ABSTRACT

Title of Dissertation: THE IMMUNOREGULATION OF INTERLEUKIN-27 IN AFRICAN TRYPANOSOME INFECTION

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Interleukin (IL)-27 is a cytokine with diverse impacts on regulation of vertebrate T helper Type 1 (Th1) responses. Initially, it was predicted as a promoter of Th1 responses. However, it was lately identified as a potent negative regulator of T cell responses in a variety of disease models, including infection with viruses, bacteria, and intracellular parasites. The extracellular protozoan parasites, African trypanosomes, cause a chronic debilitating disease associated with persistent inflammation. Using this infection model, we aim to identify novel immunoregulatory functions of IL-27 on innate and adaptive immunity. Here we demonstrate that IL-27 receptor deficient (IL-27R$^{-/-}$) mice infected with African trypanosomes display excessive production of IFN-γ by CD4^{+} T cells, exacerbated liver pathology, and dramatically shortened survival as compared with infected wild-type mice. Depletion

of $CD4^+$ T cells or neutralization of IFN-γ ameliorates the liver pathology and extends the survival of infected IL-$27R^{-/-}$ mice.

Our further interest is in deciphering the mechanisms of how $CD4^+$ T cells and IFN-γ shape the monocyte-featured innate immunity in African trypanosome infected IL-$27R^{-/-}$ mice. Blood monocytes typically consist of a heterogenous population of $Ly6C^+$ and $Ly6C^-$ monocytes. $Ly6C^+$ monocytes can give rise to inflammatory TNF-α/iNOS producing dendritic cells (Tip-DCs) and anti-inflammatory macrophages. Here we find that IL-$27R^{-/-}$ mice exhibit a higher frequency of $Ly6C^+$ monocytes recruitment to the liver, where they preferentially differentiate into Tip-DCs. This is coincided with impaired development of $Ly6C^-$ monocytes and macrophages in the liver. Depletion of $CD4^+$ T cells or neutralization of IFN-γ in infected IL-$27R^{-/-}$ mice diminishes the recruitment of $Ly6C^+$ monocytes, and their differentiation into Tip-DCs in the liver. This is accompanied by the greatly enhanced counts of $Ly6C^-$ monocytes and macrophages following antibody treatments. Further evidences show that 1) IFN-γ produced by $CD4^+$ T cells induces cell death of $Ly6C^-$ monocytes which perturb the development of Tip-DCs in infected IL-$27R^{-/-}$ mice and 2) cell intrinsic IFN-γ signaling drives $Ly6C^+$ monocytes to differentiate into Tip-DCs in infected IL-$27R^{-/-}$ mice. Thus, our data identify IL-27 signaling as a novel immunoregulator to prevent $Ly6C^+$ monocytes from differentiation into Tip-DCs through suppressing $CD4^+$ T cells to secrete IFN-γ.

THE IMMUNOREGULATION OF INTERLEUKIN-27 IN AFRICAN TRYPANOSOME INFECTION

by

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Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2018

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Dedication

To my parents, my wife and my son.

To my Master mentor Dr. Xuebo Liu, and Ph.D. mentor Dr. Meiqing Shi.

Thank you all for making my dreams come true.

Acknowledgements

I would like to present my genuine gratefulness to my mentor, Dr. Meiqing Shi. Thank you for opening a new chapter for my life and inspiring me the meaning of being a great scientist. Thank you for presenting five years of freedom, patience and encouragement to let me explore the unknown with joy. Your dedication and passion to science is what I aspire to endeavor. I would also like to acknowledge my dissertation committee members: Dr. Georgiy Belov, Dr. Utpal Pal, Dr. Zhengguo Xiao, and Dr. Xiaoping Zhu. I really appreciate your generosity of invaluable time, thoughtful guidance and constructive insights.

I express my thanks to Dr. Xiaoping Zhu and Dr. Stefan Magez for their collaboration with Dr. Shi's lab and inputs to my manuscript. Thank you to Mr. Kenneth Class for your kindness and professionalism to help me on every aspect of flow cytometry. Thanks to Dr. Jinjun Xu for working extremely closely with me on this project. To members of Dr. Shi's lab, thank you for making my lab life enjoyable and gratifying. Special thanks to Yanli Chen and Ashley Strickland for reviewing the dissertation.

Thanks to my Master mentor Dr. Xuebo Liu for supporting and encouraging me to chase the dream to study abroad. And you set an exceptional example for me in leadership, responsibility and scientific rigor.

Thanks to my family for your unlimited support and love.

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List of Abbreviations

AhR	Aryl hydrocarbon receptor
AP-1	Activator protein 1
ATP	Adenosine triphosphate
CD	Cluster of differentiation
C/EBPβ	CCAAT-enhancer-binding proteins
c-Maf	Avian musculoaponeurotic fibrosarcoma, a transcription factor
CSF-1R	Colony stimulating factor 1 receptor
Foxp3	Forkhead box P3
GATA3	GATA Binding Protein 3
ICAM-1	Intercellular Adhesion Molecule 1
IFN	Interferon
Ig	Immunoglobulin
iNKT	Invariant natural killer T
IRF	Interferon regulatory factor 4
JNK	c-Jun N-terminal kinases
KLF	Krueppel-like factor 4
LFA-1	Lymphocyte function-associated antigen 1
MAPK	Mitogen-activated protein kinases
MHC	Major histocompatibility complex
MNP	Mononuclear phagocyte
MyD88	Myeloid differentiation primary response 88
NF-κB	Nuclear factor-κB

NR4A1	nuclear receptor subfamily 4, group A, member 1
PGE2	Prostaglandin E2
PI3K	Phosphoinositide 3-kinase
ROR	RAR-related orphan receptor
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOCS	Suppressor of cytokine signalling
T-bet	T-box protein expressed in T cells, also called as TBX21
TGF-β	Transforming growth factor beta
Th	T helper
TLR	Toll-like receptor
TRIF	TIR-domain-containing adapter-inducing interferon-β
Tyk2	Tyrosine kinase 2
WT	Wild type

CHAPTER 1: INTRODUCTION

Overview

The biology of interleukin (IL)-27

IL-27 and signaling

Interleukin (IL)-27, a member of the IL-12 family cytokines, is a heterodimeric cytokine consisting of Epstein-Barr virus-induced gene 3 (EBi3) and IL-27p28 subunits (Fig. 1.1) (1). Janus kinase (JAK)-signal transducer and activator of transcription (STAT) as well as mitogen activated protein kinase (MAPK) signaling can be activated upon IL-27 is engaged with its receptor which is composed of gp130 and IL-27Rα (or WSX-1 or TCCR) (Fig. 1.1) (2). Activated IL-27/IL-27R signaling exhibits both pro-inflammatory and anti-inflammatory properties by orchestrating the activities of a broad range of cells of innate and adaptive immunity.

The subunits of IL-27

The EBi3 subunit of IL-27 was first characterized in B lymphocytes infected with Epstein-Barr virus by Mark Birkenbach and colleagues in 1996 (3). This hematopoietin receptor family member is highly related to the p40 subunit of the IL-

12. IL-27p28, the other subunit of IL-27, was identified with similar sequences and structure motifs to IL12p35 and IL12p19 while performing computational sequence modeling in 2002 by the Robert Kastelein's lab, and was designated p28 for its sharp molar mass illuminated by the SDS-PAGE (1). EBi3 and IL-27p28 are secreted separated and then complex together to form IL-27 with a covalent bond, rather than a disulfide bond as that of IL-12 and IL-23 (1). In addition, EBi3 can also partner with IL12p35 to form IL-35, another member of IL-12 family cytokines (4). Similarly, IL-27p28 can bind with Cytokine-like Factor 1 to induce the IL-6R signaling (5). Importantly, the complex of EBi3 and IL-27p28, but not individual subunits, is indispensable to initiate the IL-27/IL-27R signaling (1).

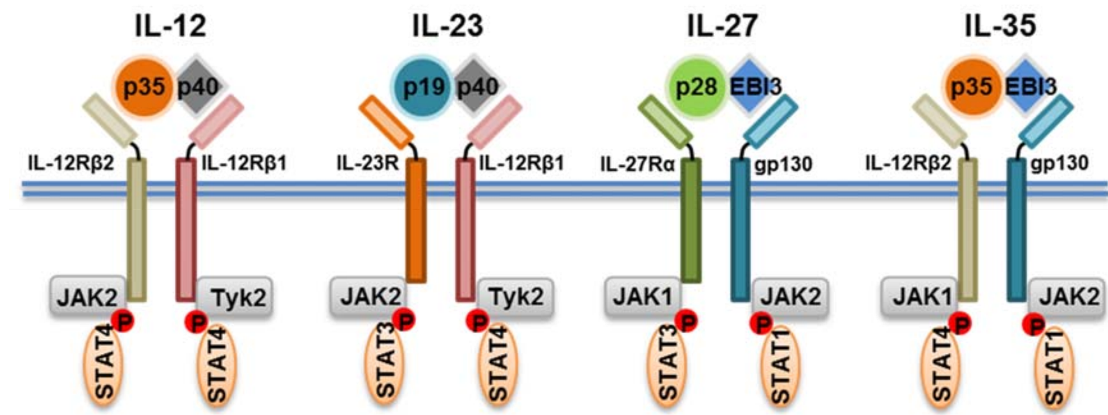

Fig. 1.1 IL-12 family cytokines, receptors, and signaling pathways

Regulation of IL-27 expression

Initial studies recognized that antigen presenting cells, like dendritic cells (DCs), macrophages and B cells, are the major cellular sources of IL-27 in response to activation of pattern recognition receptors (PRRs), including TLR3, TLR4 and TLR7 and TLR9 (6-8). Recently, iNKT cells were also documented to secrete IL-27 in response to alpha-GalCer stimulation (9). More recently, in a murine malaria infection model, a new subset of $FoxP3^-$ regulatory $CD4^+$ T cells was described to produce IL-27 upon T cell receptor activation (10). However, despite all the observations, the regulation of IL-27 expression remains poorly studied and lacks convincing *in vivo* data.

