53-mediated immunosuppression, particularly by modulating p53 transcriptional activation of an expression signature responsible for promoting

tolerance following physiologic cell death. Recently, p53 signaling during apoptosis was demonstrated to promote immune tolerance via PD-1H juxtacrine signaling while undergoing phagocytosis (Yoon et al., 2015). Indeed, PD-1H was overexpressed in iASPP-deficient lung tumor cells (**Figure 12L**) and pancreatic cancer cells (**Figure 20J**), suggesting that iASPP might inhibit p53 transcriptional activation of PD-1H, although more precise ChIP-seq experiments are necessary to determine if iASPP alters p53 binding to the *PD-1H* promoter. Future experiments involving blockade of PD-1H with mAb in combination with iASPP-deficient cancer are necessary to demonstrate a mechanistic role of PD-1H in iASPP-mediated adjuvanticity. Nevertheless, these results suggest that PD-1H might be the tumor cell-intrinsic molecule responsible for non-cell autonomous immunosuppression via homophillic intermolecular interactions with PD-1H on wild type macrophages and CD4⁺ T cells.

Alternatively, pseudo-physiologic apoptosis might also promote tolerogenic phagocytosis via immunosuppressive DAMPs, such as HMGB1 (Kang et al., 2013; Liu et al., 2015) and IL-1 α (Di Paolo and Shayakhmetov, 2016; Grivennikov et al., 2010; Hong et al., 2014), and general immunosuppression via tolerogenic molecules such as S100 family members, extracellular potassium, CXCL1, CD47, externalized cardiolipin or phosphatidylserine, MFG-E8, and thrombospondin, (Acharyya et al., 2012; Balasubramanian et al., 2015; Birge and Ucker, 2008; Coffelt et al., 2015; Eil et al., 2016; Hiratsuka et al., 2006; Jinushi et al., 2007; Majeti et al., 2009; Poon et al., 2014; Sistigu et al., 2014; Suzuki et al., 2012; Wang et al., 2015), although associations between p53 transcriptional activity and these molecules have not been demonstrated. Interestingly, iASPP-deficient keratinocytes expressed markedly

increased S100A8 and S100A9 (K. Chung, unpublished), which is also expressed by apoptotic cancer cells and can both promote cancer cell metastatic properties and recruit immunosuppressive MDSCs (Coffelt et al., 2015; Hiratsuka et al., 2006; Sinha et al., 2008; Wang et al., 2013b). Further investigation of the expression profiles of iASPP-deficient tumors is necessary to determine if these potential cell-intrinsic mechanisms, in addition to PD-1H, are responsible for promoting the non-cell autonomous tolerance observed *in vivo*.

T cell-intrinsic functions of iASPP

Distinct from – albeit complementary to – its immunogenic role in transformed cancer cells, iASPP expression in CD4⁺ T cells can also promote immunity. Although iASPP expression is greatest in the salivary gland, skin, and testis, iASPP is also expressed lowly in lymphocytes (Notari et al., 2011; Uhlén et al., 2015). iASPP-deficient CD4⁺ T cells were less sensitive to TCR mediated stimulation, expressed greater PD-1 upon stimulation, and iASPP-deficient T_{reg} cells exhibited more potent immunosuppression (**Figure 9**). Analogous to the phenotypes observed following tumor cell-intrinsic loss of iASPP, these data suggest that iASPP in CD4⁺ T cells has the orthogonal function of mitigating p53-mediated immunosuppression. p53 exerts numerous tolerogenic pressures on CD4⁺ T cells, such as inhibition of antigen-specific activation and STAT3-mediated Th17 polarization, and transcriptional activation of the *Foxp3* promoter (Kawashima et al., 2013; Watanabe et al., 2014; Zhang et al., 2011b). Additionally, the recently identified p53 transcriptional target PD-1H (Yoon et al., 2015) also has significant tolerizing influence on CD4⁺ T cells, such as inhibition of TCR-responsive activation (Wang et al., 2011) and iT_{reg} differentiation (Wang et al., 2017), although whether p53 transcriptionally activates

PD-1H in CD4⁺ T cells remains unknown. I propose here that iASPP mitigates CD4⁺ T cell suppression by inhibiting p53 transcriptional activation of the tolerizing molecule PD-1H. Indeed, iASPP deficient CD4⁺, T_{reg}, and $\gamma\delta$ T cells expressed elevated levels of surface PD-1H (**Figure 5**). Although the effects of germline deletion of iASPP on autoimmune disease severity were not as pronounced as the effects of tumor-specific deletion of iASPP on tumorigenesis, *iASPP*^{-/-} mice exhibited significantly increased immune tolerance that largely phenocopied the consequences of treatment with a PD-1H agonist (Flies et al., 2014). However, the experiments presented here do not provide definitive evidence of the mechanism of iASPP-mediated adjuvanticity, as demonstration of indirect iASPP inhibition of PD-1H-mediated tolerance requires ChIP-seq experiments demonstrating iASPP influence of p53 transcriptional activation of PD-1H in CD4⁺ T cells.

Although loss of iASPP resulted in many of the expected oppositional phenotypes of p53 deficiency, particularly in CD4⁺ T cells (**Figure 9**), some notable phenotypic differences were observed. Aged p53-deficient mice develop spontaneous systemic autoimmunity characterized by splenomegaly, IgG aggregation in the kidney, autoreactive CD4⁺ T cells (Zhang et al., 2011b), and autoantibodies (Campbell et al., 2012). Although detailed immunophenotyping revealed that the aged iASPP-deficient immune system is generally tolerogenic (**Figure 4**), iASPP-deficient mice had no major phenotypic abnormalities or lymphocyte aggregates observable by gross anatomy and histology (**Figure 2**). Additionally, p53-deficient CD4⁺ T cells exhibit preferential Th17 polarization due to increased sensitivity to IL-6 induced STAT3 phosphorylation (Zhang et al., 2011b) and concurrent decreased T_{reg} polarization due to loss of direct p53 transcriptional activation of *FoxP3* (Kawashima

et al., 2013). PD-1H itself is also necessary for iT_{reg} but not nT_{reg} differentiation, leading to the maintenance of a peripheral pool of iT_{reg} cells (Wang et al., 2017). Although iASPP-deficient mice had increased peripheral total T_{reg} cells *in vivo*, iASPP-deficient naive CD4⁺ T cells had unaltered rates of polarization towards any T helper subset *in vitro* (**Figure 10**). Lastly, p53-deficient macrophages exhibit more sensitive STAT1 (Zheng et al., 2005) and NF-κB activation (Komarova et al., 2005; Murphy et al., 2011), leading to increased secretion of IL-1, IL-6, IL-12, and TNF-α. Recently, p53 transcriptional activation of Batf3 in intratumoral monocytic MDSCs was demonstrated to be essential for the development of anti-tumoral Ly6c⁺CD103⁺ DCs (Sharma et al., 2018). Although cytokine secretion was not examined here, iASPP-deficient macrophages and DCs exhibited normal activation following STAT1 or NF-κB pathway stimulation with IFN-γ or LPS, respectively (**Figure 9J**).

