IMMUNE REGULATION OF AUTOINFLAMMATION CAUSED BY CELLULAR STRESS

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B.Sc., McGill University, 2009

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Experimental Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

August 2015

Abstract

During chronic inflammation and tissue injuries, various danger-associated molecules can be released and are able to potentiate inflammation and T cell responses. Among the many possible danger signals, I focused on studying high concentrations of extracellular ATP since it has been implicated in a variety of autoinflammatory diseases. ATP activates the inflammasome in macrophages, stimulates dendritic cell (DC) maturation, and inhibits regulatory T cell (Treg) function. However, how ATP regulates Toll-like receptor (TLR) responses in intestinal epithelial cells (IECs), which represent the front line of enteric defense, remains unclear. Therefore, I examined how ATP modulates TLR responses in IECs and found that it enhanced the response of IECs to a TLR1/2 ligand Pam_3CSK_4 primarily through the P2X7 purinergic receptor, leading to increased DC maturation and antigen-specific T cell proliferation. Furthermore, intra-rectal delivery of ATP lowered the activation threshold of epithelial cells to endogenous TLR ligands, making IECs more prone to immune activation.

Since ATP is an important molecule that can potentiate inflammatory responses, the second aim of the study was to investigate if Tregs, including $Foxp3^+$ Tregs and IL-10 producing Tr1 cells, can regulate ATP induced inflammasome activation and IL-1β production. I found that Tr1 cells inhibited the production of *Il1b* mRNA, inflammasome-mediated activation of caspase-1, and secretion of mature IL-1β, in an IL-10 dependent manner. Surprisingly, $Foxp3^+$ Tregs, despite the production of IL-10, failed to inhibit IL-1β production. The important role of IL-10 in regulating inflammasome activation was further illustrated in the monosodium urate induced peritonitis model, where IL-10R-deficient mice

had an increased influx of peritoneal neutrophils compared to wild type mice. Moreover, IL-1β production from macrophages derived from *Nlrp3*^{A350V} knock-in mice, which carry a mutation found in cryopyrin associated periodic syndrome patients, was suppressed by Tr1 cells, but not Foxp3^{+} Tregs. Using an adoptive transfer model, I found Tr1 cells can protect against weight loss in mice expressing a gain-of-function mutation in NLRP3.

Collectively, these data demonstrated the complex regulation of host response to cellular stress signal ATP, and that IL-10 producing Tr1 cells may have unique therapeutic effects in controlling ATP-mediated inflammasome activation via IL-10-mediated suppression.

Preface

Chapter 1 was based on extensive literature review about the relevant topics and edited by Dr. Levings and Dr. Steiner. Chapter 2 described the detailed methodology used throughout the study.

Research chapter 3 has been published and the figures and text were reprinted from *The European Journal of Immunology*, 2012 Dec; 42(12), Yao Y, Levings MK, Steiner TS, ATP conditions intestinal epithelial cells to an inflammatory state that promotes components of DC maturation, page 3310-21, Copyright (2012), with the permission from Wiley and Sons (license number 3526580516503). I designed and conducted all the experiments, performed the data analysis, and wrote the manuscript. Dr. Levings and Dr. Steiner aided in the experimental design, supervised the research, and provided critical edits to the manuscript.

Chapters 4 and 5 were conducted in collaboration with Dr. Hal Hoffman at the departments of Pediatrics at UCSD, and Rady Children's hospital, San Diego, California, USA. Parts of the chapters have been published and the figures and text were reprinted from the article "Tr1 cells, but not Foxp3$^+$ Regulatory T Cells, Suppress NLRP3 Inflammasome Activation via an IL-10-Dependent Mechanism" in *The Journal of Immunology*, 2015 Jul; 195(2): page 488-97, Copyright (2015) with permission from The American Association of Immunologists, Inc.. I wrote the manuscript, conducted the experiments, and prepared all the figures in chapters 4 and 5 except for the western blot figures of IL-1β and caspase 1. Jens Vent-Schmidt performed the western blot assays and prepared the graphs. May Wong assisted the *in vitro* and *in vivo* experiments under my supervision, including mouse

genotyping, co-cultures of macrophage and T cell, T cell phenotyping, peritonitis experiments, and T cell adoptive transfer experiments in NLRP3 transgenic mice. I performed the analysis for these experiments. Matthew D. McGeough from Dr. Hoffman's lab prepared bones from NLRP3 MWS mice and shipped them to us. Dr. Hoffman helped the design of the *in vivo* experiments for NLRP3 transgenic mice. Intravenous injection of T cells was performed by Dr. Scott Patterson. Dr. David Owen helped analyze the histology for the NLRP3 transgenic mice. Dr. Levings and Dr. Steiner supervised the research, provided critical suggestions to the direction of the project, and aided the preparation of the manuscript.

Animal research was performed under the approval of UBC Animal Care and Use Committee (Protocol numbers: A11-0272, A13-0194, A10-0105, A10-0214, A11-0066)

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4HT	4-hydroxytamoxifen
7-AAD	7-amino-actinomycin D
A20	Tumor necrosis factor alpha-induced protein 3
ADP	Adenosine diphosphate
AHR	Aryl hydrocarbon receptor
Aldh1a2	Retinaladehyde dehydrogenase 2
AMP	Adenosine monophosphate
AP-1	Activator protein 1
APC	Antigen presenting cells
ATP	Adenosine 3' triphosphate
BzATP	2′(3′)-O-(4-Benzoylbenzoyl)adenosine 5′-triphosphate
CAPS	Cryopyrin-associated periodic syndrome
cIAP	1/2 cellular inhibitor of apoptosis 1 and 2
CD	Crohn's disease
CINCA	Chronic infantile neurological cutaneous and articular syndrome
CTLA-4	Cytotoxic T lymphocyte antigen 4
CRP	C reactive protein
CXCL	Chemokine (C-X-C motif) ligand
DAMP	Danger/damage associated molecular pattern
DC	Dendritic cells
DIRA	Deficiency of the IL-1 receptor antagonist
Blimp	PR domain zinc finger protein 1
BMDC	Bone marrow derived dendritic cell
BMDM	bone marrow derived macrophage
BSA	Bovine serum albumin
DSS	Dextran sulfate sodium
EAE	Experimental autoimmune encephalomyelitis
ESR	Erythrocyte sedimentation rate
FACS	Fluorescence-activated cell sorting
FCAS	Familial cold autoinflammatory syndrome
FliC	*E. coli* H18 flagellin
FMF	Familiar Mediterranean fever
Foxp3	Forkhead box P3
GFP	Green fluorescent protein
GVHD	Graft versus host disease
HMGB-1	High mobility group box protein 1
HSCT	Hematopoietic stem cell transplant
IBD	Inflammatory bowel disease
ICOS	Inducible T-cell costimulator
IEC	Intestinal epithelial cells
IFN	Interferon
IL	Interleukin

IL-1Ra	IL-1 receptor antagonist
IP	Intraperitoneal
IPEX	Immunedysregulation polyendocrinopathy, enteropathy, X-linked
IRAK-M	Interleukin-1 receptor associated kinase-M
IRF	Interferon regulatory factor
IV	Intravenous
LAP	Latency-associated peptide
LAG-3	Lymphocyte-activation gene 3
LP	Lamina propria
LPS	Lipopolysaccharide
mAbs	Monoclonal antibodies
MHC	Major histocompatibility complex
MLN	Mesenteric lymph nodes
MLR	Mix lymphocyte reaction
MSU	Monosodium urate
MWS	Muckle-Wells syndrome
MyD88	Myeloid differentiation factor 88
MyD88s	MyD88 short
NFκB	Nuclear factor kappa B
NLRP3	NACHT, LRR and PYD domains-containing protein 3
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
NOMID	Neonatal-onset multisystem inflammatory disease
OVA	Ovalbumin
PAM	Pam_3CSK_4
PAMP	Pathogen associated molecular pattern
PD-1	Programmed death-1
RA	Retinoic acid
RAG	Recombination activating gene
RFP	Red fluorescent protein
SAA	Serum amyloid A
SCID	Severe combined immunodeficiency
ssDNA/RNA	Single stranded DNA/RNA
SIGIRR	Single immunoglobulin IL-1 receptor related molecule
SNP	Single nucleotide polymorphisms
SOCS	Suppressors of cytokine signaling
SPF	Specific pathogen free
TCR	T cell receptor
TGF	Transforming growth factor
Th cell	T helper cell
TIM3	T-cell immunoglobulin and mucin domain 3
TIR	Toll/IL-1R
TIRAP	TIR domain-containing adapter protein
TLR	Toll-like receptor
TNBS	2,4,6-trinitrobenzene sulphonic acid
TNF	Tumor necrosis factor
Tr1 cell	IL-10 producing type 1 regulatory cell

TRAM	TRIF-related adaptor molecule
TRAPS	Tumor necrosis factor receptor associated periodic syndrome
Tregs	Regulatory T cells
TRIF	TIR domain-containing adapter inducing interferon β
TSLP	Thymic stromal lymphopoietin
UC	Ulcerative colitis
UTP	Uridine 5 ′-triphosphate

Acknowledgements

I am forever grateful for both of my supervisors Dr. Megan Levings and Dr. Ted Steiner who are inspiring and influential role models in their own successful scientific careers. Dr. Levings is an excellent mentor with her expertise in T cell immunology. She has fostered a vibrant research environment in the lab and has provided tremendous support in everyway possible throughout my PhD studies. Dr. Steiner has been equally supportive with his vast knowledge on clinical applications and expertise in innate immunity. He has always been approachable and encouraging as a mentor and a dear friend. In addition, I am fortunate to have a great supervisory committee with Dr. Colby Zaph, and Dr. Stuart Turvey. I truly appreciate their fantastic scientific discussions, insightful suggestions and thoughtful career advices.

I thank May Wong for her diligence on providing technical assistance for the inflammasome project. I thank my current colleagues, Jonathan Han, Kate Macdonald, Jens Vent-Schimdt, Anne Pescenaker, Romy Heoplli, and previous lab members, Megan Himmel, Alicia Mcmurchy, and Adele Wang for fruitful scientific discussions and friendship. I would like to express my gratitude to Dr. Gijs Hardenberg and Dr. Scott Patterson for their mentorship on the animal experiments; and Dr. Sabine Ivison, for her mentorship when I first joined the lab. I would also like to thank Arnawaz Kifayet from the Steiner lab, Rosa Garcia, Jessica Huang, and Jana Gillies from the Levings lab for their effort in making the lab a lovable place to work at. I thank Lixin Xu who provided support for cell sorting and flow cytometry. My research was funded by a Doctoral Award and Transplant Training Program

Award from the Canadian Institutes of Health Research, and an Affiliated Scholarship from the University of British Columbia.

Dedication

I dedicate this dissertation to my family, especially my parents and in-laws for their unconditional support throughout my studies; to my cousin Yufei for her companionship and friendship; to my husband Yiteng for his love and patience especially during challenges of graduate school and life; and to my beautiful little boys who have brought so much happiness into my life.

Chapter 1: Introduction

Our bodies are constantly exposed to a variety of immune insults including pathogenic microbes and cancerous cells. Accordingly, a high level of immune surveillance and homeostatic mechanisms are in place to ensure appropriate responses to both beneficial and pathogenic organisms. The innate branch of the immune system is critical for early detection, containment and elimination of many pathogens, as well as maintaining tolerance to commensal microbes. On the other hand, the adaptive immune system receives instructions from the innate immunity to conduct appropriate effector responses depending on the type of microbes and the severity of the infection. Equally important to the effector mechanisms is the ability to regulate and/or shutdown the response when needed, which is largely directed by a subset of specialized $CD4^+$ T cells called regulatory T cells or Tregs.

One inevitable consequence of the immune response to pathogen is collateral damage of self-tissue and the release of endogenous danger signals, which can potentiate inflammation and, when not properly controlled, drive a variety of autoinflammatory diseases. Thus, a better understanding of how the immune system exerts its regulation in the context of tissue damage, and how this affects the complex crosstalk between the innate and adaptive immune systems, is highly important.

1.1. The recognition of foreignness: from "self-non-self" theory to "danger theory" and beyond.

1.1.1. Discovery of Toll-like receptors (TLRs)

In 1989, Charles Janeway proposed that the ability to distinguish between foreign molecules found on pathogens and self-antigens relies largely on the ability to recognize conserved molecular motifs (1). The corresponding receptors on the host are called pattern recognition receptors (PRRs) (1). This "self-non-self" theory has provided a conceptual framework for our current understanding of innate immune recognition, and it was confirmed by the subsequent discoveries of Toll in Drosophila (2), and Toll-like receptors (TLRs) in mammals (3). These are a family of germline-encoded invariant PRRs that are found either on the cell surface or intracellularly in the endosomes (4). They are single-pass type I membrane proteins containing an extracellular leucine-rich repeat domain that is critical for recognizing conserved structural components, or pathogen associated molecular patterns (PAMPs), such as lipoproteins, peptidylglycans, Gram-negative bacterial lipopolysaccharide (LPS), viral double-stranded RNA, bacterial unmethylated DNA, and bacterial flagellin (5) (Fig. 1.1).

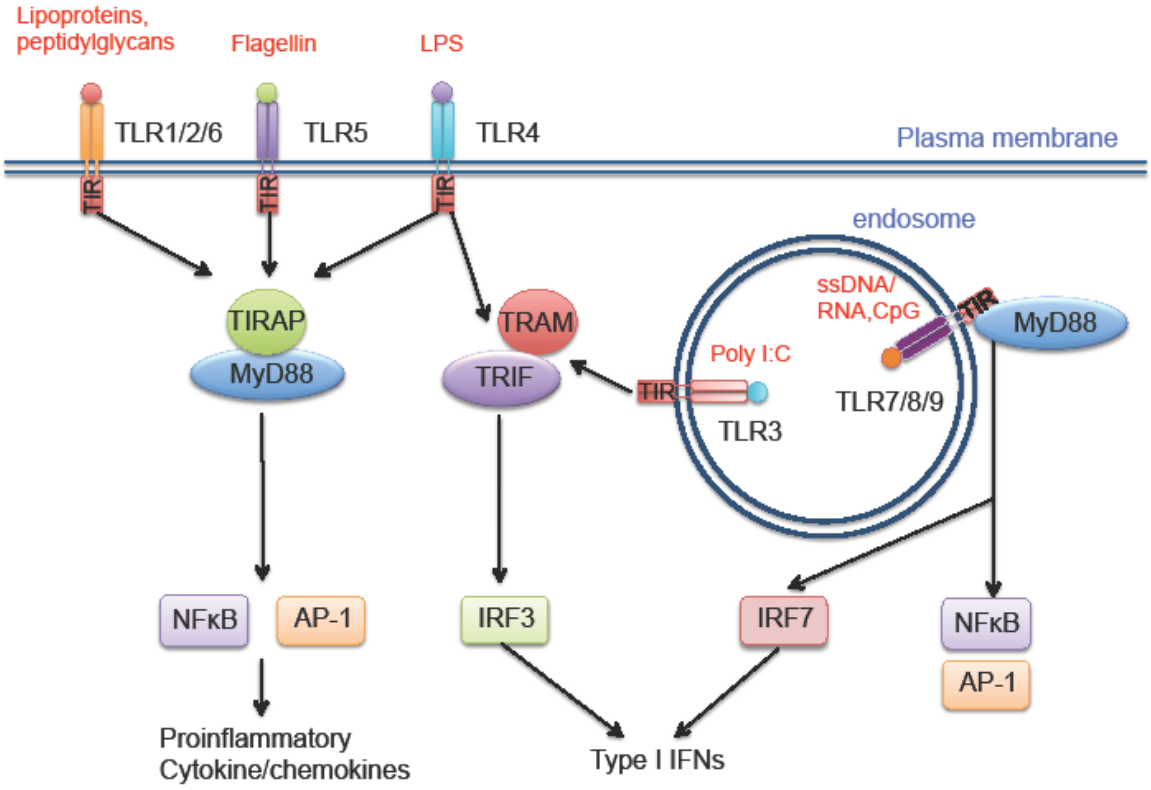


Figure 1.1 TLR ligation mediated MyD88-dependent and TRIF-dependent pathways. Ligand binding initiates the dimerization of Toll-like receptors and the downstream signaling cascade. The corresponding ligands for different TLRs are indicated in red. LPS: lipopolysaccharide; ssDNA/RNA: single stranded DNA or RNA. Most TLRs, as indicated in the figure, can signal through adaptor protein MyD88 via their intracellular TIR domains, leading to the activation of NFκB and MAPK pathways, and the transcription of proinflammatory cytokines/chemokines. TLR3 and TLR4 can signal through TRIF-dependent pathway, resulting in the activation of IRF3 (interferon regulatory factor 3) and the production of type I interferons (IFNs). TLR7/8/9 can also signal though MyD88, and in addition to activating NFκB and AP-1, they can induce the production of type 1 IFNs through activation of IRF7 (interferon regulatory factor 7).

Despite different ligand specificities due to their varying extracellular leucine-rich repeat domains, TLRs all feature a high degree of homology in the cytoplasmic Toll/IL-1R (TIR) domain that is important for the recruitment of downstream adaptor molecules, namely, myeloid differentiation factor 88 (MyD88), TIR domain-containing adapter inducing interferon β (TRIF), TRIF-related adaptor molecule (TRAM), and TIR domain-containing

adapter protein (TIRAP) (6). These different adaptor proteins are important for mediating distinct chemokine/cytokine inflammatory responses via the MyD88-denpendent pathway, or type I interferon responses via the TRIF-dependent pathway (6). Most TLRs, with the exception of TLR3, mediate signal transduction via the MyD88-dependent pathway, which is analogous to IL-1 receptor signaling, resulting in activation of activator protein 1 (AP-1) and nuclear factor kappa B (NFκB) (6, 7). TLR4 is unique in that it can utilize both MyD88-dependent and TRIF-dependent pathways (8).

TLRs are primarily expressed on sentinel cells that include immune cells such as macrophages, dendritic cells, and monocytes, as well as non-immune cells, such as fibroblasts and epithelial cells. Upon recognition of these PAMPs, TLRs can alert the immune system by causing cells to secrete inflammatory cytokines/chemokines, recruit and/or activate phagocytic cells, and in some case prime the adaptive immune response (4, 5, 7, 9). Indeed, TLR activation is critical for the defense against a variety of microbial infections, including viruses, such as, herpes simplex virus; gram positive bacteria, such as, *Streptococcus pneumoniae* and *Staphylococcus aureus*; gram negative bacteria, such as, *Salmonella typhimurium*, and *Escherichia coli*; fungi, such as, *Candida albicans*; and protozoa, such as, *Toxoplasma gondii* (10). The important role of TLRs in host defense is supported by several observations in TLR-deficient animals, where immune responses against microbial compounds are severely compromised. For example, C3H/HeJ mice with a loss-of-function TLR4 mutation resulting in impaired LPS responses are highly susceptible to gram negative bacteria (11). TLR9-deficient mice suffer more severe disease compared to wild type upon *Streptococcus pneumoniae* infection due to impaired uptake and killing of the bacteria by macrophages (12). Deficiency in TLR5 can result in dysregulated microbiota,

spontaneous colitis, and increase the susceptibility to colitis in various infection models (13-17). Overall, these studies suggest a key role of TLR innate immune mediated protection against a wide variety of pathogens and in immune homeostasis.

1.1.2. A regulatory role of TLR response

While the inflammatory role of TLRs is important for pathogen clearance and host defense, there is a growing body of literature showing that the activation of TLRs can be self-limiting and anti-inflammatory. The most well-known example is endotoxin tolerance, which was described almost 70 years ago (18). After exposure to LPS, macrophages demonstrate a decreased capacity to produce pro-inflammatory cytokines after stimulation with TLR2, 3, 4, 5, and 7 agonists (18-20). This tolerogenic effect correlates with proteolytic degradation of TLRs, and the activation of several TLR signaling inhibitors, such as MyD88 short (MyD88s), interleukin-1 receptor associated kinase-M (IRAK-M), suppressors of cytokine signaling 1 (SOCS1), single immunoglobulin IL-1 receptor related molecule (SIGIRR), and tumor necrosis factor alpha-induced protein 3 (A20) (7). Moreover, activation of TLRs is essential for cell survival during the inflammatory response as well as tissue repair following damage to the epithelium. For example, TLR5 activation leads to suppression of apoptosis in murine intestinal epithelial cells via the expression of anti-apoptotic survival proteins, such as cellular inhibitor of apoptosis 1/2 (cIAP1/2), and A20 (21). In addition, activation of TLR2 and TLR5 by heat-inactivated *Staphylococcus aureus* is able to induce accelerated tissue repair in lung epithelium, characterized by increasing cell survival and proliferation of the undamaged epithelial cells, operating mainly through epidermal growth factor receptors (22). Within the gut, homeostasis of epithelial cells is regulated by TLR recognition of commensal microbiota (23). For instance, IEC-specific

deficiency of MyD88 results in spontaneous inflammation in the small intestine (24). On the other hand, a series of studies have shown that exogenous administration of the TLR5 ligand flagellin can protect mice against lethal γ-irradiation and chemical injury (25, 26), and ensure efficient vaccine response against influenza A virus (27, 28).

Due to the apparently contradictory inflammatory and anti-inflammatory facets of TLR stimulation, significant questions remain regarding how TLR responses vary under different environmental settings and whether the response can be shifted from pathogenic to protective in chronic inflammatory diseases.

1.1.3. The evolution of the "danger theory"

According to the "self-non-self" theory, any microorganisms should be perceived as foreign and attacked by the host immune system. Yet, in sites such as the gastrointestinal tract, where the body hosts trillions of commensal microorganisms, the immune system does not undergo constant activation under homeostatic conditions despite continuous and copious exposure to microbes. Therefore, this "self-non-self" theory does not explain all the aspects of how the host immune system recognizes foreign matter. The recognition needs to be context dependent, which led to the proposal of the "danger theory" by Polly Matzinger in 1994 (29). She proposed that an immune response is primarily triggered when the cell perceives "danger signals" or "alarm signals" that are released by the body's own cells rather than in the recognition of foreign molecular structures. This theory helped to explain scenarios like autograft rejection, where "self" graft (i.e. a patient's own tissue) undergo rejection because the body sees the surgical procedure and tissue damage as danger; and tolerance to the fetus or commensal bacteria, where foreign matter is indeed tolerated (29). Moreover, this theory provided a satisfactory explanation for why resting dendritic cells

(DCs) can be activated by autologous necrotic cells that generate danger signals, but not by apoptotic cells that do not produce damage signals (30, 31). However, this concept also raises many concerns and questions: namely, how should "danger" signals be defined; what are the molecular traits of these signals; and how does the body respond to damage caused by the immune system itself.

With these questions in mind, more recent studies led to the identification of various endogenous danger signals, such as uric acid (32, 33), high concentration of adenosine 3'-triphosphate or ATP (34, 35), heat shock proteins (36), high mobility group box protein 1(HMGB1) (37), IL-1α (38), IL-33 (39), and degradation products of the extracellular matrix (40, 41). With the advance of our knowledge in these endogenous danger signals, we are now able to give a more defined description of these danger/damage associated molecular patterns or DAMPs as molecular structures that are normally "invisible" to the immune system, but released in the setting of cellular damage (42). DAMPs can either be recognized by PRRs or other receptors, resulting in induction or potentiation of inflammation and adaptive immune responses (42).

Depending on the cellular source of the damage signals, they can be divided into two categories: primary and secondary signals (42, 43). Primary signals are available during tissue damage and released directly from the damaged cells; secondary signals or feedback molecules are endogenous molecules released from activated immune cells in response to inflammation and are able to amplify or modulate the immune response in an autocrine or paracrine manner (42, 43). Some molecules, such as ATP and IL-1α, can act as both primary and secondary signals to amplify the immune response.

1.1.4. High concentrations of extracellular ATP are a "damage signal"

Accumulating evidence suggests that ATP contributes to the pathogenesis of a variety of inflammatory disorders, such as asthma, graft-versus-host disease and inflammatory bowel disease, suggesting it is an endogenous danger signal that promotes inflammation (44-48). Under physiological conditions, the extracellular ATP concentration is maintained at a very low level (10-100nM) by ectonucleotidase that actively hydrolyze ATP into adenosine (49). During cellular damage and inflammation, ATP can be immediately released into the extracellular space, either from necrotic cell death or through an active transport process via the activation of platelets and phagocytes, thereby attaining local concentrations in the millimolar range (34). At these concentrations, ATP acts as an important danger signal by activating low affinity receptors, such as the P2X7 ionotropic receptor, alerting immune cells to the presence of tissue damage and mobilizing them to the site of injuries to fight pathogens and clear cellular debris (50, 51).

The activation of P2X7 receptor can mediate a variety of inflammatory responses. First, ATP alone has been shown to skew the maturation of dendritic cells by preferentially up-regulating a subset of maturation markers while inhibiting TLR activation induced cytokine production (52-54). These DCs are then capable of directing T cell differentiation into Th1 or Th2 cell depending on other cytokine signals (52-54). Moreover, ATP is well known for its role in activating the inflammasome complex together with TLRs and the subsequent production of IL-1β and IL-18 in monocytes, macrophages, and DCs. This is believed to occur following ATP engagement of P2X7 receptor, leading to potassium efflux and the lowering of potassium concentration, thus facilitating the assembly of the inflammasome complex and activation of caspase 1(55). The inflammasome pathway will be

discussed in more detail later in chapter 1 and in chapter 4 and 5. In addition to being an inflammasome activator by itself, autocrine secretion of ATP is critical for inflammasome activation by crystalline structures, such as monosodium urate (MSU) and silica, via pannexin-1 hemichannels and several other P2 purinergic receptors (55, 56).

Although the "danger theory" was first proposed to center around DCs as the primary cells receptive to danger signals (57), accumulating evidence suggested that other cells, including stromal cells, such as epithelial cells and fibroblasts (58-61), and other immune cells, such as macrophages, eosinophils, and T cells, can also be a direct target (62-66). How these cells are affected remains an active area of research. Secondly, the original aim of the danger theory primarily focused on explaining the triggers of adaptive immune responses (29, 57, 67, 68), but we now know that danger signals play a critical role in modulating innate immune responses; examples include HMGB-1, which can directly trigger inflammatory responses and tissue repair, and assembly of inflammasomes (68). Furthermore, the danger hypothesis alone fails to explain some complex scenarios, such as how our immune system tolerates commensal microorganisms. The danger hypothesis presumes that we remain tolerant to commensal microbes because they fail to provoke damage while the pathogenic microbes are usually invasive and cause tissue damage. We now know that this tolerance is not due to passive non-recognition, but rather a highly active and coordinated process that involves different regulatory immune cells and tightly controlled TLR responses to promote barrier integrity and tissue regeneration (9, 69, 70). The next question is how to incorporate both the "self-non-self" theory and the danger theory to understand complex immune interactions in the gut and specifically ask whether danger signals changes TLR responses in different cellular compartments and what are the

downstream immune consequences. Furthermore, whether danger signals can render the regulatory immune cells, such as regulatory T cells, tolerogenic DCs, and alternatively activated macrophages, ineffective in suppressing inflammation; and if so, what are the molecular mechanisms and consequences.

1.2. The role of regulatory T cells in the control of the innate immune responses

Regulatory T cells (Tregs) constitute an essential part of the central and peripheral tolerance machinery that negatively regulates innate and adaptive immune responses. Within the $CD4^+$ T cell compartment, the best understood subsets of Tregs are $Foxp3^+$ Tregs (71, 72) and IL-10 producing type 1 regulatory (Tr1) cells (73) (Fig. 1.2).

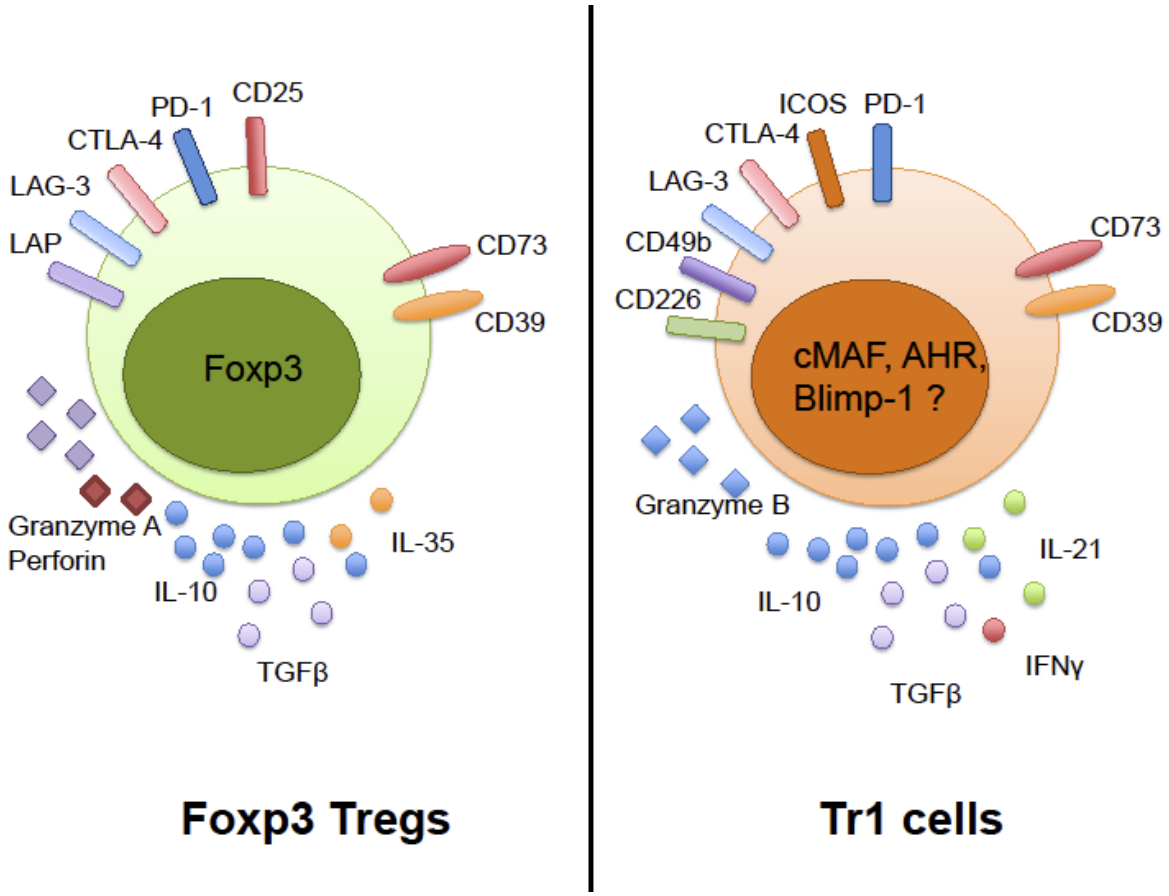


Figure 1.2 The suppressive mechanisms by two major subsets of regulatory T cells. Foxp3$^+$ Tregs are characterized by constitutive expression of CD25 and transcription factor Foxp3. Tr1 cells can be distinguished by their characteristic cytokine secretion pattern with high levels of IL-10, and intermediate to low amounts of IFNγ, TGFβ, and IL-21. In addition, Tr1 cells can also be identified by co-expression of surface molecules CD49b and LAG-3. Three transcription factors, cMAF, AHR, and Blimp-1 has been shown to regulate IL-10 production in Tr1 cells, but it remains unknown if they are the *bona fide* transcription factors for Tr1 cells. Tr1 cells can suppress the innate immune system via a variety of mechanisms, including expression of surface inhibitory molecules (CTLA-4, LAP, ICOS, PD-1, CD25, and CD226), ectoenzymes (CD39 and CD73), soluble inhibitory cytokines (IL-10, TGFβ, IL-21, and IL-35), and effector enzymes (granzyme A, B, and perforin). As shown in the figure, some of these are common mechanisms utilized by both subsets (LAG-3, CTLA-4, and IL-10), while some are unique for Foxp3 Tregs (IL-35), or Tr1 cells (IFNγ, and IL-21).

1.2.1. Immune function of Foxp3$^+$ Tregs

Foxp3$^+$ Tregs were first identified based on the expression of CD25, a high affinity IL-2 receptor (74), followed by the milestone discovery of Foxp3 being the lineage defining,

master transcription factor that controls the function and stability of these cells (75, 76). Foxp3$^+$ Tregs can have either intra-thymic or extra-thymic origins, and both subsets require continuous TCR signaling during induction and maintenance for their *in vivo* suppressive function (77, 78). So far, there are no markers available to reliably distinguish between thymic versus peripherally-derived cells and they likely employ similar molecular mechanisms for their suppressive function (79). The importance of Foxp3 in directing the development of suppressive Foxp3$^+$ Tregs is illustrated by the phenotype of scurfy mice and in patients with IPEX (immunedysregulation polyendocrinopathy, enteropathy, X-linked), which have genetic mutations in *FOXP3*. Both humans and mice with *FOXP3* mutations have devastating multi-organ autoimmunity and allergy (80). In addition to the simple expression of Foxp3 protein, epigenetic regulation of the *FOXP3* locus is important for maintaining the stability and lineage identity of the cells. Notably, under inflammatory conditions, they can adapt and up-regulate transcription factors that are typically associated with other T helper cell lineages (72).

While the role of Tregs as potent suppressor of the activation, proliferation and differentiation of effector T cells is well characterized, their function in modulating innate immune responses is less known. Several surface molecules have been proposed to play a role in Treg-mediated suppression of innate immunity, including inhibitory surface molecules cytotoxic T-lymphocyte antigen-1 (CTLA4), and lymphocyte activating gene-3 (LAG-3), as well as ecto-endonucleases CD39, and CD73 (77). CTLA-4 has been shown to down-regulate the costimulatory molecules CD80 and CD86 on DCs by transendocytosis. Accordingly, Treg-selective CTLA-4 knockout mice have increased susceptibility to several T cell mediated autoimmune diseases (81). LAG-3 is another molecule used by Tregs to

control the activation of antigen presenting cells (APCs). LAG-3 is homologous to CD4 but binds to MHC II with higher affinity. Engagement of CD4 by LAG-3 results in inhibition of DC maturation by down-regulation of costimulation molecules (82), and decreased T cell activation and proliferation (83-85). The ATP degrading ectoenzymes, CD39 and CD73 are highly expressed especially on murine Foxp3$^+$ Tregs, enabling the rapid degradation of ATP into adenosine, which can in turn suppress both the activation of DCs, and T cell proliferation (86).

In addition to expression of inhibitory surface molecules, Foxp3$^+$ Tregs also express anti-inflammatory cytokines, such as IL-10, TGFβ, and IL-35 (87), although in order to express these cytokines Tregs need to be in contact with other cells, such as conventional CD4$^+$ T cells, plasmacytoid DCs, or CD8$^+$ T cells (88). IL-10 is a potent anti-inflammatory cytokine that can act directly on T cells to inhibit IL-2 and IFNγ production, and on APCs to suppress inflammatory cytokines and costimulatory molecules (89, 90). TGFβ can also exert similar effects by inhibiting cytokine production and is known to be essential for immune homeostasis (91). Finally, Tregs have also been shown to directly kill myeloid APCs via secretion of perforin and granzyme A (92).

1.2.2. The biology of Tr1 cells

Tr1 cells are memory CD4$^+$ T cells with an extra-thymic origin that lack expression of Foxp3 (73). Upon activation, Tr1 cells exhibit a characteristic cytokine expression profile with high amounts of IL-10, low or intermediate amounts of IFNγ, TGFβ, IL-21, and minimal amounts of IL-2, IL-4 and IL-17 (73). Similar to Foxp3$^+$ Tregs, TCR activation is required for the suppressive function of Tr1 cells, but they can mediate bystander suppression once activated via cytokine dependent mechanisms (93). Another feature of Tr1

cells that resembles Foxp3$^+$ Tregs is their poor proliferative capacity *in vitro*, in part due to autocrine production of IL-10 that suppresses proliferation (94). However, addition of exogenous IL-2 and IL-15 restore the proliferation and expansion of Tr1 cells *in vitro*, allowing for further functional and mechanistic studies (95).

Unlike Foxp3$^+$ Tregs, no lineage defining transcription factors have been identified for Tr1 cells, although cMaf, AHR, and Blimp-1 have all been implicated in the production of IL-10 in *in vitro* and *in vivo* differentiated Tr1 cells (73, 96, 97). The suppressive mechanism of Tr1 cells is also distinct from Foxp3$^+$ Tregs. Tr1 cells suppress adaptive immune responses and activation of APCs largely through the production of immunosuppressive cytokines, primarily IL-10, and in some cases TGFβ (94, 98-100). Other mechanisms have been proposed for their inhibitory function of Tr1 cells *in vitro*, such as the expression of inhibitory receptors that down-regulate T cell activation, namely, CTLA-4, ICOS (inducible T cell costimulator), PD-1 (programmed death-1), LAP (latency-associated peptide), ectoenzymes, CD39, and CD73, and effector molecules that mediate cytolysis, namely, granzyme B (73). However, the relative contribution of these different effector mechanisms for the overall suppressive function of Tr1 cells remains to be defined, and the secretion of IL-10 remains a hallmark of Tr1 cell suppressive activity.

1.2.3. Foxp3$^+$ Treg-based cellular therapy

Based on their suppressive function and antigen specific immunomodulatory ability, Foxp3$^+$ Tregs and Tr1 cells have become attractive therapeutic options for treating autoimmune diseases, inflammatory diseases and other immune-mediated pathologies. The hope is that therapy with these cells could minimize the use of global immunosuppressive

regimens that have well-known associated risks of cancer and infection, and possibly establish antigen-specific long-term tolerance.

The success of using $Foxp3^+$ Tregs to restore immune tolerance and dampen effector T cell responses has been shown in a variety of animal models, including inflammatory bowel disease (101, 102), rheumatoid arthritis (103), type 1 diabetes (104), GVHD (105-107), and allograft transplantation (108, 109).

Based on extensive pre-clinical data, clinical trials using $Foxp3^+$ Tregs as cellular therapy have been initiated. Because of the low frequency of $Foxp3^+$ Tregs (2-5% of $CD4^+$ T cells in peripheral blood of adults), several strategies have been developed to boost Treg numbers. For example, treatment with low doses of IL-2 can induce/expand $Foxp3^+$ Tregs *in vivo*, or they can be expanded or differentiated *ex vivo* prior to reinfusion (110). Due to recent technical advances in selection of markers for purification, and expansion culture conditions, the expansion of Tregs is the most common method adopted in the clinical setting (111). Ongoing research is investigating how to consistently generate Tregs in multiple good manufacturing practice-compatible facilities. In addition, there is need for research into how to determine the safety and the stability of these cells when delivered into an inflammatory setting.

Fortunately, results from the initial reports of phase I/II studies showed feasibility and safety of Treg therapy in preventing the development of graft versus host disease (GVHD) (112-114). Another recent report from Theil *et al.* showed that Tregs cell therapy maybe beneficial for patients with ongoing GVHD (115): five patients with refractory chronic GVHD received *ex vivo* expanded $CD25^{hi}CD127^{lo}Foxp3^+$ Tregs from a third party, resulting in an increased frequency of circulating Tregs and decreased T cell activation (115).

Importantly, two out of five patients showed clinical improvements and the other three maintained stable disease with no other adverse immune reaction (115).

In addition, a recent study was conducted in pediatric patients with type 1 diabetes (116), who received autologous expanded $CD25^{hi}CD127^{lo}Foxp3^{+}$ Tregs. Similar to the study by Theil *et al.* (115), an increased frequency of Tregs was observed in the peripheral blood after Treg transfer, and most patients responded to the therapy and showed increased circulating C-peptide levels, reflecting increased beta-cell mass (116). Other ongoing clinical trials, in particular, the ONE Study (www.onestudy.org), will evaluate the safety and feasibility of various cell types, including $Foxp3^{+}$ Tregs and Tr1 cells, in living-donor kidney transplantation, which will give us more insights on how these cells can regulate acute inflammation in solid organ transplantation.

1.2.4. Adoptive transfer of Tr1 cells in animal models

Since the discovery of Tr1 cells in severe combined immunodeficiency (SCID) patients who successfully established split-chimerism following hematopoietic stem cell transplant (HSCT) with HLA-mismatched donors (98), it has become clear that Tr1 cells have the important ability to regulate immune homeostasis and maintain peripheral tolerance. The first T cell transfer study using Tr1 cells as a cellular therapy was conducted in a murine model of colitis (94). In that study, ovalbumin (OVA) specific Tr1 cells were generated by repetitive stimulation of naïve $CD4^{+}$ T cells obtained from DO11.10 transgenic mice, which carries the T cell receptor transgene recognizing a specific epitope on OVA protein, in the presence of high doses of IL-10. The resulting T cells had a Tr1 characteristic cytokine profile with high amounts of IL-10 and low amounts of IL-2 and IL-4. These *in vitro* generated OVA-specific Tr1 cells were then co-transferred with $CD45RB^{hi}CD4^{+}$ cells into

mice fed either normal diet or OVA-supplemented diet. The data showed a protective effect of Tr1 against colitis only in OVA fed mice, suggesting that Tr1 cells can effectively dampen naïve T cell induced intestinal inflammation and that *in vivo* antigen specific activation is required for the suppressive capacity of these cells. Along the same line, Tr1 cells generated in mixed lymphocyte reaction cultures against donor cells in the presence of IL-10 and TGFβ protect mice against developing GVHD in a model of HLA-mismatched HSCT (117). In addition, collagen-II specific Tr1 clones expanded in the presence of IL-10 reduce disease activity in both acute and chronic antigen-induced arthritis (118). Furthermore, the potency of antigen-specific Tr1 cells has been elegantly shown in a murine model of islet transplantation, where antigen-specific Tr1 cells are better at protecting islet allograft rejection than polyclonal Tr1 cells (119). Additional evidence for the suppressive capacity of antigen-specific Tr1 cells is in celiac disease, a common disorder in the small intestine driven by dysregulated T cell responses to a wheat gluten antigen, gliadin (120). Gliadin-specific Tr1 clones can strongly inhibit APC maturation and activation of gliadin-specific T cell responses through the production of IL-10 and TGFβ (121, 122).

More recently, other novel agents such as chemokines CXCL11 and CXCL12 (123, 124), and extracellular matrix component high molecular weight hyaluronan (125), have been shown to redirect the polarization of Th1 cells into IL-10 producing Tr1 cells. Injection of CXCL11-Ig fusion protein can rapidly suppress ongoing experimental autoimmune encephalomyelitis (EAE) in an IL-10-dependent manner (123, 124); hyaluronan induced Tr1 cells can protect the mice from T cell transfer colitis (125). These data suggest therapeutic agents may represent novel ways of inducing Tr1 cells *in vivo*.

1.2.5. The use of Tr1 cells as therapy in human clinical trials

In addition to animal studies, data from human proof-of-principle phase I/II trials with Tr1 cellular therapy showed promising results. The first reported Tr1 cell therapy was a dose escalation study conducted in patients with refractory Crohn's disease that failed standard treatments (126). In the study, OVA-specific Tr1 cells were generated by stimulating patient PBMCs with OVA in the presence of IL-2 and IL-4 to enrich for the OVA-specific T cells. The resulting $CD4^+$ T cells were then cloned and expanded with feeder cells, followed by selecting OVA-specific Tr1 clones based on OVA-stimulated IL-10 production. Patients received a single injection of different dosage of OVA-Tr1 cells and ingested an OVA-enriched diet to ensure appropriate migration and activation of Tr1 cells. The infusion of Tr1 cells seemed safe and well tolerated in all patients, and 40% of patients showed significant, but time-limited, clinical improvements correlating with antigen-specific hyporesponsiveness. Another clinical trial was performed in patients undergoing haploidentical HSCT therapy and aimed at evaluating if Tr1 cells can prevent GVHD (127). In this study, Tr1 cells were generated by culturing donor T cells with recipient APC in a mixed lymphocyte reaction in the presence of IL-10. Four out of twelve patients that received Tr1 cells benefited from the treatment with long-term tolerance and high frequency of circulating Tr1 cells in the absence of immunosuppression, while the remaining patients failed to respond to Tr1 therapy and developed moderate acute GVHD.

Despite the promising results in terms of safety and potential efficacy of Tr1 cellular therapy, there are obvious challenges to overcome. First, the methodology to efficiently generate a pure population of Tr1 cells remains a major challenge (127). Second, developing a better understanding of the mechanisms of action will allow refinement of protocols and

strategies to minimize potential complications such as infection. Third, developing biomarkers that can be used to select patients and track outcomes will be essential to guide this highly personalized therapy.

1.3. The role of stress signals in autoinflammatory diseases

1.3.1. How to define autoinflammatory disease

The term autoinflammation was first proposed in 1999 to distinguish from autoimmunity after discovering the genetic basis for familiar Mediterranean fever (FMF) and tumor necrosis factor receptor associated periodic syndrome (TRAPS)(128). Autoinflammation describes self-directed chronic inflammatory responses and tissue damage primarily driven by innate cells, such as neutrophils, macrophages, and monocytes, without a clear association with HLA molecules or autoantibodies (129, 130). Although autoinflammatory diseases originally described uncommon monogenic immune disorders, such as FMF, TRAPS, and cryopyrin associated periodic disorders (CAPS), the spectrum of these diseases is continuously expanding and now also includes some polygenic diseases with underlining innate immune-driven pathogenesis, such as Crohn's disease, gout, pseudogout, degenerative disease (for example, osteoarthritis), and some neurological diseases (129, 130).

The most common pathway that is perturbed in many monogenic and polygenic autoinflammatory diseases is inflammasome activation, resulting in increased production of IL-1β (131). Under homeostatic conditions, inflammasome activation in mature macrophages requires two signals: signal 1 is provided by TLR ligation, leading to the transcription of pro-IL-1β, pro-IL-18, and NLRP3; signal 2 involves sensing of endogenous/exogenous danger

signals by NLRP3, resulting in the assembly of the inflammasome complex and processing pro-IL-1β, pro-IL-18 into their mature forms (Fig. 1.3).