The up-regulation of IL-27 has been noted in the context of viral, bacterial, protozoan, and fungal infections. However, the two individual subunits of IL-27 are encoded by distinct gene loci (1). The expression of the subunit IL-27p28 in macrophage was found to be dependent on the activation of TLR4. Upon activation, TLR4 recruits the adaptor proteins MyD88 and TRIF, followed by the translocation of NF-κB (11), AP-1 (12) or IRF3 (13) to the promoter region of the IL-27p28. Additionally, IFN-γ can prime and polarize macrophages to secrete distinct cytokines,

including regulatory IL-27 through activating JNK, MAPK and PI3K signaling in macrophages (14). IFN-γ can also induce the IRF1 and IRF8 binding to the promoter region of the IL-27p28 (15). Similarly, IFN-α can induce IRF-1 binding to the IFN-stimulated response element (ISRE) of the IL-27p28 gene promoter (16). The expression of the subunit EBi3 is induced by the activation of TLR2, TLR3 and TLR9 signalings which recruit MyD88 to initiate the translocation of NF-κB and PU.1 to the promoter region of EBi3 (8).

Compared to the up-regulation, less is known about the down-regulation of IL-27 production. Four distinct classes of molecules were found to inhibit the gene expression of the two subunits of IL-27. Firstly, extracellular ATP was described in human dendritic cells suppressing the production of the IL-27p28, and to a lesser extent for the EBi3 (17). Secondly, in different conditions, C5a, a byproduct of Complement activation, limited the IL-27 production (6, 18-20) through activation of PI3K and JNK signaling pathways (19, 20). However, the specificity of C5a to suppress IL-27 is questionable, as the expression of other members of IL-12 family cytokines were inhibited as well in the same setting. Thirdly, histamine, a potent mediator of allergic inflammation, can also suppress the IL-27 production at the

mRNA and protein level upon TLRs stimulation (21). Similar to C5a, the inhibition effect of histamine is not specific to IL-27, but is also applicable to IL-12. Finally, PGE2, as a prostanoid, was also identified as a potent inhibitor of IL-27 expression in bone marrow–derived DC (BMDC) (22) and THP-1 cells (23). More recently, *in vivo* data showed that PGE2 exhibits an inhibitory effect on IL-27 expression through limiting the expression and binding of IRF1 to the p28 promoter in splenic cDCs (24).

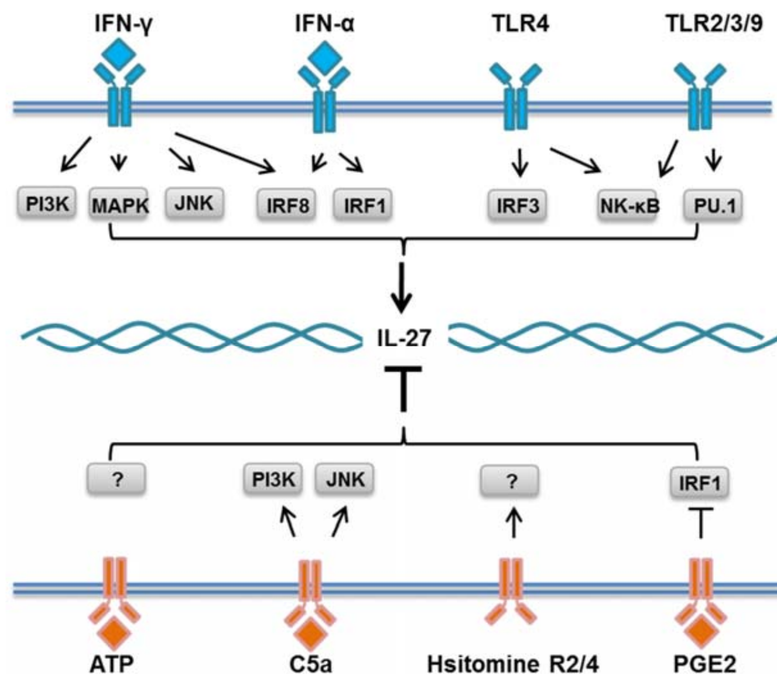

Fig. 1.2 Signaling pathways in regulation of IL-27 expression

The receptor and signaling

IL-27 selectively binds to its heterodimeric receptor composed of IL-27Rα (WSX-1 or TCCR) and gp130 (1, 25, 26). IL-27Rα is recognized as the ligand

binding chain, while gp130 is mainly for signal-transduction (2). Of note, gp130 is shared by the receptors of other cytokines, such as IL-35 and IL-6, and widely expressed by various immune and non-immune cells. In contrast, the expression of IL-27Rα is more restricted to innate and adaptive immune cells, including T cells, B cells, and NK cells etc. (2, 27, 28). Expression of IL-27Rα is also detectable in tumor cell lines, such as HeLa cells and cancer cells of patients with acute myeloid leukemia (29, 30).

Upon engagement with IL-27R, IL-27 initiates phosphorylation of Jak-STAT, followed by translocation of activated STAT1 or STAT3 to the nucleus. Phosphorylated STAT1 or STAT3 instructs the lineage commitment of cells through modulating the expression of transcription factors, for example enhancement of T-bet to drive Th1 cells (31) as well as inhibition of GATA3 to prevent Th2 (32) or RORγt to prevent Th17 (33, 34). In addition to the Jak-STAT pathway, IL-27 signaling can also activate MAPKp38 to induce expression of the transcription factor AP-1 or c-Maf leading to the enhancement of IL-10 expression (35). Furthermore, the enhancement of c-Maf induced by the MAPKp38 can synergize with AhR to optimize the expression of IL-10 (36).

Immunoregulation of T cells by IL-27

IL-27, a potent immunomodulatory cytokine, displays both pro-inflammatory and anti-inflammatory properties in disparate settings of experimental models or disease conditions. This can be attributed to the ubiquitous expression of the IL-27R on various types of cells, resulting in indirect or direct effects on the differentiation and function maintenance of distinct cell types. In this section of the Chapter 1, I will mainly focus on the immunoregultory roles of IL-27 on T cell immunity. Additionally, emerging immuoregulatory properties of IL-27 in myeloid cells will be discussed.

Pro-inflammatory roles of IL-27 in T cells

As mentioned above, subunits and the receptor of IL-27 display numerous similarities with other IL-12 family cytokines and their receptors. It is well known that IL-12 is a critical pro-inflammatory cytokine in the development of T helper (Th1) responses. Hence, when IL-27 was discovered in 2002, it was expected to be a pro-inflammatory cytokine similar to IL-12. This notion was initially confirmed by two independent groups by infecting IL-27R deficient mice with intracellular pathogens, namely *Leishmania major* and *Listeria monocytogenes* (25, 26). As compared with the wild type (WT) mice, the infected IL-27R knock-out mice

succumbed remarkably earlier due to defects in Th1 responses which are essential to control the infections. Later on in 2002, Kastelein and colleagues described the pro-inflammatory properties of IL-27 in naive $CD4^+$ T cells (Th0 cells) solidifying the idea that IL-27 is a potent pro-inflammatory cytokine which can promote the proliferation of Th0 cells but not memory $CD4^+$ T cells (Th1 cells) and induce the production of IFN-γ by Th0 cells in the presence of IL-12 (1). The idea that IL-27 promotes the development of Th1 was also observed in a model of experimental arthritis (37). In this experimental model, the diminished arthritis in IL-27R knock-out mice was associated with lower frequency of IFN-γ producing $CD4^+$ T cells and attenuated systematic level of IFN-γ which promoted the progression of disease and pathology. On the basis of the discoveries listed above, detailed mechanisms were documented by several groups that the IL-27R/STAT1/T-bet signaling pathway polarized Th0 cells to Th1 cells by inducing the up-regulation of IL-12Rβ2, which rendered Th0 cells the responsiveness to the Th1 promoting IL-12 signaling (32, 38, 39). Furthermore, the observations that IL-27 induces the elevated expression of adhesion molecules like ICAM-1 and LFA-1 on Th0 cells indicates the potential effects of IL-27 on manipulating the activation of the Th0 cells via accelelating the

transmigration of Th0 cells to the lymphoid organs and facilitating the interactions with antigen presenting cells (35).