These discrepancies might be due to differences in the fundamental nature of p53 pathway manipulation in *iASPP*^{-/-} versus *p53*^{-/-} mice. p53 deficient mice experience decreased flux through the immunosuppressive arm of p53 transactivation resulting in appreciable spontaneous autoimmune disease in aged mice (Campbell et al., 2012; Zhang et al., 2011b), while iASPP deficient mice experience increased p53-mediated immunosuppression that might not manifest in appreciable differences from wild type mice in the absence of immunological challenge, especially given the low incidence of spontaneous autoimmune disease in aged wild type mice. Notably, oppositional phenotypes were observed after immunological challenge with models of autoimmune disease (**Figure 7**), suggesting that iASPP does indeed decrease flux through the immunosuppressive arm of the p53 pathway. Another potential explanation is that although iASPP loss results in supraphysiological expression of

p53 target genes such as PD-1H (**Figure 5**), these genes are already expressed at functionally saturated levels in wild type cells and only their deficiency following p53 loss results in appreciable phenotypes. However, this is unlikely to be the case given the striking effects on iASPP loss on tumor immunosurveillance (**Figure 14**). Therefore, I hypothesize that congruent rather than oppositional phenotypes of iASPP and p53 manipulation might bear closer resemblance, which can be tested by experimentally overexpressing iASPP *in vivo*. Alternatively, phenotypic discrepancies might also be explained by the well-documented cell-type specific functionality of iASPP, wherein iASPP will not translocate to the nucleus and inhibit p53 transcriptional activity in all cell types (Lu et al., 2013; Notari et al., 2011, 2015; Slee et al., 2004), potentially explaining the null result observed in iASPP deficient macrophages.

p53-independent functions of iASPP

Although the data presented here suggest that iASPP promotes adjuvanticity by antagonizing p53-mediated tolerogenic phagocytosis, both iASPP and p53 have additional independent influences on the immune system. p53 functions in a positive feedback loop to drive type I IFN-mediated antiviral immunity (Takaoka et al., 2003), and can suppress STAT1 and NF- κ B mediated inflammation (Komarova et al., 2005; Zheng et al., 2005). Similarly, iASPP can inhibit NF- κ B subunit RelA/p65-mediated inflammation and pro-proliferative functions (Falik-Zaccai et al., 2017; Herron et al., 2005; Laska et al., 2007). Direct binding between iASPP and RelA/p65 (Yang et al., 1999a) and ensuing regulation even in the absence of p53 (Laska et al., 2009) indicate that iASPP inhibition of RelA/p65 is functionally orthogonal to its inhibition of p53. It is therefore tempting to hypothesize that the accelerated tumorigenesis

observed in the absence of iASPP is due to increased RelA/p65 promotion of survival, but the lack of acinar to ductal metaplasia observed following iASPP loss *in vitro* (**Figure 12**) suggests that iASPP is a cell-intrinsic oncogene and its inhibition of p53 pro-apoptotic function dominates the effects of inhibiting RelA/p65 anti-apoptotic activity. Notably, these results do not preclude the possibility that iASPP might instead promote RelA/p65 anti-apoptotic activity indirectly by preventing p53 inhibition of NF- κ B, although this is unlikely given that iASPP modulates the transcriptional activity but not expression of p53 (Bergamaschi et al., 2003) while p53 is thought to independently inhibit NF- κ B transcriptional activity (Komarova et al., 2005). RelA/p65 expression in mature T_{reg} cells was recently described to be essential for maintenance of T_{reg} function, identity, and immune tolerance (Oh et al., 2017). Therefore, iASPP inhibition of NF- κ B mediated immune tolerance in T_{reg} cells is an exciting p53-independent mechanism of iASPP immunological influence, although iASPP likely also has T_{reg} cell-extrinsic roles in loss of immune tolerance as tumor cell-specific deletion of iASPP promotes tolerogenic immune responses as well (**Figure 14**). Lastly, I have not investigated the potential inhibitory effect of iASPP on p53-mediated type I IFN responses or, independently, RelA/p65-mediated cytokine release. However, as iASPP had largely anti-tolerogenic effects, its inhibition of p53 or NF- κ B-mediated inflammation is likely negligible *in vivo*, although again this does not preclude the possibility of iASPP indirect promotion of NF- κ B cytokine release via inhibition of p53. Precise dissection of the potential immunomodulatory effects of secondary iASPP interactions with RelA/p65 might be achieved through utilization of more precise genetic disruption of iASPP, for example using the recently generated *iASPP*^{F815A} mouse in which the protein phosphatase 1

(PP1) binding site of iASPP is mutated and is no longer able to inhibit p53-mediated apoptosis (Llanos et al., 2011).

This study identifies iASPP as a novel component of the maintenance of the critical immune homeostasis between tolerance and autoimmunity. Loss of iASPP in cancer cells, CD4⁺ T cells, or with germline deletion elicited strikingly elevated immune tolerance. These data suggest that iASPP and p53 serve as master regulators of the immunological phenotypes of dying cells and thereby orchestrate the ensuing innate and adaptive immune responses to cell death. Although many signaling mediators such as DAMPs and 'eat-me signals' have been previously identified, the underlying pathways responsible for discriminating between physiologic apoptosis and unnatural cell death have remained previously uncharacterized. While this study demonstrates iASPP promotion of non-physiologic cell death adjuvanticity in the context of autoimmune disease and cancer, the normal physiologic role of iASPP in providing adjuvant co-stimulation remains unclear. Identification of the upstream mediators of iASPP expression or activity is necessary to conceptually link unnatural cell death and iASPP adjuvanticity. Likewise, understanding the reasons why iASPP does not function to suppress p53 immunosuppression in cases of physiologic apoptosis could lend insight to potential upstream regulators of iASPP and p53. Maintenance of the delicate balance between immunity and tolerance is essential for the prevention of both autoimmune disease and cancer, and therefore future investigation of iASPP and its regulatory mediators provides an exciting novel opportunity to identify therapeutic targets with multi-disease implications.

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APPENDIX 1: Protocols

Acinar cell culture

Mice: 6-9wk old

Acinar cultures

1. Prepare per pancreas: 30 mL HBSS 5% FBS, 20 mL HBSS 30% FBS
2. Mix 9vol collagen w/ 1vol 10x RPMI1640 on ice, add 1:10 4.2% NaHCO₃
3. Add 250 μ L/well collagen gel to 24 well plate on ice, place at 37°C for >1hr
4. Dissect pancreas into HBSS, wash, mince in 5 mL cold HBSS, into 15mL tube
5. Add 100 μ L 10 mg/mL collagenase P, mix, shake at 37°C for 20 min
6. Add 5 mL cold HBSS 5% FBS, spin 2000 rpm 3', wash by spinning 1500 rpm 3' 3x w/ 5 mL cold HBSS 5% FBS
7. Gently pipette using wide-bore P1000 through 100 μ m mesh, wash mesh w/ 5 mL HBSS 5% FBS
8. Pipette cell suspension on top of 20 mL HBSS 30% FBS, spin 1000 rpm 3'
9. Count, resuspend for 2,000 acini/well (8,000 acini/mL) in **3D Medium**
10. Mix 9vol collagen w/ 1vol 10x RPMI1640 on ice; add equal parts cell suspension, plate mixture in pre-coated 24 well plate at 0.5 mL/well, incubate at 37°C for 1hr
11. Add 1mL warm **3D Medium** $\pm$ 50ng/mL (2000x from 100 μ g/mL stock) TGF- α $\pm$ 1 μ M 4-OHT (1:10,000 from 10mM stock)
12. Replace media after 24hr (NB: careful not to aspirate discs), then every 48hr, analyze on days 3, 5

RNA prep

1. Wash collagen disc w/ HBSS, transfer 4 wells to 15 mL tube w/ 4 mL HBSS w/ 200 μ g/mL (50x; 80 μ L of 10 mg/mL stock) collagenase P, incubate for 30 min at 37 °C, spin 2000 rpm for 3'
2. Resuspend in 1 mL cold HBSS, transfer to eppendorf, spin 5000 rpm for 3'
3. Isolate RNA