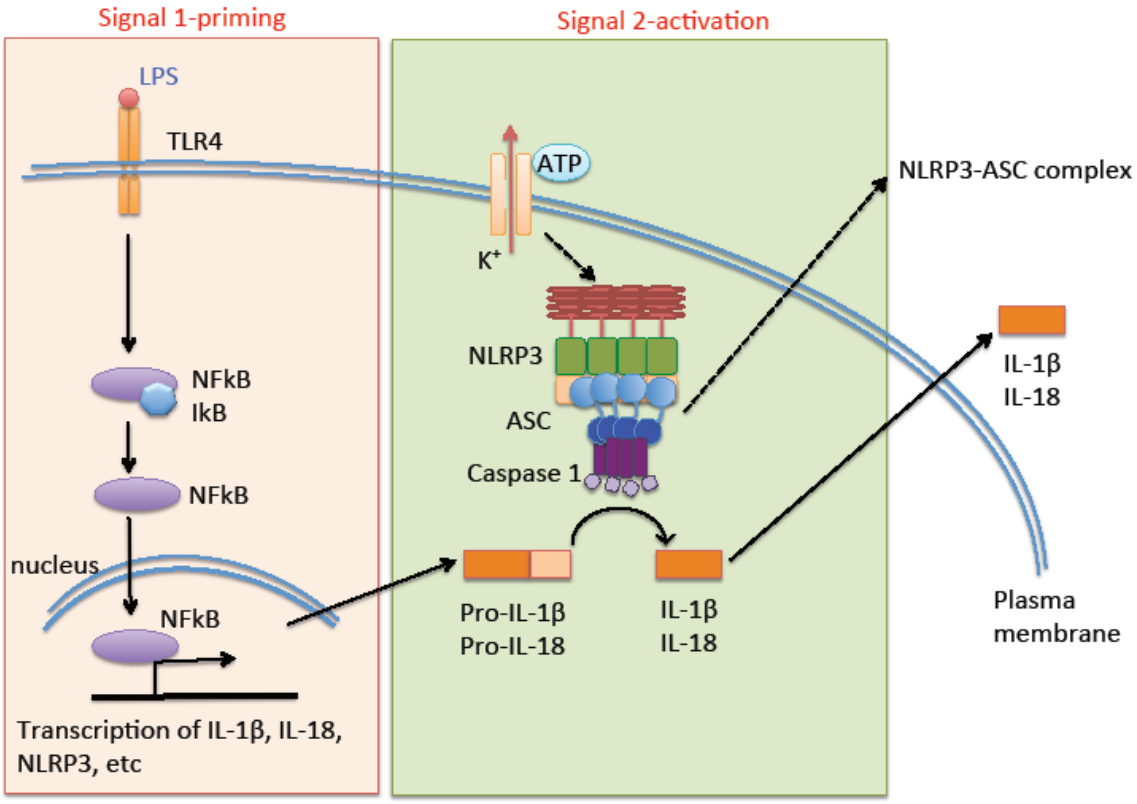


Figure 1.3 Schematic of inflammasome activation.
Signal 1: LPS binding initiates TLR4 signaling and induces activation of NFκB and transcription of pro-IL-1β, pro-IL-18, and NLRP3. Signal 2: High concentration of ATP activates ionotropic P2X7 receptor, resulting in potassium efflux, and the oligomerization of NLRP3, ASC and pro-caspase 1. Upon assembly of the NLRP3 inflammasome, pro-caspase 1 is brought together in close proximity to each other and can auto-cleave the neighboring pro-caspase 1 into active caspase 1, which can process pro-IL-1β, pro-IL-18 into their mature forms and released into extracellular space. Under certain circumstance, NLRP3-ASC complex can also be directly secreted outside the cells.

In monogenic diseases, the increased production of IL-1β is often associated with over-activation of the inflammasome pathway as a result of gain-of-function mutations in inflammasome components (NLRP3 in CAPS), or loss-of-function mutations in negative

regulators (IL-1ra in DIRA, or deficiency of the IL-1 receptor antagonist). In polygenic diseases, this maybe due to the accumulation of DAMPs as a result of tissue damage that stimulates inflammasome pathways, or dysregulated innate immunity, such as deficiency in autophagy that indirectly activates inflammasome pathways (132). It is worth noting that inflammasome activation has not only been implicated in autoinflammatory diseases, but also affects neurodegenerative disease such as Alzheimer's disease, metabolic disease such as type 2 diabetes, and atherosclerosis (133), making it a widely perturbed pathway that drives a vicious cycle of inflammation.

1.3.2. Cryopyrin associated periodic syndrome (CAPS)

Cryopyrin associated periodic syndrome is a systemic inflammatory disease that is comprised of three forms ranging from mild to severe clinical presentations: familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS), and chronic infantile neurological cutaneous and articular syndrome (CINCA), also known as neonatal-onset multisystem inflammatory disease (NOMID) (133). As described earlier, CAPS is primarily driven by autosomal dominant mutations in NLRP3 that results in a lowered threshold of activation and tendency to oligomerize spontaneously, leading to activation and increased conversion of pro-IL-1β into its mature form (131). CAPS patients develop chronic systemic inflammation characterized by peripheral blood and tissue neutrophilia, and increased circulating C reactive protein (CRP), serum amyloid A (SAA), and erythrocyte sedimentation rate (ESR) (133).

Despite the clear evidence for the molecular mechanism of disease, it has been challenging to detect high levels of IL-1β and IL-18 in these subjects, presumably because these cytokines bind to plasma proteins or cell membranes (134). Recently, two groups have

independently shown that NLRP3 can aggregate in the form of specks, or large protein complexes, with its adaptor protein ASC, and these specks can be directly released into the extracellular space upon inflammasome activation and remain active outside the cell for a prolonged period of time (135, 136). Indeed, extracellular ASC specks have been found at elevated levels in the serum of CAPS patients, which may act as danger signals themselves to amplify the conversion of pro-IL-1β (135, 136). Thus detection of these "specks" may be a more reliable way of measuring inflammasome activity in the clinic once high affinity antibodies are developed (135, 136).

The definitive proof that CAPS is an IL-1 driven disease is the striking and immediate clinical response to IL-1 targeted therapies in these patients, which alleviates virtually all the clinical symptoms (137-139). To date, three anti-IL-1 agents have been used for the treatment of CAPS patients: anakinra, canakinumab, and relonacept. Anakinra is a recombinant selective IL-1 receptor antagonist (IL-1RA) that targets both IL-1β and IL-1α and was first reported to be markedly effective in treating CAPS patients. Despite great clinical efficacy, one major limitation is the short half-life of anakinra, meaning that daily injections are required, which can result in injection site reactions and poor compliance (140). Canakinumab, is a human monoclonal antibody targeted against IL-1β; rilonacept, also known as IL-1 Trap, is a dimeric protein consisting of the 2 ligand binding domains of the IL-1 receptor linked by the Fc region of human IgG1 antibody (139). These two drugs are equally effective and both received approval for the treatment of CAPS with the advantage of a long-half life that require one injection every 2 months as compared to daily injection with anakinra (139, 140). However, for managing short-term need of IL-1 blockade, such as inflammatory flares, anakinra outweighs the other two drugs to avoid prolonged and

unnecessary IL-1 blockade that may increase the risk for bacterial infection (139). Overall, CAPS can be very well managed by these IL-1-targeted therapies. One area of active research is to expand the indications of this treatment to other chronic inflammatory disease, such as type 1 and type 2 diabetes, rheumatoid arthritis, inflammatory bowel disease, and gouty flares in patients who are refractory to standard treatment (138, 139).

1.3.3. The role of the inflammasome in inflammatory bowel disease (IBD)

Inflammatory bowel disease, comprised of Crohn's disease (CD) and ulcerative colitis (UC), is a chronic incurable inflammatory disorder that affects the gastrointestinal track in genetically susceptible individuals (69, 141, 142). Although the etiology of the disease is unclear, the pathogenesis is heavily driven by inappropriate immune responses directed against intestinal commensal bacteria (69, 143). While the disease primarily affects the colonic mucosa in UC, in CD the inflammation can affect the entire gastrointestinal tract and be transmural, leading to complications such as stricture, fistula and obstruction (69, 143). CD is frequently associated with inappropriate activity of Th1 and/or Th17 cells, and a parallel dysfunction of regulatory T cells (144, 145). In addition, dysregulated innate immune responses, such as autophagy, ER stress, and inflammasome, also have major roles in the disease (70), and patients with CD have higher levels of pro-inflammatory cytokines in the intestine, such as IL-1β (146). In fact, Crohn's disease is the first polygenic autoinflammatory disease with a genetically defined defect in innate immune pathways, namely, NOD2, autophagy, ER stress, and inflammasome (130, 143). Among these, dysregulated activation of inflammasome complex will be discussed in more detail here.

In humans, there is accumulating evidence showing a genetic association of NLRP3 to IBD in different ethnic groups, including individuals of European descent, and Chinese

(147-150); however, some of these associating single nucleotide polymorphisms (SNPs) were not replicated in larger cohort of UK population (151). In mouse models where colitis is induced via chemical disruption of the epithelial barrier, namely dextran sodium sulfate (DSS), deficiency in NLRP3 results in either protection (152, 153) or exacerbation (154-157) of the disease, which maybe a reflection of different microbiota between different animal facilities or the variation of immune activation depending on the dose of DSS.

Despite the conflicting data with NLRP3, there is compelling evidence supporting the pathogenic role of the downstream proinflammatory cytokine IL-1β. IL-1β is a potent modulator of both innate and adaptive immune responses. It can directly activate DCs and macrophages, promote neutrophil migration and T cell activation and survival (158). In addition, it is a key cytokine that drives the differentiation of "pathogenic" Th17 cells that have been shown to exacerbate the disease in models of IBD and EAE (159-162).

Several clinical reports have shown high levels of IL-1β secretion in colonic lamina propria mononuclear cells from patients with active IBD (163, 164). The production was found to be restricted to the active lesion sites, rather than control biopsies in the same patient, suggesting a role in driving local inflammation (165). The increased colonic IL-1β production is also found in several animal models of colitis, including chemical induced colitis, T cell transfer colitis, and spontaneous colitis developed in IL-10-deficient mice (166-170). The elevated IL-1β production could be due to elevated production of danger signals as a result of tissue damage, such as ATP (171), uric acid (172) or dysregulated pathways such as autophagy (173) and NOD2 signaling (174). These data suggest that increased activation of inflammasome maybe critical in driving the intestinal inflammation in IBD.

Supporting this notion, mice lacking IL-10 have upregulated expression of caspase 1 and IL-1β and develop spontaneous colitis, and treatment with caspase 1 inhibitor inhibits IL-17 production and reduces disease severity (170). In line with this finding, mice deficient in IL-1 receptor 1 are protected in the model of $CD45RB^{hi}CD4^{+}$ T cell transfer of colitis (169). Moreover, IL-1β blockade with anti-IL-1β antibody significantly decreases granulocyte recruitment and accumulation of IL-17 produced in innate lymphoid cells in *Helicobactor hepaticus* induced colitis in *Rag2-/-* mice (169).

In contrast to a clear pathogenic role of IL-1β in IBD, the role of IL-18 in the gut is more controversial. On one hand, IL-18 deficient mice showed attenuated colitis induced by DSS and 2,4,6-trinitrobenzene sulphonic acid (TNBS) (175-177); but on the other hand, IL-18 is essential for barrier integrity as illustrated in NLRP3 deficient mice suffering more severe disease upon DSS treatment due to a protective role of IL-18 and recombinant IL-18 reverses the disease severity (154).

Overall, given the important role of IL-1β in the pathogenesis of IBD, it would be important to investigate the efficacy of IL-1 targeted therapy in the disease.

1.4. Synopsis of research questions

While it is clear that TLR activation can induce both inflammatory and anti-inflammatory responses, the molecular switch that can determine the outcome remains unclear. A better understanding of how tissue derived damage signals modulate the innate immune responses can help us dissect the mechanism that drives chronic inflammation in a variety of autoinflammatory diseases. Since danger signals are predominantly studied in dendritic cells and macrophages, an outstanding question that merits investigation is how

other cell types, especially stromal cells, respond to damage signals, and how this response affects the crosstalk between innate and adaptive immunity.

Thus, my first research aim presented in chapter 3 was to investigate the role of danger signals, in particular, high concentrations of extracellular ATP, in modulating responses to TLR stimulation in epithelial cells. Due to the difficulty in culturing primary IECs, I performed *in vitro* assays using an IEC cell line to study how ATP changes TLR activation induced cytokine/chemokine responses. I also examined the purinergic receptors responsible for the ATP response on IECs. To further explore the downstream immune effects, I developed coculture assays using DCs treated with IEC conditioned media and $CD4^+$ T cells and asked if cytokines/chemokines or other soluble factors expressed by stress exposure to IECs affect DC maturation, and if so, if these DCs have enhanced ability to promote T cell proliferation to commensal antigens. To further validate the *in vitro* findings, I delivered ATP intra-rectally to mice and asked if ATP administration altered TLR response on IECs to endogenous TLR ligands produced by commensal bacteria.

Given the importance of cellular stress in potentiating inflammation, the second aim of my research was to examine if Tregs can modulate immune responses to cellular stress signals. Despite numerous studies showing the efficacy of $Foxp3^+$ Tregs and Tr1 cells in the models of autoimmunity and inflammatory diseases, their ability to regulate innate immune responses upon encounter of a danger signal remained to be defined. Another outstanding question was whether $Foxp3^+$ Tregs versus Tr1 cells have a unique therapeutic advantage in regulating stress responses. Therefore, with these questions in mind, I examined the ability of $Foxp3^+$ Tregs and Tr1 cells to modulate inflammasome activation, a key stress response pathway involved in many inflammatory and metabolic diseases. I conducted *in vitro* assays

to directly compare the suppressive ability of both Treg subsets to inflammasome activation and sought to pinpoint the mechanism of regulation in chapter 4. The results were further validated in chapter 5 using two mouse models, the monosodium urate induced peritonitis, and a murine model of CAPS.

Chapter 2: Materials and methods

2.1. Animals

Foxp3^{GFP} reporter (generation F11) (178), C3H/HeJ, *Foxp3*^{RFP}, *IL-10*^{GFP} reporter, *B6.PL-Thy1*^{a}*CyJ, B6.129S2-Il10rb* ^{tm1Agt}*/J (referred* to as *Il10rb*$^{-/-}$*), Nlrp3*$^{L351PNeoR/+}$, *B6.Cg-Tg (cre/ESR1)5Amc/J*(referred to as CAG-Cre), BALB/c and C57Bl/6 mice were purchased from Jackson laboratories(Bar Harbor, Maine, USA). *Foxp3*^{RFP}*IL-10*^{GFP} reporter mice were generated by crossing *Foxp3*^{RFP} reporter mice with *IL-10*^{GFP} mice. *Foxp3*^{GFP}*Thy1.1* reporter mice were generated by crossing *Foxp3*^{GFP} reporter mice with *B6.PL-Thy1*^{a}*CyJ* mice. *Nlrp3*$^{A350V/+}$ *CreT* mice were generated by crossing *Nlrp3*$^{A350VneoR/+}$ knock-in mice to CAG-Cre Tamoxifen inducible mice (this crossing was done at UCSD by Dr. Hal Hoffman and the bones used in the study were shipped to us); *Nlrp3*$^{L351P/+}$*CreT* mice were generated by crossing *Nlrp3*$^{L351PNeoR/+}$ knock-in mice to CAG-Cre mice, and the first generation from this cross was used for the *in vivo* experiments (179). It is worth noting that it is an autosomal dominant mutation and a singly copy of the NLRP3 transgene is sufficient to induce disease (179). All mice were bred in-house except where noted and maintained under specific pathogen-free conditions at the animal facility at the Jack Bell Research Center (in studies described in Chapter 3) or Child and Family Research Institute (in studies described in Chapter 4 and 5). The University of British Columbia (UBC) Animal Care and Use Committee and the University of California San Diego Institutional Animal Care and Use approved the experiments described in this study.

2.2. Cell Isolation, differentiation and culturing

2.2.1. IEC cell line-Mode-K cells

Mode-K cells are an immortalized mouse epithelial cell line that was kindly provided by Dr. Karen Madsen (University of Alberta), and cultured from passages 20 – 30. The cells were grown in HyQ DMEM/High glucose with 5% heat-inactivated FBS, nonessential amino acids, penicillin, streptomycin (both at 100 μg/ml and from Sigma, St. Louis. MO), and passaged three times weekly. For stimulation of cells, Mode-Ks were seeded at 2×10^5/ml in 24 well polystyrene plates and used for experiments after 24 hours when the cells were 70% confluent.

2.2.2. HEK 293 T cells

HEK 293T cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and grown in HyQ DMEM/High Glucose with 10% heat-inactivated FBS, penicillin, streptomycin, and nonessential amino acids. The cells were passaged three times per week. For transfection experiments, the cells were seeded at 7×10^4/ml in antibiotic-free media on a black 96-well plate and used for experiments after 24 hours.

2.2.3. Bone marrow-derived dendritic cells (BMDCs) and bone marrow-derived macrophages (BMDMs)

Bone marrow dendritic cells were generated from C3H/HeJ, C57/Bl6, and *Nlrp3*$^{A350V/+}$ *CreT* mice, cultured using a protocol developed by Lutz *et al.* (180). Briefly, bone marrow cells were flushed out of the femur and tibia, and cultured at a density of 2.5×10^5/ml in RPMI-1640 media containing mouse recombinant GM-CSF (eBioscience),

10% heat-inactivated FBS, 10 mM HEPES (StemCell), 2 mM L-glutamine, 50 μM β-mercaptoethanol (Sigma), penicillin and streptomycin. The cells were cultured in 100 mm^2 ×20mm polystyrene dishes for 7 days with half of the media changed on day 3 and day 6. On day 7 the BMDCs in suspension were carefully lifted and washed once before use. The adherent BMDMs were washed once with PBS, scraped off the plate, and resuspended in media. Expression of the *Nlrp3*^{A350V} transgene was induced by adding 4-hydroxytamoxifen (0.4μg/ml, Sigma) 24 hours before experiment.

2.2.4. Isolation of total or different subsets of $CD4^+$ T cells

$CD4^+$ T cells from spleens and lymph nodes were isolated from C3H/HeJ, *Foxp3*^{GFP}, *Foxp3*^{RFP}*IL-10*^{GFP} reporter or C57/Bl6 mice using a mouse anti-CD4-negative selection kit according to the manufacturer's instructions (StemCell Technologies, Vancouver, BC, Canada) and over 90% purity of $CD4^+$ cells was obtained after each isolation.

To isolate $CD25^-CD4^+$ and $CD25^+CD4^+$ cells, the cells were labeled with CD25-PE and anti-PE magnetic beads and passed over a magnetic column according to the manufacturer's instruction (Miltenyi Biotec).

To isolate $Foxp3^+$ Tregs, $CD44^{hi}Foxp3^-$ Tr1 and $CD44^{int/lo}$ $Foxp3^-$ naïve T cells, total $CD4^+$ cells were isolated by CD4 negative selection kit as above. The cells were then stained with CD4-efluor450 and CD44-APC, and then sorted into $Foxp3^+$ Tregs, $CD44^{hi}Foxp3^-$ Tr1 cells, and $CD44^{int/lo}Foxp3^-$ naïve T cells on a BD FACSAria to >95% purity (Fig. 2.1).

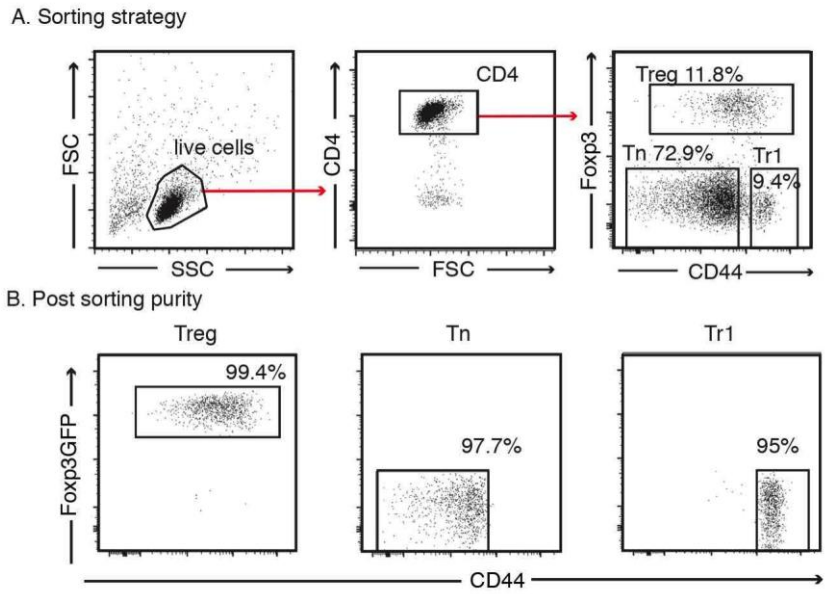


Figure 2.1 Sorting strategy and post sorting purity.
$CD4^+$ T cells were isolated from spleens and lymph nodes of *Foxp3*^{GFP} reporter mice by magnetic bead separation. The cells were then stained for CD4 and CD44, followed by flow cytometric sorting. **(A)** Gates were first set on total live cells, then $CD4^+$ cells. $CD4^+$ cells were then separated into $Foxp3^+$ Tregs, $CD44^{int/lo}Foxp3^-$ naive T conventional cells, and $CD44^{hi}Foxp3^-$ Tr1 cells. **(B)** Post sorting purity of Tregs, Tn, and Tr1 cells on the basis of CD44 and Foxp3 expression. Representative of 6 experiments.

In BALB/c mice, magnetic sorted CD4 T cells were then stained for CD25, CD45Rb, and CD44, and isolated into Tregs, Tr1 cells and naïve T cells on FACSAria (Fig. 2.2A). The purity of the cells was also confirmed after 4 days of anti-CD3/28 monoclonal antibody stimulation (Fig. 2.2B).

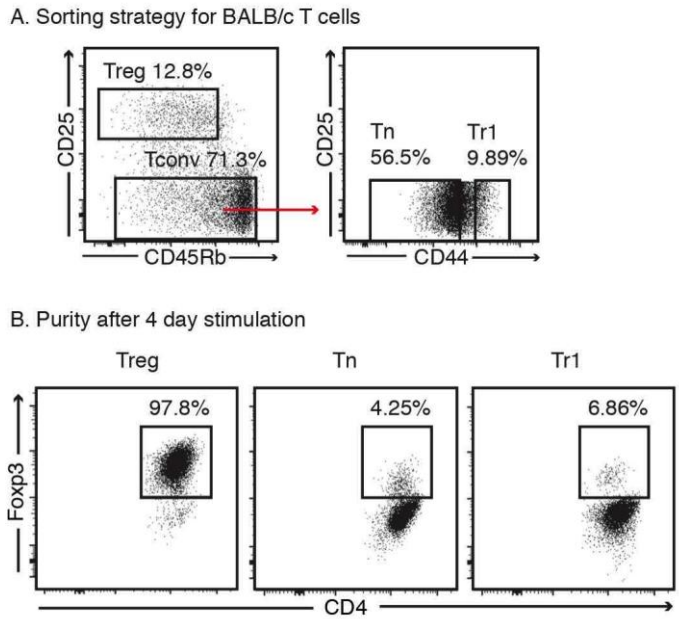


Figure 2.2 Sorting strategy and post sorting purity after 4 days of polyclonal stimulation for T cells isolated from BALB/c mice.
(A) The cells were first gated on live CD4 T cells and separated based on CD45Rb and CD25 to sort out $CD45Rb^{lo}CD25^{+}$ Tregs, and $CD25^{-}$ T conventional cells (Tconv). Tconv cells were then sorted into $CD44^{hi}$ Tr1 cells and $CD44^{int/lo}$ naïve T conventional cells. **(B)** Post sorting purity of Tregs, Tn, and Tr1 cells on the basis of Foxp3 expression. Representative of 3 experiments.

2.2.5. Isolation of $CD11c^{+}$ DCs from the spleen

Splenic DCs were isolated from mouse spleens. Briefly, freshly isolated spleens were cut into less than $1mm^2$ fragments and digest in spleen dissociation medium (StemCell) for 30 minutes at 37℃. The cells were then filtered and enriched for $CD11c^{+}$ DCs using mouse CD11c positive selection kit according to the manufacturer's instructions (StemCell).

2.2.6. Isolation of $CD90^{-}$ antigen presenting cells from the spleen

Total splenocytes were collected and resuspended in 10ml of PBS and layered onto 4ml of Lymphoprep (StemCell). The cells were spun for 15 minutes at 1800rpm with no break. The plasma-Lymphoprep interphase was then collected and followed by CD90

positive selection (StemCell). The CD90 depleted fraction was used as antigen presenting cells for the suppression assay as described later in **2.3.7**.

2.2.7. Isolation of colonic epithelial cells and lamina propria mononuclear cells (LPMCs)

Colons were cut longitudinally and washed 3 times with ice-cold PBS/0.1% bovine serum albumin (BSA). The tissue was then transferred in PBS /10% FCS/0.15% dithiothreitol (DTT), followed by shaking at 37 ℃ for 20 minutes. After incubation, the tube was vortexed vigorously, and the supernatant was discarded (to further remove mucus and feces). The tissue fragments were incubated in PBS containing 1.3mM EDTA on a rocker for 50 minutes at 37 ℃; the epithelial cells in the supernatant were filtered through 100μm nylon mesh filters, spun down and resuspend in TriZol for RNA extraction. The remaining pieces of tissue were then incubated in pre-warmed RPMI/10% FCS/15mM HEPES and cut into 1mm pieces followed by three 20 minute digestions with 0.2mg/ml type VIII collagenase (Sigma) in a 37 ℃ shaker incubator as described in (178). After each period, the digested tissue pieces were votexed vigorously and the supernatant filtered through 100μm nylon mesh filters. The three fractions collected were pooled and kept on ice until gradient separation. The LP cells were then spun down and resuspended in Percoll 30 (30% Percoll 100 in PBS/0.1% BSA), layered on 45%-70% Percoll gradient (Amershan Bioscience, Upplsa, Sweden), and centrifuged at 1800 rpm for 20 minutes with no brake or acceleration. The LPMCs were collected from the 40% to 75% interface and washed twice with PBS/0.1%BSA prior to subsequent assays.

2.3. Stimulation and treatment of cells

2.3.1. Stimulation of Mode-K cells and treatment of Mode-K supernatants

Mode-K cells were stimulated with freshly prepared solutions of ATP, UTP (Uridine 5´-triphosphate), or BzATP (3'-Benzoylbenzoyl adenosine 5´-triphosphate) (all from Sigma) for 20 minutes followed by 100 ng/ml Pam_3CSK_4 (PAM; InvivoGen, San Diego, CA). In some experiments cells were incubated with KN-62 (Sigma) for 30 minutes prior to addition of ATP. Supernatants were collected after 24 hours of stimulation and analyzed for KC and IL-6 by ELISA (Duo-set, R&D, Minneapolis, MN for KC and OptEIA, BD Biosciences, San Jose, CA, for IL-6) according to the manufacturers' instructions. Results are expressed as fold increase in cytokine concentration compared to PAM alone in each experiment. Total mRNA was isolated after 1 or 6 hours of Mode-K stimulation based on the peak of induction, and mRNA for TGFβ, TSLP, aldh1a2 and IL-15 were quantified with RT-PCR as described below.

For DC experiments, supernatants from Mode-K cells stimulated as above were incubated with 20 U/ml apyrase (Sigma) for 30 minutes at 37 ℃ to neutralize any residual ATP. ATP concentrations in Mode-K supernatants before and after apyrase treatment were measured using a luminescent ATP assay kit (SUNY, Buffalo, NY) according to the manufacturer's instructions.

2.3.2. Transfection of HEK 293 T cells

HEK 293T cells were maintained in antibiotic free media 24 hours before transfection. The cells were then transfected using Lipofectamine 2000 (Invitrogen) according to manufacturer's instruction with the following conditions, per well: pEGFP-N1 (Clontech) 1 ng, pEF6-hTLR2 or pEF6-hTLR5 5 ng, and salmon-sperm DNA to total 100

ng. The TLR5 construct was a gift from Alan Aderem (University of Washington) and the TLR2 construct was generated as described previously (181). The transfection efficiency was determined by percentage of GFP^+ cells as observed by fluorescence microscopy or by flow cytometry.

2.3.3. BMDC conditioning and activation

BMDCs were incubated for 24 hours with medium alone or conditioned cell supernatants, with or without 15 ng/ml of PAM. After 24 hours, cells were harvested for FACS analysis using the following antibodies: CD80-PE, MHC class II, and CD11c-APC. Cell viability was confirmed using 7-AAD (7-amino-actinomycin D). To measure the cytokine responses in BMDCs, total RNA was isolated after conditioning with Mode-K supernatants for 24 hours. In some experiments, DCs were pulsed with *E. coli* H18 flagellin (FliC) (182) in the presence of differentially treated Mode-K supernatants for 24 hours prior to coculture with T cells isolated from FliC immunized C3H/HeJ mice (refer to **2.3.4** for the immunization of FliC).

2.3.4. BMDC and T cell coculture experiments

To expand flagellin-specific T cells, we injected 10 μg of FliC intraperitoneally into C3H/HeJ mice, followed by two booster immunizations with 1 μg of FliC at two-week intervals. $CD4^+$ splenocytes were isolated from these immunized mice and stained with CFSE prior to coculture. C3H/HeJ BMDCs were pulsed with or without 10 μg/ml of FliC for 24 hours in the presence of different conditioned supernatants as described. DCs were then washed and cultured with isolated T cells at a ratio of 1:5 (DC/T cell) in a 96-well round bottom plate. The proliferation of T cells was analyzed after 3 days by flow cytometry.

2.3.5. Polyclonal stimulation and differentiation of CD4 T cells

After sorting, Foxp3^{+} Tregs, CD44^{hi} Foxp3^{-} Tr1 cells, and CD44$^{int/lo}$ Foxp3^{-} naïve T cells were plated at 1X10^{6}/ml and stimulated with immobilized anti-CD3 (10μg/ml), soluble anti-CD28 (2μg/ml), and rhIL-2 (200 unit/ml). For some experiments, IL-27 (50ng/ml, eBioscience) was added to the CD44$^{int/lo}$ Foxp3^{-} naïve T cells to generate IL-27 differentiated Tr1 cells. To differentiate Th1 cells, 10ng/ml of IL-12 (eBioscience) were added to the CD44$^{int/lo}$ Foxp3^{-} naïve T cells. On day 3, the cells were lifted and re-plated at 1X10^{6}/ml when cytokines and media were replenished, and supernatants were harvested for experiments on day 4 or 5.

2.3.6. T cell and BMDM cocultures

For co-cultures, pre-activated CD4 Tregs, Tr1 or Tn cells (according to **2.3.5**) or their conditioned media were added to BMDM for 30 minutes or 16 hours, respectively, at the ratio or concentration indicated in the figure legends. In some cases, 10μg/ml of IL-10 blocking antibody (JES5-2A5, eBioscience) or isotype control (IgG1κ, eBRG1, eBiscience) were added 30 minutes before adding T cells. The cultures were then stimulated with LPS (10ng/ml) for 5 hours, with addition of ATP (5mM) for the final 1 hour. Supernatants were collected for analysis by ELISA and cells were lysed for analysis of mRNA or protein expression by Western blot as described below.

2.3.7. T cell suppression assay

CD25^{-}CD4^{+} T responder cells were isolated according to **2.2.4**, labeled with cell proliferation dye eFluor670 (eBioscience) and cultured alone or with the indicated ratio of Tregs or Tr1 cells in the presence of irradiated CD90 depleted splenocytes (*as per* **2.2.6**) as

antigen presenting cells and 1μg/ml of anti-CD3. Proliferation was analyzed by flow cytometry after 3 days.

2.4. Sample analysis

2.4.1. RNA isolation and Quantitative Reverse-transcription Polymerase Chain Reaction (RT-PCR)

In chapter 3, RNA was isolated using Trizol (Invitrogen, Carlsbad, USA). RNA was quantified and 500-1000 ng from each sample was treated with DNase I, followed by reverse-transcription using RevertAid H-minus first strand cDNA synthesis kit. The cDNA was then quantified by real-time PCR using SYBR green based detection on an Opticon thermal cycler. Each reaction was performed in duplicate. The mRNA levels of β-actin for each sample were used for normalization and the fold induction for each cytokine compared to unstimulated control was calculated based on the $2^{-\Delta\Delta Ct}$ method. All reagents, except as noted, were obtained from Fermentas (Burlington, ON, Canada).

In chapter 4, RNA was isolated from T cells or BMDMs using E.N.Z.A Total RNA isolation kit according to manufacturer's instruction (OMEGA bio-tek, Norcross, GA, USA), and followed by reverse transcription using qscript cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD, USA). RT-PCR was performed on an Applied Biosysem 7500 Fast Real-Time PCR System. Data were normalized to 18s rRNA using the 2^{-ddCt} method. All primers are listed in table 2.1.

Table 2.1 List of primers

Gene Name	Forward primer (5'-3')	Reverse primer (5'-3')
18S	CAAGACGGACCAGAGCGAAA	GGCGGGTCATGGGAATAAC
Aldh1a2	CATGGTATCCTCCGCAATG	GCGCATTTAAGGCATTGTAAC
Actb	TATCGCTGCGCTGGTCGTCG	TGCTCTGGGCCTCGTCACCC
Ahr	AGCCGGTGCAGAAAACAGTA	CCAGGCGGTCTAACTCTGTG
Maf	AGGATGGCTTCAGAACTGGC	GGTCTCCACCGGTTCCTTTT
Il1b	TGTAATGAAAGACGGCACACC	TCTTCTTTGGGTATTGCTTGG
Il6	CCTCTCTGCAAGAGACTTCCATCCA	AGCCTCCGACTTGTGAAGTGGT
Il10	CAGAGCCACATGCTCCTAGA	TGTCCAGCTGGTCCTTTGTT
Il12a	CTGTGCCTTGGTAGCATCTATG	GCAGAGTCTCGCCATTATGATTC
Il12b	GGAAGCACGGCAGCAGAATA	AACTTGAGGGAGAAGTAGGAATGG
Il15	ACATCCATCTCGTGCTACTTGT	GCCTCTGTTTTAGGGAGACCT
Il23a	TCCCTACTAGGACTCAGCCAAC	GAACTCAGGCTGGGCATCT
Prdm1	CTGTACAAGCTGCCCCCAAG	TAAGGATGCCTCGGCTTGAA
Tgfb1	GGTGGACCGCAACAACGCCAT	GGGGTTCGGGCACTGCTTCC
Tslp	AGGCTACCCTGAAACTGAG	GGAGATTGCATGAAGGAATACC

2.4.2. Western Blotting for caspase 1 and pro-IL-1β

Macrophages were lysed in Laemmli buffer and proteins were denatured and separated on either 12% or 15% SDS-PAGE for analysis of IL-1β or Caspase-1, respectively. Separated proteins were transferred and probed with antibodies against IL-1β (R&D Systems), Caspase-1 (Santa Cruz Biotechnology) and β-Actin (Cell Signaling). Proteins were detected by HRP conjugated donkey anti-goat (Santa Cruz Biotechnology) or goat-anti-rabbit IgG (Dako), followed by SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) on CL-X Posure Films (Thermo Scientific) according to the manufacturer's instructions.

2.4.3. Flow cytometry

The detailed information of the antibodies is listed in **Table 2.2**. Dead cells were excluded using Fixable Viability Dye (FVD) efluor780 (eBioscience) or 7-AAD. For intracellular cytokine staining, the cells were stimulated with 50 ng/mL phorbol myristate acetate (Calbiochem, Mississauga, ON) and 1 μg/mL ionomycin (Sigma Aldrich) for 5 hours, with the addition of 10 μg/mL brefeldin A (Sigma Aldrich) for the last 4 hours. Intracellular staining was performed using Foxp3 staining buffer set (eBioscience). Flow cytometry was performed on FACSCalibur (BD), FACSCanto (BD), LSR II (BD) or Fortessa (BD), and analysis was performed on Flowjo vX0.7(Treestar).

Table 2.2 List of FACS antibodies used in the study

Name of the antibody	Clone	Fluorochrome	Supplier
CD4	RM4-5	FITC, PE, PerCP-cy5.5, PE-Cy7, APC, APC-Cy7, eFluor450, Brilliant Violet 605	BD, eBioscience, Biolegend
CD11b	M1/70	PE	BD
CD11c	N418	PE-Cy7, APC	eBioscience
CD25	PC61.5	APC	eBioscience
CD39	24DMS1	PE	eBioscience
CD44	IM7	APC	eBioscience
CD45Rb	A16	PE	BD
CD49b	HMa2	APC, PE	Biolegend
CD73	TY/11.8	PE	eBioscience
CD80	16-10-A1	APC, PE	eBioscience
CD90.1(Thy1.1)	HIS51	PE	eBioscience
CD90.2(Thy1.2)	53-2.1	PerCP, eFluor450	eBioscience
CTLA-4	UC10-4F10-11	APC	BD
F4/80	BM8	PE-Cy7	eBioscience
Foxp3	FJK-16S	FITC, PE, APC	eBioscience
Gr1	Rb6-8C5	FITC, APC	eBioscience
Helios	22F6	PE, FITC	BD
IFNγ	XMG1.2	PE-Cy7	eBioscience
IL-17	eBio17B7	FITC	eBioscience

LAG3	C9B7W	PerCP	Biolegend
LAP	TW7-16B4	PerCP	eBioscience
Name of the antibody	Clone	Fluorochrome	Supplier
MHC-II(I-$A^{k)}$	10-3-6	FITC	Santa Cruz
PD-1	J43	PE, APC	eBioscience
TIM-3	8B.2C12	PE, APC	eBioscience
Nrp1	BAF566	Biotin	R&D systems

2.4.4. ELISA

KC Duo-set ELISA(R&D, Minneapolis), IL-6 OptELISA (BD Biosciences), IL-1β ELISA (Biolegend), IL-10 ELISA (eBioscience) and IFNγ ELISA (eBioscience) were performed following manufacturer's instructions.

2.5. *In vivo* experiments

2.5.1. Intra-rectal delivery of ATP

Six- to eight-week-old mice were used for the experiments in Chapter 3, section **3.2.6**. 100 µL volumes were administered by inserting 4cm of intramedic polyethylene tubing (BD Biosciences) into the rectum to isofluorane-anesthetised mice as described previously (59). ATPγS enemas contained 100 µL of 10 mM ATPγS in 50mM Tris-HCl, pH 7.4, adjusted to 1 ml with PBS. Animals were euthanized after 18 hours and colons (excluding cecum) and MLNs collected. Colonic epithelial cells and lamina propria cells were collected as described in **2.2.7**. Isolated IECs were put into TriZol followed by RNA analysis. Isolated LPMC and lymphocytes from MLNs were stained for 7-AAD, CD11c-PE-Cy7, CD80-APC, and MHC II (I-A^k)-FITC followed by FACS analysis.

2.5.2. Adoptive transfer of T cells and injection of IL-10 in MSU induced peritonitis

Eight- to twelve-week-old mice were used for these experiments. Tregs, Tn, and Tr1 cells were isolated as described in **2.2.4**. 1 million cells or 400ug/ml of IL-10 were injected into the peritoneal cavity for 16 hours prior to the injection of 100ug of Monosodium urate (MSU, invivogen). Peritoneal lavage was performed after 5 hours with 5 ml of PBS. The cells collected were analyzed by flow cytometry.

2.5.3. Adoptive transfer of T cells in tamoxifen treated *Nlrp3*$^{L351P/+}$*CreT* mice

Three million Tr1 cells were injected intravenously into *NLRP3*$^{L351P/+}$*CreT* or *NLRP3*$^{L351P/+}$*WT* mice at the same time or 24 hours before the induction of disease. Tamoxifen was dissolved in vehicle consisting of 9:1 Sunflower seed oil from *Helianthus annus* (Sigma) and anhydrous ethanol to a final concentration of 20mg/ml. To induce the expression of Cre recombinase, 2.5ul per gram of tamoxifen (Calbiochem) per gram body weight was injected daily for 4 consecutive days, and the mice were monitored daily for activity and weights. After 24 hours of tamoxifen injection, about 50µl of blood was collected from saphenous vein. When the mice were euthanized, blood were collected by cardiac puncture and placed into a tube containing 20µl of heparin sodium salt solution (Sigma). The serum was collected for cytokine analysis using Th1/2/17 CBA kit (BD Bioscience) or IL-1β, and KC mouse flex set (BD Bioscience). The blood cells were treated 2ml of ammonium chloride solution (Stemcell) to lyse the red blood cells, and followed by flow analysis. Spleens were collected and the cells were harvested. The cells were either stained and analyzed by flow cytometry immediately, or stimulated with PMA and ionomycin for 1 hour with brefeldin A added for an additional 4 hours before flow analysis.

Mouse skin, liver, and colon biopsies were preserved in formalin, embedded in paraffin sections and stained with hematoxylin and eosin (H&E) staining for histological analysis.

2.6. Statistical analysis

Statistical analyses were performed in GraphPad Prism6. For multiple comparisons, groups were analyzed by one-way ANOVA followed by Tukey's Multiple Comparison Test. For non-parametric data, groups were analyzed by the Mann Whitney's test. Other tests are mentioned in the figure legends. Significance was set at *P* less than 0.05. Results are expressed as mean ± SEM or as mentioned.

Chapter 3: ATP conditions intestinal epithelial cells to an inflammatory state that promotes components of DC maturation.

3.1. Introduction

Intestinal epithelial cells (IECs) coordinate the dynamic interactions between luminal microbes and local immune cells and represent the front line of enteric defense (69). In addition to acting as a physical barrier to prevent passage of luminal contents, IECs are crucial for maintaining intestinal homeostasis by sampling the luminal microenvironment, integrating signals received from pattern recognition receptors and local immune cells, and secreting factors that regulate adaptive immunity by priming intestinal dendritic cells (183). Under steady state conditions, IECs maintain a hyporesponsive state to commensal flora by secreting factors such as thymic stromal lymphopoietin (TSLP), TGFβ, and retinoic acid (RA), which together facilitate the development of tolerogenic DCs and regulatory T cells (184, 185). During enteric infection, however, TLR-mediated activation of IECs can initiate robust inflammatory responses. How IECs discriminate between TLR ligands presented by invasive pathogens versus commensal microbes remains largely unknown (186).

One way that IECs may respond to pathogens is by recognizing specific danger signals like extracellular ATP (67, 187). Importantly, low expression of CD39 that results in defective ATP degradation is associated with increased risk for Crohn's disease (45). Consistent with this finding, CD39 deficient mice have increased susceptibility to DSS-induced colitis (45). The association of ATP and enhanced inflammation in the gut has also lead to the recent identification of commensal bacteria that actively produce ATP in the

lumen (188, 189), providing another important source of ATP in addition to epithelial injuries as a result of inflammation (190). In the context of IBD where patients have weaker intestinal barrier function and bacterial leakage across the mucus layer, intestinal epithelial cells represent the important first responder to cellular stress and microbial ligands. Therefore it is essential to understand how ATP regulates inflammatory responses to TLR activation in IECs.

Previous work in our laboratory showed that ATP regulates the inflammatory response to flagellin-TLR5 ligation in human IECs and that rectal administration of ATP enhanced flagellin-mediated inflammation during DSS colitis (59). In this study, we extend our findings to murine IECs and utilize an *in vitro* co-culture model with supernatants from IECs and bone marrow derived dendritic cells (BMDCs) to test how stressed IECs regulate DC maturation. Furthermore, we explored *in vivo* whether colorectal administration of ATP alone, in the absence of DSS colitis, is able to modulate how IECs respond to commensal microbes. We hypothesized that simultaneous exposure to ATP and TLR ligands would modulate how IECs respond to TLRs and thus affect DC maturation and ultimately determine how T cells respond to commensal antigens.

3.2. Results

3.2.1. ATP modulates the production of cytokines and chemokines by TLR1/2-activated IECs.

We previously showed that ATP alters TLR5 signaling in Caco-2 human IECs, and sought to determine whether this phenomenon applied to murine IECs as well. Since Mode-

K IECs do not express TLR4 and respond minimally to TLR5 ligand, we stimulated the cells with TLR1/2 agonist Pam_3CSK_4 (PAM) together with different concentrations of ATP. We focused on pro-inflammatory mediators and measured secretion of the neutrophil chemoattractant KC (Cxcl1) and IL-6 by ELISA, both of which have been previously reported to be secreted at high levels by Mode-K cells upon TLR stimulation (191). As shown in figure 3.1A, 1 mM ATP significantly augmented TLR1/2 activation-induced production of KC (from 29.9 ng/ml ± 1.41 to 73.3 ng/ml ± 2.67), while ATP alone did not induce KC expression. A similar increase in IL-6 production was observed with ATP at a lower concentration (from 156.4 pg/ml ± 20.04 to 259.2 pg/ml ± 9.49, Fig. 3.1B). Furthermore, the ATP-induced KC secretion was completely blocked by treatment with apyrase, which degrades ATP into ADP and AMP (from 73.3ng/ml ± 2.67 to 32.3 ng/ml ± 1.97, Fig. 3.1A), indicating this effect requires ATP and not its hydrolytic products.

Since IECs are known to produce tolerogenic factors that maintain immune tolerance to commensal microbes in the intestinal tract (184, 185), we also asked whether ATP modulated the expression of anti-inflammatory mediators. We thus measured mRNA expression of *Tgfb1, Tslp,* and *Aldh1a2* (encodes for retinaldehyde dehydrogenase 2), a rate-limiting enzyme in the synthesis of retinoic acid. ATP increased TLR1/2-mediated expression of mRNA encoding for each of these proteins (Fig. 3.1 C, D, and E). Since TGFβ and retinoic acid can promote Th1 and Th17 response in a proinflammatory milieu (192, 193), such as in the presence of large amounts of IL-6 or IL-15, they may act to reduce tolerance in the presence of cellular injuries.