In addition to Th0 cells, IL-27 can also promote the development of cytotoxic T lymphocytes (CTL). Similar to the effects of IL-27 on naive $CD4^+$ T cells, Yoshimoto and colleagues (40) documented that IL-27 activated multiple STATs and augmented the expression of T-bet, IL-12Rβ2 and granzyme B in naive $CD8^+$ T cells *in vitro*. In synergy with IL-12, IL-27 enhanced the IFN-γ production and clonal expansion of naive $CD8^+$ T cells. Moreover, the IFN-γ production capacity and cytotoxic activity of naive $CD8^+$ T cells were induced by IL-27 through STAT1/T-bet/IL-12Rβ2 signaling cascade. This idea was further confirmed in human $CD8^+$ T cells, where IL-27 advanced the human naive $CD8^+$ T cells to highly efficient cytotoxic cells through STAT1 induced up-regulation of SOCS1 and SOCS3 (41). Similary to what was observed in naive $CD4^+$ T cells, IL-27 induced the expression of adhesion molecules indicating an impact on trafficking of naive $CD8^+$ T cells (42). The pro-inflammatory role of IL-27 on $CD8^+$ T cells was also documented in infections of *Toxoplasma gondii* and Influenza in which IL-27R is pivotal for induction of the effector CTLs (43, 44).

Furthermore, another pro-inflammatory facet of IL-27 is displayed by its ability to hijack the development and function of regulatory T cells (Treg and Tr1) which are immunosuppressive and generally prevent the induction and proliferation of effector T cells. Huber (45) and Neufert (46) independently documented the capacity of IL-27 in regulating the generation of the TGF-β induced Treg cells *in vitro*. Their results demonstrated that IL-27 signaling induced the phosphoralation of STAT1 or STAT3, resulting in dampened acquisition of $CD25^{+}FoxP3^{+}$ Treg phenotype as well as the suppressive function. Compelling *in vivo* data obtained through tracking the phenotype of the transferred IL-$27R^{-/-}$ Th0 cells revealed that IL-$27R^{-/-}$ Th0 cells preferentially acquired the Treg phenotype compared to the IL-$27R^{+/+}$ Th0 cells (47). They also found that disease progression in mice receiving IL-27R deficient T cells was diminished, indicating that IL-27 impaired the development and suppressive functions of Treg (47).

Anti-inflammatory role of IL-27 in T cells

Compared to the pro-inflammatory effects, the inhibitory facets of IL-27 were widely appreciated in infections of a broad range of pathogens, including *Toxoplasma gondii*, *Leishmania donovani*, *Trypanosome cruzi*, *Mycobacterium tuberculosis*,

Parainfluenza and Influenza, as well as in autoimmune diseases. In addition, the Chapter 2 of my dissertation demonstrated, for the first time, the anti-inflammatory role of IL-27 in the extracellular parasites, *Trypanosome brucei brucei* and *Trypanosome congolense* (discussed in Chapter 4). From all of the distinct models of infections, it was found that IL-27/IL-27R signaling antagonizes the Th1, Th2 and Th17 responses and promotes the development Tr1 and Treg.

<u>IL-27 and Th1</u>

In 2003, the anti-inflammatory role of IL-27 was first documented by Christopher Hunter's lab by infecting mice with the intracellular pathogen *T. gondii* (48). In this experimental model, infected IL-27R knock-out mice controlled the parasitemia, but developed over-activated Th1 immunity characterized by persistent hyper-activation of $CD4^+$ T cells and excessive production of IFN-γ, which resulted in liver pathology and shortened survival of the infected hosts. Similarly, excessive production of IFN-γ by $CD4^+$ T cells in IL-27R deficient mice were noted in infections with *T. cruzi* (49), *P. bergheri* (50), *L. donovani* (51), *L. major* (52), and *M. tuberculosis* (53). Of note, in *T. cruzi* infection, IL-27$R^{-/-}$ mice displayed severe necrosis in the liver and hyper-activity of both Th1 and Th2 responses, but prolonged

parasitemia contrary to *T. gondii* infection. The persistent parasitemia was attributed to the enhanced levels of Th2 cytokines which favor the growth of the parasites. The increased production of IFN-γ by hyper-activated Th1 cells resulted in tissue damage. Overall, this study in *T. cruzi* infection described, for the first time, that IL-27 suppresses varying subsets of T cells.

The detailed mechanisms that IL-27 antagonizes the Th1 immunity are still not fully understood, and may vary considering distinct contexts of infections and immune responses. The inhibitory effects of IL-27 on hyper-activity of Th1 responses can be explained by the down-regulation of the pro-inflammatory cytokines, like IL-2 (54). In addition, a broader mechanism is that IL-27 facilitates the transition of IFN-γ^{+}CD4^{+} T cells into IFN-γ^{+}IL-10^{+}CD4^{+} T cells, consequently creating a negative feedback loop to the aberrant Th1 responses (50, 55). Furthermore, the fact that depletion of CD4^{+} T cells diminished the lethal effects in IL-27 deficient mice indicates that IL-27 directly suppresses Th1 responses (48, 49). However, it is possible that IL-27 indirectly manipulates the induction of T cell responses through impairing the priming activity of antigen presentating cells, which will be comprehensively addressed in a later section.

IL-27 and Th2

The debate about IL-27 and Th2 immunity was first started with the conflicted reports and data interpretation of *Leishmania major* infected IL-27R knock-out mice (25, 56, 57). A key point to murine *L. major* infection to be clarified is that Th1 inflammation impairs the growth of the parasites, while Th2 displays an opposite effect. The early studies (25, 56) revealed that *L. major* infected IL-27R deficient mice have impaired Th1 responses and early motility, based on which it was concluded that IL-27 was essential to the development of Th1 responses. However, more detailed data showed subsequently that the IL-27 dependent induction of Th1 inflammation is restricted to conditions in which IL-4 is excessively produced (57). Neutralization of IL-4 in infected IL-27R knock-out mice restored the level of IFN-γ, diminished the aberrant persistence of parasites and shortened survival of the IL-27R deficient mice. That is to say, the early death of the infected IL-27R knock-out mice is truly due to the hyper-activation of Th2 responses, rather than the impaired development of Th1 responses. The essential suppressive role of IL-27 on Th2 immunity was also documented in Helminth infection which biased the immunity to a Th2 inflammation in IL-27R knock-out mice (58). Another important model concerned with the regulation of Th2 responses is asthma. IL-27R deficient mice

developed exacerbated experimental allergic asthma which was attributed to the excessive production of Th2 cytokines and aberrant development of Th2 cells (32, 40, 59). The underlying mechanism was that IL-27/IL-27R signaling induced the phosphorylation of STAT1 which reduced the expression of GATA3, an essential transcription factor for Th2 immunity (32, 59).

IL-27 and Th17

In addition to the inhibitory roles of IL-27 on Th1 and Th2 development and functional acquisition, it was found that IL-27 exerts potent suppressive effects on Th17 cells. Generally, Th17 cells are extensively linked to autoimmune diseases. IL-27R deficient mice developed more severe experimental autoimmune encephalomyelitis (EAE) (60, 61). Without IL-27 signaling, excessive production of IL-17 as well as IL-22 and aggressive development of Th17 cells were observed. This notion was also applicable to the colitis model, in which IL-27 biased Th0 cells away from the generation of IL-17 producing $CD4^+$ T cells (62) and administration of rIL-27 diminished the development of Th17 cells (63). In the infection model of *T. gondii*, IL-27/IL-27R signaling impaired the development of effector function of Th17 cells (34). Similarly, IL-27 knock-out mice infected with *Listeria*

monocytogenes produced significantly higher levels of IL-17 and IL-22 (33). As compared to the inhibitory effects of IL-27 on the lineage commitment to and acquisition of Th17 cells, the effects of IL-27 on committed Th17 cells are controversial. This could be due to distinct characteristics of human and murine $CD4^+$ T cells.

The mechanism by which IL-27 suppresses Th17 cells occers in multiple ways. It was documented that IL-27 limits the expression of the transcriptional factors RORα and RORγt which are critical for Th17 lineage commitment (33, 60). Furthermore, for the inhibitory role of IL-27 in IL-17 production, it was found that IL-27 signaling activates STAT1 and occasionally STAT3 (34, 64) or promotes the production of IL-10 which re-shape the microenvironment to an anti-inflammatory status (65).

IL-27 and Regulatory T cells

In addition to antagonizing the development and functional acquisition of the distinct groups of T helper cells, IL-27 exerts inhibitory functions by inducing the development of regulatory cells which indirectly impairs the aggressive immune

responses. Regulatory T cells consist of Tr1 and conventional Treg, both of which are capable of producing IL-10 and exert immunosuppressive functions, while the former is in a FoxP3 negative phenotype which is a hall maker for the conventional Tregs (66).

Vijay K Kuchroo's lab did pioneering work on the regulation of Tr1 by IL-27 (36, 67, 68). It was reported that IL-27 can induce the differentiation of Tr1 both *in vitro* and strikingly in an *in vivo* EAE model. The underlying molecular basis of the regulation was found to be that IL-27/IL-27R signaling induces the expression of the transcription factor AhR which acts in synergy with c-Maf to transactivate the IL-10 promoter and switch conventional T cells into IL-10 producing Tr1 cells.

In contrast to Tr1, the impact of IL-27 on promoting Treg development and phenotype maintenance has been revealed by Booki Min's group in the past two years (69-71). It was reported that IL-27 displays an essential role in enhancing Treg function in models of T cell–induced colitis, graft-versus-host disease (GvHD), and EAE. In the setting of T cell-induced colitis, IL-27R deficient Tregs were less effective at limiting colitogenic T-cell expansion and inflammatory cytokine

expression compared to the WT Tregs. The molecular mechanism was that IL-27 induced the expression of Lag3, a negative regulator of inflammation, and enhanced the immune suppressive function in both the murine and human Tregs (70, 71). More striking data was obtained using Treg-specific IL27R$^{-/-}$ mice in an EAE model (69) in which it was found that IL-27 essentially targets FoxP3^{+} Tregs and resolves the autoimmune inflammation in the central nervous system (CNS). This was attributed to the fact that IL-27 is required to maintain the stability of FoxP3 expression which is critical to maintain the suppressive phenotype.