Histology

1. Fix w 4% PFA for 30 min at RT, transfer collagen disc to vial w/ 4% PFA, incubate O/N at 4°
2. Tissue process as normal

3D Medium

RPMI 1640

1% FBS

1% P/S

0.1 mg/mL STI (100x from 10mg/mL in ddH₂O, filtered and stored at -20°)

1 µg/mL Dex (2000x from 25 mg in 12.5 mL EtOH, stored at -20°)

10x RPMI

1 pkg RPMI powder

2 g NaHCO₃

100 mL ddH₂O

pH to 7.2

Heat mix

Filter, store at 4° for 2 months

4.2% NaHCO₃

4.2g NaHCO₃

100 mL ddH₂O

Filter, store at 4°

Collagenase P

100 mg Collagenase P

10 mL ddH₂O

Filter, aliquot, store at -20°

4-OHT (10mM)

10 mg 4OHT

2.58 mL EtOH

Store at -20°

Tissue Preparation

RNA

Snap freeze in LN₂, store at -80°

Tail tip

One piece of ear sample + 100ul TDB + 1ul Qiagen Proteinase K, 55C for 2hrs or longer, 95C for 10'

Use 0.2-0.5ul digestion in 10ul PCR.

Serum

1. Cull mouse, pull out eyeball, collect blood in Eppendorf, can also collect from vena cava using syringe, store at RT for 1hr
2. Spin at 1500g for 15min at 4°, no brake, store super at -80°
3. ELISA for IL-6,12,17, TNFa

Tissue ELISA

1. Weigh organ
2. Homogenize in 50 µL/mg RPMI 1% Tx100, centrifuge at 5000g for 10min
3. Store super at -80°C
4. ELISA for IL-1,6

IF and IHC prep

Day 1:

1. Perfuse lobe w/ PLP buffer (made fresh in dark and on ice, see recipe), aim for obvious vessels, typically where lobes are attached to each other
2. Place in 15mL tube filled w/ 5-10 mL of PLP buffer shielded from light
3. Place at 4° O/N on shaker (in cold room)

Day 2:

If IHC:

1. Gently dry off PLP w/ kim wipe, place in plastic cassette
2. Place cassette in beaker filled with 70% ethanol

If thick IF:

1. Gently dry off PLP w/ kim wipe
2. Perfuse w/ 30% sucrose
3. Place at 4° on shaker for 8h
4. Gently dry off 30% sucrose in PBS w/ kim wipe (important)
5. Perfuse w/ 30% OCT in PBS
6. Place in peel-away mold pre-filled with OCT, place block on wet ice
7. After 15-30min, place on dry ice to snap freeze, then store at -80°

FACS:

Important: Perfuse mouse w/ PBS

Spleen/Thymus:

1. Store in RPMI w/ 1% HI FCS + L-Glut + P/S on ice
2. Gently mash on wire mesh in petri dish, spin at 1500 rpm for 5min
3. RBC lysis w/ 1mL for 1:30 @RT, quench w/ media, filter through 100um into 15mL tube, spin
4. Resuspend in <1000ul, adjust to 10x10⁶ cells/mL

LN:

1. Store in RPMI w/ 1% HI FCS + L-Glut + P/S on ice
2. Grind w/ frosted microscope slides over petri dish
3. Spin at 1500 rpm for 5min, resuspend in <300ul, adjust to 10×10^6 cells/mL

Pancreas/Lung/Kidney/Heart

1. Place lobe in 5 mL HBSS w/ collagenase IV (1:50 = 1mg/mL, 125 U/mL) + DNase (Roche, 1:250 = 40 U/mL) + HEPES (1:40 = 25 mM) + 0.5% HI FCS (1:200)
2. gentleMACS
 - a. Pancreas = "m_imp_tumor_04"
 - b. Lung = "m_imp_tumor_01"
 - c. Kidney = "multi_E_01"
 - d. Heart = "m_heart_01"
3. Place in incubator-rotator (in chemical room) for 45 min at 37°
4. gentleMACS (again)
 - a. Pancreas = "m_imp_tumor_04"
 - b. Lung = "m_imp_tumor_01"
 - c. Kidney = "multi_E_02"
 - d. Heart = "m_heart_02"
5. Filter through 70-100um filter, smash, wash
6. Spin at 1700 rpm for 10min
7. RBC lysis w/ 1 mL for 1:30 @RT, quench, spin
8. Resuspend in <200ul, adjust to 10×10^6 cells/mL

HBSS	20 mL	100 mL
C IV	400 μ L	2 mL
DNase	80 μ L	400 μ L
HEPES	500 μ L	2.5 mL
HI FCS	100 μ L	500 μ L

Liver/Brain

1. Store in RPMI w/ 1% HI FCS + L-Glut + P/S on ice
2. Gently mash through 100 μ m filter, spin at 1500 rpm for 5min
3. Resuspend in 2.5 mL 40% Percoll in PBS, underlay w/ 1.5 mL 70% Percoll in RPMI
4. Spin 2000rpm for 25min, no brake
5. Discard fat from top layer, collect interphase in new tube in PBS
6. Spin 1500 rpm for 5min, RBC lysis w/ 1mL for 1:30 @RT, quench w/ media, spin
7. Resuspend in <300 μ L, adjust to 10×10^6 /mL

40%	70%
45 mL PBS	13.5 mL RPMI
30 mL Percoll	31.5 mL Percoll

Lamina Propria

1. Longitudinal open colon, wash colon in PBS
2. Cut into 0.5 cm pieces and incubate in Ca/Mg-free PBS w/ EDTA (1:100=5mM) for 15 min at 37°C, dump super
3. Incubate in RPMI w/ 5% FCS, 20mM HEPES, P/S, Collegenase VIII (100U/mL)
4. gentleMACS "m_imp_intestine_01"
5. Place in incubator-rotor for 60 min at 37°C
6. gentleMACS "m_imp_intestine_01" (again)
7. Filter through 70-100um filter, smash, wash
8. Spin at 1700 rpm for 10min
9. Resuspend in 2.5 mL 40% Percoll in PBS, underlay w/ 1.5 mL 70% Percoll in RPMI
10. Spin 2000rpm for 25min, no brake
11. Discard fat from top layer, collect interphase in new tube in PBS

PBS	20 mL
EDTA	200 μ L

RPMI-FCS	16 mL
HEPES	320 μ L
CVIII	2x 640 μ L

12. Spin 1500 rpm for 5min, RBC lysis w/ 1mL for 1:30 @RT, quench w/ media, spin
13. Resuspend in <300 μ L
14. Count, adjust to 10×10^6 /mL

4x (0.2 M) PB Stock:

1. Dissolve 1.794 Grams of Sodium Phosphate Monobasic Monohydrate in 25 mls dH₂O.
2. Dissolve 12.35 Grams of Sodium Phosphate Dibasic Anhydrous in 188 mls dH₂O.
3. Add #1 and #2 in a cylinder and top off to 500 mL with dH₂O
4. Check pH=7.6. Do not correct with acid or base.
5. This may be stored at room temp for up to 6 months.

2x (0.2M) Lysine Stock:

1. Dissolve 18.2g of L-lysine hydrochloride in 100 ml dH₂O
2. Adjust pH to 7.4 with 5-10N NaOH
3. Add dH₂O to 500mL
4. This may be stored at 4 Degrees C. for up to 3 weeks.