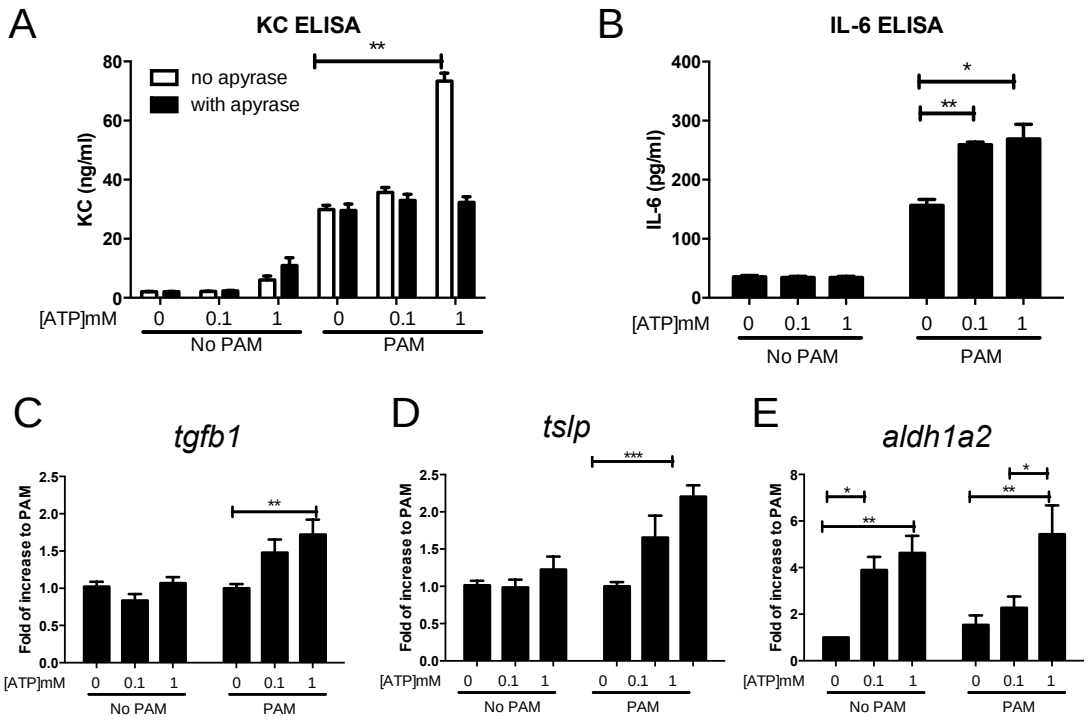


Figure 3.1 ATP enhances the cytokine/chemokine production in intestinal epithelial cells.
Mode-K cells were pre-treated with ATP at the indicated concentrations with/without apyrase for 20 minutes followed by 100 ng/mL Pam_3CSK_4 (PAM) for 24 hours **(A, B)** or 6 hours **(C, D, E)**. KC **(A)** and IL-6 **(B)** concentrations in supernatants were measured by ELISA and results are expressed as fold changes compare to PAM alone in each individual experiment (to correct for variations in absolute cytokine amounts between experiments). *Tgfb1*, *Tslp*, and *Aldh1a2* mRNA **(C, D, E)** are expressed as the fold change compared to PAM alone in each experiment. The results are compiled from at least three separate experiments. *$p < 0.05$, **$p < 0.01$, *** $p<0.001$ as shown.

3.2.2. ATP acts primarily through the P2X7 receptor on IECs.

Because most of the biological effects of extracellular ATP are mediated through P2 purinergic receptors (194), we explored which P2 receptors mediated the pro-inflammatory effect on the IECs. To study this, we first tested two different ATP analogues, UTP (uridine 5 ′-triphosphate) and BzATP (2′(3′)-O-(4-Benzoylbenzoyl)adenosine 5′-triphosphate), both of which have preferential binding to different P2 receptors. As shown in figure 3.2A, BzATP,

a preferential P2X7 receptor agonist (195), potently enhanced KC production (BzATP 3.4 ± 0.51 fold compared to ATP 3.1 ± 0.57 fold). In contrast, UTP, which is a relatively selective P2Y receptor agonist, only minimally increased KC production (Fig 3.2A, 1.36 ± 1.58 fold). In keeping with these results, KN-62, a non-competitive antagonist for P2X7, completely blocked the enhancement of KC secretion by ATP (Fig. 3.2B). These data indicate that the effects of ATP on IECs are largely mediated through the P2X7 receptor and not P2Y receptors.

Since the P2X7 receptor is known for the important downstream effect of inflammasome activation and IL-1β processing (196), we then asked whether paracrine release of IL-1β is responsible for the inflammatory effect of ATP in Mode-K cells, as has been reported in T84 human IECs (197). We did not detect any production of IL-1β by Mode-K cells at the protein level as a result of ATP and PAM treatment (data not shown). Moreover, when we treated Mode-K cells with IL-1ra in concentrations sufficient to block IL-1 receptor signaling (Fig. 3.2C), we did not inhibit KC production by Mode-Ks (Fig. 3.2D), suggesting that neither extracellular IL-1α nor IL-1β is responsible for the reported Mode-K phenotype that we observed.

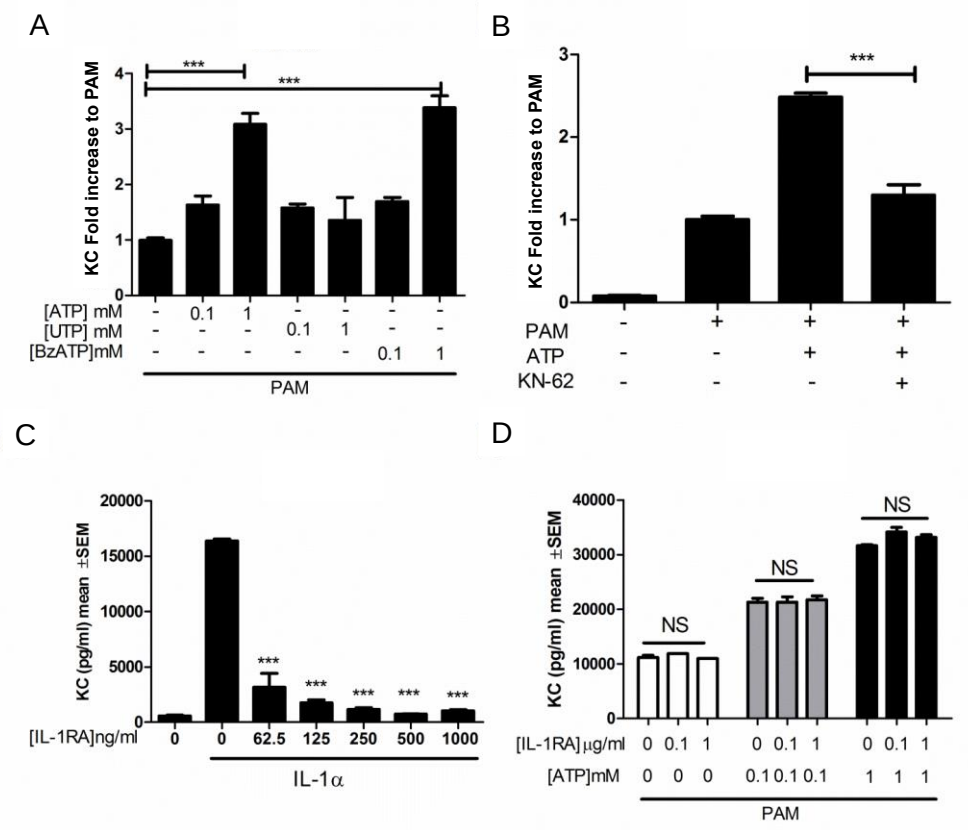


Figure 3.2 The inflammatory effect of ATP is mediated primarily through the P2X7 receptor and the effect is independent of IL-1.
Mode-K cells were pre-incubated with KN-62 (K) for 30 minutes, followed by ATP, UTP or BzATP for 20 minutes and PAM. Supernatants were collected after 24 hours of stimulation and KC concentration was measured by ELISA and expressed as fold of increase to PAM treated cells **(A, B)**. **(A)** BzATP mimics ATP in enhancing KC production. **(B)** KN-62 inhibits enhanced KC production by ATP. **(C)** Dose-response study of inhibition of IL-1α (10ng/ml) signaling by IL-1RA in Mode-K cells **(D)** Failure of IL-RA at effective concentrations to inhibit the stimulation of KC production by ATP in Mode-K cells **(C, D)**. The results are compiled from three separate experiments. *** $p<0.001$ compared to IL-1α treated Mode-K cells or as shown (one-way ANOVA followed by Tukey's Multiple Comparison Test).

3.2.3. Supernatants from ATP+PAM treated IECs enhance expression of CD80 on DCs.

In order to define the immunological consequence of stressed IECs on DC maturation, we exposed BMDCs to supernatants from IECs that were treated with PAM in

the absence or presence of ATP for 24 hours, then analyzed expression of CD80 and MHC II on $CD11c^+$ cells by flow cytometry.

Because the ATP+PAM treated IEC supernatants contained residual ATP and PAM after overnight culture, we neutralized the remaining ATP with the ATP degrading enzyme apyrase. After the treatment, the concentrations of ATP were reduced from 1 mM ATP and 100 μM ATP to 1.5±1.1 μM and 0.9±0.3 μM, respectively (Fig. 3.3).

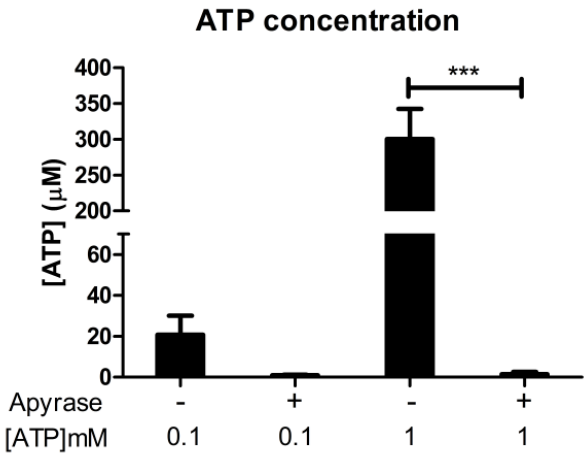


Figure 3.3 Apyrase effectively neutralizes residual ATP in IEC supernatants.
Mode-K cells were stimulated with/without 100 μM or 1 mM ATP for 24 hours. Supernatants were then incubated with 20 U/ml of apyrase for 30 minutes at 37℃. The concentration of ATP was quantified with a luminescent ATP assay. The result was averaged based on three separate experiments. *** $p<0.001$.

Next, we determined the residual TLR1/2-stimulatory activity in ATP+PAM treated IEC supernatants using an IL-8 release bioassay in TLR1/2-transfected HEK-293T cells. The TLR1/2 agonist activity present in the conditioned supernatants was equivalent to 15 ± 5 ng/ml of PAM (Fig. 3.4). Therefore, when we stimulated BMDCs, we added 15 ng/ml of PAM to each control supernatant if there was no PAM in it. DCs treated with PAM alone in the absence of IEC supernatants served as our matured DC control.

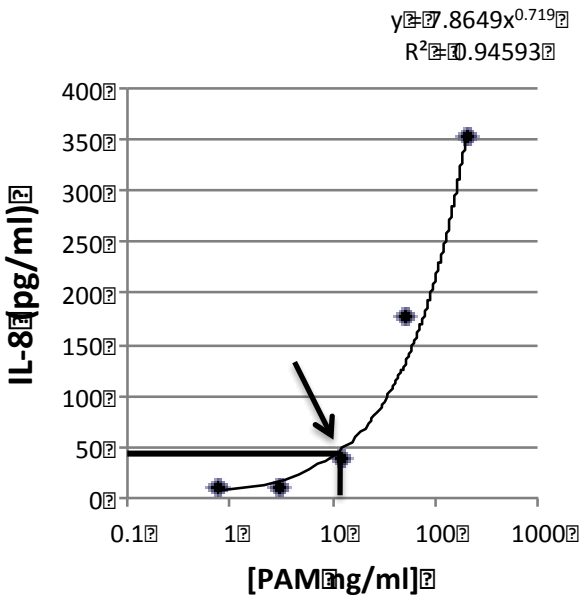


Figure 3.4 Bioassay for residual TLR1/2 agonist activity in conditioned IEC supernatants.
HEK-293T cells were transfected with TLR2 and pEGFP as a transfection control. After 48 hours, conditioned Mode-K supernatant was added in parallel with different concentrations of Pam_3CSK_4 as standards. After 24 hours, supernatant was analyzed for IL-8 secretion. Based on the calculation, the level of TLR2 agonist activity in conditioned Mode-K supernatants was equal to 15 ± 5 ng/ml, corresponding to approximately 48 pg/ml of IL-8. The result was averaged based on two separate experiments.

As shown in figures 3.5 A and 3.5 B, ATP+PAM treated IEC supernatants enhanced CD80 expression compared to supernatants from untreated IECs or IECs treated with either PAM or ATP alone. Only simultaneous ATP+PAM treated IEC supernatants were able to induce a significant increase of CD80, as opposed to single stimulus-treated IECs. In contrast to CD80, we did not observe any effect on the expression of class II MHC molecules (Fig. 3.5 B and 3.5 C).

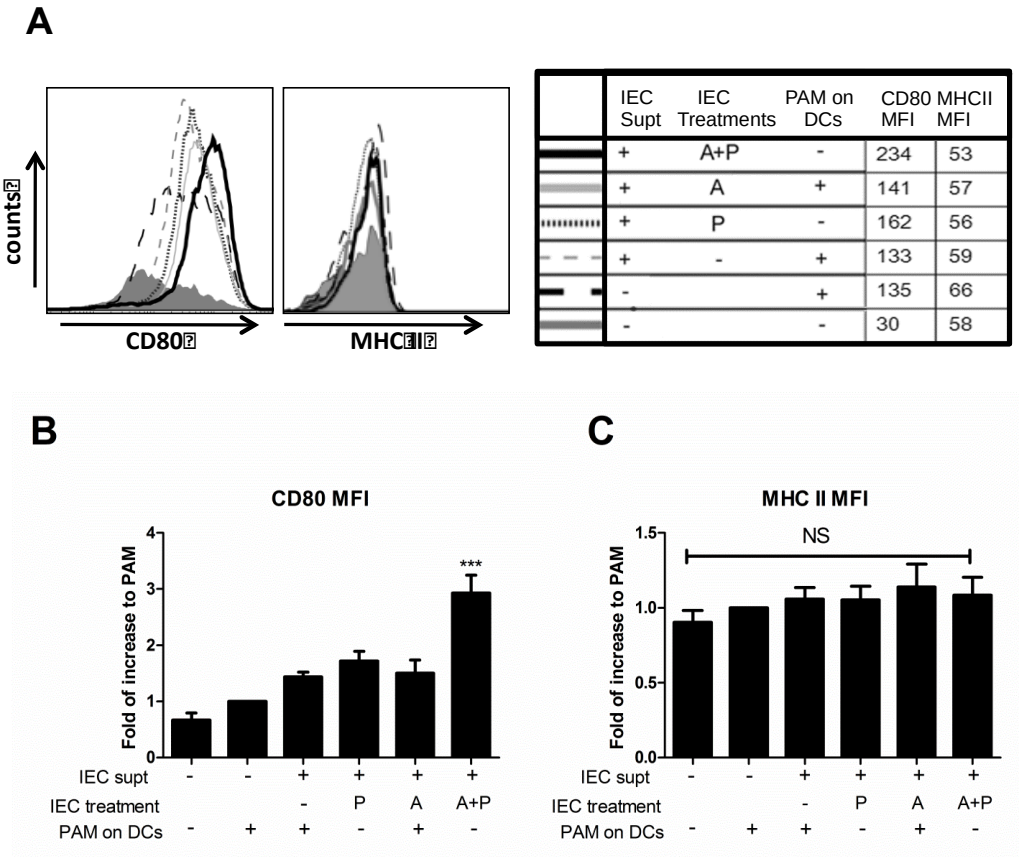


Figure 3.5 ATP treated IECs enhance BMDC CD80 expression.
Mode-K cells were stimulated with following: ATP (A) 1 mM, Pam_3CSK_4 (P) 100 ng/ml, or both (A+P) for 24h. IEC supernatants were collected and placed on BMDCs for 24 h. Apyrase was added to ATP-treated Mode-K supernatants to remove residual ATP. To match for the PAM concentration in the ATP+PAM treated IEC supernatants, 15 ng/ml of PAM was added onto the BMDCs which were treated with IEC supernatants that do not contain PAM. DCs were surface stained for MHCII ($I\text{-}A^k$) and CD80 and mean fluorescence intensity (MFI) calculated. **(A)** Representative CD80 and MHC II histogram from one of 4 experiments. **(B)** ATP+PAM treated IEC supernatants induced significantly higher CD80 expression than all other supernatants (***$p<0.001$ compared to all other groups). **(C)** ATP+PAM treated IEC supernatants did not induce MHC II expression. NS=not significant. Data expressed as mean ± SEM from 4 independent experiments normalized to the MFI of PAM matured DCs in regular media in each experiment. Statistical analysis was performed using one-way analysis of variance and Dunnett's post-test.

To exclude the possibility that ATP metabolites (such as AMP or adenosine) stimulate CD80 expression, we compared DCs stimulated with apyrase-degraded ATP to unstimulated DCs in the presence or absence of PAM and did not find any significant induction (Fig. 3.6).

Overall, these data suggest that conditioned media from stressed IECs cause a robust up-regulation of CD80 expression on BMDCs.

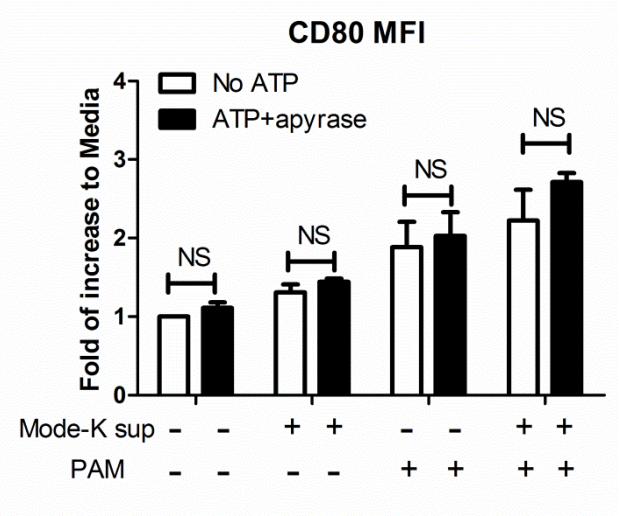


Figure 3.6 ATP degradation products do not induce CD80 expression in BMDCs. ATP solution was treated with apyrase for 30minutes before putting onto BMDCs in the presence of different supernatants as shown. DCs were conditioned for 24 hours and analyzed for CD80 expression by flow cytometry. The result was averaged based on three separate experiments.

We next measured CD80 expression in response to the UTP or BzATP stimulated Mode-K cell supernatants. We observed a similar effect of BzATP to ATP but not UTP in modulating CD80 expression on BMDCs (Fig. 3.7A), while KN-62 completely blocked the downstream effect of ATP on BMDC CD80 expression (Fig. 3.7B). Furthermore, IL-1RA did not inhibit CD80 induction on BMDCs by stressed IEC supernatants (Fig. 3.7C), confirming the effect is independent of extracellular IL-1.

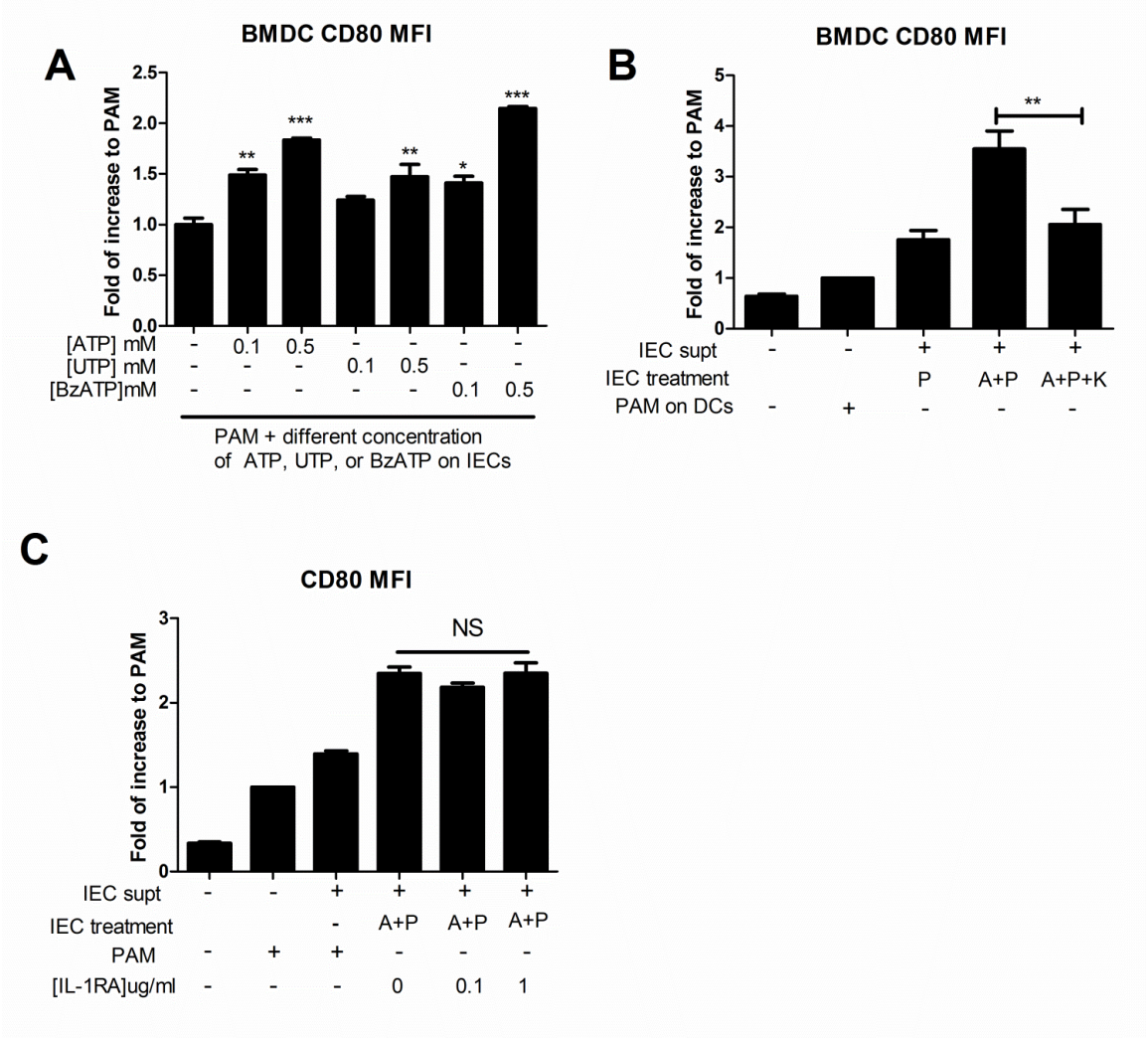


Figure 3.7 The ability of ATP treated Mode-K cells to induce CD80 on BMDC depends primarily on the P2X7 receptor and is independent of IL-1β secretion.
(A-C) Stimulated Mode-K supernatants were treated with apyrase and put onto DCs. CD80 MFI was calculated as described previously. Data expressed as mean ± SEM from 3 independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. NS=not significant (one-way ANOVA followed by Tukey's Multiple Comparison Test). **(A)** Mode-K cells were stimulated with different concentrations of ATP, UTP or BzATP for 20 minutes followed by PAM. **(B)** Mode-K cells were pre-incubated with/without KN-62 (K) for 30 minutes, followed by ATP for 20 minutes and PAM. **(C)** Different concentrations of IL-1RA were added to the supernatants prior to putting onto DCs.

We next tested the effects of stressed IEC supernatants on other DC populations. We found that ATP/Pam stressed Mode-K cells enhanced CD80 expression on C57Bl/6 BMDCs (data not shown), which are not deficient in TLR4 signaling. We found a similar trend in

CD80 expression in C3H/HeJ splenic DCs (Fig. 3.8A), and C3H/HeJ BMDCs derived in the presence of retinoic acid (Fig. 3.8B), the latter of which are reported to exhibit tolerogenic properties resembling *ex vivo* intestinal DCs (198).

In summary, these data suggest that multiple subsets of DCs can upregulate their CD80 expression upon exposure to soluble factors released by stressed epithelial cells, and that this effect depends on P2X7 receptor expression on IECs but is independent of IL-1β.

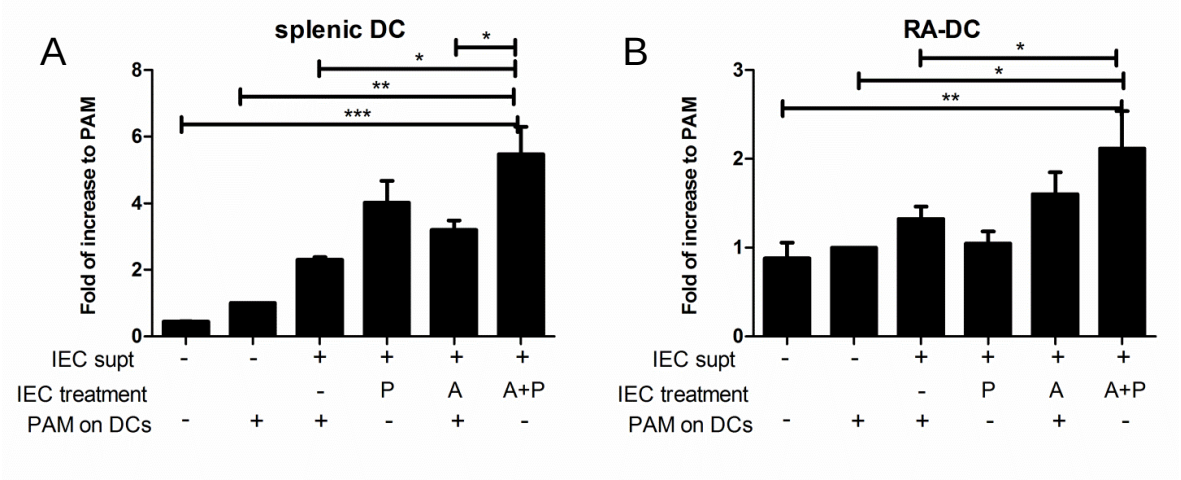


Figure 3.8 Increase CD80 expression was observed for splenic DC and retinoic acid treated BMDCs.
Mode-K cells were stimulated with the following: ATP (A) 1 mM, Pam_3CSK_4 (P) 100 ng/ml, or both (A+P) for 24h. IEC supernatants were collected, neutralized with apyrase and placed on BMDCs for 24 hour. To match for PAM concentration in the ATP+PAM treated IEC supernatants, 15 ng/ml of PAM was added onto the BMDCs that were treated with IEC supernatants that did not contain PAM. DCs were surface stained for CD11c and CD80, and mean fluorescence intensity (MFI) of CD80 were calculated. **(A)** IEC stress increase CD80 expression on splenic DCs from C3H mice. **(B)** 1 μM retinoic acid was added onto BMDCs on day 3 during in a 7-day differentiation process. Data expressed as mean ± SEM from 3 independent experiments normalized to the MFI of PAM matured DCs in regular media in each experiment.*p<0.05, **p<0.01, ***p<0.001. (one-way ANOVA followed by Tukey's Multiple Comparison Test)

3.2.4. Stressed IEC supernatants promote production of pro-inflammatory cytokines from DCs.

To further characterize how stressed IECs affect DCs, we examined cytokine production from these cells. To measure the cytokines released exclusively from the DCs, we analyzed the mRNA expression after conditioning with IEC supernatants and found that IECs exposed to PAM and ATP had a significant increase in the mRNA expression of *Il12a*, *Il12b*, *Il6*, *Il23a*, and *Tgfb1*, but not *Il10* or *Il15*, compared to controls (Fig. 3.9). Similar to what we observed in Mode-K cells, there was also increased expression of *Aldh1a2* in DCs (Fig. 3.9F), suggesting a likely increase in retinoic acid production. Such a cytokine environment would be predicted to favor the expansion of Th1 and Th17 cells.

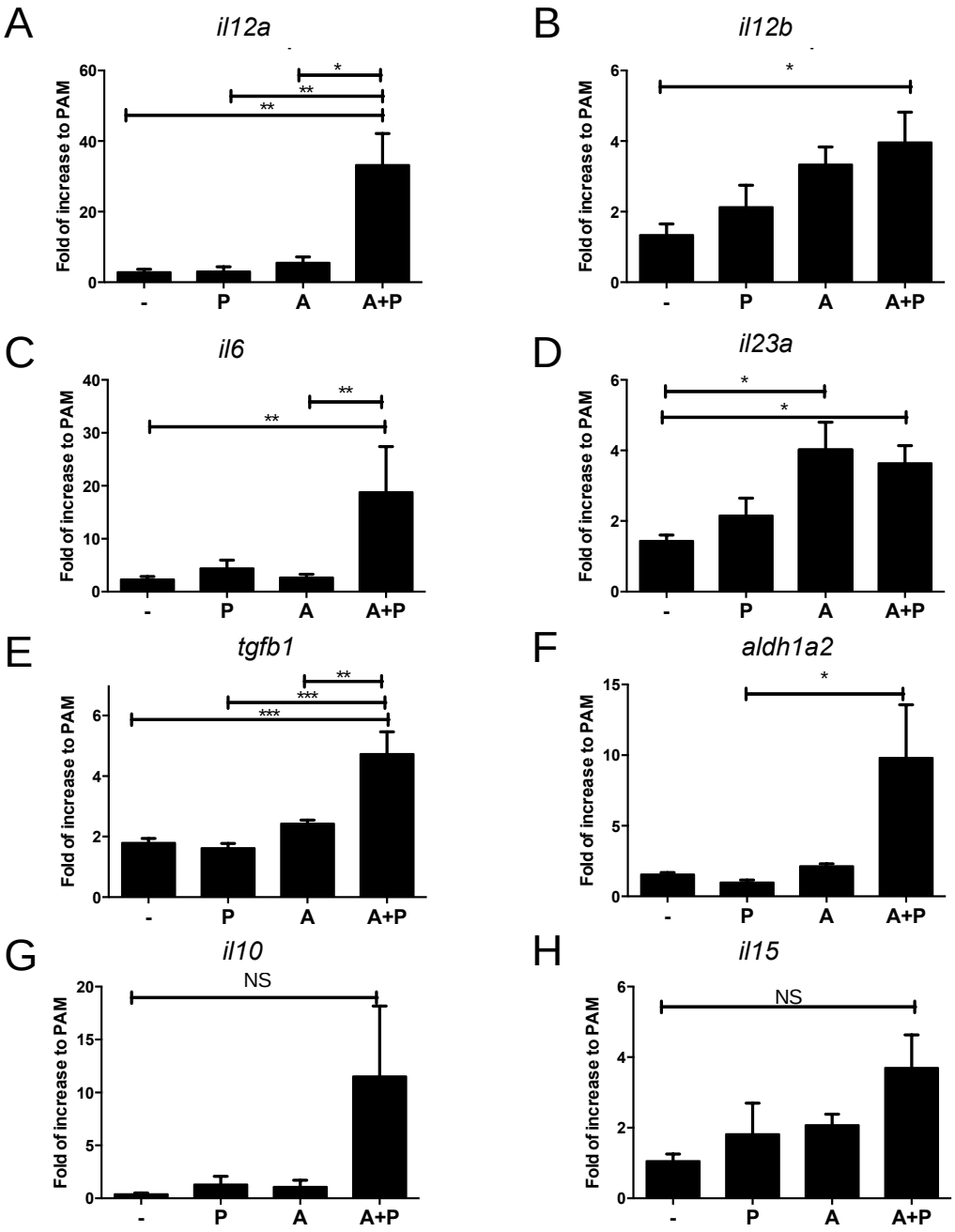


Figure 3.9 ATP+PAM treated IEC supernatants modulate BMDC cytokine production. Supernatants from Mode-K cells treated with 1 mM ATP (A), 100 ng/ml Pam (P), or both (A+P) were removed, treated with apyrase and mixed with BMDCs. BMDC RNA was isolated at 24 hours, reverse-transcribed and quantified with real-time PCR. Data are expressed as means ± SEM from 5 independent experiments normalized to the PAM treated DCs in each experiment. X-axis labeled as different treatments on Mode-K cells. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. NS=not significant.

3.2.5. ATP + PAM treated IEC supernatants support flagellin-specific T cell proliferation and expansion of Th1/17 cells.

We next assessed the ability of BMDCs exposed to stressed-IEC supernatants to stimulate a recall T-cell response to the model intestinal antigen, flagellin. First, to generate memory T cells that react to flagellin, we immunized C3H/HeJ mice with the *E. coli* H18 flagellin (FliC) and isolated $CD4^+$ T cells from the spleen. C3H/HeJ BMDCs were incubated with FliC in the presence of different conditioned supernatants overnight prior to co-culture with these $CD4^+$ T cells. As shown in figure 3.10C, DCs conditioned with supernatants from IECs exposed to ATP and PAM stimulated a significant increase in FliC-specific T cell proliferation compared to conditions without ATP. As predicted from the cytokine production profile from DCs, ATP + PAM IEC supernatant-treated DCs supported the expansion of $IFN\gamma^+$ $CD4^+$ T cells (Fig. 3.10A and D), and $IL\text{-}17^+$ $CD4^+$ T cells (Fig. 3.10B and E) compared to conditions without ATP. Using a fixable viability dye, we confirmed that all the supernatants induce similar levels of cell death therefore excluding the possibility that this is simply due to selective cell survival (data not shown). These data support our hypothesis that exposure of IECs to both ATP and PAM drives the proliferation of proinflammatory T cells and facilitates the expansion of Th1 and Th17 cells.

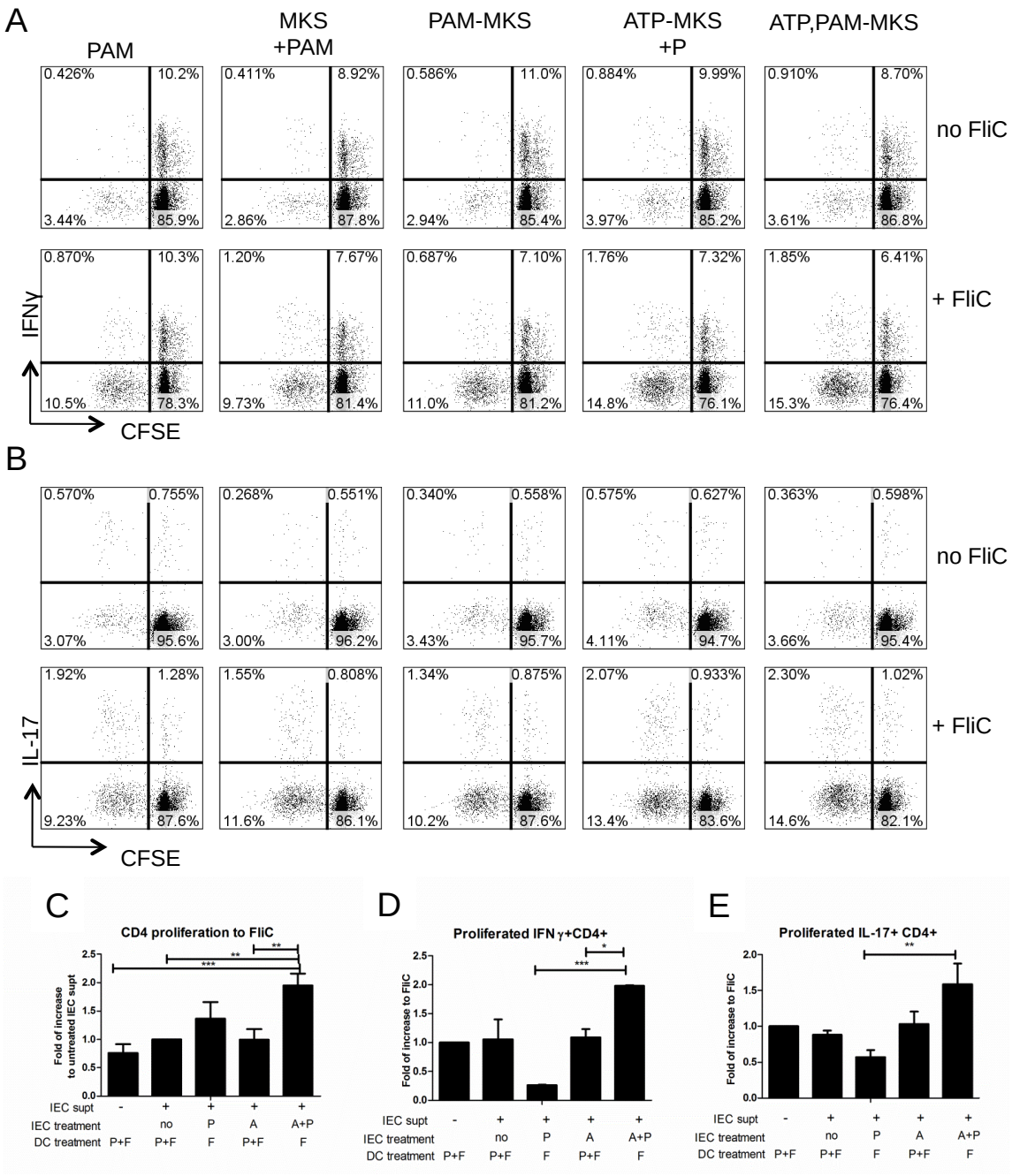


Figure 3.10 ATP+PAM treated IEC supernatants increase T cell recall response to flagellin.

(A-E) BMDCs were pulsed with or without *E. coli* flagellin (FliC) 10 μg/ml for 24 hours in media alone, untreated Mode-K supernatants, or ATP and/or Pam-treated Mode-K supernatants. The DCs were then washed and cocultured with CFSE stained splenic $CD4^+$ T cells isolated from FliC-immunized *C3H* mice. DCs and T cells were cocultured for 72 hrs and the proliferation and intracellular cytokine production were analyzed for the $CD4^+$ T cells. **(A, B)** Representative FACS plots for flagellin or no flagellin pulsed DCs in different

supernatants (**C**) The percentage of proliferated FliC-specific T cells were calculated by subtracting proliferating T cells ($CFSE^-$) in FliC treated versus untreated DCs in the same Mode-K conditioned supernatants. From that, the value was normalized to the unstimulated IEC supernatant to account for experimental variance. Results expressed as mean ± SEM from five independent experiments. (**D**, **E**) The percentage of IFNγ or IL-17 producing T cells was calculated by subtracting proliferating cytokine producing T cells ($CFSE^-IFN\gamma^+$, or $CFSE^-IL\text{-}17^+$) in FliC treated versus untreated DCs. From that, the value was normalized to the unstimulated DCs to account for experimental variance. Results expressed as mean ± SEM from three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (one-way ANOVA followed by Tukey's Multiple Comparison Test)

To determine whether stressed IEC-conditioned DCs would affect primary responses in naïve T cells, we performed a mixed lymphocyte reaction, incubating conditioned C3H/HeJ DCs with splenic T cells from *Foxp3*^{GFP} reporter mice. We found that DCs conditioned with ATP+PAM-treated IEC supernatants increased T cell proliferation with a greater percentage of proliferating cells producing IFNγ (Fig. 3.11). We did not observe any significant modulation in the percentage of total $Foxp3^+CD4^+$ T cells (Fig. 3.11C and F). Together, these results suggest that stressed IECs can condition DCs to enhance both primary and memory T cell responses.

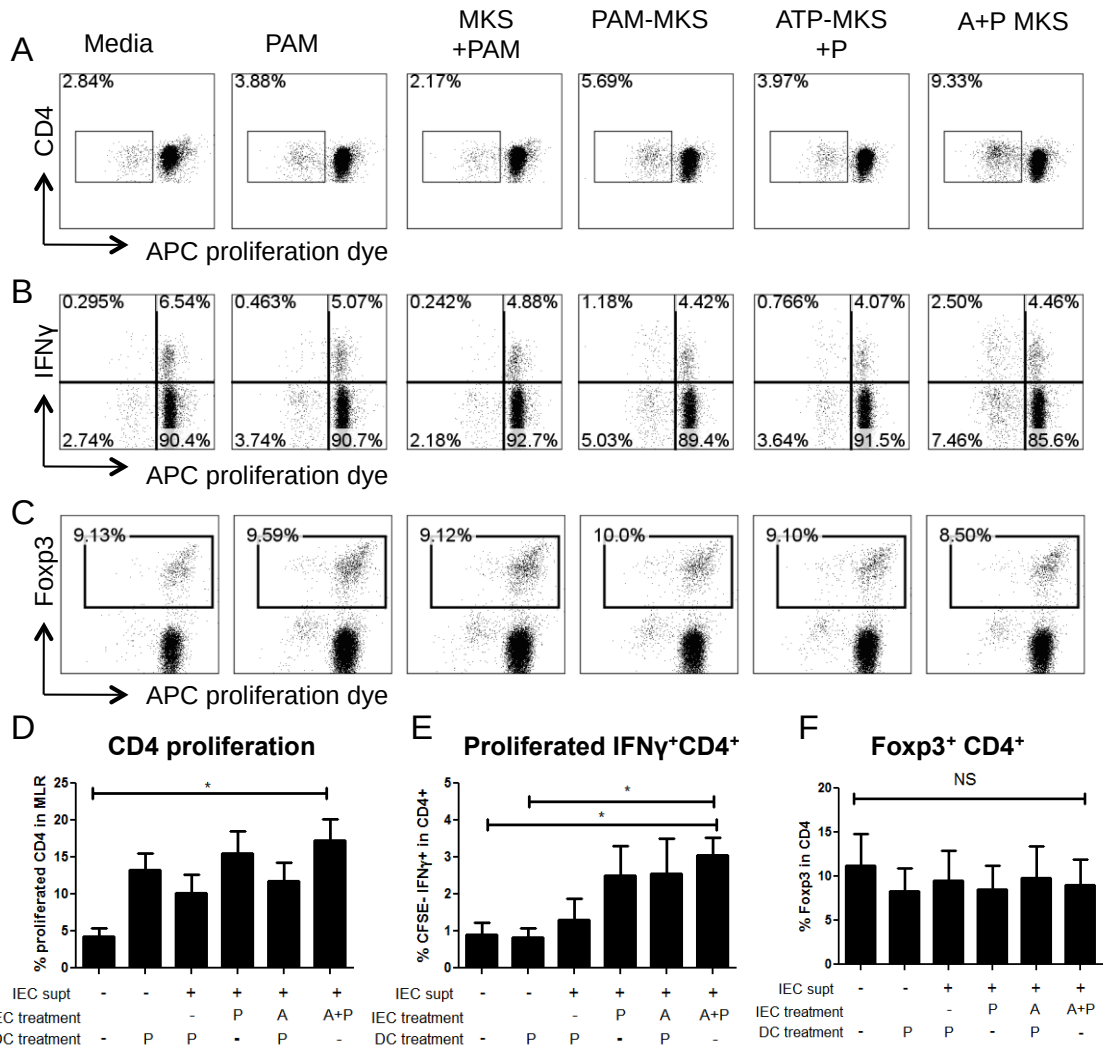


Figure 3.11 ATP+PAM treated IEC supernatants enhance polyclonal T cell proliferation.

Mode-K cells were stimulated with the following: ATP (A) 1 mM, Pam_3CSK_4 (P) 100 ng/ml, or both (A+P) for 24h. IEC supernatants were collected, neutralized with apyrase and placed on BMDCs for 24 h. C3H/HeJ BMDCs were conditioned in media alone, PAM, or different Mode-K supernatants with matching concentrations of PAM in each supernatants. The DCs were then washed and cocultured with APC proliferation dye stained splenic $CD4^+$ T cells isolated from a *Foxp3*^{GFP}(C57/Bl6) reporter mouse. DCs and T cells were cocultured for 72 hrs and analyzed for T cell proliferation and intracellular cytokine production. (A, B, C) Representative FACS plots from one of the three experiments. (D, E, F) Averaged data from three experiments. *$p<0.05$, NS= not significant. (one-way ANOVA followed by Tukey's Multiple Comparison Test)

3.2.6. Colorectal injection of ATP enhances proinflammatory cytokine production by IECs and the maturation of DCs in vivo.

To investigate whether ATP alters the IEC responses to endogenous TLR ligands presented by commensal microbes, we injected ATPγS, a non-hydrolysable form of ATP intra-rectally, and harvested colons 18 h later. We found that ATPγS was able to significantly increase the expression of *Cxcl1* and *Il6* mRNA in IECs (Fig. 3.12 A and B). To identify whether this treatment induced CD80 expression in intestinal DCs, we analyzed the $CD11c^+$ DC population in lamina propria (LP-DC) and mesenteric lymph nodes (MLN-DC). We found a trend towards an increased CD80 in LP-DCs after 18 hours (Fig. 3.12C) and MLN-DCs after 48 hours (Fig 3.12D), although the difference was not statistically significant.