Immunoregulation of Myeloid cells by IL-27

DCs are the so-called "most potent" antigen presenting cells in priming T cells as well as balancing effector and regulatory T cells. Studies by different groups identified the immunosuppressive role of IL-27 by driving DCs into a tolerogenic phenotype (72, 73). DCs treated with IL-27 displayed lower expression of co-stimulatory molecules, including CD80, CD86, CD40 and MHCII, indicating a lesser potential of antigen presentation capability to activate T cells (72, 73). In addition, IL-27 biased DCs into an immunoregulatory phenotype with up-regulated IL-10 production while down-regulated IL-12 production (72). The immunoregulatory role

of IL-27 on DCs was confirmed through co-culturing naive $CD4^+$ T cells with DCs pre-treated with IL-27. It was documented that IL-27-conditioned DCs preferably directed naive $CD4^+$ T cells into Tr1 cells, but not Th1 or Th17 cells. Lastly, adoptive transfer of DCs conditioned with IL-27 elicited less pathogenic IFN-γ and IL-17 producing cells, coincided with increased production of immunomodulatory cytokines like IL-10 and TGF-β (72). In addition to the priming activity of DCs on $CD4^+$ T cells, IL-27R deficient DCs co-cultured with NK cells induced higher IFN-γ production and elevated expression of perforin and granzyme B by NK cells than that of co-cultured with wild-type DCs (73).

IL-27 was also found to manipulate hematopoiesis, a process giving rise to lymphocytes like $CD4^+$ T cells, and myeloid cells, like DC and monocytes. Transgenic mice expressing IL-27 displayed higher rates of myelopoiesis in the bone marrow and spleen (74). Development of monocytes and neutrophils during myelopoiesis is critical to cripple invading pathogens. More recently, in a setting of malaria infection model, as compared to the IL-27R deficient mice, mice with sufficient IL-27/IL-27R signaling developed increased proliferation of myeloid progenitor cells and enhanced generation of neutrophils which essentially controlled

the blood stage malaria infection (75). Overall, IL-27 is one of the finite factors that are able to orchestrate the hematopoiesis.

In addition to the enhanced generation of neutrophils by IL-27, more recently, IL-27 was found to polarize the neutrophils which had infiltrated into the intracerebral hemorrhage-injured brain from a phenotype of releasing inflammatory molecules into one producing beneficial molecules (76).

In summary, despite myeloid cells were the major cellular sources of IL-27, the effects of IL-27 in regulating the innate immunity in context of infections, cancers or autoimmune diseases are not well described.

African trypanosomes and host immune responses

African trypanosomes are extracellular protozoan parasites. These parasites cause debilitating diseases, namely Human African Trypanosomiasis (HAT) and Animal African Trypaonosomiasis (AAT). These diseases are predominantly restricted to the sub-Saharan Africa and are of great social and economic importance

for 37 countries with an area approaching 10 million km^2 affected (77, 78). HAT and AAT are transmitted by the tsetse fly (Glossina species) of which the habitat is coincided with the distribution of the diseases. Numerous strategies, including vector control or using trypanocidal drugs, have been implemented to control the diseases (79-81), but the efficacy is limited primarily due to the fact that the parasites co-evolved with the host and developed efficient immune evasion mechanisms to avoid completely elimination by orchestrating the host immune responses in many aspects (82-84). Next, I will overview the parasites, the diseases as well as the immune responses elicited upon infection.

African trypanosomes-The parasites

African trypanosomes are a group of protozoan parasites under the genus of *Trypanosoma*, consisting of *T. brucei*, *T. congolense*, *T. evansi*, and *T. vivax*. *T. brucei* contains three subspecies: *T. brucei brucei*, *T. brucei gambiense*, and *T. brucei rhodesiense*. This genus of parasites can infect either humans (causing HAT) or domestic or wild animals (causing AAT). By infecting laboratory animals, *T. brucei brucei* and *T. congolense* have been extensively studied in the model of Murine African Trypanosomiasis.

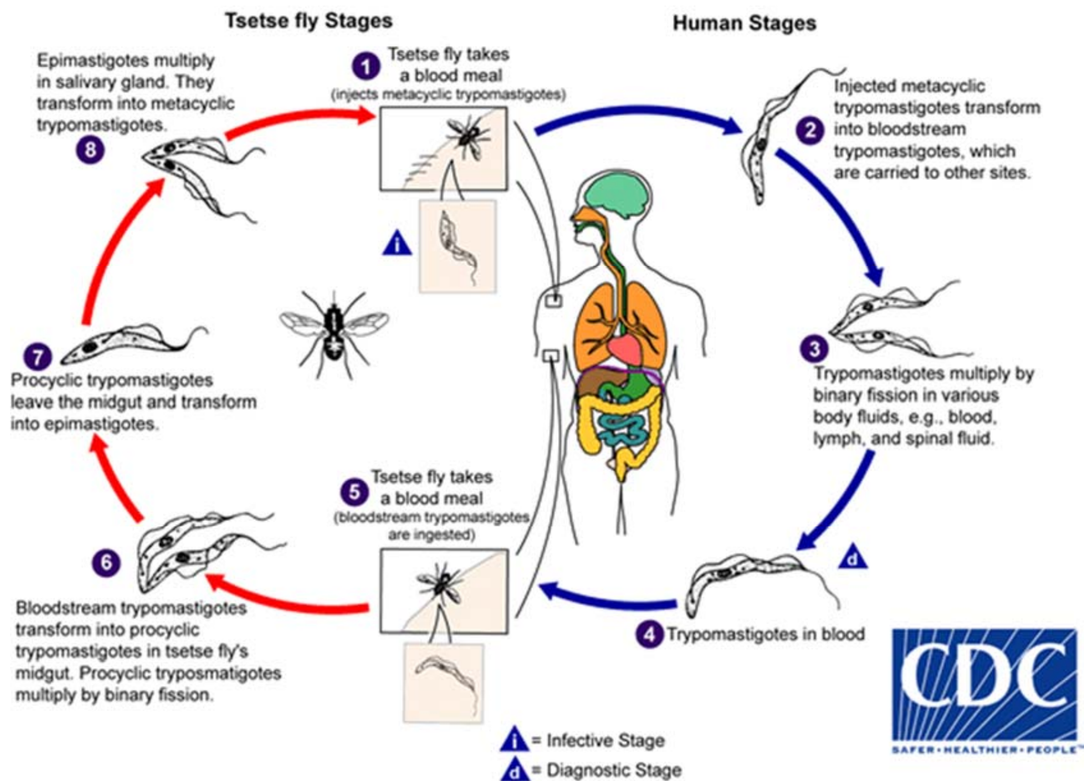

Fig. 1.3 The life cycle of African trypanosomes (CDC)

African trypanosomes have a sophisticated life cycle rotating between tsetse fly and the mammalian host (85, 86), during which the parasites experience critical transformations in terms of morphology, surface protein expression and even cell metabolism in order to survive in the different environments of distinct hosts (87). As shown in Figure 1.3, starting from a blood meal, the infected tsetse fly injects the metacyclic parasites into the mammalian host. Upon entering the lymphatic system and then passing into the blood, the parasites transform into the bloodstream

trypomastigotes which replicate by binary fission. The progenies in the bloodstream are infective and can be ingested during a blood meal by the Tsetse fly. The transformation from the trypomastigotes into the procyclic trypomastigotes takes place in tsetse fly's midgut where the procyclic parasites multiply by binary fission. The procyclic trypomastigotes will then leave the midgut and transform into the epimastigote forms which will eventually reach the salivary glands and continue the replication. Meanwhile, they transform into the metacyclic form and infectious to the mammalian hosts until a blood meal is made by the Tsetse fly. Of note, it takes about 3 weeks to complete the transformation from the bloodstream trypomatigotes to the metacyclic forms in the fly.

African trypanosomes are typically 8 to 50 µm in length and 2µm in diameter. As the typical eukaryotic cells, they have multiple organelles, namely the nucleus, mitochondria, endoplasmic reticulum, Golgi apparatus, and ribosomes. Additionally, they possess the kinetoplast, an unusual organelle, which is composed of copious circular DNA and serves as a single large mitochondrion to support cell metabolism. As mentioned above, during transformation, the surface protein expression of the

parasites is switched from procyclins, while in the midgut of the fly, to variant surface glycoproteins (VSG), while in the bloodstream of the mammalian host (88).

Of note, 10^7 copies of the VSG molecules are packed on the surface of the bloodstream parasites (89), and the VSG serves as a shielding molecule for the cell membrane of the parasites from host attacks. In addition, the genome of African trypanosomes contains more than 1000 different VSG genes per cell which is tightly regulated so that only a single VSG gene is expressed at a time (90). The sophisticatedly regulated expression of VSG gene then affords the parasite extensive means to evade the host defense and cause diseases in humans or animals.