Making Fresh PLP: (20mL, scale as needed)

	20 mL	40 mL	100 mL	125 mL
0.2M PB	5 mL	10 mL	25 mL	31.25 mL
4% PFA	5 mL	10 mL	25 mL	31.25 mL
0.2M Lysine	10 mL	20 mL	50 mL	62.5 mL
NaIO ₄	40 mg	80 mg	200 mg	250 mg

TDB

50 mM KCl
10mM Tris-HCl (pH 9)
0.1% Tx100
0.4 mg/mL ProtK

FACS buffer

2.5 mL heat inactivated FBS (30' @56°)
2.5 mL 20% NaN₃
500 mL PBS

10x ACK Lysis

8.02 g NH₄Cl
0.84 g NaHCO₃
0.29 g EDTA
100 mL H₂O
pH=7.2-7.4

1x ACK Lysis

4.01 g NH₄Cl
420 mg NaHCO₃
145 mg EDTA
500 mL H₂O
pH=7.2-7.4

HEPES (1M)

23.83 g HEPES

100 mL dH₂O

pH 7.4

Collagenase (Gibco 17104-019, 1gm)

Resuspend in 20 mL HBSS, for 50 mg/mL

Digestion

20 mL HBSS

400 µL Collagenase IV (50 mg/mL)

100 µL HI-FCS

500 µL HEPES (1M)

80 µL DNase (10,000 U/mL)

16% PFA

40 g PFA

250 mL H₂O

60°C

+1N NaOH to dissolve

pH 7.2

FACS

1. Process tissues
2. Adjust to 10^7 cells/mL, stain 100 μ L, spin 1500 rpm for 3'
3. *Opt:* for cytokine stains, add Cell Stim Cocktail (TNB-4975, 1:500) + BD GolgiPlug (555029, 1:1000) in RPMI (10% HIFCS, P/S, L-glut) 200 μ L/well for 3-6h @37°
4. Wash 2.5x w/ FACS buffer
5. *Opt:* Fc Block (BD553142, 1:50 = 10 μ g/mL) 60 μ L/ well, 5' on ice, spin
6. Surface stain + viability dye (50-100 μ L/well) 30' on ice
7. Wash 2.5x w/ FACS buffer
8. *If intracellular:*
 - a. Perm/Fix (1vol concentrate + 3vol diluent), 30' @RT or O/N @4° (no longer!), spin
 - b. Perm buffer (1:10 in dH₂O), spin, repeat, spin (don't leave!)
 - c. *Opt:* Fc Block (BD553142, 1:50 = 10 μ g/mL) in Perm buffer 100 μ L/ well, 15' on ice, spin
 - d. Intracellular stain (50-100 μ L/well) in Perm Buffer 30' on ice
 - e. Wash 0.5x w/ Perm Buffer
9. Wash 2x w/ FACS buffer, resuspend in 250-300 μ L
10. *Opt:* Streptavidin stain 10' on ice; wash 2.5x w/ FACS buffer

FACS buffer

2 mL heat inactivated FBS (30' @56°)
2 mL 10% NaN₃
400 mL PBS

10x ACK Lysis

8.02 g NH₄Cl
0.84 g NaHCO₃
29 mg EDTA
100 mL H₂O
pH=7.2-7.4

1x ACK Lysis

4.01 g NH₄Cl
420 mg NaHCO₃
14.5 mg EDTA
500 mL H₂O
pH=7.2-7.4

Aqua viability dye

100 μ L DMSO to 1 vial
Store -20°

Example FACS Panels

APCs

Fluorophore	Target	Dilution
FITC	L/D	1000
PerCPcy5.5	Ly6G	250
PE	CD86/CD80	100
PEcy5	CD24	600
AF594	CD49b	50
Pecy7	CD68	250
APC	PD1H	100
AF700	MHCII	250
BV421	CD11c	100
BV510	CD11b	100
BV650	CD19	150
BV711	F4/80	100
BV785	Ly6C	500

CD4/CD8

Fluorophore	Target	Dilution
FITC	CD45	100
PerCPcy5.5	IFNγ	50
PE	GATA3	50
PEcy5	CD4	300
PE594	Helios	100
PE-Cy7	Tim3	100
APC	CD8a	200
APCcy7	CD3	100
AF700	CD62L	150
BV421	IL17	50
BV510	TNFα	50
BV605	Tbet	50
BV650	RORγT	50
BV711	PD-1	100
BV785	CD44	200

Treg/Tgd

Fluorophore	Target	Dilution
FITC	CD4	100
PCPcy5.5	CCR8	100
PE	TCRgd	100
PEcy5	Foxp3	100
PE594	CTLA4	100
PE-Cy7	CD103	100
APC	PD1H	100
APCcy7	CD3	100
AF700	CD62L	150
BV421	KLRG1	100
BV510	CD25	50
BV605	CD80	50
BV711	PD1	100
BV785	CD44	200

Th subset

Fluorophore	Target	Dilution
FITC	CFSE	
PerCPcy5.5	IFNγ	50
PE	GATA3	50
PEcy5	Foxp3	100
PE594	CTLA4	100
PE-Cy7	Tim3	100
APC	PD1H	100
APCcy7	CD3	100
AF700	CD62L	150
BV421	CD4	100
BV605	Tbet	100
BV650	RORγT	100
BV711	PD-1	100
BV785	CD44	200

Treg

Fluorophore	Target	Dilution
FITC	CFSE	
PE	CD25	100
PEcy5	Foxp3	100
PE594	CTLA4	100
PE-Cy7	CD103	100
APC	PD1H	100
APCcy7	L/D	1000
AF700	CD62L	150
BV421	CD4	100
BV605	Tbet	100
BV650	RORγT	100
BV711	PD-1	100
BV785	CD44	200

In vitro macrophage

Fluorophore	Target	Dilution
FITC	L/D	1000
PerCPcy5.5	IL12p40	50
PE	CD86/CD80	100
Pecy7	CD68	250
APC	PD1H	100
AF700	MHCII	250
BV421	CD11c	100
BV510	CD11b	100
BV711	F4/80	100

In vitro T cell

Fluorophore	Target	Dilution
FITC	CFSE	
PE594	CTLA4	100
PE-Cy7	Tim3	100
APC	PD1H	100
APCcy7	CD3	100
AF700	CD62L	150
BV421	CD4	100
BV510	CD8	100
BV711	PD-1	100
BV785	CD44	200

FACS analyzer (BD X20 Fortessa)

Samples

1. Polystyrene tubes (BioRad Titertube 223-9391 or BD round bottom 5mL)
2. Volume b/w 200-500 μ L (NB: 600-800 μ L for unstained)

Setting Up

1. *If first user:*
 - a. Check waste is empty
 - b. Turn on cytometer (green switch on right side), then computer
 - c. Wait 10' for lasers to warm up
 - d. Remove dH₂O tube w/ sip open, press Prime, wait for Standby, then repeat
 - e. Run dH₂O for 10', then press Standby
 - f. Start BD FACSDiva and login (egarren, hockey123)
2. Run H₂O tube on "High" for 2'