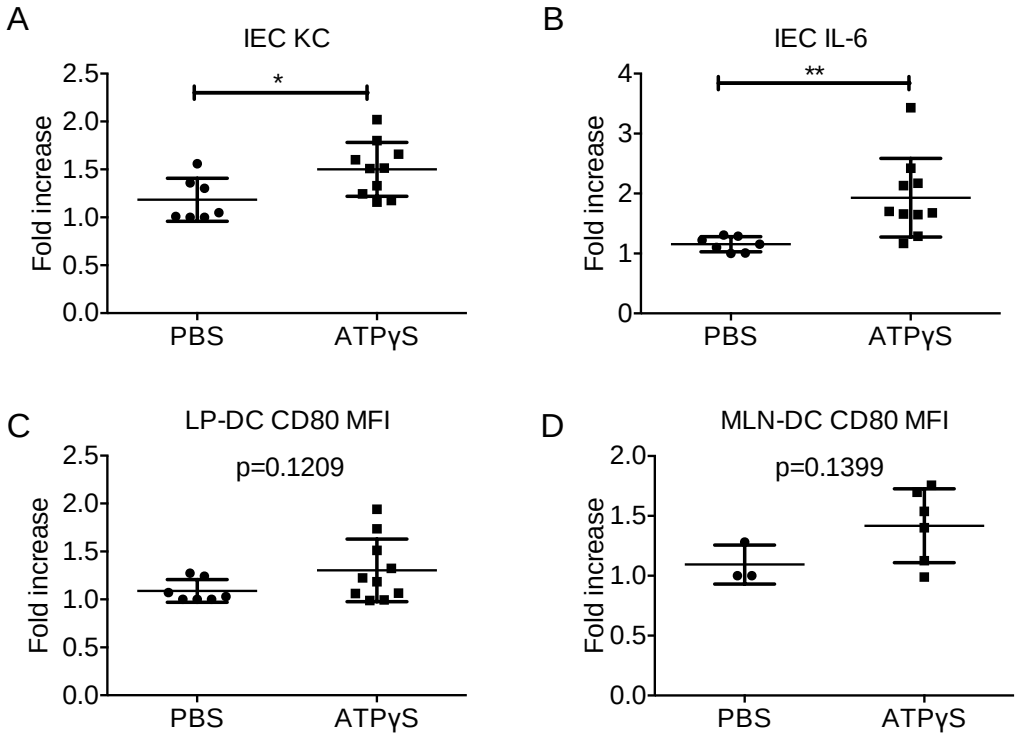


Figure 3.12 Colorectal administration of ATPγS increases IEC cytokine production and DC maturation.
Mice were given either ATPγS or PBS enemas, and euthanized 18 h later. The epithelial cells were isolated from the mouse colon, and RNA was extracted from each sample and analyzed by RT-qPCR. **(A)** IL-6, **(B)** KC(cxcl1). Error bars represent standard deviation. *p<0.05, **p<0.005 (t-test). **(C)** Lamina propria DCs were isolated and the expression of CD80 was analyzed on 7-AAD$^-$CD11c$^+$ population, and expressed as fold increase to normalize the difference between experiments. Error bars represent standard deviation. p=0.1209 (t-test) **(D)** Mice were given enemas daily for 2 days, and euthanized on day 3. Mesenteric lymph nodes were collected and cells analyzed by flow cytometry. The expression of CD80 was analyzed on 7-AAD$^-$CD11c$^+$ population and expressed as fold increase compared to one PBS treated mouse. Error bars represent standard deviation. p=0.1399 (t-test).

3.3. Discussion

There is increasing appreciation that stressed cells can release danger signals to alert the immune system and shape the downstream immune response (50, 67, 187). As an important extracellular signal of stress and injuries, ATP on its own can direct the maturation of DCs and facilitate the differentiation of naive T cells (171, 199, 200), while little is known

about its effect on IECs. Our previous published work suggests that the ability of epithelial cells to sense ATP is one way that IECs can distinguish between homeostatic and dangerous conditions. In this work, we sought to prove that ATP not only affects IEC chemokine production, but also switches them from being tolerogenic to being proinflammatory. Herein, we demonstrated that ATP and TLR1/2 stimulation together promote an inflammatory response in IECs, leading to secretion of soluble factors that increased expression of CD80 and transcripts of pro-inflammatory cytokines by DCs. The resulting DCs cause an enhanced stimulation of IFNγ– and IL-17-expressing $CD4^+$ T cells specific for the important intestinal antigen flagellin. Furthermore, we showed that ATP enemas can lead to increased IL-6 and KC production in colonic IECs in the absence of exogenous TLR stimulation, suggesting that ATP is able to change how IECs respond to endogenous TLR ligands. Together our data support the notion that sensing extracellular ATP is one mechanism IECs use to turn on proinflammatory responses (Fig. 3.13).

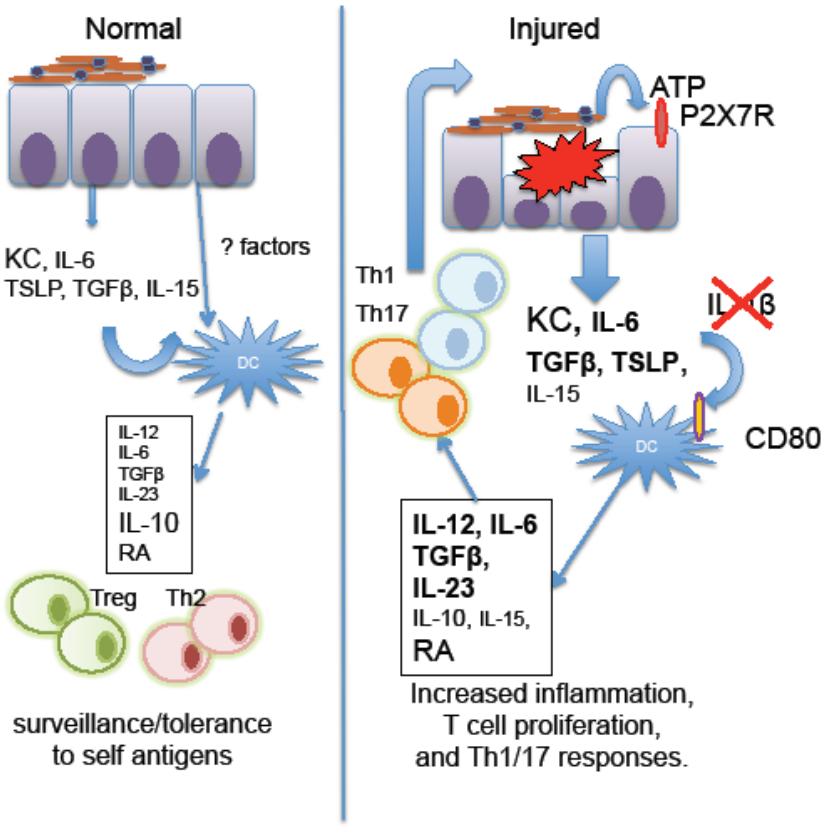


Figure 3.13 Proposed model for the effect of IEC stress on intestinal immunity. Left: normal mucosa. Unstressed IECs respond weakly to TLR ligands from intestinal bacteria (shown in lumen) and secrete basal KC (to recruit neutrophils). The IECs also condition DCs via factors such as TSLP and TGFβ to produce tolerance and regulatory T cells. Right: acutely injured mucosa. High concentrations of extracellular ATP in concert with TLR ligands induce increase KC, IL-6, TGFβ and TSLP. This leads to increased DC maturation and increased IL-12, IL-23, IL-6, and TGF β, and promotes Th1 and Th17 polarization. Increased RA production in the presence of IL-15 further enhances the Th1 and Th17 responses.

The increased cytokine/chemokine production in Mode-K IECs upon TLR1/2 ligation is similar to what we have observed in Caco-2 cells with TLR5 stimulation (59) and the effect was largely mediated by the P2X7 receptor. This suggests that the ATP effect does not depend on specific TLR stimulation and it is likely to be a more universal effect. Future studies are warranted to test whether ATP has a similar effect on augmenting other TLR responses in Mode-K cells and primary intestinal epithelial cells. We selected Pam_3CSK_4 for

these experiments because Mode-K cells, derived from C3H/HeJ mice, lack functional TLR4 and show minimal responses to TLR5. The involvement of the P2X7 receptor on IECs is not surprising as it has been implicated in a variety of chronic inflammatory diseases, where P2X7 activation is tightly linked to inflammasome assembly and IL-1β production (46-48, 200, 201). In contrast to these studies, we did not observe any IL-1β production by Mode-K IECs and blocking IL-1 receptor signaling by IL-1RA did not inhibit the enhanced cytokine production by IECs, suggesting this effect is unlikely to be mediated through paracrine release of either IL-1α or IL-1β. Most recently, the P2X7 receptor has been found to positively regulate MyD88 dependent NFκB activation (202), which offers a possible mechanistic explanation for the heightened TLR response in IECs. However, it should be noted that the specificity for BzATP and KN-62 for P2X7 is not perfect, and other P2X receptors (particularly P2X4) may also be affected by these compounds. While there are no specific P2X4 agonists or antagonists available, we reported that P2X4 and P2X7 can both augment TLR5 signaling in Caco-2 cells, suggesting that both receptors could be operational here as well (59). In order to confirm the involvement of P2X7 receptor in regulating ATP response, future studies can be conducted to look at whether intra-rectal delivery of ATP fail to regulate cytokine/chemokine response in P2X7 receptor knockout mice (available from Jackson Laboratory, stock number 005576).

To investigate the biological relevance of the ATP effect on IECs, we characterized the phenotypic changes of BMDCs conditioned with IEC supernatants. We showed that only when Mode-K cells were treated with a TLR2 ligand plus ATP were they able to induce a robust increase in CD80 on BMDCs. In contrast, we did not observe any induction of MHC II, likely because the GM-CSF that is present in the culture media stimulates maximum

expression of MHC II (203). This is supported by our observation that IFNγ treated BMDCs expressed similar levels of MHCII (data not shown).

Further analysis of the BMDCs conditioned with TLR1/2 ligand and ATP stressed-IEC supernatants showed a large increase in both Th1 and Th17 polarizing cytokine transcripts, namely *Il12a, Il12b, IL23a, Il6, Tgfb1*. This finding further supports our conclusion that the phenotypic change is not due to a direct effect of ATP on DCs since ATP stimulation of DCs is reported to inhibit IL-12 production (52). In contrast, when DCs are cultured with healthy IEC supernatants, they demonstrated decreased expression of IL-12 and IL-23 and increased IL-10 (184, 185). One limitation of the study is that we did not measure the cytokine production from these DCs at the protein level since the Mode-K cell supernatants used to condition the DCs may already contain some of these cytokines at various amounts. Therefore, we only analyzed the transcripts of various cytokines by qPCR to exclusively measure the mRNA expression from DCs. However, the results from our DC-T cell cocultures suggested that these DCs have the ability to facilitate the expansion of Th1/Th17 responses, indicating that the DCs can make the Th1/17 polarizing cytokines *in vitro*.

The increase in aldh1a2 expression in stressed IEC-conditioned DCs compared to the controls was somewhat surprising since RA is generally believed to promote tolerance rather than inflammation. Depaolo *et al*. have recently shown that retinoic acid can further enhance Th1/17 development in the presence of IL-15 via DC dependent mechanisms (192). Notably, IL-15 expression is increased in inflamed mucosa of IBD patients (204-206) and it is able to induce IFNγ and TNFα production in lamina propria T cells isolated from IBD patients but not in healthy controls (205, 206). In our co-culture system, we confirmed IL-15 transcript

expression both by Mode-K cells (data not shown) and BMDCs as shown by previous studies (207, 208). The level of IL-15, however, was not significantly altered by PAM and/or ATP. The lack of induction may be due to the nature of the stimuli as IL-15 is not known to be induced by PAM but rather to CpG, LPS and IFNγ (208, 209). Together with the enhanced costimulatory molecule CD80 expression, the increase in Th1 and Th17 polarizing cytokine production suggests a more mature phenotype of these DCs as a result of epithelial cell stress.

In light of the phenotypic changes on BMDCs resulting from epithelial cells stress, we hypothesized that these DCs would have increased capacity to activate T cells. We tested this hypothesis using our model antigen flagellin since anti-flagellin responses have important implications in the pathogenesis of inflammatory bowel disease (210-212). In addition to its well-characterized role in TLR5 activation, flagellin has been identified as a dominant antigen in patients with Crohn's disease (CD) (210, 213, 214), with about half of patients producing serum antibodies against flagellin (213, 214). The increased flagellin antibodies in these patients suggest activation of flagellin-specific T cells. Supporting evidence from a murine model has shown that a flagellin-specific $CD4^+$ T cell clone can induce colitis when adoptively transferred into immune deficient mice (215). The antigenic properties of flagellin have been further explored in *TLR5-/-* mice, and we have shown that flagellin enemas exacerbate DSS induced colitis independent of TLR5 (216). To examine whether this anti-flagellin response is subject to regulation in the context of cellular damage, we tested whether ATP stimulation of IECs can modulate anti-flagellin responses. Our data suggest that, in the context of cellular stress such as ATP, TLR stimulation of IECs lead to further enhancement of inflammation, resulting in enhanced flagellin presentation by DCs as

shown by increased $CD4^+$ T cell proliferation. Furthermore, this enhanced T cell proliferation was also observed in a mixed lymphocyte reaction with naive $CD4^+$ T cells, an effect, which could be due to the increased production of IL-6 and IL-12 by the DCs. It is worth noting that we have not tested other foreign antigens in the coculture system, such as influenza, tetanus or ovalbumin, which would reflect primary responses instead of re-activation of endogenously primed T cells as in the case of flagellin response. Future experiments are warranted to examine how epithelial cells modulate primary T cell responses in the context of cellular stress.

Our *in vitro* findings also indicated that ATP stimulated IECs can facilitate the activation of Th1 and Th17 cells where aberrant Th1/17 responses have shown to be pathogenic in IBD (217, 218). The increased Th17 response was consistent with observations by Atarashi *et al.* (171), who showed that colonic administration of ATP exacerbated colitis in a T cell transfer model and that this was mediated through increased Th17 polarization and activation of a distinct subset of lamina propria DCs expressing $CD11c^{low}CD70^{high}$. Increased Th1 and Th17 responses have also been reported in CD39 null mice, which lack efficient ATP degradation (219, 220). Despite seeing an increased percentage of Th1 and Th17 cells, we failed to observe any significant population of $Foxp3^+$ cells in the proliferated cells due to the low sensitivity of the flagellin specific antigen response in this non-transgenic system. To further investigate this question we used an MLR system with responder Foxp3-eGFP T cells, and found that the proportion of $Foxp3^+$ regulatory T cells did not change in the presence of stressed IEC supernatant. These data suggest that that ATP causes a skewing towards proinflammatory cells without a concomitant increase in regulatory cells.

In our *in vivo* model, we found that ATPγS increased production of IL-6 and KC mRNA from primary IECs, confirming the *in vitro* data. We also found a trend towards increased expression of CD80 expression in the lamina propria DCs and mesenteric lymph node DCs. The inconsistent ability of ATP alone to induce a significant increase in CD80 might be due to the insufficient diffusion of ATP across the mucosal barrier when the mice are not colitic, or not enough TLR ligand under the non-inflamed state to reach the epithelial cells to initiate a strong IEC response as seen in an *in vitro* system. Indeed, we found that IL-6 was more highly upregulated than KC, consistent with our *in vitro* data showing IL-6 expression is facilitated at lower concentrations of ATP than KC. Despite that, our results suggest that ATP alone is able to modulate how IECs respond to endogenous TLR ligands, which may in turn affect DC maturation. Our finding is consistent with previous reports on CD39 knockout mice, suggesting deficiency in ATP degradation exacerbates DSS induced colitis (45).

In conclusion, our studies provide evidence that cellular stress signals are able to activate epithelial cells, leading to enhanced DC activation and intensified T cell responses. Since IBD is driven partly by over reactive T cell responses to commensal antigens (183, 221), and heightened Th1/17 responses are pathogenic in Crohn's disease (141, 215), our results constitute a significant advance in understanding how the chronic cycle of intestinal inflammation in IBD is maintained.

Chapter 4: Tr1 cells, but not Foxp3$^+$ Tregs suppress inflammasome activation via an IL-10 dependent mechanism

4.1. Introduction

Both Foxp3$^+$ Tregs and Tr1 cells are extensively studied in models of autoimmunity, metabolic diseases and infectious diseases for their ability to negatively regulate adaptive immune responses by interfering with the activation of antigen presenting cells and other T cells (73, 96, 119, 144, 222-225). In addition to their ability to control adaptive immune responses, they are also known in regulating innate immunity primarily on APCs. Foxp3$^+$ Tregs and Tr1 cells are known to down-regulate APC maturation and antigen presentation by inhibiting CD80/86 expression in a CTLA-4 and/or LAG-3 dependent manner (73, 87). In addition, Tr1 cells and Foxp3$^+$ Tregs can kill myeloid APCs by expressing CD226 and granzyme B (90, 226), or granzyme A, B and perforin, respectively (87, 92). However, the relative ability of Tregs or Tr1 cells to control inflammasome activation, an important part of the innate immune response during tissue injury, is largely unknown.

Inflammasomes are multi-protein complexes activated in response to danger signals. Upon activation, the complex enables the cleavage and maturation of key pro-inflammatory proteins, IL-1β and IL-18, initiates pyroptosis, and provides a link between innate and adaptive immunity (227, 228). Unregulated inflammasome activation can lead to a variety of immune-cell driven pathologies. For example, gain-of-function mutations in the NLRP3 inflammasome that lower its activation threshold result in inappropriate IL-1β production and cryopyrin-associated periodic syndromes (229, 230). In addition, hyper-activity of the

NLRP3 inflammasome underlies inflammation in many common chronic diseases, including type 2 diabetes, neurodegenerative diseases, and inflammatory bowel disease (231-234).

Notably, many of these common chronic inflammatory diseases are also associated with changes in immunoregulatory cells, leading us to hypothesize that $Foxp3^+$ Tregs and/or Tr1 cells could have a role in modulating inflammasome activity. Several lines of indirect evidence support the possibility that $Foxp3^+$ Tregs and or Tr1 cells may regulate inflammasome activation and IL-1β production via production of IL-10. First, type 1interferons inhibit IL-1β production in a mouse model of EAE by stimulating autocrine production of IL-10 from macrophages (235). Second, mice that lack IL-10 have a significantly elevated inflammasome activation and IL-1β production, resulting in severe colitis in some animal facilities (170), or antigen-induced arthritis (236). Based on these studies, we aim to investigate the ability of $Foxp3^+$ Tregs and Tr1 cells to regulate inflammasome activity and report the first evidence for a unique role for Tr1 cells in controlling this pathway.

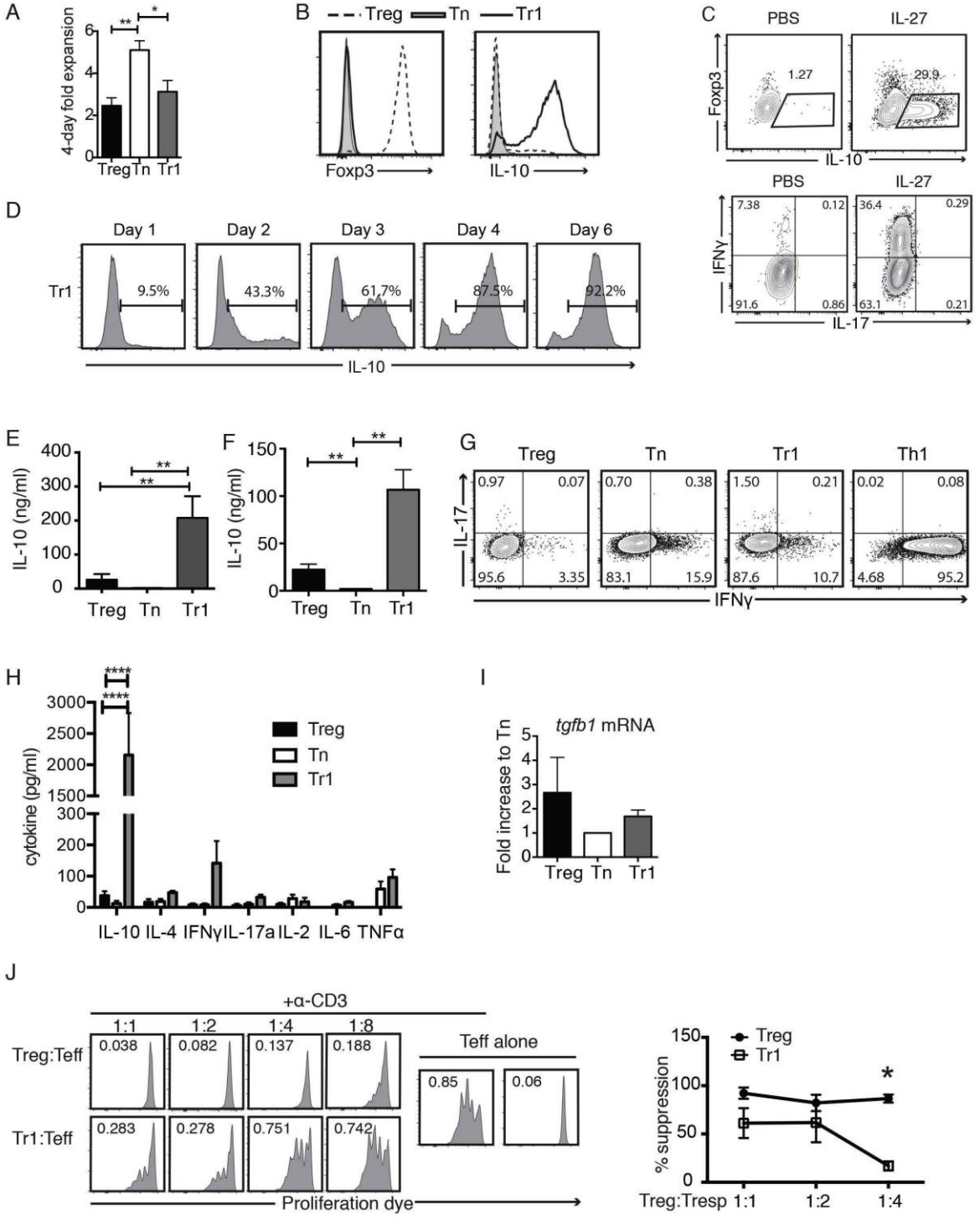
4.2. Results

4.2.1. $CD44^{hi}Foxp3^-$ T cells are enriched in Tr1 cell precursors.

A major limitation to the study of Tr1 cells has been the lack of an efficient system to isolate and/or differentiate these cells. Current methods to differentiate Tr1 cells *in vitro* involve TCR stimulation in the presence of cytokines such as IL-10, IL-27, IL-6, or IL-12 (94, 237-239), or with co-stimulation of CD46 or ICOS-L, or with tolerogenic DCs (73, 237). These methods, however, are inefficient, resulting in relatively small (i.e. <30%) proportions of IL-10-producing cells, which often co-produce high levels of IFN-γ.

Recent evidence suggests that exposure of effector memory T cells to high molecular weight hyaluronan, the ligand for CD44, can also induce IL-10 (125). We therefore speculated that expression of CD44 might identify cells that are poised to differentiate into Tr1 cells. To investigate the ability of $CD44^{hi}$ cells to differentiate into Tr1 cells, $CD44^{hi}Foxp3^{-}$ T cells (hereafter Tr1), $CD44^{int/lo}Foxp3^{-}$ naïve T cells (Tn), and $Foxp3^{+}$ Tregs (Tregs), were sorted from spleens and lymph nodes of *Foxp3*^{RFP}*Il10*^{GFP} reporter mice (Fig. 2.1). Sorted cells were activated with anti-CD3/28 mAbs and IL-2, but in the absence of any polarizing cytokines, and the fold expansion, expression of IL-10 and Foxp3 were analyzed on day 4. $CD44^{hi}Foxp3^{-}$ cells expand similarly compared to $Foxp3^{+}$ Tregs, and both grew significantly slower than $CD44^{int/lo}Foxp3^{-}$ naïve T conventional cells (Fig. 4.1A). Whereas neither Tregs nor naïve T cells contained a significant proportion of $IL\text{-}10^{+}$ cells, more than 80% of cells in the putative Tr1 cell population were $IL\text{-}10^{+}$ (Fig. 4.1B). In accordance with previous observations (237), Tn cells stimulated with IL-27 contained only ~ 30% $IL\text{-}10^{+}$ cells, of which ~37% were IFN-γ (Fig. 4.1C), and only cultures of Tregs expressed Foxp3 (Fig. 4.1B). Time course experiments showed that the induction of IL-10 in $CD44^{hi}Foxp3^{-}$ T cells occurred as early as day 1 and peaked after 4 days of stimulation (Fig. 4.1D). Due to the poor detection of IL-10 by intracellular cytokine staining (data not shown), we validated the accuracy of reporter expression by ELISA. High expression of IL-10 in Tr1 cell cultures was confirmed by measuring IL-10 in supernatants after 4 days of stimulation (Fig. 4.1E). To confirm these finds were not specific for C57Bl/6 mice, we sorted $CD4^{+}$ T cells from BALB/c mice into Tregs ($CD25^{+}CD45Rb^{lo}$), Tn ($CD25^{-}CD44^{int/lo}$), and Tr1 ($CD25^{-}CD44^{hi}$). Similar to data from C57Bl/6 mice (Fig. 2.2A), the $CD25^{-}CD44^{hi}$ Tr1 cells produce large amounts of IL-10 (Fig. 4.1F) while remaining largely Foxp3 negative (Fig. 2.2B).

Since Tr1 cells are characterized by an overall profile of cytokine production, the capacity of activated $CD44^{hi}Foxp3^{-}$ T cells to make cytokines in addition to IL-10 was examined. Consistent with the definition of Tr1 cells, activated $CD44^{hi}Foxp3^{-}$ T cells produced significantly less IFN-γ than Th1 cells (Fig. 4.1G) or Tr1 cells differentiated with IL-27 (Fig. 4.1C), and did not produce amounts of IL-2, IL-4, IL-17, TNF-α, or IL-6 that were significantly different compared to Tregs or Tn cells (Fig. 4.1H). Although we did not detect any significant TGFβ protein by ELISA, they did, however, express similar levels of *Tgfb1* mRNA compared to Tregs (Fig. 4.1I). $CD44^{hi}Foxp3^{-}$ T cells also suppressed the proliferation of $CD4^{+}$ T cells, albeit less potently than $Foxp3^{+}$ Tregs (Fig. 4.1J).


A
4-day fold expansion
Treg Tn Tr1
B
Treg Tn Tr1
Foxp3
IL-10
C
PBS
IL-27
Foxp3
IL-10
1.27
29.9
IFNγ
IL-17
7.38
0.12
91.6
0.86
36.4
0.29
63.1
0.21
D
Day 1
Day 2
Day 3
Day 4
Day 6
Tr1
9.5%
43.3%
61.7%
87.5%
92.2%
IL-10
E
IL-10 (ng/ml)
Treg Tn Tr1
F
IL-10 (ng/ml)
Treg Tn Tr1
G
Treg
Tn
Tr1
Th1
IL-17
IFNγ
0.97
0.07
95.6
3.35
0.70
0.38
83.1
15.9
1.50
0.21
87.6
10.7
0.02
0.08
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95.2
H
cytokine (pg/ml)
Treg
Tn
Tr1
IL-10 IL-4 IFNγ IL-17a IL-2 IL-6 TNFα
I
tgfb1 mRNA
Fold increase to Tn
Treg Tn Tr1
J
+α-CD3
1:1
1:2
1:4
1:8
Treg:Teff
0.038
0.082
0.137
0.188
Tr1:Teff
0.283
0.278
0.751
0.742
Teff alone
0.85
0.06
Proliferation dye
Treg
Tr1
% suppression
Treg:Tresp 1:1
1:2
1:4

Figure 4.1 CD44^{hi}Foxp3^{-}CD4^{+} T cells rapidly differentiate into Tr1 cells in vitro. **(A,B)** CD4^{+}Foxp3^{+} Tregs (Tregs), CD4^{+}CD44$^{lo/int}$Foxp3^{-} naïve conventional T cells (Tn), or CD4^{+}CD44^{hi}Foxp3^{-} cells T cells (Tr1) were sorted from *Foxp3^{RFP}IL10^{GFP}* reporter mice and stimulated with αCD3/CD28 mAbs and IL-2 for 4 days. (A) Fold expansion of cells over 4 days of stimulation (n=6, *p<0.05, **p<0.01). **(B)** Histograms show representative expression of Foxp3 and IL-10 gated on FVD^{-}CD4^{+} lymphocytes (n=3). **(C)** Tn cells were stimulated with anti-CD3/28 ± 50ng/ml of IL-27 for 4 days. The expression of IL-10 and IFNγ and IL-17 were analyzed by flow cytometry on FVD^{-}CD4^{+} cells. Representative FACS plots of 3 independent experiments. **(D)** Expression of IL-10 in Tr1 cells was analyzed daily, shown are representative histograms, n=3. **(E, F)** Amounts of IL-10 in B/6 **(E)** or BALB/c **(F)** T cell supernatants were measured by ELISA (mean ± SEM, ** p<0.001). **(E)** n=7, **(F)** n=3. **(G)** Expression of IL-17 and IFN-γ were determined in Tregs, Tn, Tr1 cells, or Th1 cells, which were Tn cells cultured in the presence of IL-12. Plots are representative of n=5. **(H)** After culturing for 4 days, T cell subsets were washed and cultured without additional stimulation or IL-2 for 24 hours. Amounts of secreted cytokines were analyzed by cytokine bead array (mean ± SEM, n=5, * p<0.05, ** p<0.01, **** p<0.0001, two way ANOVA followed by Tukey's Test comparing different T cell cytokine expression within each cytokine). **(I)** Expression of *Tgfb1* mRNA was measured after 4 days of stimulation (mean ± SEM, n=4). **(J)** The suppressive capacity of Tregs and Tr1 cells was tested by measuring the proliferation of CD4^{+}CD25^{-} T cells absence or presence of different ratios of Tregs or Tr1 cells. The proliferation of CD4^{+}CD25^{-} T cells was induced by coculture with irradiated APC in the presence of soluble anti-CD3. Shown is the average percent suppression calculated on the basis of division index (mean ±SEM, n=3, *p<0.05).

4.2.2. Tr1 cells derived from CD44^{hi}Foxp3^{-} T cells express characteristic cell surface markers and transcription factors.

We next sought to determine whether Tr1 cells differentiated from CD44^{hi}Foxp3^{-} T cells express Tr1 cell-associated surface markers (240) and/or transcription factors. Compared to Tn or Foxp3^{+} Tregs, a significantly higher proportion of Tr1 cells expressed LAG-3 and/or CD49b, from both B/6 mice (Fig. 4.2A) and BALB/c mice (Fig. 4.2B). as well as multiple additional Tr1-associated markers including CD39 and ICOS (Fig. 4.2C), although some of these markers become significantly higher after overnight resting (73). Since IL-10 has also been implicated in T cell exhaustion (241, 242), we examined the expression of PD-1 and TIM-3 and found that Tr1 cells derived from CD44^{hi}Foxp3^{-} T cells

expressed high levels of both markers (Figs. 4.2C). Compared to Foxp3^{+} Tregs, however, they did not express high levels of LAP, CD73, or Helios (Fig. 4.2C).

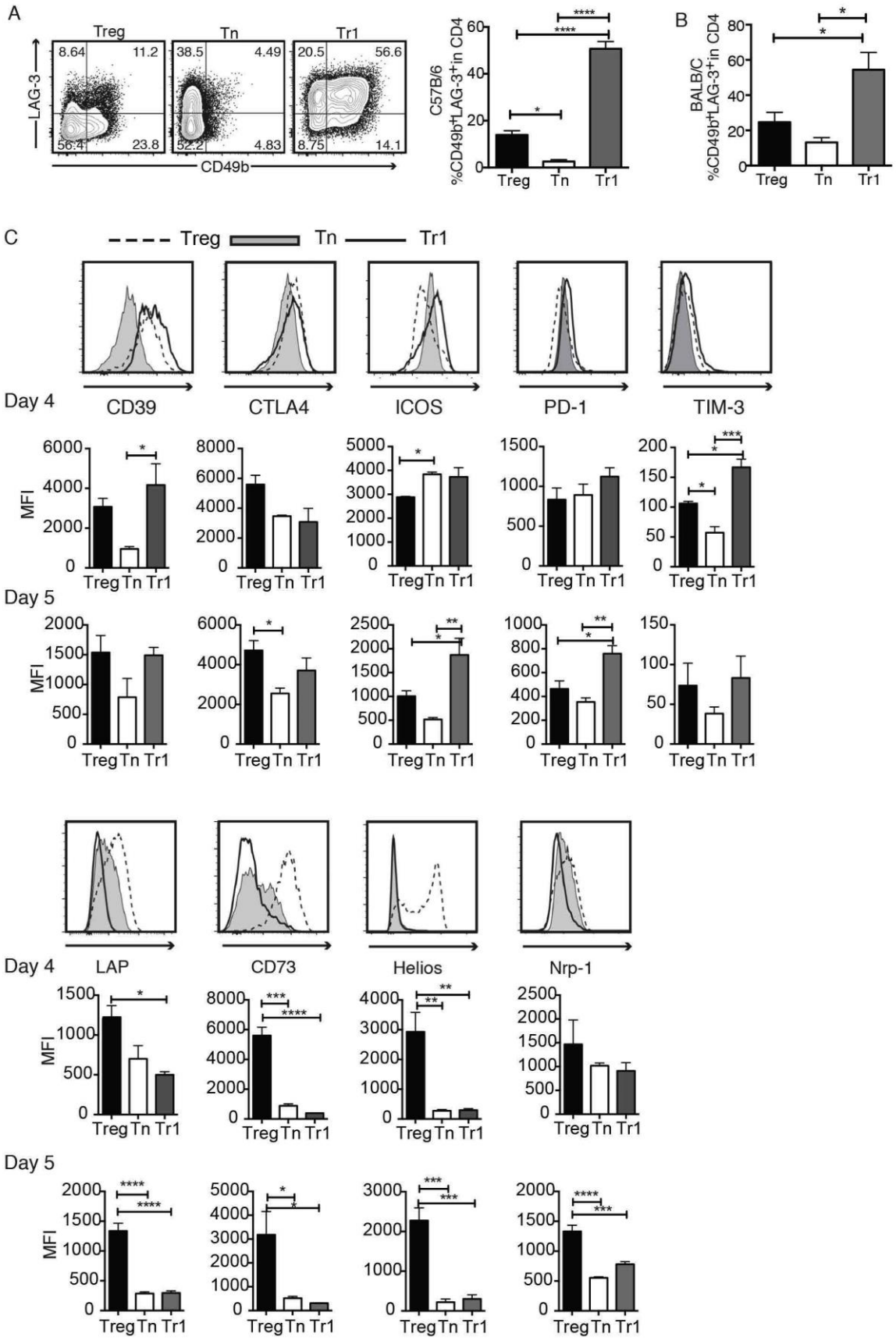
A
Treg
Tn
Tr1
8.64
11.2
56.4
23.8
38.5
4.49
52.2
4.83
20.5
56.6
8.75
14.1
LAG-3
CD49b
C57B/6
%CD49b+LAG-3+in CD4
Treg
Tn
Tr1
B
BALB/C
%CD49b+LAG-3+in CD4
C
Treg
Tn
Tr1
Day 4
CD39
CTLA4
ICOS
PD-1
TIM-3
MFI
Day 5
LAP
CD73
Helios
Nrp-1

Figure 4.2 Tr1 cells are phenotypically distinct from Foxp3$^+$ Tregs and Tn cells. **(A)** Expression of CD49b and LAG-3 was measured in T cell subsets after 4 days of activation. Shown are representative contour plots and the average percentage of CD49b$^+$LAG-3$^+$ T cells or the average MFI from 3-4 independent experiments (mean ± SEM, *** p<0.001, **** p<0.0001). **(B)** Expression of CD49b and LAG-3 in T cell subsets sorted from BALB/c mice (mean ± SEM n=3, *p<0.05). **(C)** Other Treg-associated molecules were measured in T cell subsets either after 4 days of activation (Day 4) or 4 day activation followed by 1 day resting in culture media alone (Day 5). Cells were gated on FVD$^-$CD4$^+$ lymphocytes. Shown are representative contour plots and the average percentage of CD49b$^+$LAG-3$^+$ T cells or the average MFI from 3-4 independent experiments (mean ± SEM, n=3-4, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001).

We also analyzed the expression of Tr1-associated transcription factors after 4 days of activation, and found that, in comparison to Tn, they had significantly higher expression of *Maf*, and *Prdm1* (gene for Blimp-1), and a trend towards increased *Ahr* (Fig. 4.3). In comparison to Foxp3$^+$ Tregs, only expression of *Maf* was significantly higher in Tr1 cells. Interestingly, high expression of these transcription factors was already present in *ex vivo* CD44^{hi}Foxp3$^-$ cells (Fig. 4.3), supporting the hypothesis that CD44^{hi}Foxp3$^-$ cells contain Tr1 cell precursors that are programmed to up-regulate IL-10 without the requirement for exposure to polarizing cytokines. Collectively these data show that CD44^{hi}Foxp3$^-$ T cells isolated from un-manipulated mice are poised to rapidly differentiate into Tr1 cells, representing a new and efficient way to obtain large numbers of these cells for further study.

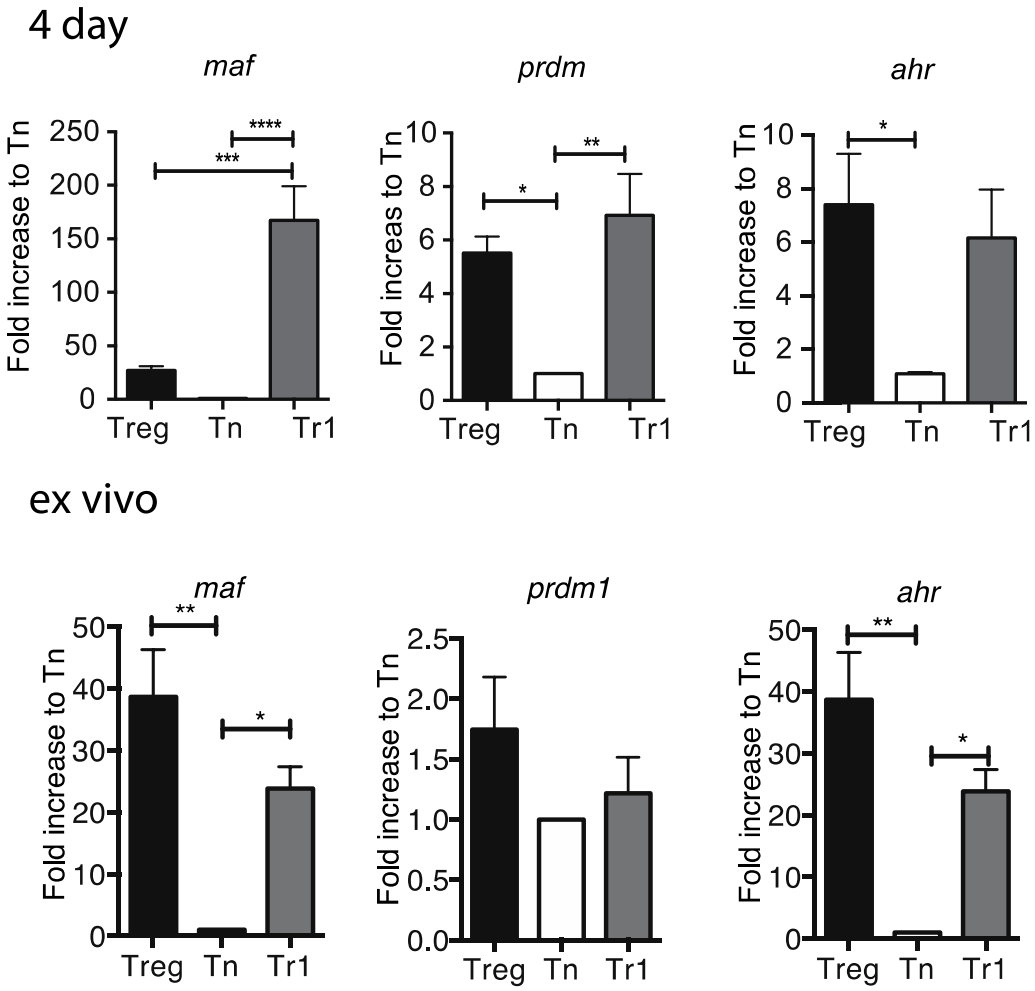

Figure 4.3 Tr1 cells have distinct transcription factor profile.
mRNA from T cells were isolated after 4 days of activation or immediately after sorting, and reversed transcribed. The mRNA expression of the indicated genes from Treg, Tn and Tr1 cells was analyzed by qPCR. Results were normalized to 18s, and expressed as fold increase to Tn. Error bars represent SEM, n=3-6, * $p<0.05$, ** $p<0.01$, ***$p<0.001$

4.2.3. Tr1 cells, but not Foxp3$^+$ Tregs, suppress inflammasome activation in macrophages via soluble factors.

To compare the ability of Tr1 versus Foxp3$^+$ Tregs to suppress the inflammasome, T cells and macrophages were co-cultured overnight, then stimulated with LPS for 4 hours followed by ATP for 1 hour. The viability of the macrophages was not decreased in the

presence of T cells (Fig. 4.4A). Surprisingly, we found that the production of mature IL-1β was significantly suppressed by Tr1 cells but not by Tregs (Figs. 4.4B, C), in a dose dependent manner (Fig. 4.4D). Similarly, we found that IL-27-differentiated Tr1 cells inhibited IL-1β release (Fig. 4.4E), suggesting that Tr1 cells derived from both sources are able to suppress the production of IL-1β. On the other hand, co-culture of macrophages with Tn cells resulted in a significant increase in inflammasome activation. Since neither LPS nor ATP alone was sufficient for the production of IL-1β in the presence of T cells (Fig. 4.4B), Tn cells do not produce factors, such as TNFα, that can bypass the requirement for either LPS or ATP. The requirement for caspase-1 in IL-1β release was confirmed by the absence of IL-1β secretion in the presence of the caspase 1 inhibitor YVAD-FMK (Fig. 4.4B).

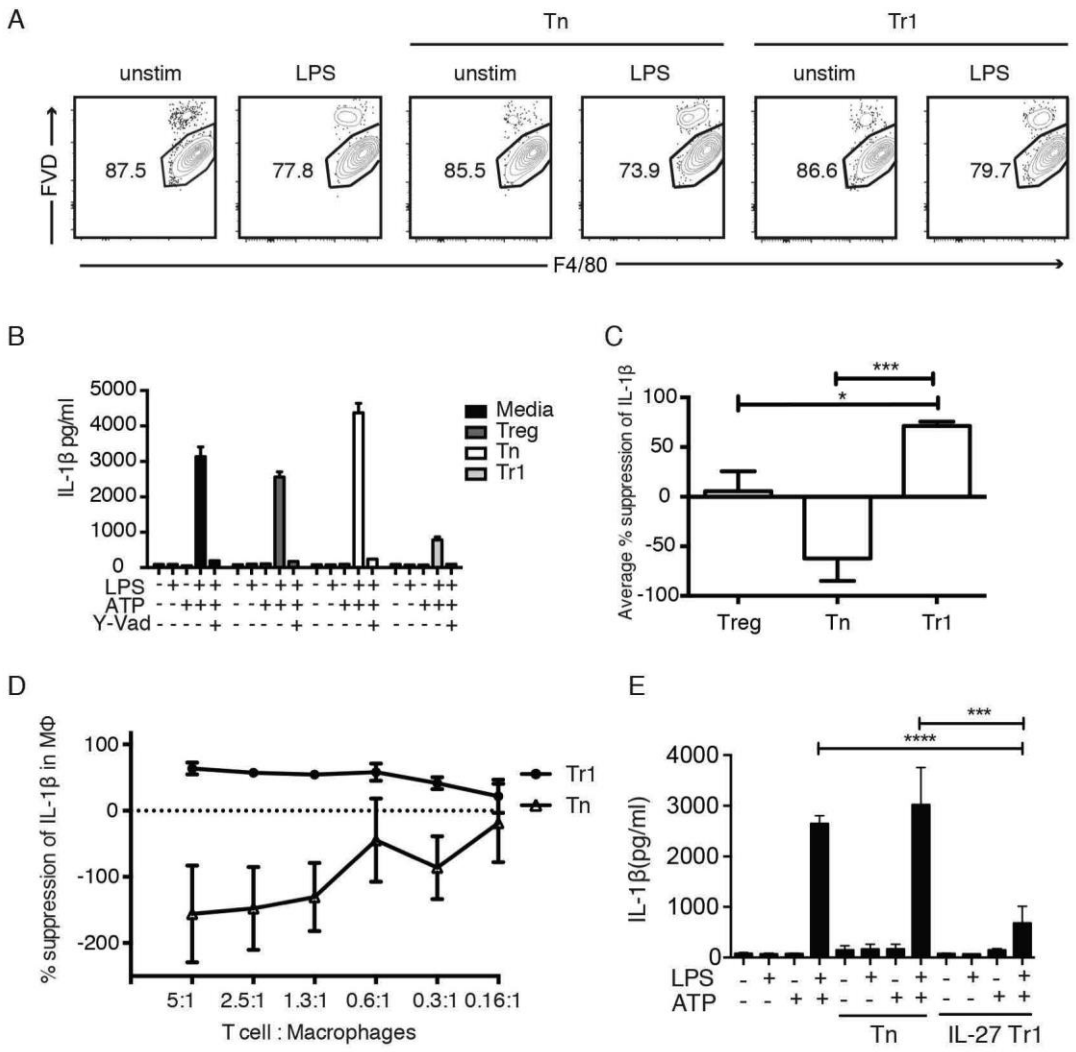

Figure 4.4 Tr1 cells but not Foxp3$^+$ Tregs suppress IL-1β production from macrophages.

(A) Tr1 or Tn cells were stimulated with anti-CD3/CD28 for 5 days and cultured with BMDM for 30 minutes. The cells were stimulated with or without LPS for 4 hours, followed by ATP for another hour. Adherent cells were collected, stained with fixable viability dye (FVD) and analyzed by flow cytometry for viability. The percentage on the graph indicates viable cells, representative of n=2. **(B, C)** After 5 days of activation, T cell subsets were co-cultured with BMDM at a ratio of 5:1 (200,000 T cells: 40,000 macrophages), with or without YVAD. LPS was added for 4 hours, followed by ATP for 1 hour, then supernatants were collected and amounts of IL-1β measured by ELISA. **(B)** Shows representative data and **(C)** shows the average percent suppression, mean ± SEM (n=6, ** $p<0.01$). **(D)** Tr1 or Tn cells were stimulated with anti-CD3/CD28 for 5 days and cultured with BMDM for 30 minutes at different ratios as indicated. The cells were stimulated with LPS for 4 hours, followed by ATP for another hour. IL-1β was measured by ELISA. Shown is the mean percent suppression ± SEM (n=3). **(E)** Tn cells were stimulated ± IL-27 for 4 days then cocultured with BMDM for 30 minutes. The cells were then stimulated with LPS and/or ATP

and the production of IL-1β was measured by ELISA. Shown in the mean±SEM, n=3, ***p<0.001, ****p<0.0001.