African trypanosomiasis-The diseases

Human African Trypanosomiasis (HAT) or sleeping sickness is fatal if patients were left untreated. There are approximately 60 million people in rural areas of Africa under risk of infection by the *T. brucei gambiense* and *T. brucei rhodesiense* (91). Currently, around 10,000 cases are reported annually to the World Health Organization (91). The HAT caused by *T. b. gambiense* represents 98% of all the reported cases and typically appears in central Africa and occasionally in western

Africa. The rest 2% of HAT cases are caused by *T. b. rhodesiense* which generally causes an acute and lethal HAT and occurs mainly in eastern and southern Africa (92). The infections caused by these two subspecies share certain common characteristics in terms of disease progression stages, i.e. the early hemolymphatic stage and the later meningoencephalitic stage. As mentioned in the life cycle, the bloodstream parasites can survive in the peripheral blood well as in the lymphatic system or spinal fluids where they maintain the infectiousness to the vector. During this stage, the patients suffer from recurring fever which results from host responses to distinct waves of parasites expressing different VSGs in the bloodstream. Distinct from *T. congolense, T. brucei* species can penetrate into the interstitial space, which affords the parasites escape the peripheral immune factors and cause tissue damage. In the later meninggoencephalitic stage, the parasites pass through the blood-brain barrier and invade the central nervous system resulting in irreversible damages due to the uncontrolled parasite proliferation in the immune privileged environment (93). Infected individuals experience a disorganized and fragmented sleep-wake cycles. Most recently, Filipa Rijo-Ferreira and colleagues reported in Nature Communication that HAT is a circadian disorder revealed with the striking observations that after infection with *T. brucei*, the circadian clock is disrupted not only at the behavioral

level of the infected mice but also at the tissue and cellular level (94). This pioneering work introduced the circadian gene regulation, a research topic at the peak of interests, to these ancient infection diseases, and revealed the nature of the disorders.

Different from *T. brucei* species, *T. congolense*, as well as *T. vivax*, are strictly intravascular parasites and among the most important causative agents of the Animal African Trypanosomiasis (AAT) or Nagana disease (95). Infected animals experience periodic fever coinciding with parasitemia waves resulting from host and parasite interactions. Including *T. brucei* species, all the parasites in genus of *Trypanosoma* are infectious to the domestic or wild animals causing the AAT which results in around US$ 5 billion economic loss annually (96). Infected animals are unsuitable for economic use, such as for diary or meat or breeding, due to the severe illness. Instead, once infection is recognized, animals are subject to euthanasia to avoid serving as a reservoir for the infectious parasites.

To eliminate the infection, various strategies have been implemented, including vector control, trypanotolerant breeds or treating livestock with trypanocidal drugs. However, success has been limited due to the high cost to local

famers and governments (96). With limited investments and political issues, it is difficult as well to comprehensively introduce trypanoresistant economic animal to such a wide range of affected lands across 37 countries (96-98). Anti-trypanosomatid drug discovery is highly needed and remains challenging due to the fact that the parasites appear to become resistant (99). There is no efficient vaccine available to prevent either HAT or AAT currently. The reason is largely due to the antigenic variation of the variant surface glycoprotein (VSG) (100). The VSG are immunogenic and sophisticatedly regulated to escape the innate and adaptive immune responses by periodically expressing a distinct VSG molecule at a given time. In addition, as mentioned above, the VSG coat masks the cell membrane of the parasites leaving the conserved components inaccessible to the neutralizing antibodies.

Immune responses to African trypanosome infection

To better combat the infection, more efforts have been made to understand the immune biology of the parasite infection and the host-parasite interactions using murine models of infection. Of note, this genus of extracellular parasites also represents one of the most intriguing models to understand the immune system and the immune regulation.

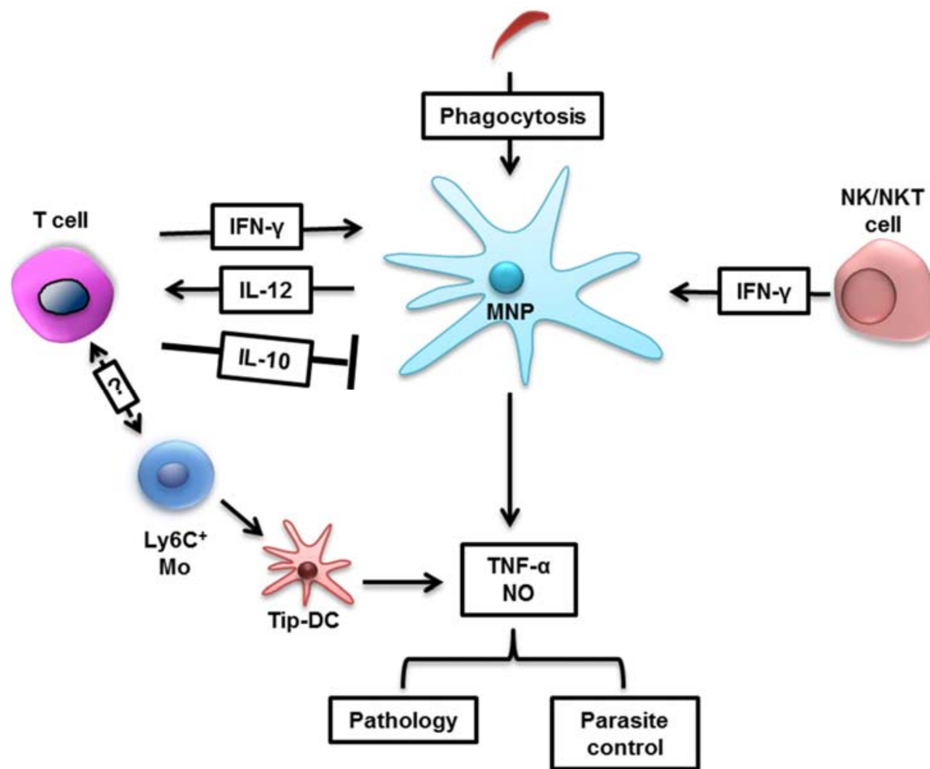

Fig. 1.4 Simplistic view of host immune responses to African trypanosomes infection

The extracellular parasites survive in the bloodstream by confronting the immune responses continuously. Liver has been recognized as the major organ to cripple the parasites through the phagocytosis by liver resident macrophages, Kupffer cells (101, 102). Depletion of Kupffer cells using clodronate liposomes, mice infected with *T. brucei brucei* or *T. congolense* display dramatically shortened survival with aggressive parasite growth compared with the control group (unpublished data), confirming an essential role of Kupffer cells in parasite clearance. The clearance of parasites by Kupffer cells is antibody dependent (103, 104). This was revealed by

infecting B cell deficient mice and IgM deficient mice. It was found that in *T. b. brucei* or *T. congolense* infected mice, B cells and IgG antibodies were crucial for limiting parasite growth, however, IgM antibodies played a marginal role in the infection. The role of complement system in phagocytosis remains poorly described. The membrane attack complex (MAC), as a result of host complement system activation, is directly formed on the surface of the invading pathogens causing complement mediated lysis. However, infected AKR mice which are genetically complement deficient, control the parasitemia as efficiently as the complement sufficient mice, indicating a redundant role of the direct complement lysis mediated by the MAC formation (105). As shown by Pan and colleagues, complement activation is required for engulfing the parasites for macrophages to engulf the parasite and complement receptor 3 (CR3) serves as a predominant receptor for complement mediated phagocytosis (106).

Upon engulfing the parasites, macrophages and myeloid lineage cells produce pro-inflammatory cytokines, such as TNF-α, nitric oxide (NO) and IL-12, and chemokines, like CCL2, which facilitate the parasite control and/or development of the innate and adaptive immunity (107-109). In addition to the antibody mediated

elimination of the parasites, TNF-α plays a significant role in limiting the parasite growth. Mice deficient in TNF-α or TNF-αR infected with either *T. brucei brucei* or *T. congolense* display impaired parasitemia control and early mortality (104, 110-113). TNF-α/TNF-αR signaling is required for the optimal production of NO in African trypanosomes infection (104). The essential role of TNF-α in parasite control was further attributed to the direct killing effects of NO on the parasites. This idea was demonstrated by using the iNOS knock-out mice bearing deficiency in NO production. iNOS deficient mice infected with *T. congolense* displayed a similar phenotype as the TNF-α or TNF-αR knock-out mice (104).

Besides TNF-α and NO, IL-12 plays an important role during infection with African trypanosomes and serves as an important immunomodulator for the Th1 development and the production of IFN-γ (114, 115). It was documented that mice with genetic deficiency in IL-12 experienced abolished production of IFN-γ in both *T. brucei brucei* and *T. congolense* infections. However, the phenotypes of mice survival and parasite control are completely different with IL-12 knock-out mice infected with *T. congolense* surviving longer while *T. brucei brucei* infected mice survive significantly shorter coincided with aberrantly persistent parasitemia. These results

are expected for *T. brucei brucei* infection, since IFN-γ is required for priming macrophages to produce the TNF-α and downstream NO (104, 115). Contrary to expectations, in *T. congolense* infection, excessive production of IFN-γ results in severe immunopathology and induces early mortality (114). Hence, the optimal inflammatory responses are essential for parasite growth control with minimal pathology. This was further supported by the fact that IFN-γ knock-out mice infected with African trypanosomes failed to control the parasitemia and survived significantly shorter (104, 116). More recently, using IFN-γ reporter mice, NK cells and NK T cells were recognized as the early producers of IFN-γ, which depicts a whole picture of the cellular source of IFN-γ by marrying the previous debates for the relative contributions of $CD4^+$ and $CD8^+$ T cells (117, 118).