New Experiment

1. "New Experiment"
 - a. Log Dec = 5 log decades
 - b. Cytometer settings
 - i. Delete parameters as needed
 - ii. Select "A" and "H" for FSC and SSC
2. Experiment > Compensation Set up > Create compensation controls
 - a. Open sub folder
 - b. "Unstained"
 - i. "Acquire"
 - ii. Parameters > Set voltages (below 10^{2.5}ish)
 - iii. NB: can change speed on machine using Low/Medium/High buttons (but keep <10K events/ sec)
 - iv. "Stop acquiring"
 - v. *Optional:* check single colors look good
 - vi. Record 10K events, "Acquire", "Record"
 - c. Single colors
 - i. Click small green arrow to left of channel name
 - ii. "Acquire", draw live gate, right click > apply to all
 - iii. Select positive population
 - iv. "Record"
 - d. Experiment > Compensation Setup > Calibrate compensation > Apply only

New Specimen

1. Rename (eg. Stain 1)
2. Click Tube 001
3. Experiment > Experiment layout, enter names of channels
4. "Global Worksheet"
 - a. Draw plot button, draw gating strategy
 - b. NB: can test using unstained / positive controls
 - c. Click plot, then draw gate
 - d. Right click > Show population hierarchy

- e. Can select populations in Inspector to gate
- f. Can also select stoping gate
5. Record (eg. 300K events), "Acquire", "Record", "Stop Recording"
6. "Next Tube", rename

Cleaning

1. Turn speed to High and press Run
2. Run FACSRinse with sip open for 1', then sip closed for 2'
3. Run FACSClean with sip open for 1', then sip closed for 2'
4. Run dH2O with sip open for 1', then sip closed for 2' (NB: check in FACSDiva that with dH2O event rate is <12 events/sec)
5. *If last user*, skip 2-4 and do following:
 - a. Run FACSClean with sip open for 1', then sip closed for 3'
 - b. Run FACSRinse with sip open for 1', then sip closed for 2'
 - c. Run dH2O with sip open for 1', then sip closed for 3' (NB: check in FACSDiva that with dH2O event rate is <12 events/sec)
 - d. Leave dH2O tube and press Standby
 - e. Quit FACSDiva
 - f. Turn off cytometer (green switch on right side)
 - g. Empty waste tank

Laser	Diva Name	Fluorochromes
488nm Blue	B 530/30	FITC, AF488, CFSE, GFP, TO-PRO-1
	B 710/50	PerCP, PCP5.5, PCPe710, 7AAD,PI
633nm Red	R 670/30	APC, AF647, AF633, TO-PRO-3
	R 730/45	AF700, APCcy5.5, DyeCycleRuby
	R 780/60	APCcy7, APC-H7, APCe780
405nm Violet	V 450/50	BV421, PacBlue, BDHorV450, DAPI, CFP, CTV
	V 525/50	BV510, BDHorV500
	V 610/20	BV605, eFluor605NC
	V 670/30	BV650
	V 710/50	BV711
	V 780/60	BV785
560nm YG	YG 586/15	PE, AF555, AF546
	YG 610/20	PI, PE594, PE-CF594, PE-Texasred, MCherry
	YG 670/30	PEcy5, 7AAD, M-Plum
	YG 710/50	PEcy5.5, PE-AF700
	YG 780/60	PEcy7

IF on Tissue

1. Cut fresh 30 μ m sections, air dry 30' @RT
2. Fixation
 - a. Acetone fix 5' @-20
 - b. or Sodium citrate (pH=6) or **Tris-EDTA-Tween** (pH=9)
 - i. Microwave: 30s (until boils), add slides, bring back to boil, leave slides until cool
 - ii. **Pressure cooker**: power=10 temp=120°, bring to boil, add in slides, put on lid, start timing after black valve rises, 3-5', turn off, run under cold water, release pressure, take lid off, run cold water in pot >5', remove slides
 - c. or 4% PFA fix
 - d. or MeOH fix 5' @-20
3. PBS 3x7'
4. Immunomix 1h @RT
5. Primary in Immunomix O/N @4°
6. 0.3% PBS 4x7'
7. Secondary in Immunomix O/N @4°
8. 0.3% PBS-Tx 4x7', DAPI (5 μ g/mL on second wash)
9. PBS 3x5'
10. *Optional*: to reduce bkgd, 0.3% Sudan Black for 10'
11. Mount w/ Vectashield, seal w/ nailpolish

Immunomix (50 mL)

5 mL serum
1 mL 10% BSA
250 μ L 10% NaN₃
150 μ L Tx100
43.75 mL PBS

IHC on Tissue

1. Dewax slides: Histoclear 70% EtOH
2. Sodium Citrate (pH=6) or **Tris-EDTA-Tween** (pH=9) or Sodium Citrate (pH=6)
 - a. Microwave: 30s (until boils), add slides, bring back to boil, leave slides until cool
 - b. **Pressure cooker:** power=10 temp=120°, bring to boil, add in slides, put on lid tightly, start timing after black valve rises, 3-5', turn off, run under cold water, release pressure, take lid off, run cold water in pot >5', remove slides
3. 3% H₂O₂ (Paul 4°) in MeOH for 15' @RT;
4. PBS 1x5'
5. 5% GS in PBS for 1h @RT
6. Primary in 5% GS O/N @4°
7. PBS 3x5'
8. Biotin Goat anti-Rabbit IgG (Vector BA-1000) 1:400 in 5% GS for 30' @RT
9. PBS 3x5'
10. Vectastain Elite ABC for 15-30' @RT
11. PBS 4x5'
12. DAB (5mL dH₂O + 2drops buffer + 4drops DAB + 2drops H₂O₂) for 1-10' @RT; into H₂O
13. Heamtoxylin for 3-10s (8s pancreas, 10s brain)
14. Running H₂O 2x2'
15. 70% EtOH ... Histoclear
16. Mount w/ Vector mounting medium

10x Tris-EDTA

12.1 g Tris Base
4.71 g EDTA (no disodium)
to 1000 mL dH₂O
pH=9.0

10x Sodium Citrate

29.4 g Tri-sodium citrate
to 1000 mL dH₂O
pH=6.0

10x Citric Acid

19.2 g Citric Acid
to 1000 mL dH₂O
pH=6.0

For 1x:

100 mL 10x Buffer
0.5 mL Tween20
to 1000 mL dH₂O

Conditional Mouse Lung Cancer Using Virus

Mice: 8-12wk

1. Prepare virus
 - a. Thaw, aliquot in 10 μ L, and store at -80°C (each freeze thaw decreases titer 10 fold)
 - b. 25×10^6 PFU/mouse (50 μ L/mouse = 500×10^6 /mL, 1:20 from 10^{10} PFU/mL); therefore per mouse: 2.5 μ L stock Ad-Cre + 47 μ L OptiMEM + 0.625 μ L CaCl₂ (1:200 from 2M stock = 10 mM final)
 - c. Incubate for 20 min at RT
2. Place virus on ice, platform, heat lamp, and bleach in hood

4 mice
10 μ L stock Ad-Cre
188 μ L OptiMEM
2.5 μ L CaCl₂

Intranasal Infection

1. Anesthetize mice (power on O₂ and re-breather, >95% O₂, hook up box and re-breather, 2L/min O₂, 3.5 isoflurane setting, put mouse in, wait for ~2 resp/min)
2. Hold mouse loosely, ventral side up, with head above feet
3. Hold protein gel loading pipette tip over one nostril and dispense 25 μ L virus dropwise; NB: don't insert pipette tip into nostril
4. Allow mouse to inhale, and recover for 5min
5. Repeat with remaining 25 μ L
6. Place mice under heat lamp to recover for 15min

NB:

1. Median survival times:
 - a. *Adenovirus*: Kras = 185d, Kras-p53 = 76d
 - b. *Lentivirus*: Kras = 266d, Kras-p53 = 170d
2. After 15wks or death: Lung/spleen/LN for FACS/IHC