Since Tr1 cells act primarily via cytokine-mediated suppression, we asked if suppression of IL-1β secretion was mediated by soluble factors. Indeed, addition of media conditioned by the T cell subsets recapitulated the effect of cell co-cultures: Tr1 cell-conditioned media potently suppressed IL-1β; Tregs had little effect; and Tn cells enhanced IL-1β (Fig. 4.5A, B). Notably, addition of rIL-10 had a similar suppressive effect (Fig. 4.5B), suggesting that the Tr1 cell mediated suppression may be IL-10 dependent. As seen with the co-cultures, the suppressive effect by Tr1 cell conditioned media was dose dependent (Fig. 4.5C). On the other hand, despite containing ~26 ng/ml of rIL-10 (Fig. 4.1D) which should result in ~80% suppression (Fig. 4.5D), conditioned media from Tregs failed to inhibit IL-1β production. Therefore Tr1 cells, but not $Foxp3^+$ Tregs, suppress IL-1β production via a cell contact-independent mechanism.

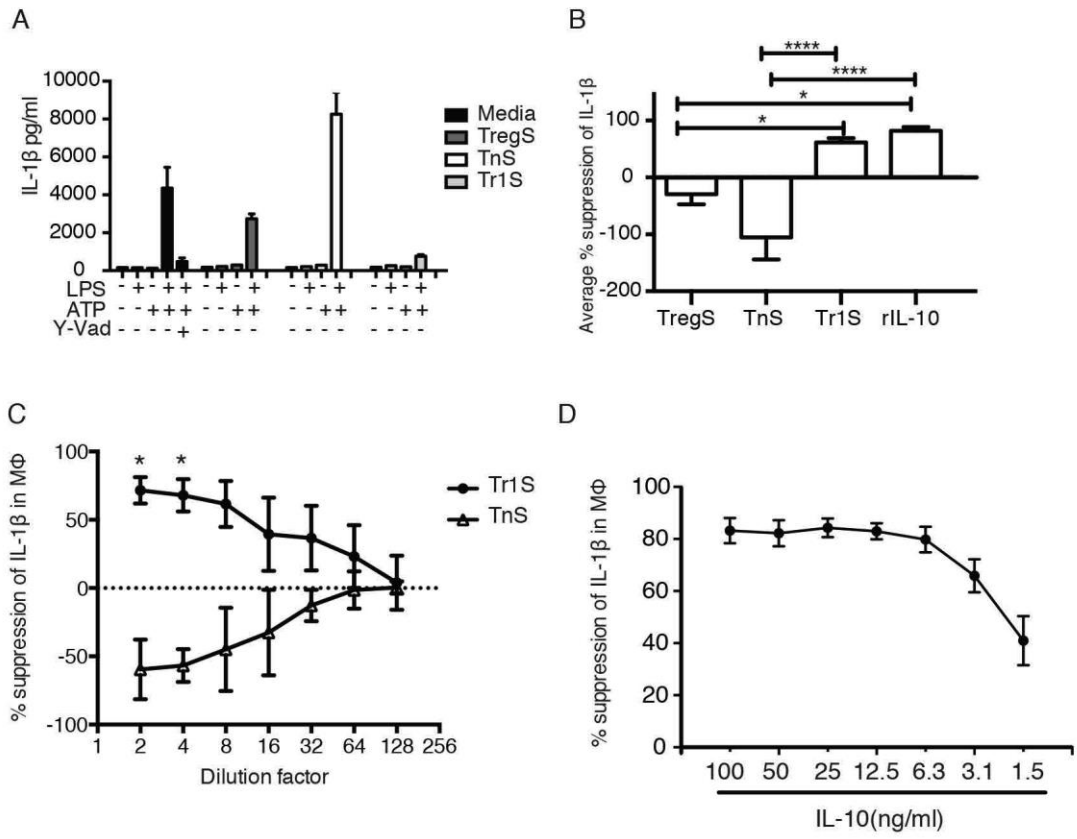

Figure 4.5 Tr1 cells suppress IL-1β production via contact independent mechanisms. T cells were activated as described in figure 4.4. **(A, B)** Macrophages were incubated with supernatants from stimulated Tregs, Tn or Tr1 cells (TregS, TnS and Tr1S, respectively) at 1:1 ratio of conditioned to non-conditioned media, or rIL-10 (100ng/ml), then stimulated as indicated. **(A)** Shows representative data and **(B)** shows the average percent suppression, mean ± SEM (n=8, * $p<0.05$, **** $p<0.0001$). BMDM were cultured with T cell supernatants at the indicated dilution **(C)**, or with the indicated amount of IL-10 **(D)**. The cells were then stimulated with LPS+ATP and the supernatants were collected and analyzed by ELISA for IL-1β production. **(C, D)** Results were shown as the mean percent suppression ± SEM (n=3-4). *$p<0.05$ determined with multiple t-tests using the Sidak-Bonferroni method.

4.2.4. Tr1 cells suppress IL-1β production from macrophages with an NLRP3 gain-of-function mutation.

To ask if Tr1 cells could also control inflammasome activation in diseases caused by hyper-activation of this pathway, we used macrophages from tamoxifen inducible *Nlrp3*$^{A350V/+}$ knock-in mice (*Nlrp3*$^{A350V/+}$ *CreT*, here referred to as *MWS CreT*), which carry a mutation corresponding to human cryopyrin associated periodic syndrome patients (179).

MWS CreT macrophages were cultured in 4-hydroxytamoxifen (4HT) to induce the expression of mutant NLRP3. As expected, these macrophages no longer required ATP stimulation for the production of mature IL-1β (Figs. 4.6A,B), confirming constitutive activity of caspase 1. Consistent with our observations in wild type macrophages, Tr1 cells, but not Foxp3^{+} Tregs, potently suppressed IL-1β production in *MWS CreT* macrophages, regardless of whether they were stimulated with LPS alone or with LPS and ATP (Fig. 4.6 A,B). A similar suppressive effect was observed with Tr1-conditioned media or recombinant IL-10 (Fig. 4.6 C,D). These data support the notion that Tr1 cells and IL-10 could be important for controlling the pathological release of IL-1β that occurs in autoinflammatory disease such as CAPS.

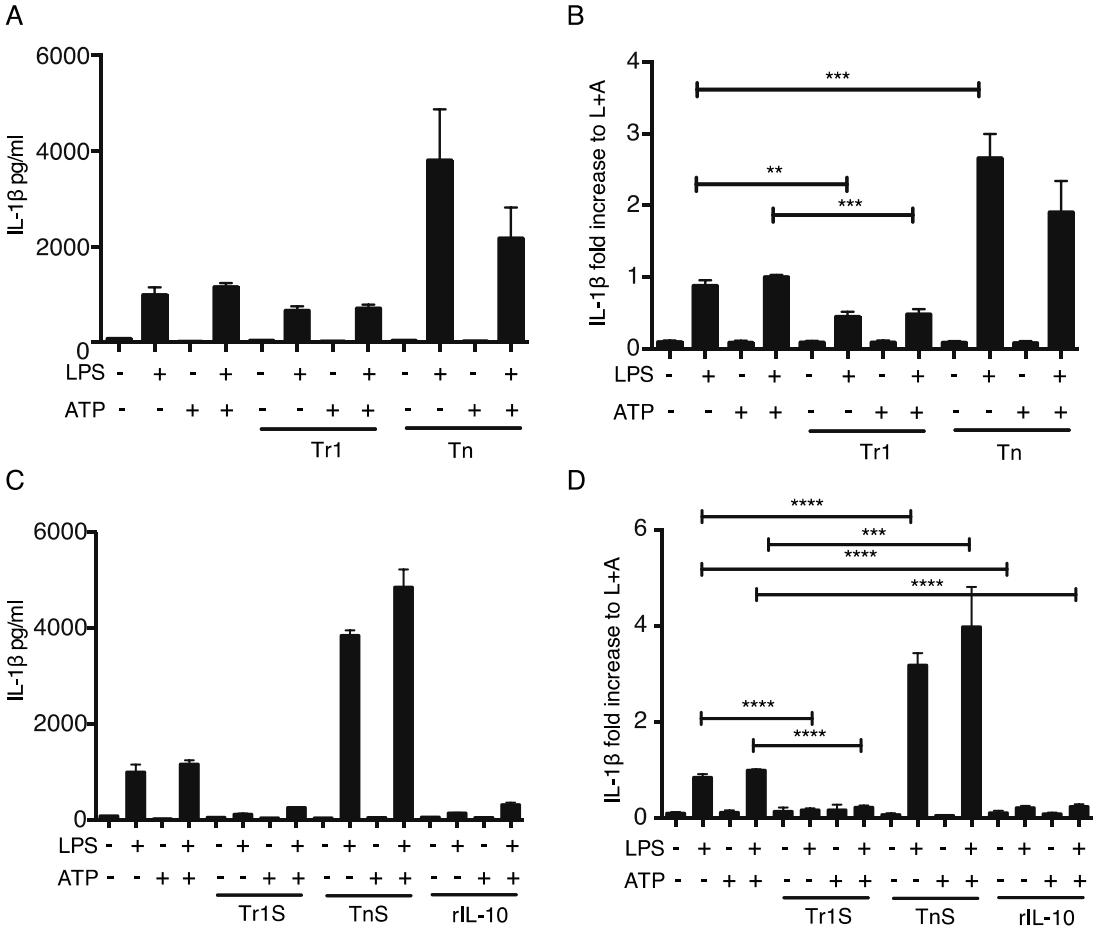

Figure 4.6 Tr1 cells and their conditioned media suppress IL-1β production from BMDM carrying NLRP3 gain-of-function mutations.
(A-D) BMDM from NLRP3 MWS CreT mice or wild type mice were treated with 4-HT for 24 hours before**,** co-culturing with T cells. Shown are representative plot (**A**, mean ± SD), averaged data (**B**, n=3, mean ± SEM) and data from cocultures using conditioned media or rIL-10 (**C**, representative data, mean ± SD; **D**, averaged data from n=3, mean ± SEM). Averaged data are shown as secreted IL-1β expressed in relation to those from BMDM stimulated with LPS and ATP, but in the absence of T cells or T cell conditioned media (**p<0.01, *** p<0.001, **** p<0.0001).

4.2.5. Tr1 cells suppress *Il1b* transcription and caspase 1 activation via an IL-10R-dependent mechanism.

We next explored the mechanism by which Tr1 cells regulate the production of IL-1β, a process known to be tightly-regulated by a two-step activation process (227, 228). The first step involves cytokine- or Toll-like receptor-mediated activation of NF-κB, leading to transcription and translation of pro-IL-1β. The second step requires inflammasome activation, then caspase-1 cleavage and activation, followed by caspase-1-mediated proteolytic activation of IL-1β. For these experiments we used media conditioned by T cells so that mRNA and protein signals were exclusively derived from macrophages. Exposure of macrophages to media conditioned by Tr1 cells, or to recombinant IL-10, inhibited the production of *Il1b* mRNA, which could be due to decreased transcription or a decrease in mRNA stability (Fig. 4.7A). Tr1 cell-mediated inhibition of pro-IL-1β protein expression was demonstrated by Western blotting (Fig. 4.7B). In addition to decreased transcripts of *Il1b*, Tr1 cell conditioned media also directly inhibited the generation of mature, cleaved caspase 1, without affecting the amount of pro-caspase 1 (Fig. 4.7C). Thus, soluble factors released by Tr1 cells affect both steps required for mature IL-1β production.

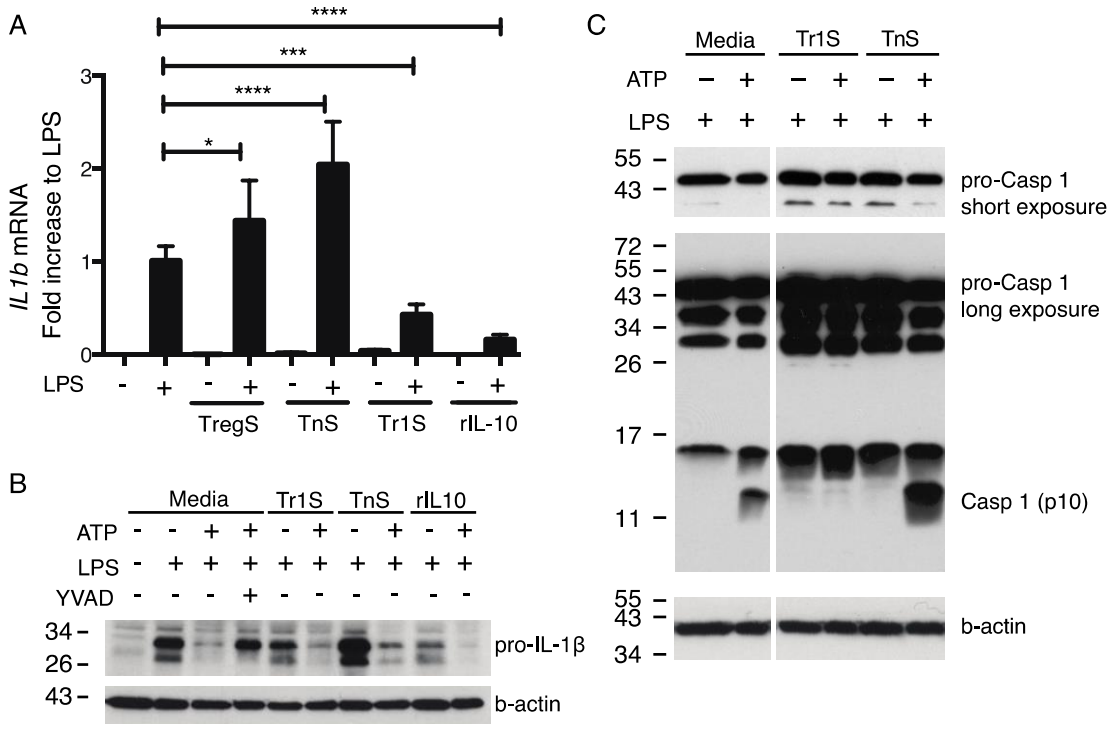

Figure 4.7 Tr1 cells inhibit the production of *I11b* mRNA and activation of caspase 1. BMDM were stimulated in the absence or presence of media conditioned by Treg, Tn, or Tr1 cells, or with rIL-10. (**A**) Expression of *Il1b* mRNA in BMDM shown as fold increase compared to stimulation with LPS but without T cell conditioned media. Mean ± SEM, n=3 ($p<0.05$). Lysates from BMDM were analyzed by Western blotting to detect (**B**) pro-IL-1β and (**C**) pro-caspase 1 (short exposure), pro- and mature caspase 1 (long exposure), and β-actin (loading control). Blots are representative of n=3.

We next investigated if the suppressive effects of Tr1 cells on IL-1β production were mediated by IL-10. We first tested the effects of Tr1 cells on macrophages derived from *Il10rb*-deficient mice and found that, in comparison to wild type macrophages, these cells were completely resistant to the suppressive effects of Tr1 cells (Fig. 4.8A,B), or their conditioned media (Fig. 4.8C,D). Moreover, antibody-mediated neutralization of IL-10 also reversed the suppressive effect of Tr1 cells (Fig. 4.8E,F). Tr1 cells also failed to reduce the

concentrations of *Il1b* mRNA (Fig. 4.9A) or cleavage of caspase 1 (Fig. 4.9B) in *Il10rb*-deficient macrophages. Thus IL-10 is necessary for Tr1 mediated suppression of mature IL-1β production.

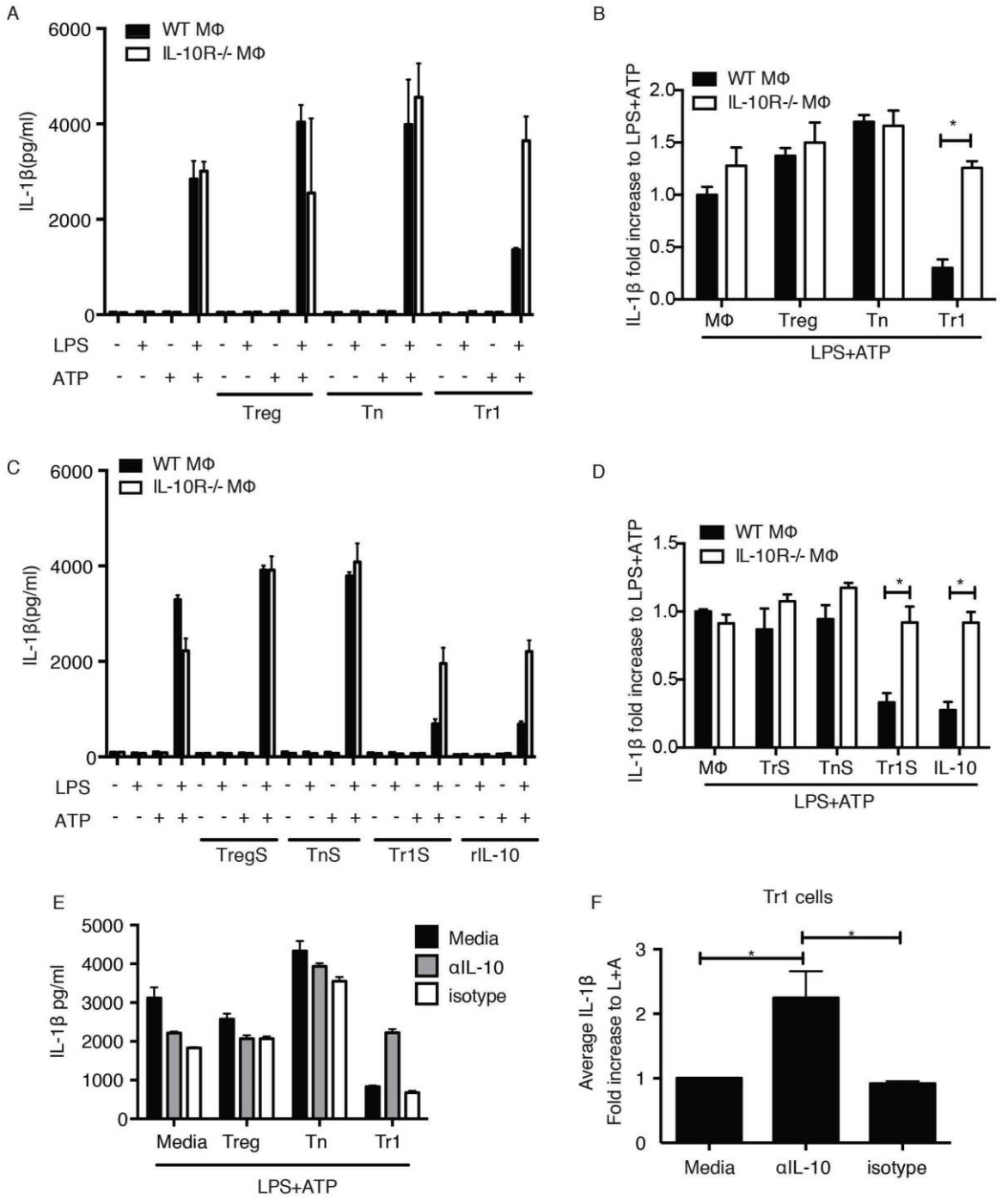

Figure 4.8 The suppression of IL-1β production by Tr1 cells is mediated by IL-10R signaling.

BMDM from WT or *Il10rb-/-* mice were co-cultured with T cells (**A, B, E, F**) or T cell conditioned media (**C-D**) in the presence of media alone, anti-IL-10 blocking antibody, or isotype antibody as indicated. **(A-F)** After stimulation with LPS and ATP, amounts of IL-1β in supernatants were measured by ELISA. Shown are representative data (**A, C**, **E**, mean ±

SD) averaged data (**B, D, F**, mean ± SEM) normalized to BMDM stimulated with LPS and ATP (*p<0.05, multiple t-test corrected for multiple comparisons using Sidak-Bonferroni method). **(E)** shows a representative experiment, **(F)** shows the average amount of IL-1β secreted by BMDM cultured with Tr1 cells in the absence or presence of α-IL-10 mAbs, or an isotype mAbs; results are normalized to fold increase to LPS+ATP and expressed as mean ±SEM of n=3, *p<0.05.

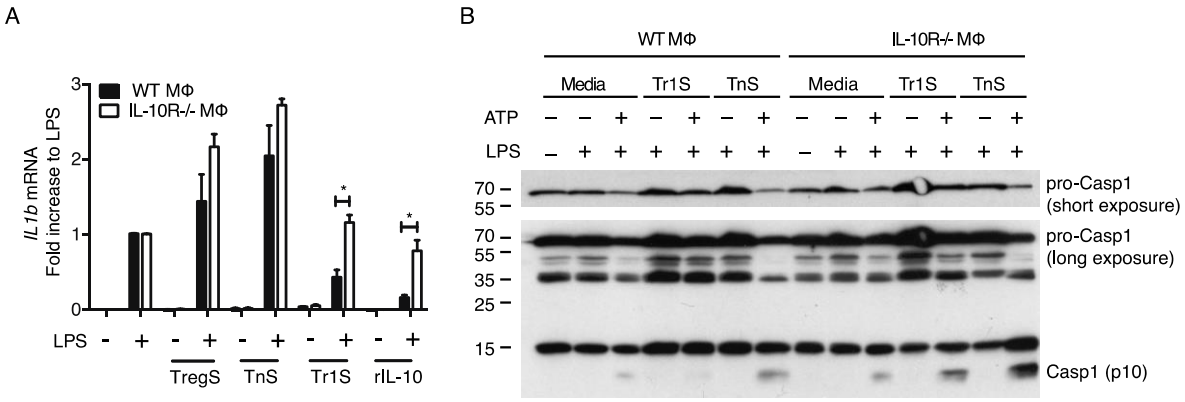

Figure 4.9 The suppression of *Il1b* transcription and caspase 1 activation is dependent on IL-10R signaling.
BMDM from WT or *Il10rb-/-* mice were co-cultured with T cell conditioned media and stimulated as indicated. (**A**) Average amounts of *Il1b* mRNA were determined by qPCR. (n=2, *p<0.05, multiple t-test corrected for multiple comparisons using Sidak-Bonferroni method). (**B**) Expression of pro-caspase 1 (top, short exposure), pro-caspase 1 and mature caspase 1 (bottom, long exposure) was measured by Western blotting, representative of n=3.

4.3. Discussion

Clinical trials with Tr1 cells or Foxp3$^+$ Tregs are being actively pursued in the hope that these cell-based therapies will offer durable and specific treatments for a variety of immune mediated diseases such as graft-versus-host disease, transplant rejection, and inflammatory bowel disease. Activation of the inflammasome contributes to the pathology in all of these diseases, yet the ability of Tr1 cells or Foxp3$^+$ Tregs to control this process is unknown. Here, we found that Tr1 cells, but not Foxp3$^+$ Tregs, suppress IL-1β production by negatively regulating both production of *Il1b* mRNA and activation of caspase-1. Furthermore, the suppression is largely IL-10 dependent as the effect is abrogated in IL-10R

deficient macrophages. Importantly, Tr1 cells suppressed IL-1β production caused by a clinically-relevant gain-of-function mutation in NLRP3, suggesting that Tr1 cells may have unique therapeutic advantages that are not shared by $Foxp3^+$ Tregs.

A major limitation for studying Tr1 cells has been the lack of an efficient system to generate relatively homogeneous populations of cells for study. Here we report a major advance and show that in vitro activation of $CD4^+CD44^{hi}Foxp3^-$ T cells from unmanipulated mice, without addition of any polarizing cytokines, results in a population of cells that is >80% positive for IL-10, has the expected profile of cytokine production ($IL\text{-}10^{hi}IFN\text{-}\gamma^{lo}IL\text{-}2^-IL\text{-}4^-$), and expresses all of the major Tr1 cell associated cell surface markers and transcription factors, including CD49b, LAG-3, cMaf, Blimp1 and AHR (73). These cells also express higher levels of Tr1 surface markers CD39, ICOS, CTLA-4, and PD-1 (73) compared to naïve T cells either after 4 days of activation or after resting when the transient upregulation of some of these markers go down again in Tn (73). However, we failed to detect expression of LAP or TGFβ protein, which is different from Tr1 cells generated by repeated antigen stimulation or nasal anti-CD3 monoclonal antibody administration (243, 244).

Since CD44 is a marker for T cell memory, in a laboratory-raised mouse CD44-expressing cells carry distinct characteristics compared to true antigen experienced memory T cells that also express high levels of CD44 (245), including more rapid and cytokine-driven in vivo proliferation independent of antigen stimulation. The emergence of these cells does not seem to rely on commensal bacterial antigens as germ-free mice also carry a similar frequency of $CD44^{hi}Foxp3^-$ cells in their $CD4^+$ T cell compartment (245). However, the function of these cells remains largely unknown. Our *in vitro* data indicated that these cells

carry a distinct cytokine expression pattern and are poised to mount immunomodulatory rather than immunostimulatory responses. We have also investigated the potential utility of CD44 as a marker for human Tr1 cells and found that human peripheral blood $CD4^+$ T cells uniformly express high levels of CD44 (data not shown), and therefore CD44 is not suitable for purifying Tr1 cells in humans.

Recently, three studies have shown that IL-10 signaling in myeloid cells is important for intestinal homeostasis as myeloid cell-selective IL-10 receptor knockout mice have increased susceptibility to colitis and intestinal pathology (246-248). On the other hand, the expression of IL-10R in T cells is dispensable these models (246-248). Consistent with the dominant role of IL-10 in Tr1 mediated suppression, we found that Tr1 cell-mediated inhibition of *Il1b* mRNA production and caspase 1 activation is completely dependent on IL-10R signaling, as macrophages from *Il10rb*-deficient mice were resistant to Tr1 cell-mediated inhibition of inflammasome activation. This was confirmed by Li. *et al.*, who showed the similar results in macrophages from lamina propria and suggested that the inability of macrophages to sense IL-10 results in increased intestinal inflammation that is largely driven by IL-1β and Th17 cells (248).

Our finding of the decreased *Il1b* mRNA production by IL-10 is consistent with some data in human monocytes, murine peritoneal derived macrophages or macrophage cell lines (89, 249, 250), but not others (236, 251, 252). This discrepancy may be attributed to various factors including the stability of IL-10, time of delivery, and different regulatory mechanisms in monocytes and macrophages. Although in our *in vitro* system recombinant IL-10 is as potent as Tr1 cells and their conditioned media, we propose that Tr1 cells may possess better

suppressive function than exogenous IL-10 *in vivo* due to their ability to deliver IL-10 continuously at the site of inflammation.

Recently, two studies found that mice lacking IL-10 have significantly elevated inflammasome activation and IL-1β production, resulting in severe colitis (236) or antigen-induced arthritis (170). However, IL-10 knockout mice have increased production of inflammatory cytokines resulting in tissue damage, which could contribute to inflammasome activation as much as the lack of macrophage exposure to IL-10. Our data complements these two studies by showing that IL-10 and Tr1-cell conditioned media can directly regulate caspase 1 activation *in vitro*, and that this effect is completely dependent on IL-10R signaling. In contrast to the effects of Tr1 cells, which are mediated by soluble factors, a previous study found that memory T cells suppressed the inflammasome via a contact-dependent mechanism, with indirect evidence for a possible role of TNF family ligands (253). Therefore the ability of different T cells to produce IL-10, and/or express inhibitory cell surface molecules, may be harnessed therapeutically to dampen inflammation.

One important question remaining is why did $Foxp3^+$ Tregs fail to suppress the inflammasome. The role of IL-10 in $Foxp3^+$ Treg-mediated suppression is controversial, with a wealth of literature arguing for or against a role for this cytokine in different in vitro assays and disease settings (87). Findings from mice with a specific deletion of *Il10* in $Foxp3^+$ Tregs suggest that these discrepancies are likely due to an essential role of IL-10 in $Foxp3^+$ Treg-mediated control of tolerance at mucosal surfaces, but not for autoimmunity (254). In our system, although $Foxp3^+$ Tregs produced significantly more IL-10 than Tn cells (average of 26.1 ± 6.3 versus 1.7 ± 0.05 ng/ml), this amount was on average 10 fold lower than the amount produced by Tr1 cells. Notably, as little as 6 ng/ml of rIL-10 efficiently suppressed

the inflammasome, so lack of sufficient IL-10 production by Foxp3$^+$ Tregs is likely not the explanation for why these cells cannot prevent activation of this pathway. Further investigation into the mechanistic basis for why Foxp3$^+$ Tregs do not control inflammasome activation is warranted.

In summary, our *in vitro* data strongly suggest a superior function of Tr1 cells in regulating inflammasome activation and the production of IL-1β, expanding our current understanding of their mechanisms of suppression and providing important justification for pursuing the use of these cells as cellular therapy for autoinflammatory diseases.

Chapter 5: Use of Tr1 cells as a cellular therapy for autoinflammatory diseases

5.1. Introduction

Tr1 cells are potent immune modulators that can actively suppress effector T cell responses in an antigen-specific manner via the secretion of IL-10 (73). The use of Tr1 cells as prophylactic treatment has been demonstrated in a variety of experimental disease models, including T cell transfer colitis (94), celiac disease (255), GVHD post HSCT (117), and islet transplantation (119). More recently, in murine models of acute and chronic collagen-induced arthritis, adoptive transfer of Tr1 cells can both prevent and cure the disease (118). Furthermore, initial clinical trials with human Tr1 cells indicated the feasibility and safety in treating patients with IBD (126) and HSCT (127). Based on these promising data, one area of interest is to expand the application of Tr1 cellular therapy to treat more immune-mediated diseases. Based on our *in vitro* data, we focused on examining two disease models where increased activation of inflammasomes drives the pathophysiology: namely, gout and CAPS.

Gout is common polygenic autoinflammatory disease with a molecular basis similar to monogenic autoinflammatory diseases (130). It can manifest as acute inflammatory arthritis flares or chronic inflammation that cause bone erosions and damage of joint structure (256). A gout attack is initiated by deposition of monosodium urate (MSU) crystals in the joint that triggers activation of the inflammasome, resulting in the release of IL-1β and chemokines, and the infiltration of inflammatory monocytes and neutrophils (33, 257). This

causes acute and extremely painful joint swelling, characteristically in the first metatarsophalangeal joint in the foot, but frequently other locations as well.

Activation of the NLRP3 inflammasome plays a key role in delivering proinflammatory signals and driving the pathogenesis of gout: MSU crystals do not trigger IL-1β production in peritoneal macrophages that lack NLRP3, ASC or caspase 1(33). On the other hand, the response to MSU seems unaffected in various TLR deficient mice, suggesting there is not a specific TLR responsible for the production of IL-1β upon MSU treatment (258). This maybe due to a compensatory role of different TLRs or TLR activation may be dispensable for MSU induced inflammation (258). In contrast, surface binding (259), phagocytosis of MSU (260) and autocrine ATP production (55) are all required for MSU-mediated responses. The involvement of IL-1β in the pathogenesis of gout is best illustrated in patients who received IL-1 targeted therapy, which showed promising clinical improvements in an open-label study (261, 262). One concern with this treatment is the side effects of IL-1 blockade that may increase the risk for serious bacterial infection in a non-life-threatening disease.

Another disease model that is ideal for testing how Tr1 cells regulate NLRP3 inflammasome-mediated disease is CAPS (described in **1.3.2**). Several groups have created transgenic mouse models harboring different gain-of-function mutations found in CAPS patients (258). Macrophages carrying these mutations, namely, $Nlrp3^{A350V}$ (263), $Nlrp3^{L351P}$ (263), $Nlrp3^{R258W}$ (264) and $Nlrp3^{D301N}$ (265), showed increased spontaneous inflammasome activation *in vitro* and only require TLR activation to stimulate the production of IL-1β (as we showed in Chapter 4). Interestingly, these mutations can cause a spectrum of disease ranging from mild to severe, closely resembling the clinical features in human diseases

(263). Notably, for unclear reasons, the mutation corresponding to the most severe human disease (NOMID) causes only a mild disease in mice, while the mildest mutation in humans (FCAS) causes a severe and rapid fatal inflammation in mice (263).

In addition to the potent activating effects on innate immune cells, dysregulated inflammasome activation also affects the adaptive immune system. For example, IL-1β promotes Th17 polarization, and these cells can also drive the pathogenesis of multiple autoinflammatory diseases (264, 266). In the light of our discovery of the superior ability of $CD44^{hi}Foxp3^{-}$ Tr1 cells to inhibit IL-1β secretion, and based on a wealth of literature on their suppressive function in dampening the activation of effector T cells, I asked if these newly identified endogenous polyclonal Tr1 cells are capable of suppressing inflammasome activation *in vivo*. I therefore established two mouse models of inflammasome activation: MSU-induced peritonitis and systemic inflammation in mice carrying gain-of-function mutations of NLRP3 (corresponding to FCAS in humans). Below I describe the results of adoptively transferring Tr1 cells on disease pathogenesis.

5.2 Results

5.2.1. Monosodium urate (MSU) induces peritonitis resulting in increased neutrophil infiltration.

The mouse model of MSU induced peritonitis has been developed to study inflammation in gout. In this model, intraperitoneal injection of MSU leads to the activation of the NLRP3 inflammasome in peritoneal macrophages, resulting in production of IL-1β and subsequently chemokines that recruit neutrophils into the peritoneal cavity (33). The

infiltration of neutrophils is dependent on the inflammasome and IL-1β, as it does not occur in mice deficient for NLRP3, ASC, caspase 1 or IL-1R1 (33).

For these experiments, the amount of MSU required to induce a robust inflammatory response in the peritoneum was first determined by injecting 500, 250, 100, 20, 4, or 0 µg of MSU in mice. It was found that 100µg of MSU crystals is the lowest dose able to elicit a robust and reproducible inflammatory response as shown by increased infiltrating $Gr1^{+}F4/80^{-}$ neutrophils in the peritoneal lavage fluid (Fig. 5.1). Despite this response, IL-1β, IL-10 and KC were unexpectedly below the detection limit by ELISA, even after concentrating the peritoneal lavage fluid (data not shown), possibly as a result of degradation by neutrophil proteases.

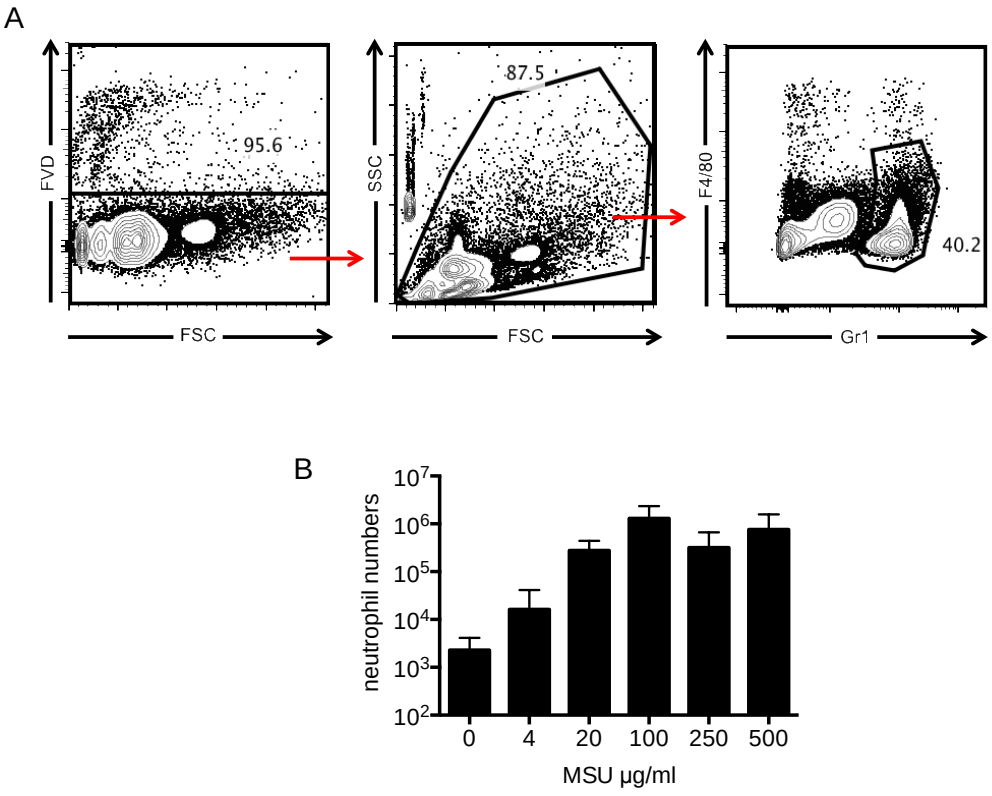

Figure 5.1 Monosodium urate crystals can induce neutrophil infiltration in a dose dependent manner.
Peritoneal lavage fluid was collected 5 hours after MSU injection. The cells were washed once, stained with FVD and antibodies to CD4, Gr1, F4/80 and CD11b and analyzed by flow cytometry. **(A)** Representative gating strategy of $Gr1^{+}F4/80^{-}$ neutrophils with the numbers indicating cell frequency in the gate. **(B)** Quantification of peritoneal neutrophil numbers (n=3 or 4 per group). Error bar represents standard deviation, pooled from three independent experiments.

5.2.2. IL-10R signaling regulates inflammasome activation *in vivo*.

Since IL-10 is critical in controlling inflammasome activation and production of IL-1β *in vitro*, we asked if IL-10 could protect mice from peritoneal inflammation *in vivo* following MSU injection. The expression of IL-10 was analyzed in endogenous $CD4^{+}$ T cells in the peritoneum without MSU treatment, and it was found that both $Foxp3^{+}$ Tregs and $CD44^{hi}Foxp3^{-}$ Tr1 cells, but not Tn, express high levels of IL-10 *ex vivo* (Figs. 5.2 A, B). It was also noted that there was an increased percentage of $CD44^{hi}Foxp3^{-}$ Tr1 cells in the peritoneum (about 57% Fig. 5.2A) compared to Tr1 cells isolated from spleens and lymph nodes (about 9.4%, Figs. 2.1). Next, 400ng of IL-10 or PBS was injected into the peritoneal cavity of wild type mice prior to the injection of MSU. Recombinant IL-10 did not inhibit neutrophil accumulation in the peritoneum (Fig. 5.2C). We then compared wild type to *Il10rb*-deficient mice in their inflammatory response after MSU injection. In wild type mice, injection of MSU resulted in a rapid increase in the absolute number of $Gr1^{+}CD11b^{+}F4/80^{-}$ neutrophils in the peritoneal lavage fluid (Fig. 5.2 D, E). As expected, $Il10rb^{-/-}$ mice were impaired in controlling this inflammasome-mediated response and had a significant increase in the number of infiltrating neutrophils (Fig. 5.2 D, E). Thus, IL-10R signaling has a critical role for regulating inflammasome activation and IL-1β production *in vivo*.

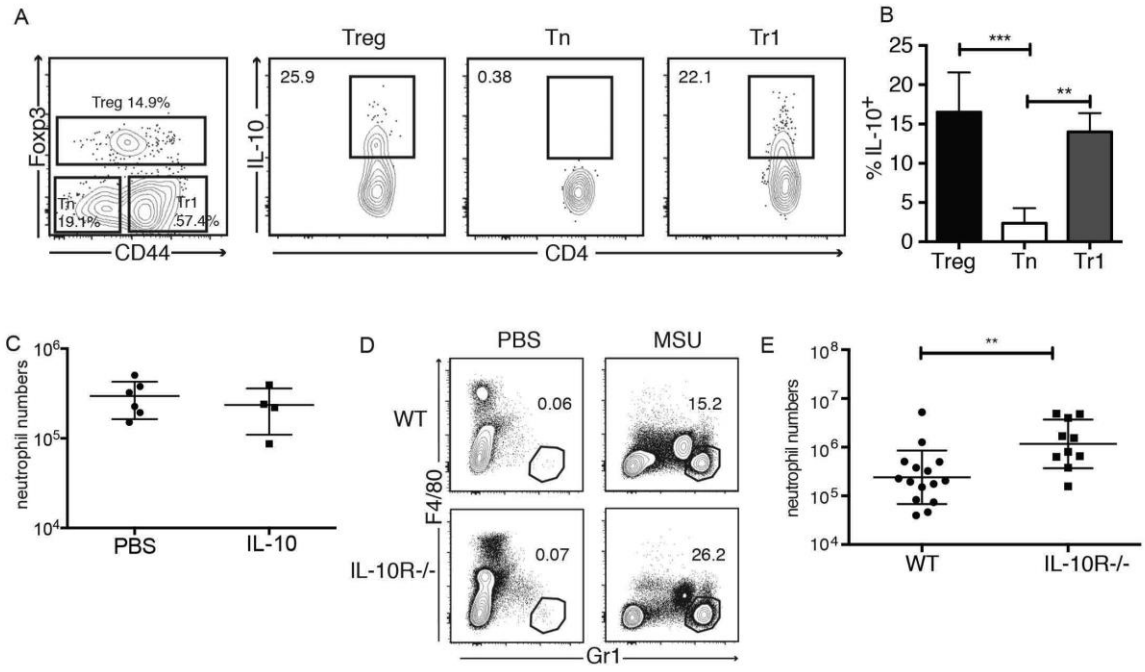

Figure 5.2 IL-10 receptor knockout mice have increased peritoneal inflammation upon monosodium urate injection.
(A, B) Peritoneal lavage was collected from *Foxp3*^{RFP}*Il10*^{GFP} reporter mice and analyzed by flow cytometry. Percent of Foxp3^{+} Tregs, CD44^{hi}Foxp3^{-} Tr1 cells, and CD44$^{int/lo}$Foxp3^{-} Tn were shown on the far left plot, and the frequencies of IL-10-GFP^{+} are indicated in the corresponding representative plots as shown on the right. **(A)** Representative FACS plots, **(B)** averaged frequency of IL-10^{-}GFP^{+} cells in each population. (n=4, **p<0.01, ***p<0.001). **(C)** Mice were injected with 400ng of IL-10 for 16 hours before the injection of 100µg MSU. Peritoneal fluid was collected 5 hours after MSU injection and the cells were analyzed by flow cytometry. The graph shows the number of Gr1^{+}F4/80^{-} neutrophils, and error bars represent standard deviation. **(D, E)** Gr1^{+}F4/80^{-} Neutrophils recovered from peritoneal lavage of wild type or IL-10R KO mice 5 hours after injection of MSU or PBS vehicle. **(D)** Representative FACS plots. **(E)** Neutrophil numbers. Error bar represents standard deviation (**p<0.01, students' t-test)

5.2.3. Tr1 cells transfer does not protect against MSU induced peritonitis.

Next, we sought to determine if Tr1 cells are able to inhibit MSU induced neutrophil migration *in vivo*. Foxp3^{+} Tregs, CD44$^{lo/int}$Foxp3^{-} Tn, and CD44^{hi}Foxp3^{-} Tr1 cells were isolated from *Thy1.1-Foxp3*^{GFP} reporter mice, and stimulated with anti-CD3/28 for 4 days as described in **2.3.5**. One million Treg, Tn, or Tr1 cells were injected into *Thy1.2* recipients, 16 hours prior to the injection of MSU. Despite of the presence of injected T cells (Fig. 5.3A),

we failed to detect any difference between the mice injected with T cells or with PBS vehicle alone (Fig. 5.3B). We then tried injecting Tr1 cells or 400ng of rIL-10 at the same time as the MSU injection and also failed to detect any suppression by Tr1 cells or IL-10 (Fig. 5.3C).

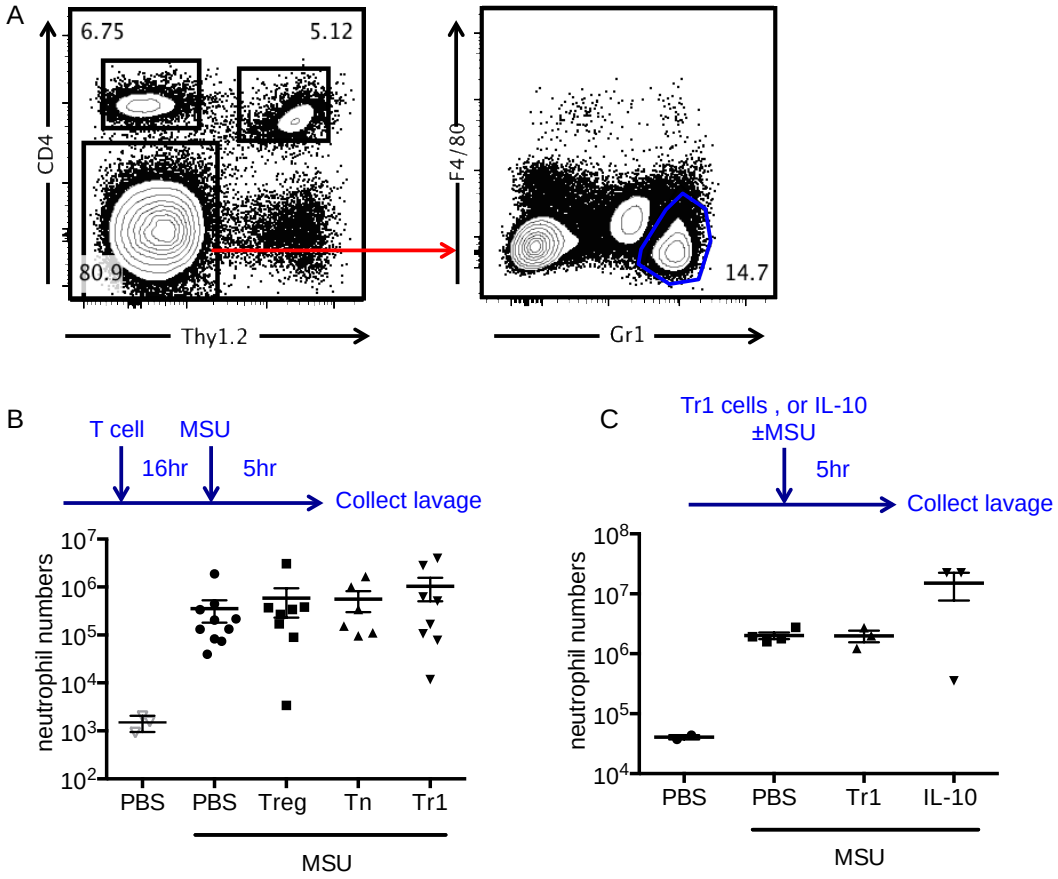

Figure 5.3 Neither Tr1 cells nor rIL-10 prevent peritonitis induced by MSU.
Foxp3 Tregs, Tn and Tr1 cells were sorted from *Thy1.1-Foxp3*^{GFP} reporter mice and stimulated for 4 days with polyclonal TCR stimulation in the presence of IL-2. One million cells were then transferred into *Thy1.2* mice 16 hours before **(A, B)** or the same time as **(C)** MSU injection. **(A)** Representative FACS plots of total live cells (left), and non-T cells (right). **(B, C)** Top shows the schematic of the injections; bottom shows the number of Gr1^{+}F4/80^{-} neutrophils.