Similar to IFN-γ, excessive production of TNF-α and iNOS can also result in tissue damage. Recently, TNF-α and iNOS producing dendritic cells, also referred as Tip-DCs, were recognized as the major cellular source of the two inflammatory molecules (109, 119). CCR2 knock-out mice experiencing impaired development of Tip-DC survived significantly longer than WT mice during infection with *T. brucei brucei* (109). The extended survival was coincided with less liver pathology,

indicating Tip-DCs are detrimental to the host if their development was improperly regulated. One potential molecule capable of regulating inflammatory responses is IL-10, a regulatory cytokine that antagonizes the production of IFN-γ and the development of the Tip-DC (109, 116, 120, 121).

Goals of the Dissertation

We believe that putting deeper insight into the immunological interface between African trypanosome and the host enables us to discover the potential novel targets and tools for more effective control and prevention of African trypanosomiasis. Interleukin (IL)-27 has been recognized as a pro- and anti-inflammatory cytokine by modulating a list of cells belonging to innate and adaptive immunity. Given the fact that various types of cells and immune factors determine the parasite control and the outcome of the disease, we aim to elucidate the potential immunoregulatory effects of IL-27 on host immune responses in the setting of African trypanosome infection. Notions and ideas obtained in this dissertation are expected to provide novel mechanisms to immunoregulation of the African trypanosome infection, but also to gain new insights into the function of IL-27.

CHAPTER 2: IL-27 SIGNALING IS CRUCIAL FOR SURVIVAL OF MICE INFECTED WITH AFRICAN TRYPANOSOMES VIA PREVENTING LETHAL EFFECTS OF CD4$^+$ T CELLS AND IFN-Γ

Abstract

African trypanosomes are extracellular protozoan parasites causing a chronic debilitating disease associated with a persistent inflammatory response. Maintaining the balance of the inflammatory response via downregulation of activation of M1-type myeloid cells was previously shown to be crucial to allow prolonged survival. Here we demonstrate that infection with African trypanosomes of IL-27 receptor-deficient (IL-27R$^{-/-}$) mice results in severe liver immunopathology and dramatically reduced survival as compared to wild-type mice. This coincides with the development of an exacerbated Th1-mediated immune response with overactivation of CD4$^+$ T cells and strongly enhanced production of inflammatory cytokines including IFN-γ. What is important is that IL-10 production was not impaired in infected IL-27R$^{-/-}$ mice. Depletion of CD4$^+$ T cells in infected IL-27R$^{-/-}$ mice resulted in a dramatically reduced production of IFN-γ, preventing the early mortality of infected IL-27R$^{-/-}$ mice.

This was accompanied by a significantly reduced inflammatory response and a major amelioration of liver pathology. These results could be mimicked by treating IL-27R$^{-/-}$ mice with a neutralizing anti-IFN-γ antibody. Thus, our data identify IL-27 signaling as a novel pathway to prevent early mortality via inhibiting hyperactivation of CD4^{+} Th1 cells and their excessive secretion of IFN-γ during infection with African trypanosomes. These data are the first to demonstrate the essential role of IL-27 signaling in regulating immune responses to extracellular protozoan infections.

Author Summary

Infection with extracellular protozoan parasites, African trypanosomes, is characterized by a persistent inflammatory immune response. It has been recently shown that maintaining the balance of the inflammatory responses via dampening M1-type myeloid cell activation is critical to guarantee control of the parasites and survival of the host. In this study, we demonstrated that IL-27 receptor-deficient (IL-27R$^{-/-}$) mice infected with African trypanosomes developed an excessive inflammatory response and severe liver immunopathology, resulting in dramatically reduced survival, as compared to infected wild-type mice. The early mortality of infected IL-27R$^{-/-}$ mice was correlated with significantly elevated secretions of

inflammatory cytokines, particularly IFN-γ, and enhanced activation of $CD4^+$ Th1 cells. Importantly, IL-10 production was not impaired in infected IL-27$R^{-/-}$ mice. Either depletion of $CD4^+$ T cells, resulting in a dramatically reduced secretion of IFN-γ, or neutralization of IFN-γ, prevented the early mortality of infected IL-27$R^{-/-}$ mice with a significantly reduced inflammatory response and a major amelioration of the liver pathology. Thus, our data identify IL-27 signaling as a novel pathway to prevent the early mortality via inhibiting hyperactivation of $CD4^+$ Th1 cells and their excessive secretions of IFN-γ during experimental infection with extracellular protozoan parasites African trypanosomes.

Introduction

African trypanosomiasis is a vector-borne parasitic disease of medical and veterinary importance. It is estimated that 170,000 people contract the disease every year, and that approximately 70 million people mainly in sub-Saharan Africa are at the risk of contracting the disease (122, 123). In addition, this disease severely limits the agricultural development by affecting domestic animals in the area (123). The causative agents of this disease are various species of genus of *Trypanosoma*, which are extracellular protozoan parasites equipped with a flagellum that emerges from the

flagellar pocket and provides the parasite with its motility (123). Upon the bite of the mammalian host by a trypanosome-infected tsetse fly, the parasites enter the blood circulation via lymph vessels and can multiply in the bloodstream and interstitial fluids of the host (83, 124). The parasites have evolved very sophisticated evasion mechanisms to survive in the chronically infected host (83, 124, 125), causing a serious disease that is often fatal without treatment (122, 123).

Due to practical and ethical reasons, mouse models have become an alternative and proven to be a cornerstone for studying African trypanosomiasis of humans and domestic animals (126). Most of studies have been performed with *T. brucei* and *T. congolense* parasites (124, 126). Based on mouse models, although the parasites circulate in the blood stream, the liver is the major place for clearance of the parasites (127-129). Recent studies demonstrated that Kupffer cells efficiently engulf trypanosomes, which is mediated by both IgM and IgG antibodies specific to the parasites (102, 130, 131). IFN-γ, mainly secreted by VSG-specific $CD4^+$ T cells (132-134) following activation by dendritic cells (135, 136), has been shown to mediate protection during African trypanosomiasis (132, 134, 137-139). Proinflammatory cytokines such as IL-12, TNF-α, as well as iNOS produced by M1-type myeloid cells

are also critical for host resistance to African trypanosomes (134, 140-144). However, excessive secretions of these inflammatory cytokines by hyperactivated myeloid cells and T cells lead to liver pathology and shorten the survival of infected mice (131, 141, 145-148). In this respect, IL-10 has been found to be essential for maintenance of the immunological balance between protective and pathological immune responses during African trypanosomiasis (131, 139, 141, 145, 146). Importantly, the role of IL-10 as an anti-inflammatory agent has been more recently confirmed in cattle, primate and human infections with African trypanosomes (149-151). It remains unknown whether, in addition to IL-10 signaling, another pathway that maintains this immunological balance exists.

IL-27, a recently identified cytokine produced primarily by macrophages and dendritic cells, is a member of the IL-12 super-family (152). The IL-27 receptor (IL-27R) complex consists of the specific IL-27Rα subunit (WSX-1) and the IL-6R subunit (gp130), and is expressed on numerous subsets of leukocytes including $CD4^+$ T cells, $CD8^+$ T cells, NK cells, monocytes, Langerhans cells, and dendritic cells (153). Earlier studies have demonstrated that IL-27, as a proinflammatory cytokine, drives naïve T cells to differentiate into Th1 cells (26, 154, 155). More recent studies

have suggested that IL-27 also has the function to inhibit immunopathology via downregulation of active $CD4^+$ T cells during infections, particularly with intracellular protozoan parasites (156-160). However, the precise mechanism of $CD4^+$ T cell-mediated immunopathogenesis in the absence of IL-27 signaling still remains incompletely understood. In addition, it is not clear so far whether IL-27 plays an important role in regulation of the immune responses during infections with extracellular protozoan parasites such as African trypanosomes. Based on previous data showing that a subset of highly activated pathological $CD4^+$ T cells produces excessive IFN-γ, and leads to immunopathology and early mortality of mice infected with *T. congolense* (131, 147, 148), we formulate a hypothesis that IL-27 signaling is, besides IL-10 signaling, another novel pathway that prevents the immunopathology and early mortality via down-regulation of the hyperactivity of $CD4^+$ T cells and their excessive secretion of IFN-γ during experimental Africa trypanosomiasis. With this in mind, we examine in this study how IL-27 signaling regulates the immune responses in mice infected with African trypanosomes.