2M CaCl₂

14.7 g CaCl₂·2H₂O
50 mL H₂O

Hepatitis

Mice: Male 8-10wk old C57BL/6

1. i.v. 15 mg/kg ConA in PBS (for 20g mouse: 100 μ L at 3 mg/mL)
2. Serum (60 μ L) at 6 and 12h for ALT/AST/ELISA, can dilute in PBS
 - a. Store at RT for 1hr
 - b. Spin at 1500g for 15min at 4°, no brake, store super at -80°
 - c. Prepare working rgnt: 5 vol Buffer R1 + 1 vol Enzyme R2
 - d. Zero spectrophotometer at 340nm w/ dH₂O
 - e. Add 1mL Working Rgnt to test tube, warm to 37° for 3min
 - f. Add 50 μ L sample, mix gently, read absorbance every min for 3min while incubating at 37°
 - g. Calculate abs/min, multiply by -1746 for U/L, expect 1000U/L (healthy 3-30 U/L)
3. After 24h: Liver/spleen for FACS/IHC/qPCR/WB
4. Picrosirius Stain
 - a. Deparaffinize and hydrate
 - b. Weigert's haematoxylin
 - c. 60min in filtered PicroSirius (0.5g Sirius red in 500mL picric acid)
 - d. 2x acidic H₂O (2.5mL glacial acetic acid in 500mL H₂O)
 - e. Rinse in dH₂O, dehydrate, mount

Genotyping

1. Each tail tip / ear clip in 99 μ L TDB + 1 μ L Prot K (final 0.4 mg/mL), 2h at 55°, 10min at 95°, store 4°
2. PCR rxn:
 - 5 μ L 2x Taq (Green or Red)
 - 0.5 μ L Primer (1:10 personal dilution)
 - 0.5 μ L DNA
 - to 10 μ L H₂O
3. PCR conditions:
 - 3' 94 °
 - 29x: 40s 94°, 40s 60°, 40s 72°
 - 3' 72°
4. 1.5 g Agarose + 150 mL TAE + 5 μ L EtBr

<u>1.5mL</u> 167 μ L primer1 167 μ L primer2 2x 583 μ L H ₂ O

XIAK: iASPP lx/lx; R26 LSL-YFP; Kras; Pdx1-Cre
XKPC: ASPP2 lx/lx; R26 LSL-YFP; Kras; Pdx1-Cre; p53
XKYC: ASPP2 lx/lx; R26 LSL-YFP; Kras; Pdx1-Cre
XBJW=XTVB=ASPP2 del
XCDW=XEGW=ASPP2 lx/lx; Nestin-CreER
XIAB=XBWQ=iASPPdel
XCNW=XIAA=XIAC=iASPPlx/lx, iASPPdel, Rosa-CreER

Pdx1-Cre: Cre F + Cre B + Actin F + Actin B
753bp = Actin, 308bp = Cre
Rosa-CreER: CreR + CreB + Actin F + ACTinB
753bp = Actin, 308bp = Cre
ASPP2 lx/lx: SDL1s + SDL2s
203bp = LoxP, 169bp = WT
iASPP lx/lx: FRANT9 + FLP2
400bp = LoxP, 265bp = WT
Kras: 2xY116 + Y117 + Y118
450bp = WT, 327 bp = Kras
R26 LSL-YFP: YFPc + YFPwt; YFPc + YFPmut
600bp = WT, 320bp = R26 YFP
P53: p53u + p53wt; p53u + p53mut
270bp = LoxP, 166 bp = WT
ASPP2 del: F4, B3, pGK
800bp = ASPP2del, 597bp = WT
iASPPdel: I8-2 + E8-2(wt); I8-2 + FLP2(ko)
600bp = WT, 700 bp = iASPPdel

TDB

12.5 mL 1M KCl
2.5 mL 1M Tris-HCl
0.25 mL TritonX100
to 250 mL H₂O

50x TAE

242 g Tris-base
57.1 mL Acetic acid (add last)
18.61 g Disodium EDTA
to 1 L H₂O

EAE

Mice: 8-10 wk female C57BL/6J

1. s.c. injection of 200 μ g MOG₃₅₋₅₅ emulsified 1:1 in 20 μ g CFA (Difco) with 400 μ g H37Ra mycobacterium tuberculosis
 - a. Per mouse: 200 μ L emulsion = 100 μ L MOG + 100 μ L CFA
 - b. Dilute MOG₃₅₋₅₅ to 2 mg/mL in PBS, store -20° (200 μ L aliquot)
 - c. Day of, dilute CFA to 4 mg/mL (1:2.5, per ms 40 μ L + 60 μ L) in IFA
 - d. Draw up 100 μ L/ms MOG in 2mL syringe using 27G needle, draw up 100 μ L/ms CFA in 2mL syringe using 20G needle, get rid of any air, attach to three way valve
 - e. Nearly close valve and emulsify for >10min until stiff
 - f. Wait >30min (4hrs) to ensure stable emulsification, draw entirely into one syringe
 - g. Inject s.c. 2x 100 μ L per mouse
 - h. NB: potential adverse effects include sc emulsion bumps at ejection site after 2d, alopecia after 5d, 10-40% of mice will clear emulsion bump, skin ulcers at injection site that will heal in 3-4d, antibiotic ointment may be applied if necessary
2. After 0.5hr, i.v. injection of pertussis toxin in PBS (200-300 ng/mouse, 100 μ L/mouse; 3 μ g/mL = 1:33 from 100 μ g/mL stock) on d0 and d2
3. Weigh and score mice daily for 2 months
 - a. Mean cumulative clinical score = sum daily mean scores
 - b. Area under curve
 - c. Relapse = increase clinical score for 3 consecutive days
 - d. Kaplan meyer
 - e. Clinical scores (blinded)
 - i. 0.5 = partial tail limpness
 - ii. 1 = tail limpness
 - iii. 1.5 = righting reflex weakness
 - iv. 2 = hind limb weakness
 - v. 2.5 = paralysis one hind limb
 - vi. 3 = paralysis of both hind limbs
 - vii. 3.5 = paralysis hind lib and one fore limb
 - viii. 4 = paralysis hind limb and both forelimb
 - ix. 5 = death
4. After 17d, 24d, 50d or death: Spleen/LN/blood for FACS/ELISA/MOG response assay, perfuse, CNS for IHC/IF/FACS
 - a. MOG response assay
 - i. Isolate spleen (WT vs KO), total splenocytes or CD4 T cell isolation kit (Miltenyi Biotec), check purity
 - ii. Wash 2xPBS, label 5x10⁶/mL with CFSE (5 μ M, 1:1,000 from 5mM stock) for 10min at 37°C, quench w/ 5x vol FBS for 5min, wash 2x
 - iii. Culture splenocytes at 10⁶/mL in RPMI 10%FCS, P/S/G $\pm$ MOG (0,5,10,15,20 μ g/mL)
 - iv. After 3d: FACS for CFSE, ELISA on supers for IL-2,6, 12, 17, TNFa, IFNg

- b. FACS
 - i. CD4, Th17, B, Mac, Treg, CXCR2+, PD-1 cells in brain/spinal cord/spleen/LN
 - c. RNAseq on splenic CD4s
- 5. *Opt*: Adoptive transfer
 - a. After 10d, culture LNs and splenocytes at $50 \times 10^6/\text{mL}$ for 72hr w/ MOG ($20\mu\text{g}/\text{mL}$) and rIL-12 ($10\text{ng}/\text{mL}$)
 - b. Wash 2x, i.p. transfer 30×10^6 cells into 6-7wk female mice