5.2.4. Cre-ER inducible *Nlrp3*^{L351P} mice develop severe systemic inflammation upon tamoxifen induction.

Since mice carrying mutations with *Nlrp3*^{L351P} develop severe systemic neutrophilic inflammation that results in neonatal or perinatal death, *Nlrp3*$^{L351PNeoR/+}$ knock-in mice were

crossed to CAG-Cre mice to generate *Nlrp3*$^{L351P/+}$*CreT*, a tamoxifen inducible knock-in strain (263, 267). Injection of tamoxifen induced rapid weight loss and poor survival in *Nlrp3*$^{L351P/+}$*-CreT* mice but not *Nlrp3*$^{L351P/+}$*-WT* mice (Fig. 5.4A, B). Of note, *Nlrp3*$^{L351P/+}$*-CreT* mice treated with vehicle alone without tamoxifen did not develop weight loss in the course of the experiment (data not shown). End-point serum cytokine analysis revealed variably increased IL-6, and KC, but no difference in IL-1β, TNFα, IFNγ or IL-10 (Fig. 5.4D). Despite normal numbers of macrophages and $CD4^+$ T cells in the spleen, there was significantly increased $Gr1^+F4/80^-$ neutrophil infiltration in *NLRP3*$^{L351P/+}$*-CreT* mice compared to *Nlrp3*$^{L351P/+}$*-WT* mice (Fig. 5.4C), which resembles the tissue neutrophilia observed in CAPS patients. Furthermore, *NLRP3*$^{L351P/+}$*-CreT* mice developed acute liver inflammation as indicated by venous thrombosis, liver infarction, and inflammatory cell infiltration that primarily consist of neutrophils, while other sites, like colon and skin were not affected (Fig. 5.5).

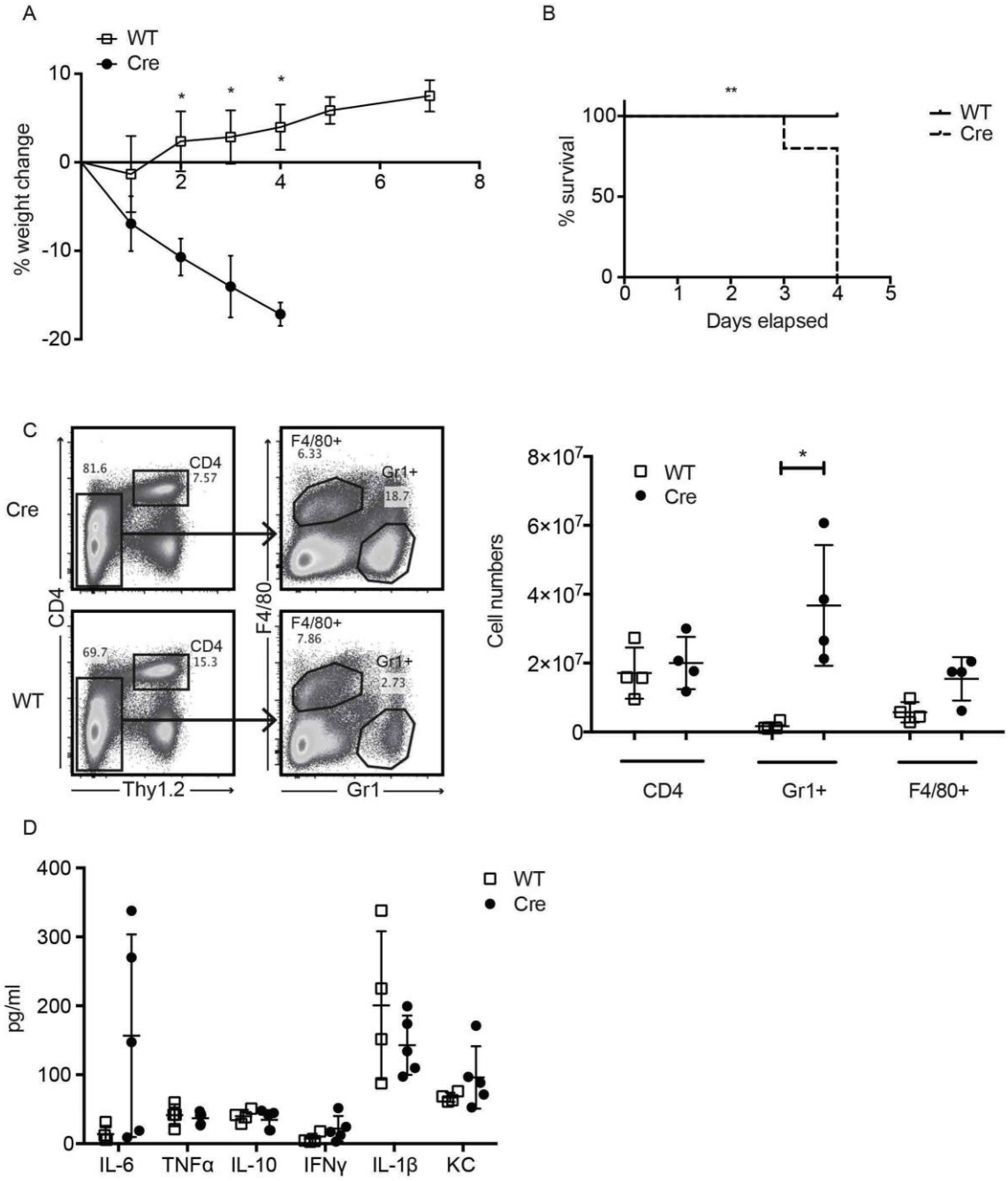

Figure 5.4 Tamoxifen injection leads to rapid neutrophil mediated inflammation in *NLRP3$^{L351P/+}$-CreT* mice.

Tamoxifen was injected IP daily for four consecutive days and the mice were weighed daily. **(A)** Percent weight change and **(B)** percent survival were indicated in *Nlrp3$^{L351P/+}$-WT* (WT, solid circle) and *Nlrp3$^{L351P/+}$-CreT* (Cre, empty square) mice. **(C)** At the end point of the experiment, spleens were harvested and the number of $CD4^+$ T cells, $F4/80^+$ macrophages, and $Gr1^+$ neutrophils was analyzed by flow cytometry. The FACS plots on the left indicate gating strategy; live cells were analyzed for the expression of Thy1.2 (all mice express

Thy1.2 congenic marker) and CD4, then Thy1.2^{-}CD4^{-} cells were analyzed for expression of F4/80 and Gr1. The numbers in the plots indicate frequencies with associated population. Right panel is the quantification of cell numbers, and each dot represent one mouse. **(A, B, C)** * p<0.05, **p<0.01, multiple t-tests corrected for multiple comparisons using Holm-Sidak method. **(D)** Serum cytokines were measured at the end point of the experiment. **(A, B, C, D)** Error bar presents standard deviation.

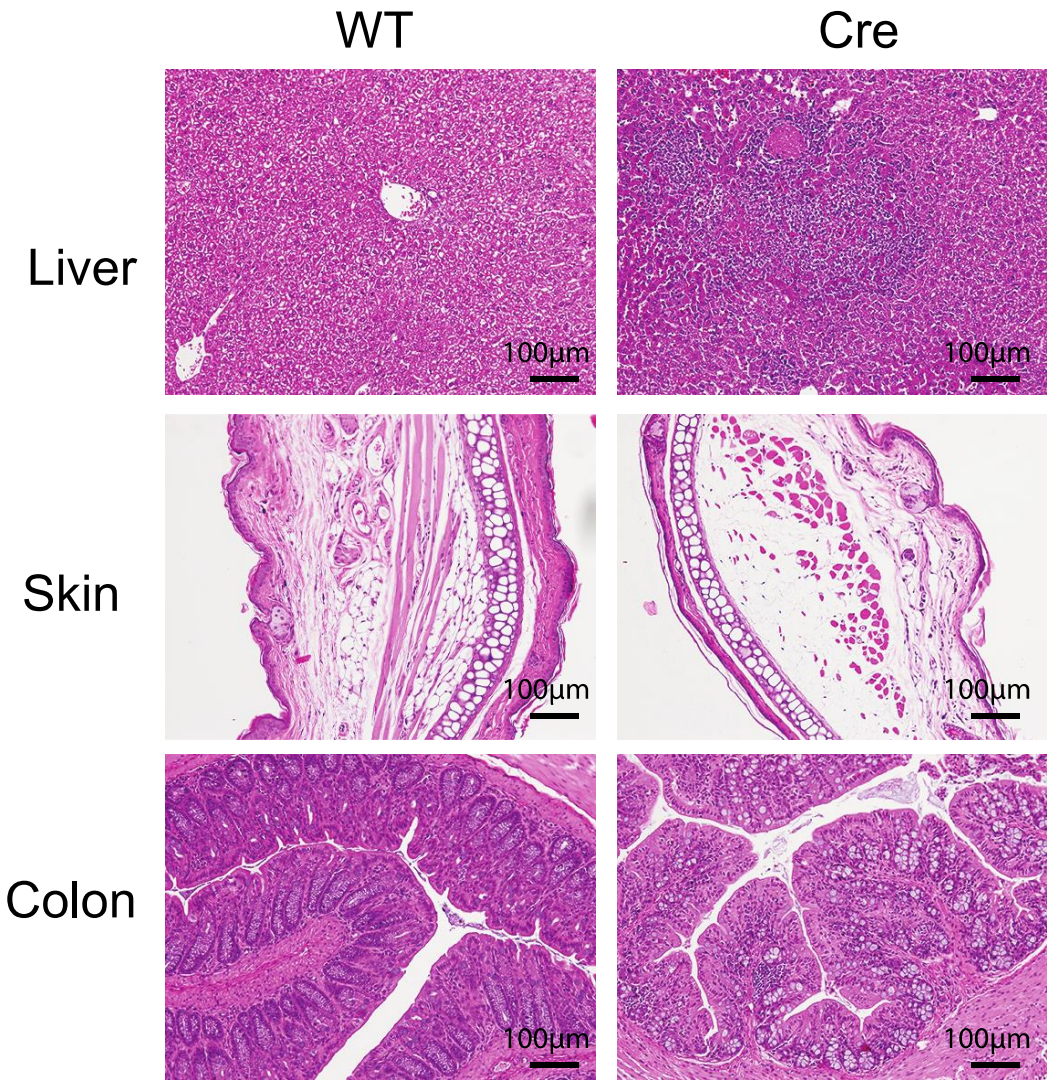

Figure 5.5 Tamoxifen treated *Nlrp3*$^{L351P/+}$-*CreT* mice develop hepatic inflammation. H&E-stained tissue sections from liver, skin and colon from *Nlrp3*$^{L351P/+}$-*WT* and *Nlrp3*$^{L351P/+}$-*CreT* mice (magnification, ×10). Sections are representative of at least 3 mice per group.

5.2.5. Adoptive transfer of Tr1 cells 24 hours prior to tamoxifen treatment does not prevent systemic inflammation in *Nlrp3*$^{L351P/+}$*CreT* mice.

In order to determine if Tr1 cells are protective in preventing systemic inflammation in the *Nlrp3*$^{L351P/+}$*-CreT* mice, 3 million Tr1 cells were injected intravenously 24 hours prior to tamoxifen. The cells were well tolerated in both wild type and *Nlrp3*$^{L351P/+}$*-CreT* mice without causing any weight loss within the first 24 hours, and no adverse effects were observed in wild type mice 4 days after the injection (Fig. 5.6A). In the *Nlrp3*$^{L351P/+}$*-CreT* mice, the ones that received Tr1 cells were marginally protected on day 2 (PBS: -7.71% ±0.044 versus Tr1 cells: -4.51% ±0.020) and day 3 (PBS: -9.97% ±0.047 versus Tr1 cells: -6.06% ±0.014); however, the protective effect was gone on day 4 (PBS: -13.4% ±0.043 versus Tr1 cells: -12.2% ±0.023) (Fig. 5.6A).

Serum cytokines were measured 24 hours and 72 hours after tamoxifen injection (Fig. 5.6B, C). At both time points, IL-6 was found to be elevated in *Nlrp3*$^{L351P/+}$*-CreT* mice compared to wild type mice, but there was no effect of injecting Tr1 cells when compared to PBS treated mice. On the other hand, there was a trend towards decreased KC in *Nlrp3*$^{L351P/+}$*-CreT* mice treated with Tr1 cells.

We then sought to determine if the injection of Tr1 cells resulted in elevated circulating IL-10 and found minimally increased serum IL-10 two days after injection of Tr1 cells (Fig. 5.6B). No significant amounts of IL-10 were detected after 4 days (data not shown). It is worth noting that the *Nlrp3*$^{L351P/+}$*-CreT* mouse with the highest amounts of circulating IL-10 at day 2 also had the least weight loss in the group.

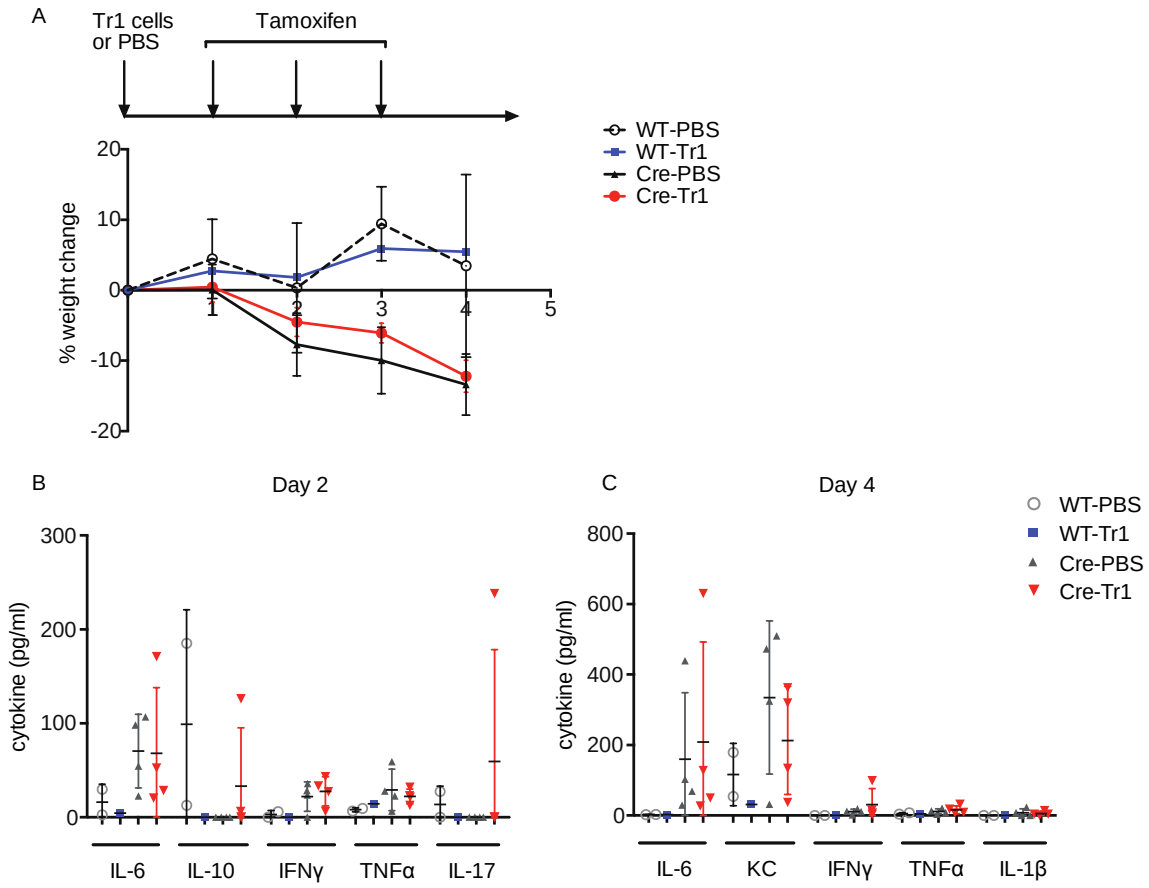

Figure 5.6 Adoptive transfer of Tr1 cells prior to tamoxifen induction does not eliminate weight loss and inflammatory cytokine production in *Nlrp3*$^{L351P/+}$*-CreT* mice. *Nlrp3*$^{L351P/+}$*-WT* (WT) mice and *Nlrp3*$^{L351P/+}$*-CreT* (Cre) mice were injected IV with either PBS or 3 million Tr1 cells pre-stimulated with polyclonal stimulation for 4 days. After 24 hours of T cell injection, the mice were treated with tamoxifen daily for 3 days as indicated in (**A**). **(A)** Injection scheme (top), and percent weight change (bottom). Serum cytokines measured 2 **(B)** or 4 days **(C)** post T cell transfer by cytokine bead array. **(A-C)** Error bar represents standard deviation.

Due to the minimal clinical protection of Tr1 cells in *Nlrp3*$^{L351P/+}$*-CreT* mice, we then asked if they engrafted properly in a competitive environment in the presence of endogenous T cells. Thy1.1^{+} Tr1 cells were transferred into *Nlrp3*$^{L351P/+}$*-CreT* (Thy1.2) or wild type (Thy1.2) hosts 24 hours before tamoxifen treatment, and the proportion of Thy1.1 and Thy1.2 CD4^{+} T cells in the spleen and blood were analyzed 4 days after T cell injection.

It was found that the transferred Thy1.1^{+} Tr1 cells constituted a significant proportion of the CD4^{+} T cells in the spleen (26.7% ±0.53, Fig. 5.7A,B) and blood (46.2% ±9.93, Fig. 5.7E, F), which is also reflected in absolute numbers (Fig. 5.7D). Consistent with the minimal protection in weight loss, the transfer of Tr1 cells did not cause any decrease in neutrophil frequency (Fig. 5.7A, C) or absolute number (Fig. 5.7D) in the spleen, or in the blood (Fig. 5.7E, G), indicating that Tr1 cells failed to down-regulate neutrophilic inflammation. Histological analysis on liver biopsies revealed similar hepatic inflammation in *Nlrp3*$^{L351P/+}$-*CreT* mice with or without Tr1 cells transfer (Fig. 5.8), and no abnormalities were observed in skin or colon sections in all the mice.

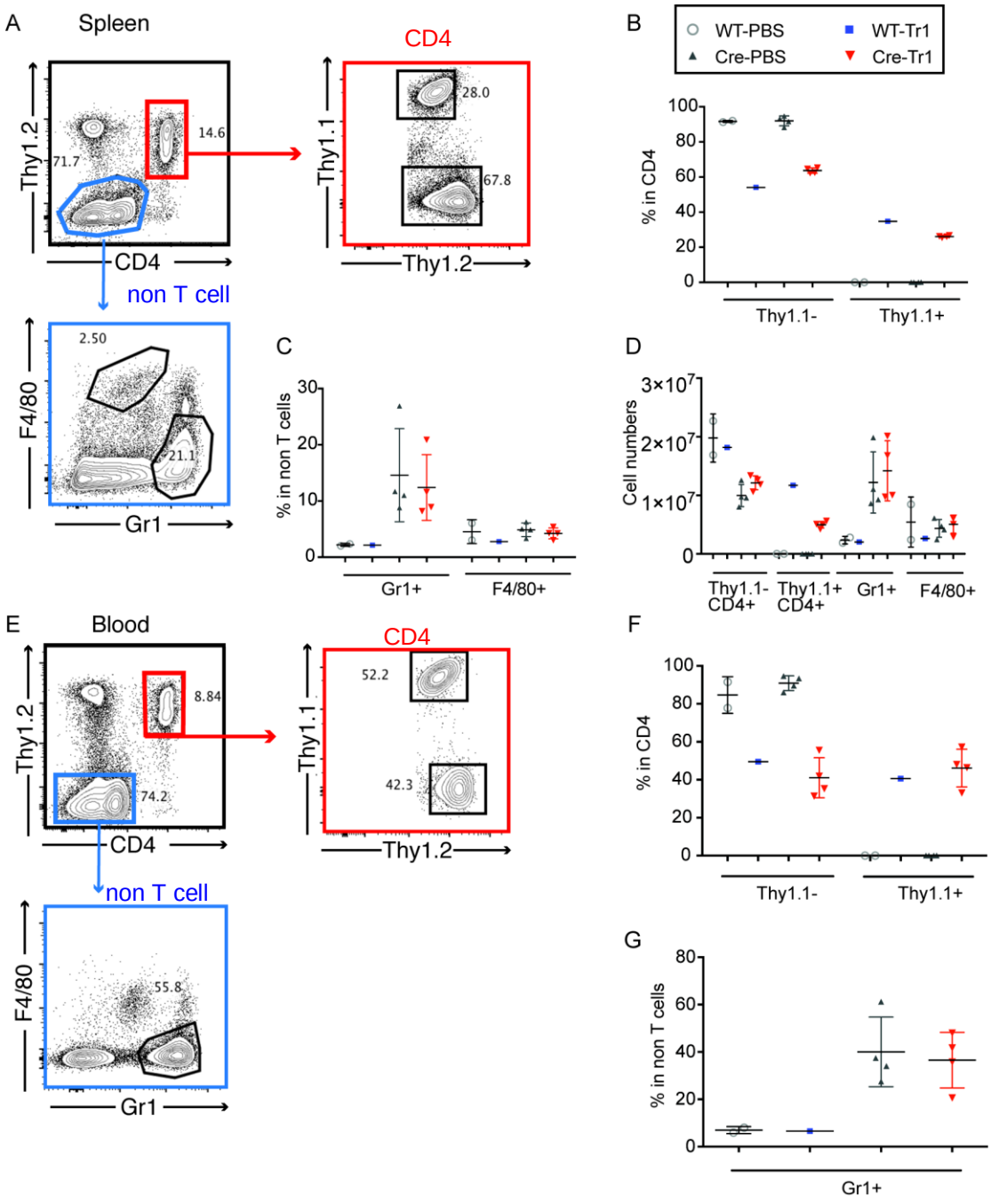

Figure 5.7 Engraftment of Tr1 cells in *Nlrp3*$^{L351P/+}$-*CreT* mice.
Thy1.1^{+}Thy1.2^{+} Tr1 cells were adoptively transferred into *Nlrp3*$^{L351P/+}$-*CreT* (Thy1.2^{+}) or wild type mice 24 hours before tamoxifen treatment as described in figure 5.5. The mice were euthanized 4 days after Tr1 cell transfer; cells from the spleens **(A-D)** and blood **(E-G)** were harvested and analyzed by flow cytometry. **(A)** Representative FACS plots from an *Nlrp3*$^{L351P/+}$-*CreT* mouse treated with Tr1 cells. Live cells were gated based on Thy1.2 and CD4 (left). Right: Thy1.2^{+}CD4^{+} T cells were then separated based on Thy1.1 into host CD4^{+} T cells (Thy1.1^{-}) and donor T cells (Thy1.1^{+}). Bottom: Thy1.2^{-}CD4^{-} cells (non-T cells) were

analyzed based on F4/80 and Gr1. **(B)** Percentages of donor Thy1.1^{+} and host Thy1.1^{-} cells in CD4^{+} cells. **(C)** Percentages of neutrophils (Gr1^{+} cells) and macrophages (F4/80^{+} cells) in Thy1.2^{-}CD4^{-} cells (denoted as non-T cells). **(D)** Numbers of Thy1.1^{+}, Thy1.1^{-} CD4^{+} T cells, neutrophils (Gr1^{+}), and macrophages (F4/80^{+}) in the spleen. **(E)** Representative FACS plots on blood cells with the same gating strategy as **A**, except no distinct F4/80^{+} macrophage population was found or analyzed. **(F)** Percentages of donor (Thy1.1^{+}) and host T cells (Thy1.1^{-}) in CD4^{+} T cells. **(G)** Percentage of Gr1^{+} neutrophils in non-T cells. **(B, C, D, F, G)** Error bars represent standard deviation.

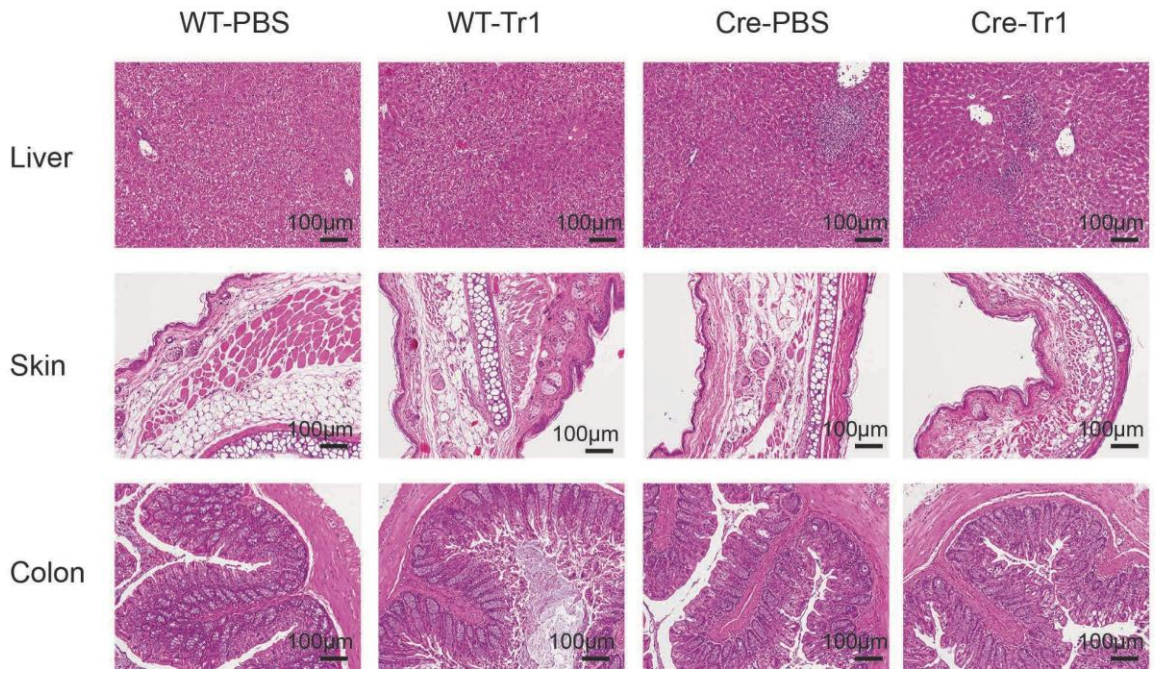

Figure 5.8 Liver inflammation in *Nlrp3*$^{L351P/+}$*-CreT* mice with and without adoptive transfer of Tr1 cells.
H&E-stained liver, skin and colon sections (magnification, ×10) of *Nlrp3*$^{L351P/+}$*-CreT* (Cre) or wild type (WT) mice treated with tamoxifen for 3 days with PBS or Tr1 cells as indicated in the schematic in figure 5.6A. Representative of at least 3 mice per group.

Based on their superior ability to suppress inflammasome activation induced IL-1β *in vitro* as shown in Chapter 4, we were surprised to find minimal protection of these cells *in vivo*. Therefore, we asked if the transferred Tr1 cells maintained the characteristic Tr1 cytokine profile *in vivo* or shifted to produce more proinflammatory cytokines in the presence of systemic inflammation. To investigate this question, total splenocytes were isolated at the end point of the experiment and stimulated with PMA and ionomycin 5 hours

before FACS analysis. Similar to observations *in vitro*, 5-20% of Tr1 cells produced IFNγ (Fig. 5.9A, B); less then 1% were positive for IL-17 (data not shown); and they did not up-regulate Foxp3 expression *in vivo* (Fig. 5.9A, B). Furthermore, injection of Tr1 cells did not alter the production of IFNγ in host $CD4^+$ T cells (Fig. 5.9A, C), however, there was a trend towards a decreased frequency of $Foxp3^+$ Tregs in the spleen (Fig. 5.9A, C). Therefore, these data suggest that adoptive transfer of Tr1 cells 24 hours prior to tamoxifen injection does not fully prevent systemic inflammation in *Nlrp3*$^{L351P/+}$*-CreT* mice and Tr1 cells did not upregulate IFNγ or IL-17 upon adoptive transfer.

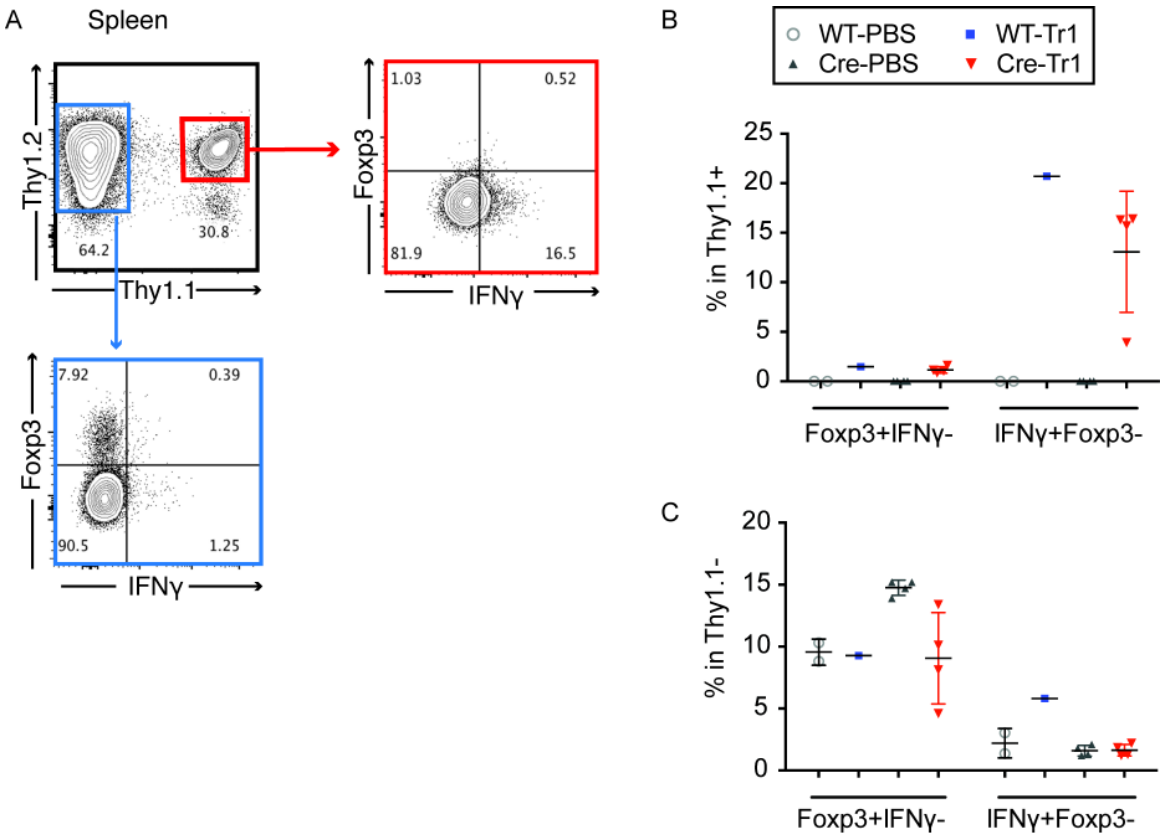

Figure 5.9 Expression of IFNγ and Foxp3 in donor and host $CD4^+$ T cells in the spleen. $Thy1.1^+$ $Thy1.2^+$ Tr1 cells were adoptively transferred into *Nlrp3*$^{L351P/+}$*-CreT* ($Thy1.2^+$) or wild type mice ($Thy1.2^+$) and treated with tamoxifen for 3 days. On day 4, the mice were euthanized, and the splenocytes were stimulated with PMA and ionomycin for 5 hours with

brefeldin A added in the last 4 hours. The cells were staining for FVD, Thy1.1, Thy1.2, IFNγ, Foxp3, and IL-17 and followed by FACS analysis. **(A)** Representative FACS plots from an *Nlrp3*$^{L351P/+}$*-CreT* mouse treated with Tr1 cells. Live CD4^{+} cells were gated on Thy1.2 and Thy1.1 (left). Thy1.1^{+}CD4^{+} and Thy1.1^{-}CD4^{+} T cells were then separated based on Foxp3 and IFNγ. Percentages of Foxp3^{+}IFNγ^{-} and Foxp3^{-}IFNγ^{+} cells from donor Thy1.1^{+}CD4^{+} cells **(B)** and host Thy1.1^{-}CD4^{+} cells **(C)**.

5.2.6. Adoptive transfer of Tr1 cells at the same time as tamoxifen induction is more effective at reducing systemic inflammation in *Nlrp3*$^{L351P/+}$*CreT* mice.

Next, we asked if Tr1 cells would be more effective if injected at the time when the inflammation is induced as the Tr1 cells may be more activated *in vivo* when there is already inflammation at the time of engraftment. To investigate this, Thy1.1^{+} Tr1 cells were adoptively transferred into *Nlrp3*$^{L351P/+}$*-CreT* (Thy1.2) or wild type (Thy1.2) hosts immediately following tamoxifen injection and the treatment showed a trend towards less weight loss compared to *Nlrp3*$^{L351P/+}$*-CreT* mice at all the time points (Fig. 5.10A). Furthermore, transfer of Tr1 cells decreased serum KC levels significantly, and there was a trend towards decreased IL-6 levels (Fig. 5.10B). Similar to previous observation, injected Tr1 cells repopulated the lymphoid compartment efficiently and constituted about 30% of CD4^{+} cells in the spleen (Fig. 5.10C) and about 50% of CD4^{+} cells in the blood (Fig. 5.10D). Overall, these results suggested that injection of Tr1 cells at the time of disease induction might provide better protection against systemic inflammation in *Nlrp3*$^{L351P/+}$*-CreT* mice compared to if injected prior to disease induction.

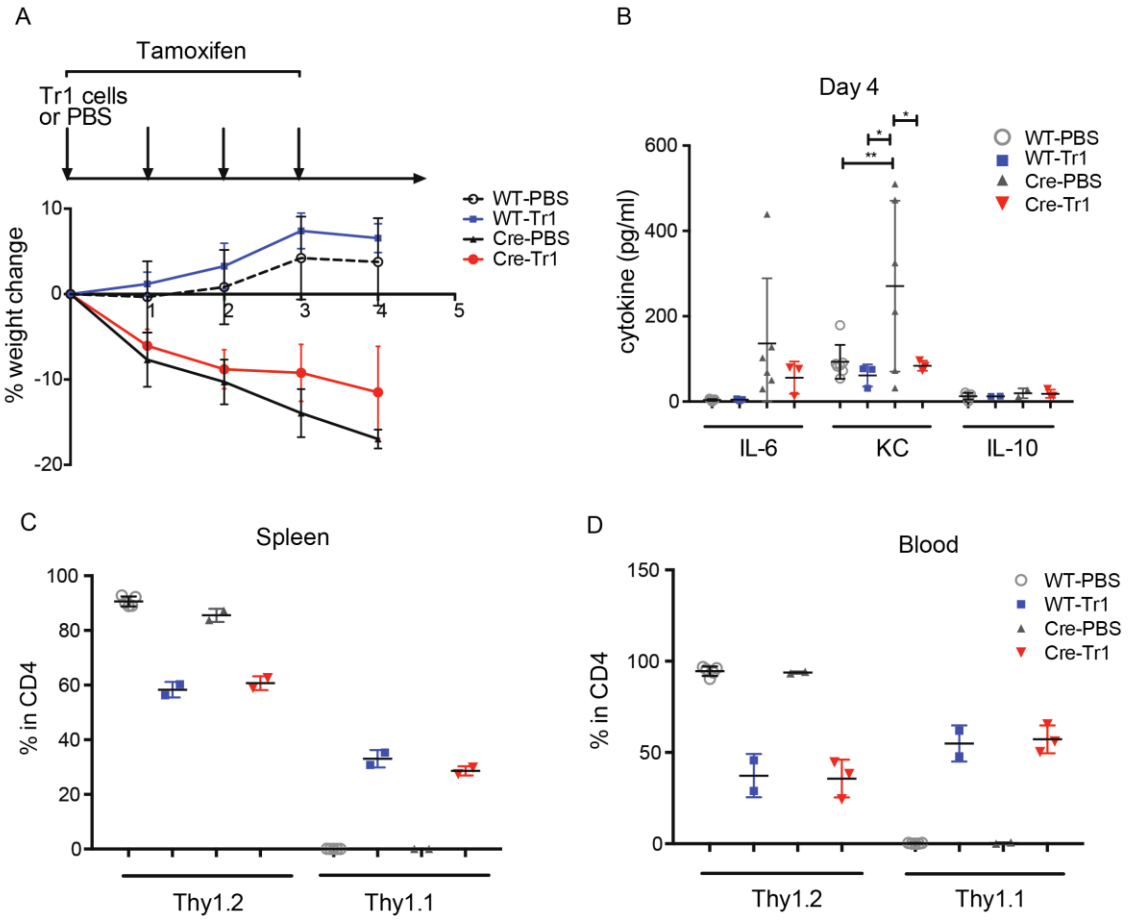

Figure 5.10 Injection of Tr1 cells at the time of tamoxifen injection partially protect the mice from systemic inflammation.
Nlrp3$^{L351P/+}$*-WT* (WT) mice and *Nlrp3*$^{L351P/+}$*-CreT* (Cre) mice were injected IV with either PBS or 3 million Tr1 cells, followed by tamoxifen IP injection immediately as indicated in (**A**). **(A)** Injection scheme (top), and percent weight change (bottom). **(B)** Serum cytokines 4 days after T cell transfer as by cytokine bead array. Percentages of donor Thy1.1^{+} and host Thy1.2^{+} cells in CD4^{+} cells from the spleen **(C)** and blood **(D)** on day 4. **(A-D)** Error bar represents standard deviation.

5.3. Discussion

The therapeutic potential of Tr1 cells has been explored in various disease models (73), and in humans, with two proof-of-principle trials in patients who received a hematopoietic stem cell transplant (127), or had Crohn's disease (126). However, it is unclear if Tr1 cells can regulate inflammasome-driven disease *in vivo*. Here, we explored the

possibility of using Tr1 cells in treating autoinflammatory disorders specifically looking at MSU induced peritonitis, and a mouse model of CAPS. Although we did not find any protective effect of $CD44^{hi}Foxp3^{-}$ Tr1 cells in MSU-induced peritonitis, we were able to show that IL-10 receptor signaling is critical in regulating MSU induced inflammation *in vivo*. Furthermore, adoptive transfer of Tr1 cells appears to be protective in preventing systemic inflammation in a murine model of CAPS if administered at the right time.

IL-10 is a potent anti-inflammatory cytokine that regulates the activation of various immune cells involved in innate and adaptive immunity. Importantly, we found that both $Foxp3^{+}$ Tregs and Tr1 cells can produce high levels of IL-10 in the peritoneum; the endogenous IL-10 can suppress local inflammation induced by MSU as illustrated in increased neutrophil infiltration in IL-10R-/- mice. However, administration of recombinant IL-10, either at the same time or prior to MSU injection, failed to prevent inflammation, which could be explained by its short half-life and low bioefficacy (268). It is likely that novel delivery methods will be required to improve its efficacy. The same challenge of IL-10 administration was seen in clinical trials with IBD patients, where IL-10 had minimal efficacy and high variability depending the patient cohort (269). Several factors need to be taken into consideration, including the current delivery method either with daily subcutaneous injections or intravenous injections that results in low intestinal IL-10 concentration, and the fact that IL-10 fails to suppress all of the inflammatory cytokines in patients with chronic and active disease (269). Tr1 cells may be able to overcome these shortfalls and provide a better option in delivering site-specific and antigen-specific suppression through locally concentrated secretion of IL-10 as well as other suppressive mechanisms.

Although IL-10 is required for suppressing MSU-induced peritoneal inflammation, we failed to detect any significant protective effect of transferring in Tr1 cells. This may be due to insufficient transfer of cells in immune competent mice as endogenous T cells are also found in the peritoneal space. Indeed, we were unable to detect any IL-10 protein by ELISA in the peritoneal lavage after transferring Tr1 cells. On the other hand, since Tr1 cells can also produce low/intermediate amounts of TNFα and IFNγ, it is possible these cells may exacerbate inflammation if too many are infused. Therefore, a future study would be to define a suitable dose of Tr1 cells in order to optimize the suppressive effects while not increasing the pre-existing inflammation.

In addition to the challenge in dose finding, the MSU-induced peritonitis model has other limitations. First, it is a model of acute inflammation that may be insensitive to the anti-inflammatory cytokines produced by Tr1 cells. Second, the model is measured on the basis of neutrophil migration, which is a complex biological process several steps downstream of IL-1β production, that can be influenced by many factors. Last but not least, MSU forms insoluble crystals in suspension, leading to variability in the injected dose that could mask effectiveness of the treatment. With these concerns, we concluded that, despite its simplicity, this model is not ideal to test the effectiveness of Tr1 cells at suppressing the inflammasome.

We therefore sought to develop another mouse model where inflammasome activation can be more directly measured and selected the mouse model of FCAS developed by Hoffman. *et al.* (263), as the disease is rapid and multiple clinical measurements could be done to carefully evaluate the efficacy of Tr1 cell treatment. After pre-treatment of Tr1 cells before tamoxifen induction, the FCAS mice showed some improvement in the course of the experiment, yet this protection was completely lost at the end point, as reflected by

comparable weight loss, neutrophil counts in the blood and spleen as well as serum IL-6 levels in PBS versus Tr1-treated mice. Since Tr1 cells may not get properly activated in the absence of inflammation and/or antigen stimulation upon *in vivo* transfer in this experimental setup, I examined whether the cells would be more protective if administered immediately after tamoxifen induction. Indeed, the Tr1 cells were more protective as shown by decreased weight loss at all time point examined, as well as decreased serum KC levels. These results indicated that the timing of Tr1 cell transfer could critically affect treatment outcome.

Since the Tr1 cells were unlikely going to receive any further *in vivo* stimulation in our experimental setting, the IL-10 was most likely produced because of the 4-day *in vitro* TCR stimulation prior to the injection. Therefore, one possibility is that by delivering Tr1 cells at the same time of disease induction maximized the exposure of IL-10 to inflammatory macrophages and dampened IL-1β production. To further improve the suppressive function of Tr1 cells, *in vivo* antigen stimulation might be required. Indeed, *in vivo* antigen stimulation was necessary for the beneficial effect of Tr1 cells in many disease models, including T cell transfer colitis (94), GVHD in HLA-mismatched HSCT (117), and islet transplantation (119). Future experiments will need to be conducted using $CD44^{hi}Foxp3^{-}$ cells derived from TCR transgenic mice to enable *in vivo* antigen stimulation with minimal effects on endogenous T cells.

One interesting observation was the remarkable ability of these polyclonal Tr1 cells to repopulate upon *in vivo* transfer, especially in the blood where remarkably half of the $CD4^{+}$ T cells were derived from the injected Tr1 cells. This was consistent with previous observations by Younes. *et al.*, where $CD44^{hi}Foxp3^{-}$ T cells can undergo rapid proliferation and expansion *in vivo* (245). Furthermore, the injected Tr1 cells maintained the expected

expression of IFNγ, IL-17, and Foxp3 after 4 days of transfer as observed *in vitro* (in chapter 4). The cytokine data, which showed serum levels of IL-10 only increased marginally 2 days post injection and was undetectable after 4 days, suggested that either production of this cytokine may not be optimal in this experimental design or the rapid degradation of IL-10 impeded its detection.

Indeed, one limitation of the current study is the difficulty in measuring the kinetics of IL-10 production from Tr1 cells *in vivo*, as IL-10 is difficult to measure by intracellular cytokine staining. A method that is commonly used to overcome this challenge is to derive Tr1 cells from $Il10^{GFP}$ reporter mice. However, GFP has a longer half-life (more than 24 hours) compared to IL-10 (approximately 3 hours), meaning that expression of GFP may persist even if IL-10 does not (270). It is possible to use other novel IL-10 reporter mice, such as ITIB mice (271), which are developed based on a reporter enzyme β-lactamase that cleaves a membrane permeable substrate that will fluoresce when the enzyme gets activated. The advantage of this reporter system over GFP is that the reporter enzyme β-lactamase has a similar half-life as IL-10 enabling faithful detection in real time.

Overall, IL-10 receptor signaling clearly regulates inflammasome activation *in vitro* and *in vivo* in a model of MSU-induced peritonitis. Adoptive transfer of polyclonal Tr1 cells was safe at least in the first 4 days that were evaluated and the cells were able to repopulate efficiently in an immune competent mouse. Furthermore, the data showed for the first time that Tr1 cells have some promising protective effect in a mouse model of CAPS. These results also indicated that the time of delivery and other factors such as *in vivo* antigen stimulation may be required for the maximal protective effect of these cells.

Chapter 6: Conclusions

Endogenous danger signals play a critical role in creating a microenvironment that unleashes and/or modulates innate and adaptive immune responses, adding another layer of complexity in the regulation of host immunity. The main goals of my research have been to: investigate the cellular interaction between innate and adaptive immunity when damage signals are exposed in the context of autoinflammatory diseases; identify potential targets of the pathways initiated by cellular stress; and examine regulatory mechanisms that maybe beneficial for dampening stress responses.