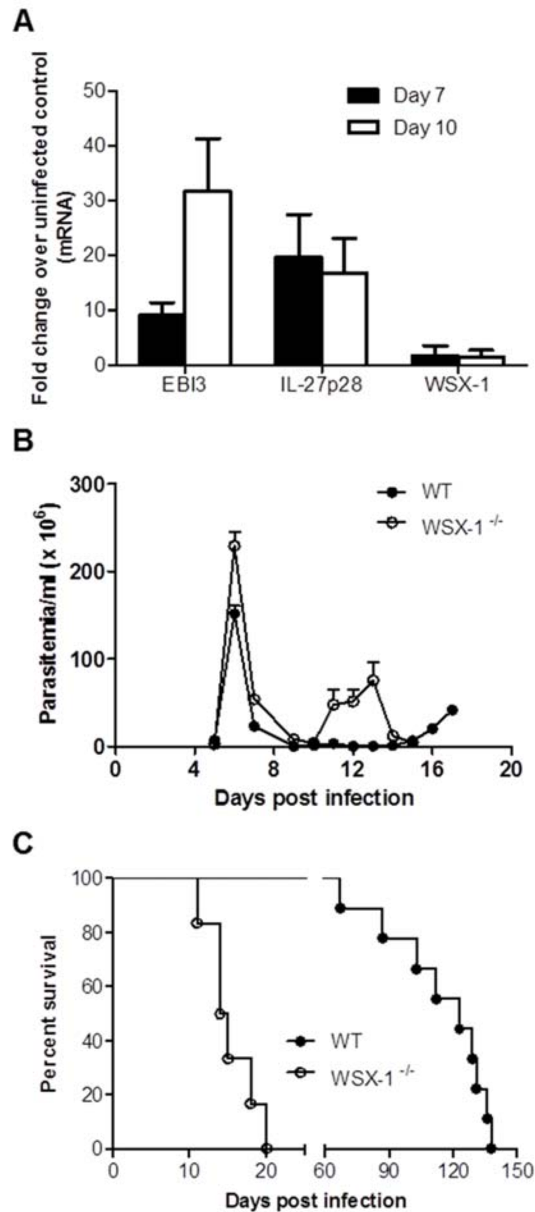

Fig. 2.1 Enhanced expression of IL-27 and its crucial role in survival of mice infected with *T. congolense*.

(A) mRNA expression levels of IL-27p28, EBI3 and WSX-1 in the liver of wild-type mice infected with *T. congolense* on day 7 and 10 versus day 0 (uninfected). (B) Parasitemia of IL-27R$^{-/-}$ (WSX-1$^{-/-}$) and wild-type mice (n=6-9) infected with *T. congolense*. (C) Survival of IL-27R$^{-/-}$ and wild-type mice (n=6-9) infected with *T. congolense*. Data are presented as the mean ± SEM. The results presented are representative of 3 separate experiments.

Results

1. IL-27 signaling is crucial for survival of mice infected with *T. congolense*

To evaluate the role of IL-27 signaling during African trypanosomiasis, we first determined whether infection led to increased expression of this cytokine or its receptor. Wild-type C57BL/6 mice were infected with *T. congolense*, a species of African trypanosomes which are unable to leave the circulation and only live in blood vessels, causing fatal disease in cattle (83). The mice were euthanized at day 0, 7, and 10 after infection, as parasitemia usually peaked on day 6-7 after infection (134, 148). As the liver is the major organ for clearance of the parasites (127-129, 131), the liver was collected for measurement of mRNA levels of IL-27 and its receptor using real-time quantitative RT-PCR. mRNA levels of both subunits of IL-27 (IL-27p28 and EBI3) were upregulated in the liver of mice at day 7 and 10 after infection, compared to uninfected mice (Fig. 2.1A). In contrast, mRNA levels of IL-27 receptor (WSX-1) were not affected by the infection (Fig. 2.1A).

Next, we infected IL-27R$^{-/-}$ (WSX-1$^{-/-}$) and wild-type mice with *T. congolense* to assess whether IL-27 signaling affected the disease progression. Similar to infected

wild-type mice, infected IL-27R$^{-/-}$ mice could control the first wave of parasitemia (Fig. 2.1B). However, IL-27R$^{-/-}$ mice succumbed to the infection on day 12 to 20 after infection with a mean survival time of 14.5 days (Fig. 2.1C). In contrast, infected wild-type mice survived until day 67 to 138 days after infection with a mean survival time of 123 days (Fig. 2.1C). Compared to infected wild-type mice, the infected IL-27R$^{-/-}$ mice survived significantly shorter ($p<0.01$). These data demonstrated that IL-27 signaling is required for survival of mice infected with *T. congolense*.

2. Deficiency of IL-27 signaling results in enhanced systemic inflammatory responses in mice infected with *T. congolense*

The above results demonstrated that absence of IL-27 signaling led to earlier mortality of mice infected with African trypanosomes. As uncontrolled inflammation causes early mortality of mice infected with African trypanosomes (83, 124), we next examined the plasma levels of inflammatory cytokines and their secretions by cultured spleen cells. As shown in Fig. 2.2A, significantly higher amounts of IFN-γ, IL-12p40, and TNF-α were detected in the plasma of IL-27R$^{-/-}$ mice infected with *T. congolense*, compared to infected wild-type mice, on day 7 and 10 after infection

(p<0.01). Although the plasma level of IFN-γ in IL-27R$^{-/-}$ mice decreased on day 10 after infection probably due to clearance of the first wave of parasitemia, it was still significantly higher than that of the infected wild-type mice (p<0.01, Fig. 2.2A).

To evaluate the secretions of cytokines by spleen cells, spleen cells were collected from IL-27R$^{-/-}$ and wild-type mice on day 7 and 10 after infection with *T. congolense*, and cultured *in vitro* for 48 h. The production of IFN-γ, IL-12p40, and TNF-α by spleen cells were significantly elevated in infected IL-27R$^{-/-}$ mice, compared to infected wild-type mice (p<0.01 or <0.05, Fig. 2.2B). As recent studies have shown that IL-27 mainly regulates CD4^{+} T cell activation during infection with intracellular pathogens (156-160), we further evaluated IFN-γ-producing CD4^{+} T cells in the spleen cultures using flow cytometry. A limited and similar percentage and absolute number of CD4^{+} T cells from uninfected wild-type and IL-27R$^{-/-}$ mice produced IFN-γ after 12 h stimulation with Cell Stimulation Cocktail (containing PMA, ionomycin, and protein transport inhibitors). However, by 7 and 10 days post infection both the percentage and the absolute number of IFN-producing CD4^{+} T cells were significantly enhanced in IL-27R$^{-/-}$ mice when compared to wild-type cohorts (Fig. 2.2C).

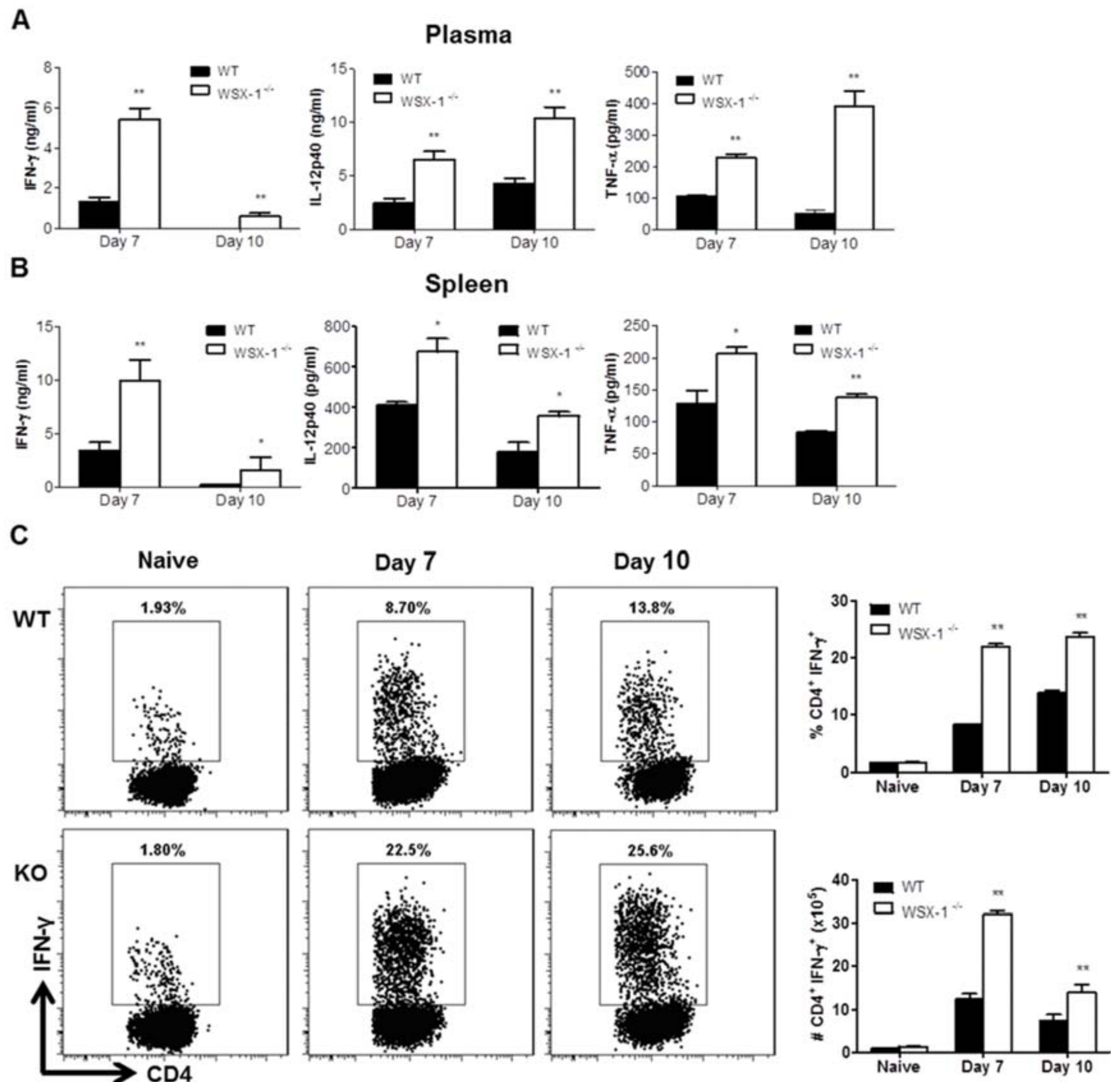

Fig. 2.2 IL-27 signaling suppresses systemic inflammatory responses in mice infected with *T. congolense*.