Macrophages

Mice: 6-8wk old

Peritoneal Macs

1. Isolate macs from peritoneum
 - a. Open and retract abdominal skin, inject 10 mL DMEM into left peritoneum through 20-27G, gently massage peritoneum
 - b. Tent peritoneum with 19G needle, withdraw slowly
 - c. Spin 400g for 10min at 4°, resuspend in DMEM
2. Culture at 10^6 /mL in DMEM w/ 10%FCS, P/S/G (96well: 200 μ L/well; 24 well: 800 μ L/well)
3. After 2hr, wash 2x w/ PBS, change media, culture until 80% confluent, change media every 3 days
4. Add $\pm$ GolgiStop (2 μ M BD) $\pm$ rm IFNg (10 U/mL = 2 ng/mL; 1:10,000 from 20 μ g/mL stock) $\pm$ LPS (200-1000 pg/mL; 1:5,000,000 from 5mg/mL stock) $\pm$ polyIC (4 μ g/mL; 1:2,500 from 10mg/mL stock)
5. After 20, 60, 90 min LPS (1 μ g/mL; 1:5,000 from 5mg/mL stock): WB for phospho-STAT1
6. After 48hr: FACS for CD11b+IL12p40+, ELISA/qPCR on supers for IL-6, 12

BMDM

1. Bone marrow
 - a. Put leg in RPMI on ice
 - b. Cut knee joint, cut off muscle, remove remaining tissue w kimwipe
 - c. Clean bone in 70% EtOH for 2min, wash in PBS, transfer to RPMI
 - d. Trim off ends w/ sharp scalpel, flush bone marrow with DMEM using 24G (tibia) or 21G (femur)
 - e. Vigorously triturate, filter and spin, 1800 rpm 10'
 - f. RBC lysis for 1:30, quench, spin
2. Culture at 500K- 10^6 /mL for 6 days in DMEM 10% FCS, P/S/G w/ rmMCSF (20 ng/mL; 1:1000 from 20 μ g/mL stock) **or** rmGMCSF (20 ng/mL; 1:1000 from 20 μ g/mL stock) on non-TC treated dishes, add fresh medium after 3d
3. Confirm BMDM by CD11b+, F4/80+
4. Culture $\pm$ rm IFNg (10 ng/mL; 1:2,000 from 20 μ g/mL stock) for 4hr, then O/N w/ $\pm$ GolgiStop (2 μ M BD) $\pm$ LPS (100 ng/mL; 1:50,000 from 5mg/mL stock) $\pm$ polyIC (20 μ g/mL; 1:500 from 10mg/mL stock)
5. After 20, 60, 90 min LPS (1 μ g/mL; 1:5,000 from 5mg/mL stock): WB for phospho-STAT1
6. Detach using ice cold 5 mM EDTA for 15' on ice
7. After 16hr: FACS for CD11b+IL12p40+, ELISA/qPCR on supers for IL-6, 12

Phagocytosis Assay

1. Bone marrow
 - a. Put leg in RPMI on ice
 - b. Cut knee joint, cut off muscle, remove remaining tissue w kimwipe
 - c. Clean bone in 70% EtOH for 2min, wash in PBS, transfer to RPMI
 - d. Trim off ends, flush bone marrow with DMEM using 27G
 - e. Vigorously pipette, smash filter and spin, 1800 rpm 10'
 - f. RBC lysis, quench, spin
2. Culture at 10^6 /mL for 6 days in DMEM 10% FCS, P/S/G w/ rmMCSF (20 ng/mL; 1:1000 from 20 μ g/mL stock) **or** rmGMCSF (20 ng/mL, 1:1000) on non-TC treated dishes, add fresh medium after 3d
3. Confirm BMDM by CD11b+, F4/80+, BMDC by CD11c+, MHCII+
4. Label BMDM w/ Cell Trace Far Red (1 μ M; 1:1,000 from 1mM stock) for 10min at 37°C, quench w/ 5x vol FBS for 5min, wash 2x, plate BMDMs at 200K/well in 24 well (50K/well in 96 well)
5. Thymocytes
 - a. Isolate thymus from <8wk old mouse,
 - b. Smash filter through 100 μ m, resuspend at 10^6 /mL, culture in RPMI 10% HI-FCS, P/S/G, 10mM HEPES, 50 μ M BME until ready
 - c. Label w/ CFSE (5 uM, 1:1,000 from 5mM stock) for 10min at 37°C, quench w/ 5x vol FBS for 5min, wash 2x, confirm >60% apoptosis by TUNEL+
 - d. $\pm$ Expose to 2 Gy IR
6. After 24h, change to phenol-free DMEM/F12 2%FCS
7. After 30min add 150 μ L of:
 - a. 4×10^6 in 24well or 1×10^6 in 96well (FACS) or 10^6 (imaging) labeled apoptotic thymocytes
 - b. or 1 mg/mL pHrodo E. Coli BioParticles
8. Live imaging every 1 min for 1hr
 - a. 20x objective, autofocus on Cy5, 3 fields per well
 - b. Don't move plate once set-up (add cells *in situ*)
 - c. Capture every 1min for first 90min, then every 5min for 24h
9. After 15, 30, 60, 90 min analyze by FACS

CD4

Mice: 4-8wk old

Anti-CD3 stimulation

1. Coat 96 well flat plates w/ $\pm$ anti-CD3 (0, 2x, 0.16-2.5 μ g/mL, 2C11, eBio; 1:400 from 1mg/mL stock) $\pm$ anti-CD28 (100, 200, 400, 1000 ng/mL, PV-1 BioXCell; 1:, 1000 from 1mg/mL stock) in PBS O/N at 4°C at 50 μ L/well, wash 3x
2. Isolate spleen (WT vs KO), CD4 T cell isolation kit (Miltenyi Biotec), check purity
 - a. Everything on ice, volume listed is minimum, scale as needed
 - b. Resuspend cells in 40 μ L **MaB** per 10x10⁶ cells
 - c. Add 10 μ L biotin-Ab CD4 cocktail per 10x10⁶ cells, mix, incubate for 5min at 4° (fridge)
 - d. Add 30 μ L **MaB** per 10x10⁶, 20 μ L anti-biotin microbeads per 10x10⁶ cells, mix, incubate for 10min at 4° (fridge)
 - e. Put LS column (Max 2000x10⁶ cells) in magnetic field (Midi or Quadro MACS, purple/orange), rinse LS column w/ 3mL **MaB**, apply cell suspension, collect effluent, wash w/ 3mL **MaB**, collect flow-through
 - f. Count, check purity (CD3, CD4)
3. Label with CFSE (2 μ M, 1:2500 from 5mM stock) for 10min at 37°C, quench w/ 5x vol FBS for 5min, wash 2x
4. Save some for d0 FACS
5. Culture at 100K cells/96well in RPMI w/ 10% FBS, 10mM HEPES, 50 μ M BME, P/S/G $\pm$ mL-2 (40 ng/mL; 1:1000 from 40 μ g/mL stock) or rh IL-2 (0, 10x, 0.01-100 ng/mL=1000U/mL; 1:100 from 10 μ g/mL stock)
6. After 0,1,2,3,4d [for WT $\pm$ rh IL-2 (10 ng/mL; 1:1000 from 10 μ g/mL stock) $\pm$ anti-CD3]: WB/qPCR for iASPP
7. After 0,2,4,6,8,10d [for $\pm$ rh IL-2 (10 ng/mL; 1:1000 from 10 μ g/mL stock) $\pm$ anti-CD3]: calculate fold expansion (recovered/initial), FACS for CFSE, CD25, G2/M/S (PI)
 - a. PI Staining
 - i. Intracellular (for cell cycle), not if for viability
 - ii. 25 μ g/mL PI for 30min at RT
8. After 3d: FACS for CFSE, ELISA/qPCR on supers for IL-2, 6, 12, 17, TNFa, IFNg, iASPP