The first aim of the study was to uncover novel molecular mechanisms by which danger signals can potentiate the immune response in a polygenic autoinflammatory disorder, namely, inflammatory bowel disease. Extensive literature has demonstrated a role for high concentration of ATP in directly regulating maturation and inflammasome activation in DCs and macrophages; however, the effect of ATP is not well appreciated in other cell types. To investigate this, I established a sequential co-culture method to measure the downstream immune effects of exposing intestinal epithelial cells to danger signals. Using this co-culture system, I found that ATP can increase the magnitude of TLR responses by increasing cytokine/chemokine production in murine intestinal epithelial cells, leading to altered DC maturation, as judged by increased expression of the costimulatory molecule CD80 and cytokines that affect T helper cell differentiation. Increased DC activation can then facilitate the proliferation and expansion of T cells specific for a commensal antigen, flagellin. Since ATP has been shown to enhance anti-flagellin responses and exacerbate DSS colitis (59),

these *in vitro* data provide a cellular mechanism for ATP-initiated stress responses on the intestinal epithelium that can re-direct TLR responses from tolerogenic to inflammatory.

This *in vitro* finding was further validated using an *in vivo* model by intrarectal delivery of ATPγS, a non-degradable analogue of ATP. By increasing the concentration of luminal ATP, without causing barrier damage or administration of exogenous TLR ligands, the cytokine responses on IECs to endogenous microbial molecules can be altered. These data support the hypothesis that cellular stress potentiates the responses to TLR ligands.

Furthermore, I found exposure of IECs to ATP creates a microenvironment favoring the expansion of Th1 and Th17 cells in the presence of DCs. Although DCs are not directly sensing ATP in our system *per se*, other studies have indicated that they can directly respond to ATP stimulation and promote a Th17 response (171). In addition to the role of ATP in DCs, direct activation of P2X7 receptor on $Foxp3^+$ Tregs can inhibit their suppressive function and skew them into IL-17 producing cells (65). Furthermore, elevated ATP induces mast cell activation and IL-1β production independent of caspase 1 and drives intestinal inflammation in an acute colitis model induced by TNBS (272). Together with our findings, these results demonstrate the complex role of ATP in mediating intestinal pathology by targeting different cell types.

Overall, these results suggested an important association between ATP driven purinergic signaling and increased or uncontrolled activation of commensal-reactive Th1 and Th17 cells observed in Crohn's disease. Therefore, targeting purinergic signaling pathways may be a beneficial treatment for IBD. Along the same line, my results also highlighted the involvement of P2X7 receptor on ATP mediated signaling in IECs, which can be targeted by pharmaceutical inhibitors. Indeed, the effectiveness of P2X7 receptor blockade has been

shown in many animal models of autoimmunity or inflammatory diseases, such as LPS induced systemic inflammation (273), neuroinflammation (274), ischemia/reperfusion injury (275, 276), liver fibrosis (277), allograft transplantation (278), and rheumatoid arthritis (279).

In IBD specifically, elevated expression of P2X7 receptor has been found in the intestinal epithelium and lamina propria in patients with Crohn's disease, and is associated with increased production of IL-1β and IL-17 in the intestine (272, 280). Consistent with this finding, prophylactic blockade of P2X7 receptor prevents experimental colitis induced by TNBS (272, 281). Furthermore, mice deficient for P2X7 receptor are protected from severe intestinal pathology in chemically induced colitis by DSS and TNBS. Based on these promising pre-clinical data, a phase II randomized, double-blinded, placebo-controlled trial has been conducted with a selective P2X7 antagonist, AZD9056, in patients with Crohn's disease (282). The drug was well tolerated with minor side effects, and showed some improvements in the CD activity index (282). Taken together, these results warrant more investigation into the therapeutic potential of targeting P2X7 receptor signaling pathways in inflammatory bowel disease.

Since immune activation by danger signals has been implicated in a variety of immune-mediated diseases, which are often associated with perturbed regulatory pathways, I next focused my research on investigating the ability of regulatory T cells, both $Foxp3^+$ Tregs and Tr1 cells, to modulate cellular stress response.

Unlike $Foxp3^+$ Tregs, which can be easily tracked and studied on the basis of expression of their the lineage-defining transcription factor, Foxp3, tools to study Tr1 cells are more limiting (73). First, the lack of lineage-defining transcription factors makes it difficult to purify these cells *ex vivo*. This challenge was partially solved with the recent

identification of surface biomarkers of Tr1 cells; however, *in vivo* stimulation with anti-CD3 is required prior to isolation either to expand the numbers and/or induce expression of CD49b and LAG3 (240). Second, the methods to polarize Tr1 cells *in vitro* are usually long and require high levels of polarizing cytokines. Furthermore, the yield is not satisfactory and often not pure making it difficult to directly assess the role of Tr1 cells.

In the process of trying to generate different subsets of murine T cells, I discovered a new method to generate a homogenous population of polyclonal Tr1 cells. Specifically, I found that polyclonal stimulation of $CD4^{+}CD44^{hi}Foxp3^{-}$ cells with IL-2, in the absence of any polarizing cytokines, results in a population that is highly enriched in Tr1 cells. Interestingly, $CD4^{+}CD44^{hi}Foxp3^{-}$ T cells were previously described as memory T cells with characteristics distinct from conventional antigen-experienced memory cells, including rapid proliferation and cytokine-driven self-renewal independent of TCR stimulation (245). In mice raised in specific pathogen free (SPF) environments (which have not been exposed to infectious agents), this population may contain a minor proportion of slowly proliferating true memory cells and a majority of rapid dividing cells that did not arise from naïve T cells (245). Furthermore, their emergence does not rely on commensal microbial or food antigens as germfree mice maintained on an elemental diet (antigen-free) have a similar frequency of $CD44^{hi}$ T conventional cells that have a TCR repertoire similar to cells isolated from mice raised in an SPF environment and fed a normal diet (245, 283-285). Notably, prior to my studies, no physiological function had been identified for these cells.

My data show that upon *in vitro* polyclonal stimulation, $CD4^{+}CD44^{hi}Foxp3^{-}$ T cells rapidly up-regulate IL-10, resulting in a population that closely resembles Tr1 cells as judged by expression of cytokines, surface markers and transcription factors, as well as

functional suppressive capacity *in vitro*. Collectively, these data strongly suggest the cells are *bona fide* Tr1 cells. My data also suggest that unconventional memory cells may be the precursor for Tr1 cells. Yet, many questions remained to be answered. For example, what are the factors that drive the differentiation of memory phenotype cells into Tr1 cells *in vivo*? Do CD44 ligands, such as an extracellular matrix component hyaluronin, play a role in regulating the *in vivo* activation of these cells? Is there an equivalent population of Tr1 precursor cells existing in humans? If so, what factors drive the differentiation of human Tr1 cells? These questions demand more studies on this population that would give us a better understanding of the biology of Tr1 cells.

It is worth noting that although the emergence of memory phenotype cells does not seem to require antigen stimulation, generation of functional Tr1 cells that express IL-10 and function as suppressor cells in vitro does require stimulation through TCR (73, 90, 222, 286). *In vivo* adoptive transfer experiments also suggest antigen stimulation is important for their suppressive capacity (94, 118-120). These data are consistent with my observation that unstimulated memory precursor cells did not produce IL-10 or any other cytokines (data not shown).

After obtaining a population of mouse Tr1 cells, I focused on investigating the ability of $Foxp3^+$ Tregs versus Tr1 cells to regulate NLRP3 inflammasome activation. The results indicated that Tr1 cells, but not $Foxp3^+$ Tregs, inhibited ATP-induced inflammasome activation via an IL-10 dependent mechanism. To my knowledge, this is the first report of a differential role for Tr1 cells versus $Foxp3^+$ Tregs in regulating a common inflammatory pathway, as previous studies showed a similar suppressive function of Tr1 cells in different disease settings, such as GVHD (105, 112, 127, 287, 288), intestinal inflammation (94, 101,

254), organ transplantation (104, 119, 289), and in an infectious model with *Aspergillus fumigatus* (222, 290).

Although Foxp3$^+$ Tregs produce amounts of IL-10 that should theoretically be sufficient to inhibit IL-1β production, they did not suppress the inflammasome to the same degree as Tr1 cells. This finding suggests that Foxp3$^+$ Tregs may have mechanisms that counteract the effect of IL-10. One possibility that worth pursuing is based on my observation that the expression of the ectoenzyme CD73, which degrades AMP into adenosine, is consistently higher in Tregs compared to Tr1 cells. Since adenosine potentiates inflammasome activation via activation of hypoxia-inducible-factor 1α (291), this mechanism may block an inhibitory effect of IL-10. Moreover, since CD73 can also be secreted within exosomes into the supernatants of Tregs (292), this mechanism could also account for why the conditioned media of Tregs also failed to suppress IL-1β production. An interesting possibility is that Foxp3$^+$ Tregs and Tr1 cells are designed to suppress different targets and have non-redundant role in mediating cell-specific and signal-specific immune responses.

Despite superior inhibitory effects on inflammasome activation *in vitro*, the effectiveness of CD44^{hi}Foxp3$^-$ Tr1 cells in suppressing inflammasome mediated inflammation was not observed in MSU induced peritonitis model and only a limited effect was observed in the FCAS mice. Since macrophages reside in close proximity with Tr1 cells within a defined volume of media *in vitro*, the amount of IL-10 produced by Tr1 cells can accumulate and potently inhibit the production of IL-1β. On the other hand, in order to suppress IL-1β production from macrophages *in vivo*, Tr1 cells need to migrate in close proximity to macrophages and secrete IL-10. This process requires efficient trafficking and

secretion of high amounts as IL-10 might be rapidly diluted and/or degraded by proteases. Indeed, we did not detect elevated IL-10 levels either in the peritoneal lavage in the peritonitis model or sera in the FCAS mice, despite the clear presence of Tr1 cells, suggesting that most IL-10 remains in the local environment and is degraded rather than systemically distributed.

The inconsistent ability of Tr1 cells to suppress systemic inflammation in FCAS mice *in vivo* may be related to the inflammatory cytokine environment that the cells were exposed to upon transfer. Future *in vitro* experiments need to be conducted to examine if these polyclonal Tr1 cells are more suppressive when exposed to inflammatory cytokines or CD40 after the 4-day TCR stimulation. The other possible way to enhance the suppressive capacity of these cells is to focus on exploring ways to enrich for antigen-specific cells during the stimulation phase: for example, culturing together with allogeneic APCs to expand the alloreactive Tr1 cells, or APCs presenting a known antigen to expand the antigen specific clones. Other possibilities include isolating $CD44^{hi}Foxp3^{-}$ cells from TCR transgenic animals so the cells can be stimulated *in vivo* with the cognate antigen. The phenotype and suppressive capacity of the antigen-reactive Tr1 cells will need to be carefully evaluated first in the *in vitro* assays, and ultimately in mouse models as described in Chapter 5.

In addition to optimizing the delivery of $CD44^{hi}Foxp3^{-}$ Tr1 cells in a timely and antigen-specific manner, another important consideration is to determine the functional stability of these cells under conditions of chronic inflammation as these cells seem to have a remarkable ability to repopulate the CD4 compartment *in vivo*. Although our results indicated a stable phenotype of Tr1 cells based on the production of inflammatory cytokines over 4-5 days, primarily due to the limitation of our choice of model, it is critical to assess their

functional stability in a long term, for example, using *NLRP3*^{A350V} mice, another NLRP3 transgenic model with a slower disease progression, or a T cell transfer colitis model.

In summary, the data in this thesis demonstrate a novel role of ATP in modulating TLR responses in intestinal epithelial cells, and uncover a unique ability of Tr1 cells, but not Foxp3^{+} Tregs to suppress inflammasome activation *in vitro*, largely mediated by IL-10 secretion. These *in vitro* studies triggered further investigations on the application of Tr1 cells as a cellular therapy for autoinflammatory diseases, such as CAPS. I showed, for the first time, that there was a promising protective effect of Tr1 cells in a murine model of CAPS, which may open up new therapeutic opportunities for this disease. More importantly, the data revealed the unique potential of Tr1 cells to dampen inflammatory responses to cellular stress signals, together with their well-characterized suppressive function in adaptive immune responses, Tr1 cells may represent a novel therapy in treating a wide range of autoinflammatory diseases.

References

1. Janeway, C. A., Jr. 1989. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harbor symposia on quantitative biology* 54 Pt 1: 1-13.
2. Lemaitre, B., E. Nicolas, L. Michaut, J. M. Reichhart, and J. A. Hoffmann. 1996. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell* 86: 973-983.
3. Medzhitov, R., P. Preston-Hurlburt, and C. A. Janeway, Jr. 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* 388: 394-397.
4. Janeway, C. A., Jr., and R. Medzhitov. 1998. Introduction: the role of innate immunity in the adaptive immune response. *Seminars in immunology* 10: 349-350.
5. Kopp, E. B., and R. Medzhitov. 1999. The Toll-receptor family and control of innate immunity. *Current opinion in immunology* 11: 13-18.
6. Kawasaki, T., and T. Kawai. 2014. Toll-like receptor signaling pathways. *Frontiers in immunology* 5: 461.
7. Takeda, K., and S. Akira. 2005. Toll-like receptors in innate immunity. *International immunology* 17: 1-14.
8. Takeda, K. 2005. Evolution and integration of innate immune recognition systems: the Toll-like receptors. *Journal of endotoxin research* 11: 51-55.
9. Kamdar, K., V. Nguyen, and R. W. DePaolo. 2013. Toll-like receptor signaling and regulation of intestinal immunity. *Virulence* 4: 207-212.
10. Mogensen, T. H. 2009. Pathogen recognition and inflammatory signaling in innate immune defenses. *Clinical microbiology reviews* 22: 240-273, Table of Contents.

11. Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton, and B. Beutler. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 282: 2085-2088.

12. Albiger, B., S. Dahlberg, A. Sandgren, F. Wartha, K. Beiter, H. Katsuragi, S. Akira, S. Normark, and B. Henriques-Normark. 2007. Toll-like receptor 9 acts at an early stage in host defence against pneumococcal infection. *Cellular microbiology* 9: 633-644.

13. Hawn, T. R., W. R. Berrington, I. A. Smith, S. Uematsu, S. Akira, A. Aderem, K. D. Smith, and S. J. Skerrett. 2007. Altered inflammatory responses in TLR5-deficient mice infected with Legionella pneumophila. *Journal of immunology* 179: 6981-6987.

14. Vijay-Kumar, M., C. J. Sanders, R. T. Taylor, A. Kumar, J. D. Aitken, S. V. Sitaraman, A. S. Neish, S. Uematsu, S. Akira, I. R. Williams, and A. T. Gewirtz. 2007. Deletion of TLR5 results in spontaneous colitis in mice. *The Journal of clinical investigation* 117: 3909-3921.

15. Vijay-Kumar, M., J. D. Aitken, A. Kumar, A. S. Neish, S. Uematsu, S. Akira, and A. T. Gewirtz. 2008. Toll-like receptor 5-deficient mice have dysregulated intestinal gene expression and nonspecific resistance to Salmonella-induced typhoid-like disease. *Infection and immunity* 76: 1276-1281.

16. Carvalho, F. A., O. Koren, J. K. Goodrich, M. E. Johansson, I. Nalbantoglu, J. D. Aitken, Y. Su, B. Chassaing, W. A. Walters, A. Gonzalez, J. C. Clemente, T. C. Cullender, N. Barnich, A. Darfeuille-Michaud, M. Vijay-Kumar, R. Knight, R. E. Ley, and A. T. Gewirtz. 2012. Transient inability to manage proteobacteria promotes chronic gut inflammation in TLR5-deficient mice. *Cell host & microbe* 12: 139-152.

17. Carvalho, F. A., I. Nalbantoglu, S. Ortega-Fernandez, J. D. Aitken, Y. Su, O. Koren, W. A. Walters, R. Knight, R. E. Ley, M. Vijay-Kumar, and A. T. Gewirtz. 2012. Interleukin-1beta (IL-1beta) promotes susceptibility of Toll-like receptor 5 (TLR5) deficient mice to colitis. *Gut* 61: 373-384.

18. Beeson, P. B., and R. Technical Assistance of Elizabeth. 1947. Tolerance to Bacterial Pyrogens : I. Factors Influencing Its Development. *The Journal of experimental medicine* 86: 29-38.

19. de Vos, A. F., J. M. Pater, P. S. van den Pangaart, M. D. de Kruif, C. van 't Veer, and T. van der Poll. 2009. In vivo lipopolysaccharide exposure of human blood leukocytes induces cross-tolerance to multiple TLR ligands. *Journal of immunology* 183: 533-542.

20. Nomura, F., S. Akashi, Y. Sakao, S. Sato, T. Kawai, M. Matsumoto, K. Nakanishi, M. Kimoto, K. Miyake, K. Takeda, and S. Akira. 2000. Cutting edge: endotoxin tolerance in mouse peritoneal macrophages correlates with down-regulation of surface toll-like receptor 4 expression. *Journal of immunology* 164: 3476-3479.

21. Vijay-Kumar, M., H. Wu, R. Jones, G. Grant, B. Babbin, T. P. King, D. Kelly, A. T. Gewirtz, and A. S. Neish. 2006. Flagellin suppresses epithelial apoptosis and limits disease during enteric infection. *The American journal of pathology* 169: 1686-1700.

22. Shaykhiev, R., J. Behr, and R. Bals. 2008. Microbial patterns signaling via Toll-like receptors 2 and 5 contribute to epithelial repair, growth and survival. *PloS one* 3: e1393.

23. Rakoff-Nahoum, S., J. Paglino, F. Eslami-Varzaneh, S. Edberg, and R. Medzhitov. 2004. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell* 118: 229-241.

24. Gong, J., J. Xu, W. Zhu, X. Gao, N. Li, and J. Li. 2010. Epithelial-specific blockade of MyD88-dependent pathway causes spontaneous small intestinal inflammation. *Clinical immunology* 136: 245-256.

25. Vijay-Kumar, M., J. D. Aitken, C. J. Sanders, A. Frias, V. M. Sloane, J. Xu, A. S. Neish, M. Rojas, and A. T. Gewirtz. 2008. Flagellin treatment protects against chemicals, bacteria, viruses, and radiation. *Journal of immunology* 180: 8280-8285.

26. Burdelya, L. G., V. I. Krivokrysenko, T. C. Tallant, E. Strom, A. S. Gleiberman, D. Gupta, O. V. Kurnasov, F. L. Fort, A. L. Osterman, J. A. Didonato, E. Feinstein, and A. V. Gudkov. 2008. An agonist of toll-like receptor 5 has radioprotective activity in mouse and primate models. *Science* 320: 226-230.

27. Oh, J. Z., R. Ravindran, B. Chassaing, F. A. Carvalho, M. S. Maddur, M. Bower, P. Hakimpour, K. P. Gill, H. I. Nakaya, F. Yarovinsky, R. B. Sartor, A. T. Gewirtz, and B. Pulendran. 2014. TLR5-mediated sensing of gut microbiota is necessary for antibody responses to seasonal influenza vaccination. *Immunity* 41: 478-492.

28. Song, L., G. Liu, S. Umlauf, X. Liu, H. Li, H. Tian, L. Reiserova, F. Hou, R. Bell, and L. Tussey. 2014. A rationally designed form of the TLR5 agonist, flagellin, supports superior immunogenicity of Influenza B globular head vaccines. *Vaccine* 32: 4317-4323.

29. Matzinger, P. 1994. Tolerance, danger, and the extended family. *Annual review of immunology* 12: 991-1045.

30. Sauter, B., M. L. Albert, L. Francisco, M. Larsson, S. Somersan, and N. Bhardwaj. 2000. Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. *The Journal of experimental medicine* 191: 423-434.

31. Gallucci, S., M. Lolkema, and P. Matzinger. 1999. Natural adjuvants: endogenous activators of dendritic cells. *Nature medicine* 5: 1249-1255.

32. Shi, Y., J. E. Evans, and K. L. Rock. 2003. Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature* 425: 516-521.

33. Martinon, F., V. Petrilli, A. Mayor, A. Tardivel, and J. Tschopp. 2006. Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* 440: 237-241.

34. Zeiser, R., O. Penack, E. Holler, and M. Idzko. 2011. Danger signals activating innate immunity in graft-versus-host disease. *Journal of molecular medicine (Berlin, Germany)* 89: 833-845.

35. Mariathasan, S., K. Newton, D. M. Monack, D. Vucic, D. M. French, W. P. Lee, M. Roose-Girma, S. Erickson, and V. M. Dixit. 2004. Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature* 430: 213-218.

36. Srivastava, P. 2002. Roles of heat-shock proteins in innate and adaptive immunity. *Nature reviews. Immunology* 2: 185-194.

37. Lotze, M. T., and K. J. Tracey. 2005. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. *Nature reviews. Immunology* 5: 331-342.

38. Werman, A., R. Werman-Venkert, R. White, J. K. Lee, B. Werman, Y. Krelin, E. Voronov, C. A. Dinarello, and R. N. Apte. 2004. The precursor form of IL-1alpha is

an intracrine proinflammatory activator of transcription. *Proceedings of the National Academy of Sciences of the United States of America* 101: 2434-2439.

39. Cayrol, C., and J. P. Girard. 2014. IL-33: an alarmin cytokine with crucial roles in innate immunity, inflammation and allergy. *Current opinion in immunology* 31: 31-37.

40. Brennan, T. V., L. Lin, X. Huang, D. M. Cardona, Z. Li, K. Dredge, N. J. Chao, and Y. Yang. 2012. Heparan sulfate, an endogenous TLR4 agonist, promotes acute GVHD after allogeneic stem cell transplantation. *Blood* 120: 2899-2908.

41. Shirali, A. C., and D. R. Goldstein. 2008. Activation of the innate immune system by the endogenous ligand hyaluronan. *Current opinion in organ transplantation* 13: 20-25.

42. Gallo, P. M., and S. Gallucci. 2013. The dendritic cell response to classic, emerging, and homeostatic danger signals. Implications for autoimmunity. *Frontiers in immunology* 4: 138.

43. Gallucci, S., and P. Matzinger. 2001. Danger signals: SOS to the immune system. *Current opinion in immunology* 13: 114-119.

44. Chen, L., and C. F. Brosnan. 2006. Exacerbation of Experimental Autoimmune Encephalomyelitis in P2X7R-/- Mice: Evidence for Loss of Apoptotic Activity in Lymphocytes. *Journal of immunology* 176: 3115-3126.

45. Friedman, D. J., B. M. KÃ¼nzli, Y. I. A-Rahim, J. Sevigny, P. O. Berberat, K. Enjyoji, E. Csizmadia, H. Friess, and S. C. Robson. 2009. CD39 deletion exacerbates experimental murine colitis and human polymorphisms increase susceptibility to

inflammatory bowel disease. *Proceedings of the National Academy of Sciences* 106: 16788-16793.

46. Lister, M., J. Sharkey, D. Sawatzky, J. Hodgkiss, D. Davidson, A. Rossi, and K. Finlayson. 2007. The role of the purinergic P2X7 receptor in inflammation. *Journal of inflammation* 4: 5.

47. Riteau, N., P. Gasse, L. Fauconnier, A. Gombault, M. Couegnat, L. Fick, J. Kanellopoulos, V. F. Quesniaux, S. Marchand-Adam, B. Crestani, B. Ryffel, and I. Couillin. 2010. Extracellular ATP is a danger signal activating P2X7 receptor in lung inflammation and fibrosis. *American journal of respiratory and critical care medicine* 182: 774-783.

48. Wilhelm, K., J. Ganesan, T. Muller, C. Durr, M. Grimm, A. Beilhack, C. D. Krempl, S. Sorichter, U. V. Gerlach, E. Juttner, A. Zerweck, F. Gartner, P. Pellegatti, F. Di Virgilio, D. Ferrari, N. Kambham, P. Fisch, J. Finke, M. Idzko, and R. Zeiser. 2010. Graft-versus-host disease is enhanced by extracellular ATP activating P2X7R. *Nature medicine* 16: 1434-1438.

49. Robson, S. C., J. Sevigny, and H. Zimmermann. 2006. The E-NTPDase family of ectonucleotidases: Structure function relationships and pathophysiological significance. *Purinergic Signal* 2: 409-430.

50. Di Virgilio, F. 2005. Purinergic mechanism in the immune system: A signal of danger for dendritic cells. *Purinergic signalling* 1: 205-209.

51. Rock, K. L., J. J. Lai, and H. Kono. 2011. Innate and adaptive immune responses to cell death. *Immunological reviews* 243: 191-205.

52. la Sala, A., D. Ferrari, S. Corinti, A. Cavani, F. Di Virgilio, and G. Girolomoni. 2001. Extracellular ATP induces a distorted maturation of dendritic cells and inhibits their capacity to initiate Th1 responses. *Journal of immunology* 166: 1611-1617.

53. Wilkin, F., X. Duhant, C. Bruyns, N. Suarez-Huerta, J. M. Boeynaems, and B. Robaye. 2001. The P2Y11 receptor mediates the ATP-induced maturation of human monocyte-derived dendritic cells. *Journal of immunology* 166: 7172-7177.

54. Schnurr, M., T. Toy, A. Shin, M. Wagner, J. Cebon, and E. Maraskovsky. 2005. Extracellular nucleotide signaling by P2 receptors inhibits IL-12 and enhances IL-23 expression in human dendritic cells: a novel role for the cAMP pathway. *Blood* 105: 1582-1589.

55. Gombault, A., L. Baron, and I. Couillin. 2012. ATP release and purinergic signaling in NLRP3 inflammasome activation. *Frontiers in immunology* 3: 414.

56. Riteau, N., L. Baron, B. Villeret, N. Guillou, F. Savigny, B. Ryffel, F. Rassendren, M. Le Bert, A. Gombault, and I. Couillin. 2012. ATP release and purinergic signaling: a common pathway for particle-mediated inflammasome activation. *Cell death & disease* 3: e403.

57. Matzinger, P. 1998. An innate sense of danger. *Seminars in immunology* 10: 399-415.

58. Hreggvidsdottir, H. S., T. Ostberg, H. Wahamaa, H. Schierbeck, A. C. Aveberger, L. Klevenvall, K. Palmblad, L. Ottosson, U. Andersson, and H. E. Harris. 2009. The alarmin HMGB1 acts in synergy with endogenous and exogenous danger signals to promote inflammation. *Journal of leukocyte biology* 86: 655-662.

59. Ivison, S. M., M. E. Himmel, M. Mayer, Y. Yao, A. Kifayet, M. K. Levings, and T. S. Steiner. 2011. The stress signal extracellular ATP modulates antiflagellin immune responses in intestinal epithelial cells. *Inflammatory bowel diseases* 17: 319-333.

60. Suwara, M. I., N. J. Green, L. A. Borthwick, J. Mann, K. D. Mayer-Barber, L. Barron, P. A. Corris, S. N. Farrow, T. A. Wynn, A. J. Fisher, and D. A. Mann. 2014. IL-1alpha released from damaged epithelial cells is sufficient and essential to trigger inflammatory responses in human lung fibroblasts. *Mucosal immunology* 7: 684-693.

61. Yao, Y., M. K. Levings, and T. S. Steiner. 2012. ATP conditions intestinal epithelial cells to an inflammatory state that promotes components of DC maturation. *European journal of immunology* 42: 3310-3321.

62. Stenfeldt, A. L., and C. Wenneras. 2004. Danger signals derived from stressed and necrotic epithelial cells activate human eosinophils. *Immunology* 112: 605-614.

63. Aswad, F., H. Kawamura, and G. Dennert. 2005. High sensitivity of CD4+CD25+ regulatory T cells to extracellular metabolites nicotinamide adenine dinucleotide and ATP: a role for P2X7 receptors. *Journal of immunology* 175: 3075-3083.

64. Heiss, K., N. Janner, B. Mahnss, V. Schumacher, F. Koch-Nolte, F. Haag, and H. W. Mittrucker. 2008. High sensitivity of intestinal CD8+ T cells to nucleotides indicates P2X7 as a regulator for intestinal T cell responses. *Journal of immunology* 181: 3861-3869.

65. Schenk, U., M. Frascoli, M. Proietti, R. Geffers, E. Traggiai, J. Buer, C. Ricordi, A. M. Westendorf, and F. Grassi. 2011. ATP inhibits the generation and function of regulatory T cells through the activation of purinergic P2X receptors. *Sci Signal* 4: ra12.

66. Cruz, C. M., A. Rinna, H. J. Forman, A. L. Ventura, P. M. Persechini, and D. M. Ojcius. 2007. ATP activates a reactive oxygen species-dependent oxidative stress response and secretion of proinflammatory cytokines in macrophages. *The Journal of biological chemistry* 282: 2871-2879.

67. Matzinger, P. 2002. The danger model: a renewed sense of self. *Science (New York, N.Y.)* 296: 301-305.

68. Pradeu, T., and E. L. Cooper. 2012. The danger theory: 20 years later. *Frontiers in immunology* 3: 287.

69. Maloy, K. J., and F. Powrie. 2011. Intestinal homeostasis and its breakdown in inflammatory bowel disease. *Nature* 474: 298-306.

70. Harrison, O. J., and K. J. Maloy. 2011. Innate immune activation in intestinal homeostasis. *Journal of innate immunity* 3: 585-593.

71. Sakaguchi, S., M. Miyara, C. M. Costantino, and D. A. Hafler. 2010. FOXP3+ regulatory T cells in the human immune system. *Nature reviews. Immunology* 10: 490-500.

72. Josefowicz, S. Z., L. F. Lu, and A. Y. Rudensky. 2012. Regulatory T cells: mechanisms of differentiation and function. *Annual review of immunology* 30: 531-564.

73. Roncarolo, M. G., S. Gregori, R. Bacchetta, and M. Battaglia. 2014. Tr1 cells and the counter-regulation of immunity: natural mechanisms and therapeutic applications. *Current topics in microbiology and immunology* 380: 39-68.

74. Sakaguchi, S., N. Sakaguchi, M. Asano, M. Itoh, and M. Toda. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains

(CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *Journal of immunology* 155: 1151-1164.

75. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299: 1057-1061.

76. Fontenot, J. D., M. A. Gavin, and A. Y. Rudensky. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nature immunology* 4: 330-336.

77. Levine, A. G., A. Arvey, W. Jin, and A. Y. Rudensky. 2014. Continuous requirement for the TCR in regulatory T cell function. *Nature immunology* 15: 1070-1078.

78. Zhu, J., and E. M. Shevach. 2014. TCR signaling fuels T(reg) cell suppressor function. *Nature immunology* 15: 1002-1003.

79. Liston, A., and D. H. Gray. 2014. Homeostatic control of regulatory T cell diversity. *Nature reviews. Immunology* 14: 154-165.

80. Chaudhry, A., and A. Y. Rudensky. 2013. Control of inflammation by integration of environmental cues by regulatory T cells. *The Journal of clinical investigation* 123: 939-944.

81. Wing, K., Y. Onishi, P. Prieto-Martin, T. Yamaguchi, M. Miyara, Z. Fehervari, T. Nomura, and S. Sakaguchi. 2008. CTLA-4 control over Foxp3+ regulatory T cell function. *Science* 322: 271-275.

82. Liang, B., C. Workman, J. Lee, C. Chew, B. M. Dale, L. Colonna, M. Flores, N. Li, E. Schweighoffer, S. Greenberg, V. Tybulewicz, D. Vignali, and R. Clynes. 2008. Regulatory T cells inhibit dendritic cells by lymphocyte activation gene-3 engagement of MHC class II. *Journal of immunology* 180: 5916-5926.

83. Workman, C. J., L. S. Cauley, I. J. Kim, M. A. Blackman, D. L. Woodland, and D. A. Vignali. 2004. Lymphocyte activation gene-3 (CD223) regulates the size of the expanding T cell population following antigen activation in vivo. *Journal of immunology* 172: 5450-5455.

84. Workman, C. J., and D. A. Vignali. 2003. The CD4-related molecule, LAG-3 (CD223), regulates the expansion of activated T cells. *European journal of immunology* 33: 970-979.

85. Workman, C. J., and D. A. Vignali. 2005. Negative regulation of T cell homeostasis by lymphocyte activation gene-3 (CD223). *Journal of immunology* 174: 688-695.

86. Deaglio, S., K. M. Dwyer, W. Gao, D. Friedman, A. Usheva, A. Erat, J. F. Chen, K. Enjyoji, J. Linden, M. Oukka, V. K. Kuchroo, T. B. Strom, and S. C. Robson. 2007. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *The Journal of experimental medicine* 204: 1257-1265.

87. Vignali, D. A., L. W. Collison, and C. J. Workman. 2008. How regulatory T cells work. *Nature reviews. Immunology* 8: 523-532.

88. Collison, L. W., M. R. Pillai, V. Chaturvedi, and D. A. Vignali. 2009. Regulatory T cell suppression is potentiated by target T cells in a cell contact, IL-35- and IL-10-dependent manner. *Journal of immunology* 182: 6121-6128.

89. de Waal Malefyt, R., J. Abrams, B. Bennett, C. G. Figdor, and J. E. de Vries. 1991. Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *The Journal of experimental medicine* 174: 1209-1220.

90. Gregori, S., K. S. Goudy, and M. G. Roncarolo. 2012. The cellular and molecular mechanisms of immuno-suppression by human type 1 regulatory T cells. *Frontiers in immunology* 3: 30.

91. Palomares, O., M. Martin-Fontecha, and R. Lauener. 2014. Regulatory T cells and immune regulation of allergic diseases: roles of IL-10 and TGF-beta. 15: 511-520.

92. Grossman, W. J., J. W. Verbsky, W. Barchet, M. Colonna, J. P. Atkinson, and T. J. Ley. 2004. Human T regulatory cells can use the perforin pathway to cause autologous target cell death. *Immunity* 21: 589-601.

93. Vieira, P. L., J. R. Christensen, S. Minaee, E. J. O'Neill, F. J. Barrat, A. Boonstra, T. Barthlott, B. Stockinger, D. C. Wraith, and A. O'Garra. 2004. IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4+CD25+ regulatory T cells. *Journal of immunology* 172: 5986-5993.

94. Groux, H., A. O'Garra, M. Bigler, M. Rouleau, S. Antonenko, J. E. de Vries, and M. G. Roncarolo. 1997. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389: 737-742.

95. Bacchetta, R., C. Sartirana, M. K. Levings, C. Bordignon, S. Narula, and M. G. Roncarolo. 2002. Growth and expansion of human T regulatory type 1 cells are independent from TCR activation but require exogenous cytokines. *European journal of immunology* 32: 2237-2245.

96. Apetoh, L., F. J. Quintana, C. Pot, N. Joller, S. Xiao, D. Kumar, E. J. Burns, D. H. Sherr, H. L. Weiner, and V. K. Kuchroo. 2010. The aryl hydrocarbon receptor

interacts with c-Maf to promote the differentiation of type 1 regulatory T cells induced by IL-27. *Nature immunology* 11: 854-861.

97. Neumann, C., F. Heinrich, K. Neumann, V. Junghans, M. F. Mashreghi, J. Ahlers, M. Janke, C. Rudolph, N. Mockel-Tenbrinck, A. A. Kuhl, M. M. Heimesaat, C. Esser, S. H. Im, A. Radbruch, S. Rutz, and A. Scheffold. 2014. Role of Blimp-1 in programing Th effector cells into IL-10 producers. *The Journal of experimental medicine*.

98. Bacchetta, R., M. Bigler, J. L. Touraine, R. Parkman, P. A. Tovo, J. Abrams, R. de Waal Malefyt, J. E. de Vries, and M. G. Roncarolo. 1994. High levels of interleukin 10 production in vivo are associated with tolerance in SCID patients transplanted with HLA mismatched hematopoietic stem cells. *The Journal of experimental medicine* 179: 493-502.

99. Barrat, F. J., D. J. Cua, A. Boonstra, D. F. Richards, C. Crain, H. F. Savelkoul, R. de Waal-Malefyt, R. L. Coffman, C. M. Hawrylowicz, and A. O'Garra. 2002. In vitro generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. *The Journal of experimental medicine* 195: 603-616.

100. Brun, V., H. Bastian, V. Neveu, and A. Foussat. 2009. Clinical grade production of IL-10 producing regulatory Tr1 lymphocytes for cell therapy of chronic inflammatory diseases. *International immunopharmacology* 9: 609-613.

101. Mottet, C., H. H. Uhlig, and F. Powrie. 2003. Cutting edge: cure of colitis by CD4+CD25+ regulatory T cells. *Journal of immunology* 170: 3939-3943.

102. Powrie, F., M. W. Leach, S. Mauze, S. Menon, L. B. Caddle, and R. L. Coffman. 1994. Inhibition of Th1 responses prevents inflammatory bowel disease in scid mice reconstituted with CD45RBhi CD4+ T cells. *Immunity* 1: 553-562.

103. Miyara, M., Y. Ito, and S. Sakaguchi. 2014. TREG-cell therapies for autoimmune rheumatic diseases. *Nature reviews. Rheumatology* 10: 543-551.

104. Jaeckel, E., N. Mpofu, N. Saal, and M. P. Manns. 2008. Role of regulatory T cells for the treatment of type 1 diabetes mellitus. *Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme* 40: 126-136.

105. Taylor, P. A., C. J. Lees, and B. R. Blazar. 2002. The infusion of ex vivo activated and expanded CD4(+)CD25(+) immune regulatory cells inhibits graft-versus-host disease lethality. *Blood* 99: 3493-3499.

106. Hoffmann, P., J. Ermann, M. Edinger, C. G. Fathman, and S. Strober. 2002. Donor-type CD4(+)CD25(+) regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation. *The Journal of experimental medicine* 196: 389-399.

107. Edinger, M., P. Hoffmann, J. Ermann, K. Drago, C. G. Fathman, S. Strober, and R. S. Negrin. 2003. CD4+CD25+ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. *Nature medicine* 9: 1144-1150.

108. Hall, B. M., N. W. Pearce, K. E. Gurley, and S. E. Dorsch. 1990. Specific unresponsiveness in rats with prolonged cardiac allograft survival after treatment with cyclosporine. III. Further characterization of the CD4+ suppressor cell and its mechanisms of action. *The Journal of experimental medicine* 171: 141-157.

109. Golshayan, D., S. Jiang, J. Tsang, M. I. Garin, C. Mottet, and R. I. Lechler. 2007. In vitro-expanded donor alloantigen-specific CD4+CD25+ regulatory T cells promote experimental transplantation tolerance. *Blood* 109: 827-835.

110. McMurchy, A. N., A. Bushell, M. K. Levings, and K. J. Wood. 2011. Moving to tolerance: clinical application of T regulatory cells. *Seminars in immunology* 23: 304-313.

111. Singer, B. D., L. S. King, and F. R. D'Alessio. 2014. Regulatory T cells as immunotherapy. *Frontiers in immunology* 5: 46.

112. Trzonkowski, P., A. Dukat-Mazurek, M. Bieniaszewska, N. Marek-Trzonkowska, A. Dobyszuk, J. Juscinska, M. Dutka, J. Mysliwska, and A. Hellmann. 2013. Treatment of graft-versus-host disease with naturally occurring T regulatory cells. *BioDrugs : clinical immunotherapeutics, biopharmaceuticals and gene therapy* 27: 605-614.

113. Trzonkowski, P., M. Bieniaszewska, J. Juscinska, A. Dobyszuk, A. Krzystyniak, N. Marek, J. Mysliwska, and A. Hellmann. 2009. First-in-man clinical results of the treatment of patients with graft versus host disease with human ex vivo expanded CD4+CD25+CD127- T regulatory cells. *Clinical immunology* 133: 22-26.

114. Brunstein, C. G., J. S. Miller, Q. Cao, D. H. McKenna, K. L. Hippen, J. Curtsinger, T. Defor, B. L. Levine, C. H. June, P. Rubinstein, P. B. McGlave, B. R. Blazar, and J. E. Wagner. 2011. Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. *Blood* 117: 1061-1070.

115. Theil, A., S. Tuve, U. Oelschlagel, A. Maiwald, D. Dohler, D. Ossmann, A. Zenkel, C. Wilhelm, J. M. Middeke, N. Shayegi, K. Trautmann-Grill, M. von Bonin, U.

Platzbecker, G. Ehninger, E. Bonifacio, and M. Bornhauser. 2015. Adoptive transfer of allogeneic regulatory T cells into patients with chronic graft-versus-host disease. *Cytotherapy*.

116. Marek-Trzonkowska, N., M. Mysliwiec, A. Dobyszuk, M. Grabowska, I. Derkowska, J. Juscinska, R. Owczuk, A. Szadkowska, P. Witkowski, W. Mlynarski, P. Jarosz-Chobot, A. Bossowski, J. Siebert, and P. Trzonkowski. 2014. Therapy of type 1 diabetes with CD4(+)CD25(high)CD127-regulatory T cells prolongs survival of pancreatic islets - results of one year follow-up. *Clinical immunology* 153: 23-30.

117. Zeller, J. C., A. Panoskaltsis-Mortari, W. J. Murphy, F. W. Ruscetti, S. Narula, M. G. Roncarolo, and B. R. Blazar. 1999. Induction of CD4+ T cell alloantigen-specific hyporesponsiveness by IL-10 and TGF-beta. *Journal of immunology* 163: 3684-3691.

118. Asnagli, H., D. Martire, N. Belmonte, J. Quentin, H. Bastian, M. Boucard-Jourdin, P. B. Fall, A. L. Mausset-Bonnefont, A. Mantello-Moreau, S. Rouquier, I. Marchetti, C. Jorgensen, A. Foussat, and P. Louis-Plence. 2014. Type 1 regulatory T cells specific for collagen type II as an efficient cell-based therapy in arthritis. *Arthritis research & therapy* 16: R115.

119. Gagliani, N., T. Jofra, A. Stabilini, A. Valle, M. Atkinson, M. G. Roncarolo, and M. Battaglia. 2010. Antigen-specific dependence of Tr1-cell therapy in preclinical models of islet transplant. *Diabetes* 59: 433-439.

120. Guandalini, S., and R. Gokhale. 2002. Update on immunologic basis of celiac disease. *Current opinion in gastroenterology* 18: 95-100.

121. Salvati, V. M., G. Mazzarella, C. Gianfrani, M. K. Levings, R. Stefanile, B. De Giulio, G. Iaquinto, N. Giardullo, S. Auricchio, M. G. Roncarolo, and R. Troncone.

2005. Recombinant human interleukin 10 suppresses gliadin dependent T cell activation in ex vivo cultured coeliac intestinal mucosa. *Gut* 54: 46-53.

122. Gianfrani, C., M. K. Levings, C. Sartirana, G. Mazzarella, G. Barba, D. Zanzi, A. Camarca, G. Iaquinto, N. Giardullo, S. Auricchio, R. Troncone, and M. G. Roncarolo. 2006. Gliadin-specific type 1 regulatory T cells from the intestinal mucosa of treated celiac patients inhibit pathogenic T cells. *Journal of immunology* 177: 4178-4186.

123. Meiron, M., Y. Zohar, R. Anunu, G. Wildbaum, and N. Karin. 2008. CXCL12 (SDF-1alpha) suppresses ongoing experimental autoimmune encephalomyelitis by selecting antigen-specific regulatory T cells. *The Journal of experimental medicine* 205: 2643-2655.

124. Zohar, Y., G. Wildbaum, R. Novak, A. L. Salzman, M. Thelen, R. Alon, Y. Barsheshet, C. L. Karp, and N. Karin. 2014. CXCL11-dependent induction of FOXP3-negative regulatory T cells suppresses autoimmune encephalomyelitis. *The Journal of clinical investigation* 124: 2009-2022.

125. Bollyky, P. L., R. P. Wu, B. A. Falk, J. D. Lord, S. A. Long, A. Preisinger, B. Teng, G. E. Holt, N. E. Standifer, K. R. Braun, C. F. Xie, P. L. Samuels, R. B. Vernon, J. A. Gebe, T. N. Wight, and G. T. Nepom. 2011. ECM components guide IL-10 producing regulatory T-cell (TR1) induction from effector memory T-cell precursors. *Proceedings of the National Academy of Sciences of the United States of America* 108: 7938-7943.

126. Desreumaux, P., A. Foussat, M. Allez, L. Beaugerie, X. Hebuterne, Y. Bouhnik, M. Nachury, V. Brun, H. Bastian, N. Belmonte, M. Ticchioni, A. Duchange, P. Morel-Mandrino, V. Neveu, N. Clerget-Chossat, M. Forte, and J. F. Colombel. 2012. Safety

and efficacy of antigen-specific regulatory T-cell therapy for patients with refractory Crohn's disease. *Gastroenterology* 143: 1207-1217.e1201-1202.

127. Bacchetta, R., B. Lucarelli, C. Sartirana, S. Gregori, M. T. Lupo Stanghellini, P. Miqueu, S. Tomiuk, M. Hernandez-Fuentes, M. E. Gianolini, R. Greco, M. Bernardi, E. Zappone, S. Rossini, U. Janssen, A. Ambrosi, M. Salomoni, J. Peccatori, F. Ciceri, and M. G. Roncarolo. 2014. Immunological Outcome in Haploidentical-HSC Transplanted Patients Treated with IL-10-Anergized Donor T Cells. *Frontiers in immunology* 5: 16.

128. McDermott, M. F., I. Aksentijevich, J. Galon, E. M. McDermott, B. W. Ogunkolade, M. Centola, E. Mansfield, M. Gadina, L. Karenko, T. Pettersson, J. McCarthy, D. M. Frucht, M. Aringer, Y. Torosyan, A. M. Teppo, M. Wilson, H. M. Karaarslan, Y. Wan, I. Todd, G. Wood, R. Schlimgen, T. R. Kumarajeewa, S. M. Cooper, J. P. Vella, C. I. Amos, J. Mulley, K. A. Quane, M. G. Molloy, A. Ranki, R. J. Powell, G. A. Hitman, J. J. O'Shea, and D. L. Kastner. 1999. Germline mutations in the extracellular domains of the 55 kDa TNF receptor, TNFR1, define a family of dominantly inherited autoinflammatory syndromes. *Cell* 97: 133-144.

129. Doria, A., M. Zen, S. Bettio, M. Gatto, N. Bassi, L. Nalotto, A. Ghirardello, L. Iaccarino, and L. Punzi. 2012. Autoinflammation and autoimmunity: bridging the divide. *Autoimmunity reviews* 12: 22-30.

130. McGonagle, D., and M. F. McDermott. 2006. A proposed classification of the immunological diseases. *PLoS medicine* 3: e297.

131. Savic, S., L. J. Dickie, M. Battellino, and M. F. McDermott. 2012. Familial Mediterranean fever and related periodic fever syndromes/autoinflammatory diseases. *Current opinion in rheumatology* 24: 103-112.

132. Yuk, J. M., and E. K. Jo. 2013. Crosstalk between autophagy and inflammasomes. *Molecules and cells* 36: 393-399.

133. Broderick, L., D. De Nardo, B. S. Franklin, H. M. Hoffman, and E. Latz. 2015. The inflammasomes and autoinflammatory syndromes. *Annual review of pathology* 10: 395-424.

134. Hoffman, H. M., S. Rosengren, D. L. Boyle, J. Y. Cho, J. Nayar, J. L. Mueller, J. P. Anderson, A. A. Wanderer, and G. S. Firestein. 2004. Prevention of cold-associated acute inflammation in familial cold autoinflammatory syndrome by interleukin-1 receptor antagonist. *Lancet* 364: 1779-1785.

135. Franklin, B. S., L. Bossaller, D. De Nardo, J. M. Ratter, A. Stutz, G. Engels, C. Brenker, M. Nordhoff, S. R. Mirandola, A. Al-Amoudi, M. S. Mangan, S. Zimmer, B. G. Monks, M. Fricke, R. E. Schmidt, T. Espevik, B. Jones, A. G. Jarnicki, P. M. Hansbro, P. Busto, A. Marshak-Rothstein, S. Hornemann, A. Aguzzi, W. Kastenmuller, and E. Latz. 2014. The adaptor ASC has extracellular and 'prionoid' activities that propagate inflammation. *Nature immunology* 15: 727-737.

136. Baroja-Mazo, A., F. Martin-Sanchez, A. I. Gomez, C. M. Martinez, J. Amores-Iniesta, V. Compan, M. Barbera-Cremades, J. Yague, E. Ruiz-Ortiz, J. Anton, S. Bujan, I. Couillin, D. Brough, J. I. Arostegui, and P. Pelegrin. 2014. The NLRP3 inflammasome is released as a particulate danger signal that amplifies the inflammatory response. *Nature immunology* 15: 738-748.

137. Jesus, A. A., and R. Goldbach-Mansky. 2014. IL-1 blockade in autoinflammatory syndromes. *Annual review of medicine* 65: 223-244.

138. Mitroulis, I., P. Skendros, and K. Ritis. 2010. Targeting IL-1beta in disease; the expanding role of NLRP3 inflammasome. *European journal of internal medicine* 21: 157-163.

139. Dinarello, C. A., A. Simon, and J. W. van der Meer. 2012. Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases. *Nature reviews. Drug discovery* 11: 633-652.

140. Vitale, A., D. Rigante, O. M. Lucherini, F. Caso, I. Muscari, F. Magnotti, M. G. Brizi, S. Guerrini, M. Patti, L. Punzi, M. Galeazzi, and L. Cantarini. 2013. Biological treatments: new weapons in the management of monogenic autoinflammatory disorders. *Mediators Inflamm* 2013: 939847.

141. Bouma, G., and W. Strober. 2003. The immunological and genetic basis of inflammatory bowel disease. *Nature reviews. Immunology* 3: 521-533.

142. Cho, J. H., and C. T. Weaver. 2007. The Genetics of Inflammatory Bowel Disease. *Gastroenterology* 133: 1327-1339.

143. Khor, B., A. Gardet, and R. J. Xavier. 2011. Genetics and pathogenesis of inflammatory bowel disease. *Nature* 474: 307-317.

144. Bollrath, J., and F. M. Powrie. 2013. Controlling the frontier: regulatory T-cells and intestinal homeostasis. *Seminars in immunology* 25: 352-357.

145. Shale, M., C. Schiering, and F. Powrie. 2013. CD4(+) T-cell subsets in intestinal inflammation. *Immunological reviews* 252: 164-182.

146. Opipari, A., and L. Franchi. 2015. Role of inflammasomes in intestinal inflammation and Crohn's disease. *Inflamm Bowel Dis* 21: 173-181.

147. Schoultz, I., D. Verma, J. Halfvarsson, L. Torkvist, M. Fredrikson, U. Sjoqvist, M. Lordal, C. Tysk, M. Lerm, P. Soderkvist, and J. D. Soderholm. 2009. Combined polymorphisms in genes encoding the inflammasome components NALP3 and CARD8 confer susceptibility to Crohn's disease in Swedish men. *Am J Gastroenterol* 104: 1180-1188.

148. Roberts, R. L., R. K. Topless, A. J. Phipps-Green, R. B. Gearry, M. L. Barclay, and T. R. Merriman. 2010. Evidence of interaction of CARD8 rs2043211 with NALP3 rs35829419 in Crohn's disease. *Genes and immunity* 11: 351-356.

149. Villani, A. C., M. Lemire, G. Fortin, E. Louis, M. S. Silverberg, C. Collette, N. Baba, C. Libioulle, J. Belaiche, A. Bitton, D. Gaudet, A. Cohen, D. Langelier, P. R. Fortin, J. E. Wither, M. Sarfati, P. Rutgeerts, J. D. Rioux, S. Vermeire, T. J. Hudson, and D. Franchimont. 2009. Common variants in the NLRP3 region contribute to Crohn's disease susceptibility. *Nature genetics* 41: 71-76.

150. Zhang, H. X., Z. T. Wang, X. X. Lu, Y. G. Wang, J. Zhong, and J. Liu. 2014. NLRP3 gene is associated with ulcerative colitis (UC), but not Crohn's disease (CD), in Chinese Han population. *Inflammation research : official journal of the European Histamine Research Society ... [et al.]* 63: 979-985.

151. Lewis, G. J., D. C. Massey, H. Zhang, F. Bredin, M. Tremelling, J. C. Lee, C. Berzuini, and M. Parkes. 2011. Genetic association between NLRP3 variants and Crohn's disease does not replicate in a large UK panel. *Inflamm Bowel Dis* 17: 1387-1391.

152. Bauer, C., P. Duewell, C. Mayer, H. A. Lehr, K. A. Fitzgerald, M. Dauer, J. Tschopp, S. Endres, E. Latz, and M. Schnurr. 2010. Colitis induced in mice with dextran sulfate sodium (DSS) is mediated by the NLRP3 inflammasome. *Gut* 59: 1192-1199.

153. Bauer, C., P. Duewell, H. A. Lehr, S. Endres, and M. Schnurr. 2012. Protective and aggravating effects of Nlrp3 inflammasome activation in IBD models: influence of genetic and environmental factors. *Digestive diseases (Basel, Switzerland)* 30 Suppl 1: 82-90.

154. Hirota, S. A., J. Ng, A. Lueng, M. Khajah, K. Parhar, Y. Li, V. Lam, M. S. Potentier, K. Ng, M. Bawa, D. M. McCafferty, K. P. Rioux, S. Ghosh, R. J. Xavier, S. P. Colgan, J. Tschopp, D. Muruve, J. A. MacDonald, and P. L. Beck. 2011. NLRP3 inflammasome plays a key role in the regulation of intestinal homeostasis. *Inflamm Bowel Dis* 17: 1359-1372.

155. Zaki, M. H., K. L. Boyd, P. Vogel, M. B. Kastan, M. Lamkanfi, and T. D. Kanneganti. 2010. The NLRP3 inflammasome protects against loss of epithelial integrity and mortality during experimental colitis. *Immunity* 32: 379-391.

156. Dupaul-Chicoine, J., G. Yeretssian, K. Doiron, K. S. Bergstrom, C. R. McIntire, P. M. LeBlanc, C. Meunier, C. Turbide, P. Gros, N. Beauchemin, B. A. Vallance, and M. Saleh. 2010. Control of intestinal homeostasis, colitis, and colitis-associated colorectal cancer by the inflammatory caspases. *Immunity* 32: 367-378.

157. Allen, I. C., E. M. TeKippe, R. M. Woodford, J. M. Uronis, E. K. Holl, A. B. Rogers, H. H. Herfarth, C. Jobin, and J. P. Ting. 2010. The NLRP3 inflammasome functions as a negative regulator of tumorigenesis during colitis-associated cancer. *The Journal of experimental medicine* 207: 1045-1056.

158. Dinarello, C. A. 2009. Immunological and inflammatory functions of the interleukin-1 family. *Annual review of immunology* 27: 519-550.

159. Sutton, C., C. Brereton, B. Keogh, K. H. Mills, and E. C. Lavelle. 2006. A crucial role for interleukin (IL)-1 in the induction of IL-17-producing T cells that mediate autoimmune encephalomyelitis. *The Journal of experimental medicine* 203: 1685-1691.

160. Chung, Y., S. H. Chang, G. J. Martinez, X. O. Yang, R. Nurieva, H. S. Kang, L. Ma, S. S. Watowich, A. M. Jetten, Q. Tian, and C. Dong. 2009. Critical regulation of early Th17 cell differentiation by interleukin-1 signaling. *Immunity* 30: 576-587.

161. Acosta-Rodriguez, E. V., G. Napolitani, A. Lanzavecchia, and F. Sallusto. 2007. Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. *Nature immunology* 8: 942-949.

162. Lee, Y., A. Awasthi, N. Yosef, F. J. Quintana, S. Xiao, A. Peters, C. Wu, M. Kleinewietfeld, S. Kunder, D. A. Hafler, R. A. Sobel, A. Regev, and V. K. Kuchroo. 2012. Induction and molecular signature of pathogenic TH17 cells. *Nature immunology* 13: 991-999.

163. Reinecker, H. C., M. Steffen, T. Witthoeft, I. Pflueger, S. Schreiber, R. P. MacDermott, and A. Raedler. 1993. Enhanced secretion of tumour necrosis factor-alpha, IL-6, and IL-1 beta by isolated lamina propria mononuclear cells from patients with ulcerative colitis and Crohn's disease. *Clinical and experimental immunology* 94: 174-181.

164. McAlindon, M. E., C. J. Hawkey, and Y. R. Mahida. 1998. Expression of interleukin 1 beta and interleukin 1 beta converting enzyme by intestinal macrophages in health and inflammatory bowel disease. *Gut* 42: 214-219.

165. Ludwiczek, O., E. Vannier, I. Borggraefe, A. Kaser, B. Siegmund, C. A. Dinarello, and H. Tilg. 2004. Imbalance between interleukin-1 agonists and antagonists: relationship to severity of inflammatory bowel disease. *Clinical and experimental immunology* 138: 323-329.

166. McCall, R. D., S. Haskill, E. M. Zimmermann, P. K. Lund, R. C. Thompson, and R. B. Sartor. 1994. Tissue interleukin 1 and interleukin-1 receptor antagonist expression in enterocolitis in resistant and susceptible rats. *Gastroenterology* 106: 960-972.

167. Okayasu, I., S. Hatakeyama, M. Yamada, T. Ohkusa, Y. Inagaki, and R. Nakaya. 1990. A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. *Gastroenterology* 98: 694-702.

168. Cominelli, F., C. C. Nast, B. D. Clark, R. Schindler, R. Lierena, V. E. Eysselein, R. C. Thompson, and C. A. Dinarello. 1990. Interleukin 1 (IL-1) gene expression, synthesis, and effect of specific IL-1 receptor blockade in rabbit immune complex colitis. *The Journal of clinical investigation* 86: 972-980.

169. Coccia, M., O. J. Harrison, C. Schiering, M. J. Asquith, B. Becher, F. Powrie, and K. J. Maloy. 2012. IL-1beta mediates chronic intestinal inflammation by promoting the accumulation of IL-17A secreting innate lymphoid cells and CD4(+) Th17 cells. *The Journal of experimental medicine* 209: 1595-1609.

170. Zhang, J., S. Fu, S. Sun, Z. Li, and B. Guo. 2014. Inflammasome activation has an important role in the development of spontaneous colitis. *Mucosal immunology* 7: 1139-1150.

171. Atarashi, K., J. Nishimura, T. Shima, Y. Umesaki, M. Yamamoto, M. Onoue, H. Yagita, N. Ishii, R. Evans, K. Honda, and K. Takeda. 2008. ATP drives lamina propria T(H)17 cell differentiation. *Nature* 455: 808-812.

172. Crane, J. K., and K. M. Mongiardo. 2014. Pro-inflammatory effects of uric acid in the gastrointestinal tract. *Immunological investigations* 43: 255-266.

173. Saitoh, T., N. Fujita, M. H. Jang, S. Uematsu, B. G. Yang, T. Satoh, H. Omori, T. Noda, N. Yamamoto, M. Komatsu, K. Tanaka, T. Kawai, T. Tsujimura, O. Takeuchi, T. Yoshimori, and S. Akira. 2008. Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production. *Nature* 456: 264-268.

174. Maeda, S., L. C. Hsu, H. Liu, L. A. Bankston, M. Iimura, M. F. Kagnoff, L. Eckmann, and M. Karin. 2005. Nod2 mutation in Crohn's disease potentiates NF-kappaB activity and IL-1beta processing. *Science* 307: 734-738.

175. Kanai, T., M. Watanabe, A. Okazawa, T. Sato, M. Yamazaki, S. Okamoto, H. Ishii, T. Totsuka, R. Iiyama, R. Okamoto, M. Ikeda, M. Kurimoto, K. Takeda, S. Akira, and T. Hibi. 2001. Macrophage-derived IL-18-mediated intestinal inflammation in the murine model of Crohn's disease. *Gastroenterology* 121: 875-888.

176. Matsunaga, H., R. Hokari, T. Ueda, C. Kurihara, H. Hozumi, M. Higashiyama, Y. Okada, C. Watanabe, S. Komoto, M. Nakamura, A. Kawaguchi, S. Nagao, A. Sekiyama, and S. Miura. 2011. Physiological stress exacerbates murine colitis by

enhancing proinflammatory cytokine expression that is dependent on IL-18. *American journal of physiology. Gastrointestinal and liver physiology* 301: G555-564.

177. Sivakumar, P. V., G. M. Westrich, S. Kanaly, K. Garka, T. L. Born, J. M. Derry, and J. L. Viney. 2002. Interleukin 18 is a primary mediator of the inflammation associated with dextran sulphate sodium induced colitis: blocking interleukin 18 attenuates intestinal damage. *Gut* 50: 812-820.

178. Hardenberg, G., Y. Yao, C. A. Piccirillo, M. K. Levings, and T. S. Steiner. 2012. Toll-like receptor 5 deficiency protects from wasting disease in a T cell transfer colitis model in T cell receptor-beta-deficient mice. *Inflammatory bowel diseases* 18: 85-93.

179. McGeough, M. D., C. A. Pena, J. L. Mueller, D. A. Pociask, L. Broderick, H. M. Hoffman, and S. D. Brydges. 2012. Cutting edge: IL-6 is a marker of inflammation with no direct role in inflammasome-mediated mouse models. *Journal of immunology* 189: 2707-2711.

180. Lutz, M. B., N. Kukutsch, A. L. Ogilvie, S. Rossner, F. Koch, N. Romani, and G. Schuler. 1999. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J Immunol Methods* 223: 77-92.

181. Ivison, S. M., N. R. Graham, C. Q. Bernales, A. Kifayet, N. Ng, L. A. Shobab, and T. S. Steiner. 2007. Protein kinase D interaction with TLR5 is required for inflammatory signaling in response to bacterial flagellin. *Journal of immunology* 178: 5735-5743.

182. Donnelly, M. A., and T. S. Steiner. 2002. Two nonadjacent regions in enteroaggregative Escherichia coli flagellin are required for activation of toll-like receptor 5. *The Journal of biological chemistry* 277: 40456-40461.

183. Artis, D. 2008. Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. *Nature reviews. Immunology* 8: 411-420.

184. Zeuthen, L. H., L. N. Fink, and H. Frokiaer. 2008. Epithelial cells prime the immune response to an array of gut-derived commensals towards a tolerogenic phenotype through distinct actions of thymic stromal lymphopoietin and transforming growth factor-beta. *Immunology* 123: 197-208.

185. Iliev, I. D., E. Mileti, G. Matteoli, M. Chieppa, and M. Rescigno. 2009. Intestinal epithelial cells promote colitis-protective regulatory T-cell differentiation through dendritic cell conditioning. *Mucosal immunology* 2: 340-350.

186. Medzhitov, R. 2007. Recognition of microorganisms and activation of the immune response. *Nature* 449: 819-826.

187. Kono, H., and K. L. Rock. 2008. How dying cells alert the immune system to danger. *Nature reviews.Immunology* 8: 279-289.

188. Ivanova, E. P., Y. V. Alexeeva, D. K. Pham, J. P. Wright, and D. V. Nicolau. 2006. ATP level variations in heterotrophic bacteria during attachment on hydrophilic and hydrophobic surfaces. *International microbiology : the official journal of the Spanish Society for Microbiology* 9: 37-46.

189. Iwase, T., H. Shinji, A. Tajima, F. Sato, T. Tamura, T. Iwamoto, M. Yoneda, and Y. Mizunoe. 2010. Isolation and identification of ATP-secreting bacteria from mice and humans. *Journal of clinical microbiology* 48: 1949-1951.

190. Yin, J., K. Xu, J. Zhang, A. Kumar, and F. S. Yu. 2007. Wound-induced ATP release and EGF receptor activation in epithelial cells. *Journal of cell science* 120: 815-825.

191. Campeau, J. L., S. Y. Salim, E. J. Albert, N. Hotte, and K. L. Madsen. 2012. Intestinal epithelial cells modulate antigen-presenting cell responses to bacterial DNA. *Infection and immunity* 80: 2632-2644.

192. DePaolo, R. W., V. Abadie, F. Tang, H. Fehlner-Peach, J. A. Hall, W. Wang, E. V. Marietta, D. D. Kasarda, T. A. Waldmann, J. A. Murray, C. Semrad, S. S. Kupfer, Y. Belkaid, S. Guandalini, and B. Jabri. 2011. Co-adjuvant effects of retinoic acid and IL-15 induce inflammatory immunity to dietary antigens. *Nature* 471: 220-224.

193. Langrish, C. L., Y. Chen, W. M. Blumenschein, J. Mattson, B. Basham, J. D. Sedgwick, T. McClanahan, R. A. Kastelein, and D. J. Cua. 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *The Journal of experimental medicine* 201: 233-240.

194. Bours, M. J., P. C. Dagnelie, A. L. Giuliani, A. Wesselius, and F. Di Virgilio. 2011. P2 receptors and extracellular ATP: a novel homeostatic pathway in inflammation. *Front Biosci (Schol Ed)* 3: 1443-1456.

195. Theatre, E., V. Bours, and C. Oury. 2009. A P2X ion channel-triggered NF-kappaB pathway enhances TNF-alpha-induced IL-8 expression in airway epithelial cells. *Am J Respir Cell Mol Biol* 41: 705-713.

196. Ferrari, D., C. Pizzirani, E. Adinolfi, R. M. Lemoli, A. Curti, M. Idzko, E. Panther, and F. Di Virgilio. 2006. The P2X7 Receptor: A Key Player in IL-1 Processing and Release. *Journal of immunology* 176: 3877-3883.

197. Cesaro, A., P. Brest, V. Hofman, X. Hebuterne, S. Wildman, B. Ferrua, S. Marchetti, A. Doglio, V. Vouret-Craviari, F. Galland, P. Naquet, B. Mograbi, R. Unwin, and P. Hofman. 2010. Amplification loop of the inflammatory process is induced by P2X7R

activation in intestinal epithelial cells in response to neutrophil transepithelial migration. *American journal of physiology. Gastrointestinal and liver physiology* 299: G32-42.

198. Feng, T., Y. Cong, H. Qin, E. N. Benveniste, and C. O. Elson. 2010. Generation of mucosal dendritic cells from bone marrow reveals a critical role of retinoic acid. *Journal of immunology* 185: 5915-5925.

199. Bles, N., M. Horckmans, A. Lefort, F. Libert, P. Macours, H. El Housni, F. Marteau, J. M. Boeynaems, and D. Communi. 2007. Gene expression profiling defines ATP as a key regulator of human dendritic cell functions. *Journal of immunology* 179: 3550-3558.

200. Yip, L., T. Woehrle, R. Corriden, M. Hirsh, Y. Chen, Y. Inoue, V. Ferrari, P. A. Insel, and W. G. Junger. 2009. Autocrine regulation of T-cell activation by ATP release and P2X7 receptors. *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 23: 1685-1693.

201. Muller, T., R. P. Vieira, M. Grimm, T. Durk, S. Cicko, R. Zeiser, T. Jakob, S. F. Martin, B. Blumenthal, S. Sorichter, D. Ferrari, F. Di Virgillio, and M. Idzko. 2011. A potential role for P2X7R in allergic airway inflammation in mice and humans. *Am J Respir Cell Mol Biol* 44: 456-464.

202. Liu, Y., Y. Xiao, and Z. Li. 2011. P2X7 receptor positively regulates MyD88-dependent NF-kappaB activation. *Cytokine* 55: 229-236.

203. Labeur, M. S., B. Roters, B. Pers, A. Mehling, T. A. Luger, T. Schwarz, and S. Grabbe. 1999. Generation of tumor immunity by bone marrow-derived dendritic cells correlates with dendritic cell maturation stage. *Journal of immunology* 162: 168-175.

204. Kirman, I., and O. H. Nielsen. 1996. Increased numbers of interleukin-15-expressing cells in active ulcerative colitis. *The American Journal of Gastroenterology* 91: 1789-1794.

205. Liu, Z., K. Geboes, S. Colpaert, G. R. D'Haens, P. Rutgeerts, and J. L. Ceuppens. 2000. IL-15 is highly expressed in inflammatory bowel disease and regulates local T cell-dependent cytokine production. *Journal of immunology (Baltimore, Md.: 1950)* 164: 3608-3615.

206. Vainer, B., O. H. Nielsen, J. Hendel, T. Horn, and I. Kirman. 2000. Colonic expression and synthesis of interleukin 13 and interleukin 15 in inflammatory bowel disease. *Cytokine* 12: 1531-1536.

207. Bas, A., M. Swamy, L. Abeler-Dorner, G. Williams, D. J. Pang, S. D. Barbee, and A. C. Hayday. 2011. Butyrophilin-like 1 encodes an enterocyte protein that selectively regulates functional interactions with T lymphocytes. *Proceedings of the National Academy of Sciences of the United States of America* 108: 4376-4381.

208. Dubois, S. P., T. A. Waldmann, and J. R. Muller. 2005. Survival adjustment of mature dendritic cells by IL-15. *Proceedings of the National Academy of Sciences of the United States of America* 102: 8662-8667.

209. Kuwajima, S., T. Sato, K. Ishida, H. Tada, H. Tezuka, and T. Ohteki. 2006. Interleukin 15-dependent crosstalk between conventional and plasmacytoid dendritic cells is essential for CpG-induced immune activation. *Nature immunology* 7: 740-746.

210. Lodes, M. J., Y. Cong, C. O. Elson, R. Mohamath, C. J. Landers, S. R. Targan, M. Fort, and R. M. Hershberg. 2004. Bacterial flagellin is a dominant antigen in Crohn disease. *The Journal of clinical investigation* 113: 1296-1306.

211. Lunardi, C., C. Bason, M. Dolcino, R. Navone, R. Simone, D. Saverino, L. Frulloni, E. Tinazzi, D. Peterlana, R. Corrocher, and A. Puccetti. 2009. Antiflagellin antibodies recognize the autoantigens Toll-Like Receptor 5 and Pals 1-associated tight junction protein and induce monocytes activation and increased intestinal permeability in Crohn's disease. *Journal of internal medicine* 265: 250-265.

212. Vijay-Kumar, M., and A. T. Gewirtz. 2009. Flagellin: key target of mucosal innate immunity. *Mucosal immunology* 2: 197-205.

213. Sitaraman, S. V., J. M. Klapproth, D. A. Moore, 3rd, C. Landers, S. Targan, I. R. Williams, and A. T. Gewirtz. 2005. Elevated flagellin-specific immunoglobulins in Crohn's disease. *American journal of physiology. Gastrointestinal and liver physiology* 288: G403-406.

214. Targan, S. R., C. J. Landers, H. Yang, M. J. Lodes, Y. Cong, K. A. Papadakis, E. Vasiliauskas, C. O. Elson, and R. M. Hershberg. 2005. Antibodies to CBir1 flagellin define a unique response that is associated independently with complicated Crohn's disease. *Gastroenterology* 128: 2020-2028.

215. Elson, C. O., Y. Cong, C. T. Weaver, T. R. Schoeb, T. K. McClanahan, R. B. Fick, and R. A. Kastelein. 2007. Monoclonal anti-interleukin 23 reverses active colitis in a T cell-mediated model in mice. *Gastroenterology* 132: 2359-2370.

216. Ivison, S. M., M. E. Himmel, G. Hardenberg, P. A. Wark, A. Kifayet, M. K. Levings, and T. S. Steiner. 2010. TLR5 is not required for flagellin-mediated exacerbation of DSS colitis. *Inflamm Bowel Dis* 16: 401-409.

217. Jyonouchi, H., L. Geng, A. Cushing-Ruby, and I. M. Monteiro. 2010. Aberrant responses to TLR agonists in pediatric IBD patients; the possible association with

increased production of Th1/Th17 cytokines in response to candida, a luminal antigen. *Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology* 21: e747-755.

218. Rovedatti, L., T. Kudo, P. Biancheri, M. Sarra, C. H. Knowles, D. S. Rampton, G. R. Corazza, G. Monteleone, A. Di Sabatino, and T. T. Macdonald. 2009. Differential regulation of interleukin 17 and interferon gamma production in inflammatory bowel disease. *Gut* 58: 1629-1636.

219. Deaglio, S., K. M. Dwyer, W. Gao, D. Friedman, A. Usheva, A. Erat, J. F. Chen, K. Enjyoji, J. Linden, M. Oukka, V. K. Kuchroo, T. B. Strom, and S. C. Robson. 2007. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *The Journal of experimental medicine* 204: 1257-1265.

220. Kunzli, B. M., P. Nuhn, K. Enjyoji, Y. Banz, R. N. Smith, E. Csizmadia, D. Schuppan, P. O. Berberat, H. Friess, and S. C. Robson. 2008. Disordered pancreatic inflammatory responses and inhibition of fibrosis in CD39-null mice. *Gastroenterology* 134: 292-305.

221. Niess, J. H. 2008. Role of mucosal dendritic cells in inflammatory bowel disease. *World journal of gastroenterology : WJG* 14: 5138-5148.

222. Bedke, T., R. G. Iannitti, A. De Luca, G. Giovannini, F. Fallarino, C. Berges, J. P. Latge, H. Einsele, L. Romani, and M. S. Topp. 2014. Distinct and complementary roles for Aspergillus fumigatus-specific Tr1 and Foxp3+ regulatory T cells in humans and mice. *Immunology and cell biology* 92: 659-670.

223. Burzyn, D., C. Benoist, and D. Mathis. 2013. Regulatory T cells in nonlymphoid tissues. *Nature immunology* 14: 1007-1013.

224. Shevach, E. M. 2011. Biological functions of regulatory T cells. *Advances in immunology* 112: 137-176.

225. Roncarolo, M. G., S. Gregori, M. Battaglia, R. Bacchetta, K. Fleischhauer, and M. K. Levings. 2006. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunological reviews* 212: 28-50.

226. Magnani, C. F., G. Alberigo, R. Bacchetta, G. Serafini, M. Andreani, M. G. Roncarolo, and S. Gregori. 2011. Killing of myeloid APCs via HLA class I, CD2 and CD226 defines a novel mechanism of suppression by human Tr1 cells. *European journal of immunology* 41: 1652-1662.

227. Davis, B. K., H. Wen, and J. P. Ting. 2011. The inflammasome NLRs in immunity, inflammation, and associated diseases. *Annual review of immunology* 29: 707-735.

228. Schroder, K., and J. Tschopp. 2010. The inflammasomes. *Cell* 140: 821-832.

229. Hoffman, H. M., A. A. Wanderer, and D. H. Broide. 2001. Familial cold autoinflammatory syndrome: phenotype and genotype of an autosomal dominant periodic fever. *The Journal of allergy and clinical immunology* 108: 615-620.

230. Agostini, L., F. Martinon, K. Burns, M. F. McDermott, P. N. Hawkins, and J. Tschopp. 2004. NALP3 forms an IL-1beta-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. *Immunity* 20: 319-325.

231. Robbins, G. R., H. Wen, and J. P. Ting. 2014. Inflammasomes and metabolic disorders: old genes in modern diseases. *Molecular cell* 54: 297-308.

232. Heneka, M. T., M. P. Kummer, and E. Latz. 2014. Innate immune activation in neurodegenerative disease. *Nature reviews. Immunology* 14: 463-477.

233. Li, H. B., C. Jin, Y. Chen, and R. A. Flavell. 2014. Inflammasome activation and metabolic disease progression. *Cytokine & growth factor reviews*.

234. Hoffman, H. M., and A. A. Wanderer. 2010. Inflammasome and IL-1beta-mediated disorders. *Current allergy and asthma reports* 10: 229-235.

235. Guarda, G., M. Braun, F. Staehli, A. Tardivel, C. Mattmann, I. Forster, M. Farlik, T. Decker, R. A. Du Pasquier, P. Romero, and J. Tschopp. 2011. Type I interferon inhibits interleukin-1 production and inflammasome activation. *Immunity* 34: 213-223.

236. Greenhill, C. J., G. W. Jones, M. A. Nowell, Z. Newton, A. K. Harvey, A. N. Moideen, F. L. Collins, A. C. Bloom, R. C. Coll, A. Robertson, M. A. Cooper, M. Rosas, P. R. Taylor, O. N. LA, I. R. Humphreys, A. S. Williams, and S. A. Jones. 2014. Interleukin-10 regulates the inflammasome-driven augmentation of inflammatory arthritis and joint destruction. *Arthritis research & therapy* 16: 419.

237. Pot, C., H. Jin, A. Awasthi, S. M. Liu, C. Y. Lai, R. Madan, A. H. Sharpe, C. L. Karp, S. C. Miaw, I. C. Ho, and V. K. Kuchroo. 2009. Cutting edge: IL-27 induces the transcription factor c-Maf, cytokine IL-21, and the costimulatory receptor ICOS that coordinately act together to promote differentiation of IL-10-producing Tr1 cells. *Journal of immunology* 183: 797-801.

238. Jin, J. O., X. Han, and Q. Yu. 2013. Interleukin-6 induces the generation of IL-10-producing Tr1 cells and suppresses autoimmune tissue inflammation. *Journal of autoimmunity* 40: 28-44.

239. Saraiva, M., J. R. Christensen, M. Veldhoen, T. L. Murphy, K. M. Murphy, and A. O'Garra. 2009. Interleukin-10 production by Th1 cells requires interleukin-12-

induced STAT4 transcription factor and ERK MAP kinase activation by high antigen dose. *Immunity* 31: 209-219.

240. Gagliani, N., C. F. Magnani, S. Huber, M. E. Gianolini, M. Pala, P. Licona-Limon, B. Guo, D. R. Herbert, A. Bulfone, F. Trentini, C. Di Serio, R. Bacchetta, M. Andreani, L. Brockmann, S. Gregori, R. A. Flavell, and M. G. Roncarolo. 2013. Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells. *Nature medicine* 19: 739-746.

241. Yi, J. S., M. A. Cox, and A. J. Zajac. 2010. T-cell exhaustion: characteristics, causes and conversion. *Immunology* 129: 474-481.

242. Schietinger, A., and P. D. Greenberg. 2014. Tolerance and exhaustion: defining mechanisms of T cell dysfunction. *Trends in immunology* 35: 51-60.

243. Oida, T., X. Zhang, M. Goto, S. Hachimura, M. Totsuka, S. Kaminogawa, and H. L. Weiner. 2003. CD4+CD25- T Cells That Express Latency-Associated Peptide on the Surface Suppress CD4+CD45RBhigh-Induced Colitis by a TGF- -Dependent Mechanism. *The Journal of Immunology* 170: 2516-2522.

244. Duan, W., T. So, A. K. Mehta, H. Choi, and M. Croft. 2011. Inducible CD4+LAP+Foxp3- regulatory T cells suppress allergic inflammation. *Journal of immunology* 187: 6499-6507.

245. Younes, S. A., G. Punkosdy, S. Caucheteux, T. Chen, Z. Grossman, and W. E. Paul. 2011. Memory phenotype CD4 T cells undergoing rapid, nonburst-like, cytokine-driven proliferation can be distinguished from antigen-experienced memory cells. *PLoS biology* 9: e1001171.

246. Zigmond, E., B. Bernshtein, G. Friedlander, C. R. Walker, S. Yona, K. W. Kim, O. Brenner, R. Krauthgamer, C. Varol, W. Muller, and S. Jung. 2014. Macrophage-restricted interleukin-10 receptor deficiency, but not IL-10 deficiency, causes severe spontaneous colitis. *Immunity* 40: 720-733.

247. Shouval, D. S., A. Biswas, J. A. Goettel, K. McCann, E. Conaway, N. S. Redhu, I. D. Mascanfroni, Z. Al Adham, S. Lavoie, M. Ibourk, D. D. Nguyen, J. N. Samsom, J. C. Escher, R. Somech, B. Weiss, R. Beier, L. S. Conklin, C. L. Ebens, F. G. Santos, A. R. Ferreira, M. Sherlock, A. K. Bhan, W. Muller, J. R. Mora, F. J. Quintana, C. Klein, A. M. Muise, B. H. Horwitz, and S. B. Snapper. 2014. Interleukin-10 receptor signaling in innate immune cells regulates mucosal immune tolerance and anti-inflammatory macrophage function. *Immunity* 40: 706-719.

248. Li, B., P. Gurung, R. K. Malireddi, P. Vogel, T. D. Kanneganti, and T. L. Geiger. 2015. IL-10 engages macrophages to shift Th17 cytokine dependency and pathogenicity during T-cell-mediated colitis. *Nature communications* 6: 6131.

249. Fiorentino, D. F., A. Zlotnik, T. R. Mosmann, M. Howard, and A. O'Garra. 1991. IL-10 inhibits cytokine production by activated macrophages. *Journal of immunology* 147: 3815-3822.

250. Wang, P., P. Wu, M. I. Siegel, R. W. Egan, and M. M. Billah. 1994. IL-10 inhibits transcription of cytokine genes in human peripheral blood mononuclear cells. *The Journal of Immunology* 153: 811-816.

251. Bogdan, C., J. Paik, Y. Vodovotz, and C. Nathan. 1992. Contrasting mechanisms for suppression of macrophage cytokine release by transforming growth factor-beta and interleukin-10. *The Journal of biological chemistry* 267: 23301-23308.

252. Panuska, J. R., R. Merolla, N. A. Rebert, S. P. Hoffmann, P. Tsivitse, N. M. Cirino, R. H. Silverman, and J. A. Rankin. 1995. Respiratory syncytial virus induces interleukin-10 by human alveolar macrophages. Suppression of early cytokine production and implications for incomplete immunity. *The Journal of clinical investigation* 96: 2445-2453.

253. Guarda, G., C. Dostert, F. Staehli, K. Cabalzar, R. Castillo, A. Tardivel, P. Schneider, and J. Tschopp. 2009. T cells dampen innate immune responses through inhibition of NLRP1 and NLRP3 inflammasomes. *Nature* 460: 269-273.

254. Rubtsov, Y. P., J. P. Rasmussen, E. Y. Chi, J. Fontenot, L. Castelli, X. Ye, P. Treuting, L. Siewe, A. Roers, W. R. Henderson, Jr., W. Muller, and A. Y. Rudensky. 2008. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity* 28: 546-558.

255. Du Pre, M. F., A. E. Kozijn, L. A. van Berkel, M. N. ter Borg, D. Lindenbergh-Kortleve, L. T. Jensen, Y. Kooy-Winkelaar, F. Koning, L. Boon, E. E. Nieuwenhuis, L. M. Sollid, L. Fugger, and J. N. Samsom. 2011. Tolerance to ingested deamidated gliadin in mice is maintained by splenic, type 1 regulatory T cells. *Gastroenterology* 141: 610-620, 620.e611-612.

256. Perez-Ruiz, F., N. Dalbeth, and T. Bardin. 2014. A Review of Uric Acid, Crystal Deposition Disease, and Gout. *Advances in therapy*.

257. Steiger, S., and J. L. Harper. 2014. Mechanisms of spontaneous resolution of acute gouty inflammation. *Current rheumatology reports* 16: 392.

258. Heymann, M. C., and A. Rosen-Wolff. 2013. Contribution of the inflammasomes to autoinflammatory diseases and recent mouse models as research tools. *Clinical immunology* 147: 175-184.

259. Hari, A., Y. Zhang, Z. Tu, P. Detampel, M. Stenner, A. Ganguly, and Y. Shi. 2014. Activation of NLRP3 inflammasome by crystalline structures via cell surface contact. *Scientific reports* 4: 7281.

260. Kingsbury, S. R., P. G. Conaghan, and M. F. McDermott. 2011. The role of the NLRP3 inflammasome in gout. *Journal of inflammation research* 4: 39-49.

261. So, A., T. De Smedt, S. Revaz, and J. Tschopp. 2007. A pilot study of IL-1 inhibition by anakinra in acute gout. *Arthritis research & therapy* 9: R28.

262. Sidiropoulos, P. I., G. Goulielmos, G. K. Voloudakis, E. Petraki, and D. T. Boumpas. 2008. Inflammasomes and rheumatic diseases: evolving concepts. *Annals of the rheumatic diseases* 67: 1382-1389.

263. Brydges, S. D., J. L. Mueller, M. D. McGeough, C. A. Pena, A. Misaghi, C. Gandhi, C. D. Putnam, D. L. Boyle, G. S. Firestein, A. A. Horner, P. Soroosh, W. T. Watford, J. J. O'Shea, D. L. Kastner, and H. M. Hoffman. 2009. Inflammasome-mediated disease animal models reveal roles for innate but not adaptive immunity. *Immunity* 30: 875-887.

264. Meng, G., F. Zhang, I. Fuss, A. Kitani, and W. Strober. 2009. A mutation in the Nlrp3 gene causing inflammasome hyperactivation potentiates Th17 cell-dominant immune responses. *Immunity* 30: 860-874.

265. Bonar, S. L., S. D. Brydges, J. L. Mueller, M. D. McGeough, C. Pena, D. Chen, S. K. Grimston, C. L. Hickman-Brecks, S. Ravindran, A. McAlinden, D. V. Novack, D. L.

Kastner, R. Civitelli, H. M. Hoffman, and G. Mbalaviele. 2012. Constitutively activated NLRP3 inflammasome causes inflammation and abnormal skeletal development in mice. *PloS one* 7: e35979.

266. Mills, K. H., L. S. Dungan, S. A. Jones, and J. Harris. 2013. The role of inflammasome-derived IL-1 in driving IL-17 responses. *Journal of leukocyte biology* 93: 489-497.

267. Brydges, S. D., L. Broderick, M. D. McGeough, C. A. Pena, J. L. Mueller, and H. M. Hoffman. 2013. Divergence of IL-1, IL-18, and cell death in NLRP3 inflammasomopathies. *The Journal of clinical investigation* 123: 4695-4705.

268. Saxena, A., S. Khosraviani, S. Noel, D. Mohan, T. Donner, and A. R. Hamad. 2014. Interleukin-10 paradox: A potent immunoregulatory cytokine that has been difficult to harness for immunotherapy. *Cytokine*.

269. Marlow, G. J., D. van Gent, and L. R. Ferguson. 2013. Why interleukin-10 supplementation does not work in Crohn's disease patients. *World J Gastroenterol* 19: 3931-3941.

270. Bouabe, H. 2012. Cytokine reporter mice: the special case of IL-10. *Scandinavian journal of immunology* 75: 553-567.

271. Bouabe, H., Y. Liu, M. Moser, M. R. Bosl, and J. Heesemann. 2011. Novel highly sensitive IL-10-beta-lactamase reporter mouse reveals cells of the innate immune system as a substantial source of IL-10 in vivo. *Journal of immunology* 187: 3165-3176.

272. Kurashima, Y., T. Amiya, T. Nochi, K. Fujisawa, T. Haraguchi, H. Iba, H. Tsutsui, S. Sato, S. Nakajima, H. Iijima, M. Kubo, J. Kunisawa, and H. Kiyono. 2012.

Extracellular ATP mediates mast cell-dependent intestinal inflammation through P2X7 purinoceptors. *Nature communications* 3: 1034.

273. Cauwels, A., E. Rogge, B. Vandendriessche, S. Shiva, and P. Brouckaert. 2014. Extracellular ATP drives systemic inflammation, tissue damage and mortality. *Cell death & disease* 5: e1102.

274. Chen, S., Q. Ma, P. R. Krafft, Q. Hu, W. Rolland, 2nd, P. Sherchan, J. Zhang, J. Tang, and J. H. Zhang. 2013. P2X7R/cryopyrin inflammasome axis inhibition reduces neuroinflammation after SAH. *Neurobiology of disease* 58: 296-307.

275. Chu, K., B. Yin, J. Wang, G. Peng, H. Liang, Z. Xu, Y. Du, M. Fang, Q. Xia, and B. Luo. 2012. Inhibition of P2X7 receptor ameliorates transient global cerebral ischemia/reperfusion injury via modulating inflammatory responses in the rat hippocampus. *Journal of neuroinflammation* 9: 69.

276. Yan, Y., J. Bai, X. Zhou, J. Tang, C. Jiang, E. Tolbert, G. Bayliss, R. Gong, T. C. Zhao, and S. Zhuang. 2015. P2X7 receptor inhibition protects against ischemic acute kidney injury in mice. *American journal of physiology. Cell physiology*: ajpcell.00245.02014.

277. Huang, C., W. Yu, H. Cui, Y. Wang, L. Zhang, F. Han, and T. Huang. 2014. P2X7 blockade attenuates mouse liver fibrosis. *Molecular medicine reports* 9: 57-62.

278. Marcillo, A., B. Frydel, H. M. Bramlett, and W. D. Dietrich. 2012. A reassessment of P2X7 receptor inhibition as a neuroprotective strategy in rat models of contusion injury. *Experimental neurology* 233: 687-692.

279. McInnes, I. B., S. Cruwys, K. Bowers, and M. Braddock. 2014. Targeting the P2X7 receptor in rheumatoid arthritis: biological rationale for P2X7 antagonism. *Clinical and experimental rheumatology* 32: 878-882.

280. Neves, A. R., M. T. Castelo-Branco, V. R. Figliuolo, C. Bernardazzi, F. Buongusto, A. Yoshimoto, H. F. Nanini, C. M. Coutinho, A. J. Carneiro, R. Coutinho-Silva, and H. S. de Souza. 2014. Overexpression of ATP-activated P2X7 receptors in the intestinal mucosa is implicated in the pathogenesis of Crohn's disease. *Inflamm Bowel Dis* 20: 444-457.

281. Marques, C. C., M. T. Castelo-Branco, R. G. Pacheco, F. Buongusto, A. do Rosario, Jr., A. Schanaider, R. Coutinho-Silva, and H. S. de Souza. 2014. Prophylactic systemic P2X7 receptor blockade prevents experimental colitis. *Biochimica et biophysica acta* 1842: 65-78.

282. Ochoa-Cortes, F., A. Linan-Rico, K. A. Jacobson, and F. L. Christofi. 2014. Potential for developing purinergic drugs for gastrointestinal diseases. *Inflamm Bowel Dis* 20: 1259-1287.

283. Min, B., A. Thornton, S. M. Caucheteux, S. A. Younes, K. Oh, J. Hu-Li, and W. E. Paul. 2007. Gut flora antigens are not important in the maintenance of regulatory T cell heterogeneity and homeostasis. *European journal of immunology* 37: 1916-1923.

284. Jiang, H. Q., M. C. Thurnheer, A. W. Zuercher, N. V. Boiko, N. A. Bos, and J. J. Cebra. 2004. Interactions of commensal gut microbes with subsets of B- and T-cells in the murine host. *Vaccine* 22: 805-811.

285. Bruno, L., H. von Boehmer, and J. Kirberg. 1996. Cell division in the compartment of naive and memory T lymphocytes. *European journal of immunology* 26: 3179-3184.

286. Levings, M. K., R. Sangregorio, F. Galbiati, S. Squadrone, R. de Waal Malefyt, and M. G. Roncarolo. 2001. IFN-alpha and IL-10 induce the differentiation of human type 1 T regulatory cells. *Journal of immunology* 166: 5530-5539.

287. Andolfi, G., G. Fousteri, M. Rossetti, C. F. Magnani, T. Jofra, G. Locafaro, A. Bondanza, S. Gregori, and M. G. Roncarolo. 2012. Enforced IL-10 expression confers type 1 regulatory T cell (Tr1) phenotype and function to human CD4(+) T cells. *Molecular therapy : the journal of the American Society of Gene Therapy* 20: 1778-1790.

288. Beres, A. J., and W. R. Drobyski. 2013. The role of regulatory T cells in the biology of graft versus host disease. *Frontiers in immunology* 4: 163.

289. Gagliani, N., S. Gregori, T. Jofra, A. Valle, A. Stabilini, D. M. Rothstein, M. Atkinson, M. G. Roncarolo, and M. Battaglia. 2011. Rapamycin Combined with Anti-CD45RB mAb and IL-10 or with G-CSF Induces Tolerance in a Stringent Mouse Model of Islet Transplantation. *PloS one* 6.

290. Bacher, P., O. Kniemeyer, A. Schonbrunn, B. Sawitzki, M. Assenmacher, E. Rietschel, A. Steinbach, O. A. Cornely, A. A. Brakhage, A. Thiel, and A. Scheffold. 2014. Antigen-specific expansion of human regulatory T cells as a major tolerance mechanism against mucosal fungi. *Mucosal immunology* 7: 916-928.

291. Ouyang, X., A. Ghani, A. Malik, T. Wilder, O. R. Colegio, R. A. Flavell, B. N. Cronstein, and W. Z. Mehal. 2013. Adenosine is required for sustained inflammasome activation via the A(2)A receptor and the HIF-1alpha pathway. *Nature communications* 4: 2909.

292. Schuler, P. J., Z. Saze, C. S. Hong, L. Muller, D. G. Gillespie, D. Cheng, M. Harasymczuk, M. Mandapathil, S. Lang, E. K. Jackson, and T. L. Whiteside. 2014. Human CD4(+) CD39(+) regulatory T cells produce adenosine upon co-expression of surface CD73 or contact with CD73(+) exosomes or CD73(+) cells. *Clinical and experimental immunology* 177: 531-543.