Naive CD4 polarization

1. Coat 96 well flat plates w/ anti-CD3 (1 μ g/mL, 2C11, eBio; 1:1,000 from 1 mg/mL stock) + anti-CD28 (1 μ g/mL, PV-1 BioXCell; 1:1,000 from 1 mg/mL stock) O/N at 4°C, wash 3x
2. Isolate spleen (WT vs KO), CD4+CD44-CD62L+ T cell isolation kit (Miltenyi Biotec), check purity
 - a. Resuspend cells in 40 μ L **MaB** per 10x10⁶ cells
 - b. Add 10 μ L biotin-Ab naive CD4 cocktail per 10x10⁶ cells, mix, incubate for 5min at 4° (fridge)
 - c. Add 20 μ L **MaB**, 20 μ L anti-biotin microbeads, 10 μ L CD44 microbeads per 10x10⁶ cells, mix, incubate for 10min at 4°

- d. Add 1mL **MaB** per 100×10^6 cells, spin 300g for 10min, resuspend in 500 μ L **MaB** per $<100 \times 10^6$ cells
- e. Put LS column in magnetic field, rinse LS column w/ 3mL **MaB**, apply cell suspension, wash w/ 3mL **MaB**, collect flow-through
- f. Count, check purity (CD3, CD4, CD44, CD62L)
3. Label with CFSE (5 μ M, 1:1,000 from 5mM stock) for 10min at 37°C, quench w/ 5x vol FBS for 5min, wash 2x
4. Culture at 10^6 /mL in RPMI w/ 10% FBS, 10mM HEPES, 50 μ M BME, P/S/G
 - a. Nothing
 - b. Th0: anti-IFN γ (5 μ g/mL; 1:200 from 1mg/mL stock), anti-IL4 (5 μ g/mL; 1:200 from 1mg/mL stock)
 - c. Th17 polarization: anti-IL-4 (5 μ g/mL; 1:200 from 1mg/mL stock), anti-IFN γ (5 μ g/mL; 1:200 from 1mg/mL stock), rhIL-23 (10 ng/mL; 1:1,000 from 10 μ g/mL stock), TGF β (1 ng/mL; 1:10,000 from 10 μ g/mL stock), rmlL-6 (0,1,5,100 ng/mL; 1:500 from 50 μ g/mL stock)
 - d. Treg: TGF β (1 ng/mL; 1:10,000 from 10 μ g/mL stock), anti-IFN γ (10 μ g/mL; 1:100 from 1mg/mL stock), anti-IL4 (5 μ g/mL; 1:200 from 1mg/mL stock)
 - e. Th1: IL-12 (10 ng/mL; 1:1,000 from 10 μ g/mL stock), anti-IL4 (5 μ g/mL; 1:200 from 1mg/mL stock)
 - f. Th2: IL-4 (10 ng/mL; 1:1,000 from 10 μ g/mL stock), anti-IFN γ (5 μ g/mL; 1:200 from 1mg/mL stock)
5. After 15min: WB for pSTAT3, pp65
6. After 48hr: qPCR (10^6) for ROR γ T, Foxp3, Tbet, GATA3
7. After 3d: FACS for CFSE, Th1, Th17, Th2, Treg, calculate fold expansion (recovered/initial)
8. ELISA on supers for IL-6, 12,17, IFN γ

Treg suppression assay

1. Isolate spleen (WT vs KO), filter 30 μ m, CD4+CD25+ Treg isolation kit, check purity
 - a. *Opt*: pre-activate by plating on anti-CD3 (2.5 μ g/mL; 1:400 from 1mg/mL stock) coated round bottom 96 well at 500K/mL in RPMI w/ 10%FCS, 10mM HEPES, 50 μ M BME, P/S/G, rhTGF β (2 ng/mL; 1:5,000 from 10 μ g/mL stock), rhIL-2 (50 U/mL=5 ng/mL; 1:2000 from 10 μ g/mL stock) for 4d, wash, count
 - b. Resuspend cells in 40 μ L **MaB** per 10×10^6 cells
 - c. Add 10 μ L MACS Reg T cell Biotin-Ab cocktail per 10×10^6 cells, mix, incubate for 10 min at 4°C (in fridge)
 - d. Add 30 μ L **MaB**, 20 μ L anti-biotin microbeads, 10 μ L CD25-PE Ab per 10×10^6 cells, mix, incubate for 15 min at 4°C
 - e. Put LD column (max 500 $\times 10^6$ cells) in magnetic field (Midi or Quadro MACS, purple/orange), rinse LD column w/ 2mL buffer, apply cell suspension, wash w/ 2x 1mL **MaB**, collect flow through (CD4s)
 - f. Spin 300g for 5min, resuspend in 90 μ L **MaB** per 10×10^6 cells
 - g. Add 10 μ L of anti-PE microbeads per 10×10^6 cells, mix, incubate for 15min at 4°C

- h. Put MS column (max 200×10^6 cells) in magnetic field (Mini or Octo MACS, teal), rinse MS column w/ 500 μ L **MaB**, apply cell suspension, wash w/ 2x 500 μ L **MaB**, collect flow through (CD4+CD25-)
- i. Put second MS column in magnetic field, equilibrate second MS column w/ 500 μ L **MaB**, remove first MS column and place over second MS column, add 1mL **MaB** to first MS column, firmly push plunger into column
- j. Wash w/ 2x 500 μ L **MaB**, collect flow through (CD4+CD25-)
- k. Remove column and place on collection tube, pipette 1mL **MaB**, firmly push plunger into column (CD4+CD25+)
- l. Count, check purity (CD3, CD4, CD25, Foxp3)
2. Label Tconv (CD4+CD25-) with CFSE (5 μ M, 1:1,000 from 5mM stock) for 10min at 37°C, quench w/ 5x vol FBS for 5min, wash 2x
3. Adjust CD4+CD25- to 500K/mL [4X] in RPMI w/ 10% FCS, P/S/G, 10mM HEPES, 50 μ M BME, 1mM sodium pyruvate, 100 mM NEAA, add 50 μ L/well (final = 25K/well) in 96well round bottom
4. Adjust Treg to 500K/mL [4X], 5x two-fold serial dilutions, add 50 μ L/well (final = 1:1, 1:2, 1:4, etc), final well no Tregs
5. Add 100 μ L/well 3000rad=30Gy splenocytes (500K/mL), labeled Cell Trace Far Red (1 μ M; 1:1,000 from 1mM stock) for 10min at 37°C, quench w/ 5x vol FBS for 5min, wash 2x, w/ soluble anti-CD3 (1 μ g/mL)
6. After 3d, FACS for CFSE, Tbet, calculate fold expansion (recovered/initial)
7. ELISA on super for IL-2, 10, IFN γ , TNFa

MaB

0.5% BSA

2 mM EDTA

PBS

Degas by vacuum at RT (attach flask to vacuum, stir at high speed for >1hr)

Store 4°C