(A) Plasma levels of IFN-γ, IL-12p40, and TNF-α in IL-27R$^{-/-}$ (WSX-1$^{-/-}$) and wild-type mice (n=4) on day 7 and 10 after infection with *T. congolense*. (B) Secretions of IFN-γ, IL-12p40 and TNF-α in the supernatant fluids of cultured spleen cells purified from IL-27R$^{-/-}$ and wild-type mice (n=4) on day 7 and 10 following infection with *T. congolense*. (C) The frequency (left and upper right) and the absolute number (lower right) of splenic IFN-γ-producing CD4^{+} T cells derived from IL-27R$^{-/-}$ and wild-type mice (n=3) on day 0, 7 and 10 after infection following 12 h *in vitro* restimulation with Cell Stimulation Cocktail (containing PMA, ionomycin, and protein transport inhibitors). Data are presented as the mean ± SEM. The results presented are representative of 2-3 separate experiments.

3. IL-27R$^{-/-}$ mice develop severe liver pathology during infection with *T. congolense*

We and others have previously shown that excessive systemic inflammatory responses of mice infected with African trypanosomes are associated with severe liver damage (131, 141, 161, 162). In addition, the liver is the primary organ of trypanosome clearance (127, 129, 131). Therefore, we next evaluated effects of IL-27 signaling on liver pathology during the course of infection with the parasites. IL-27R$^{-/-}$ mice, but not wild-type mice, showed extensive pale geographic areas highly suggestive of necrosis on day 10 after infection with *T. congolense* (Fig. 2.3A). Microscopic examination of the liver of infected IL-27R$^{-/-}$ mice revealed many large areas with loss of hepatocyte cellular architecture and an infiltration of inflammatory cells (Fig. 2.3B). By contrast, these pathological changes were not observed in the liver of infected wild-type mice (Fig. 2.3B). To further characterize the liver pathology, we measured the serum activities of alanine aminotransferase (ALT) of mice during *T. congolense* infection. As shown in Figure 2.3C, IL-27R$^{-/-}$ mice had significantly higher serum activities of ALT than wild-type mice on both day 7 and day 10 after infection ($p<0.05$), indicating death of hepatocytes and release of cytosolic enzymes. These results demonstrated that IL-27 signaling played a major

role in prevention of the liver pathology that was associated with enhanced systemic inflammatory responses.

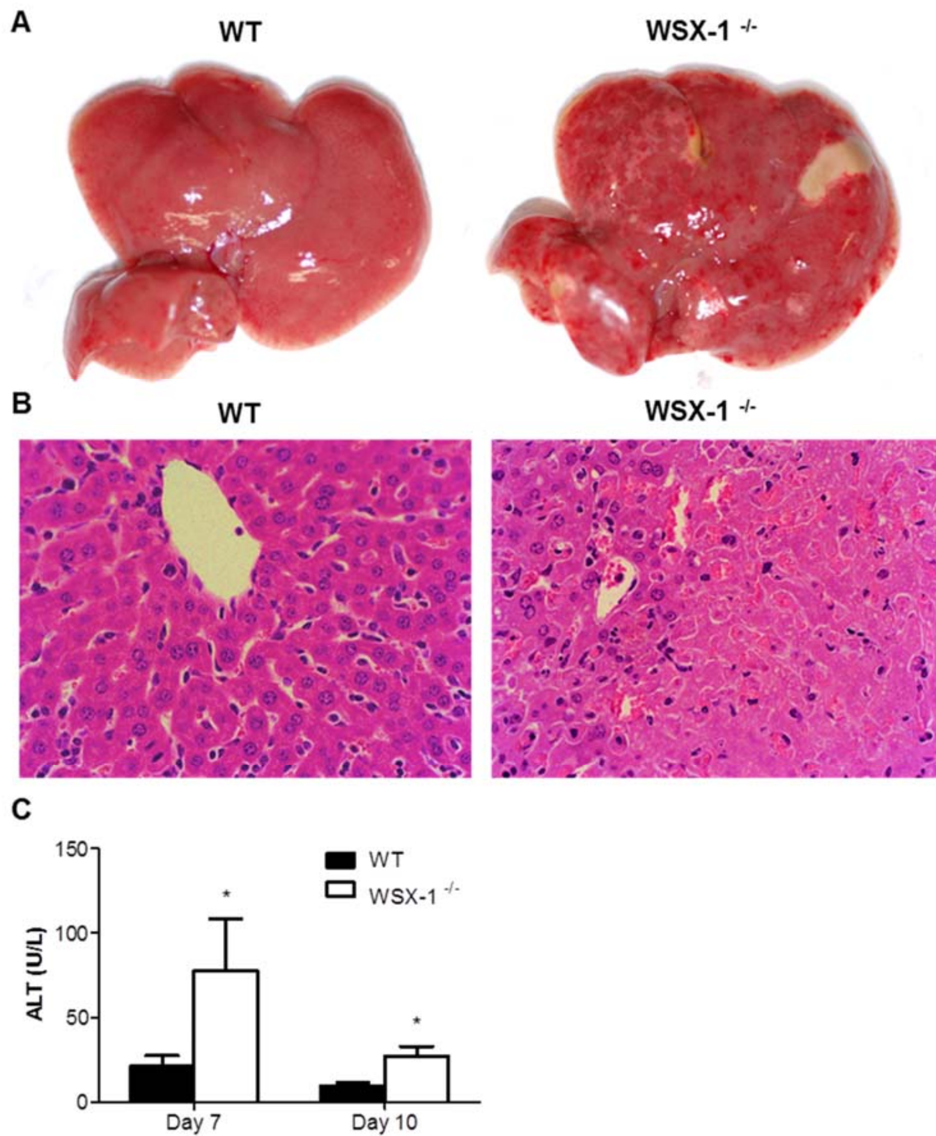

Fig. 2.3 IL-27 signaling is required to prevent liver immunopathology during infection with *T. congolense*.

(A) Macroscopic examination of liver on day 10 after infection with *T. congolense* revealed the presence of extensive pale geographic areas in IL-27R$^{-/-}$ (WSX-1$^{-/-}$), but not wild-type mice (n=4). (B) Hematoxylin and eosin staining showing loss of hepatocyte cellular architecture in the liver of IL-27R$^{-/-}$, but not wild-type mice (n=4) on day 10 after infection with *T. congolense* (original magnification ×40). (C) Serum ALT activities were assessed in IL-27R$^{-/-}$ and wild-type mice (n=4) on day 7 and 10 after infection with *T. congolense*. Data are presented as the mean ± SEM. The results presented are representative of 2 separate experiments.

4. Early mortality of IL-27R$^{-/-}$ mice infected with *T. congolense* is not due to impaired IL-10 production

It has been shown that IL-10 is crucial for survival of mice infected with African trypanosomes through limiting inflammation (131, 139). In particular, failure to control inflammatory responses in mice infected with African trypanosomes in the absence of IL-10 signaling is associated with severe liver pathology (131, 141, 146). In this regard, IL-27 has been shown to drive CD4^{+} T cells to produce IL-10 for downregulation of inflammation (163-165). The similarity of the cytokine profile and liver pathology of infected mice in the absence of IL-27 signaling and IL-10 signaling (131, 139) prompted us to examine whether IL-27 signaling prevented early mortality of mice infected with African trypanosomes via IL-10. We first compared the disease progression in the absence of IL-27 signaling with that in the absence of IL-10 signaling. *T. congolense*-infected IL-27R$^{-/-}$ mice and wild-type mice showed similar parasitemia and a significantly reduced survival after administration of anti-IL-10 receptor (IL-10R) mAb ($p<0.01$, Fig. 2.4A). Strikingly, infected wild-type mice treated with anti-IL-10R mAb survived significantly shorter than infected IL-27R$^{-/-}$ mice ($p<0.01$, Fig 2.4A), suggesting that IL-27 and IL-10 may independently regulate inflammatory responses during African trypanosomiasis. Next we compared the IL-

10 levels in plasma, and supernatant fluids of cultured spleen cells or liver leukocytes between IL-27R$^{-/-}$ and wild-type mice infected with *T. congolense*. There was no significant difference in IL-10 production in plasma and supernatant fluids of the cultures between IL-27R$^{-/-}$ and wild-type mice on day 7 after infection (Fig. 2.4B). Surprisingly, IL-27R$^{-/-}$ mice even showed significantly higher amounts of IL-10 in both plasma (up to 14 folds) and supernatant fluids of cultured spleen cells or liver leukocytes on day 10 after infection ($p<0.01$ or <0.05, Fig. 2.4B), demonstrating that secretion of IL-10 was strengthened, rather than impaired in IL-27R$^{-/-}$ mice infected with African trypanosomes, probably due to deficiency of the immune regulation mediated by IL-27 signaling in those infected IL-27R$^{-/-}$ mice. Taken together, these data suggested that early mortality of IL-27R$^{-/-}$ mice infected with African trypanosomes was not due to impaired IL-10 production.

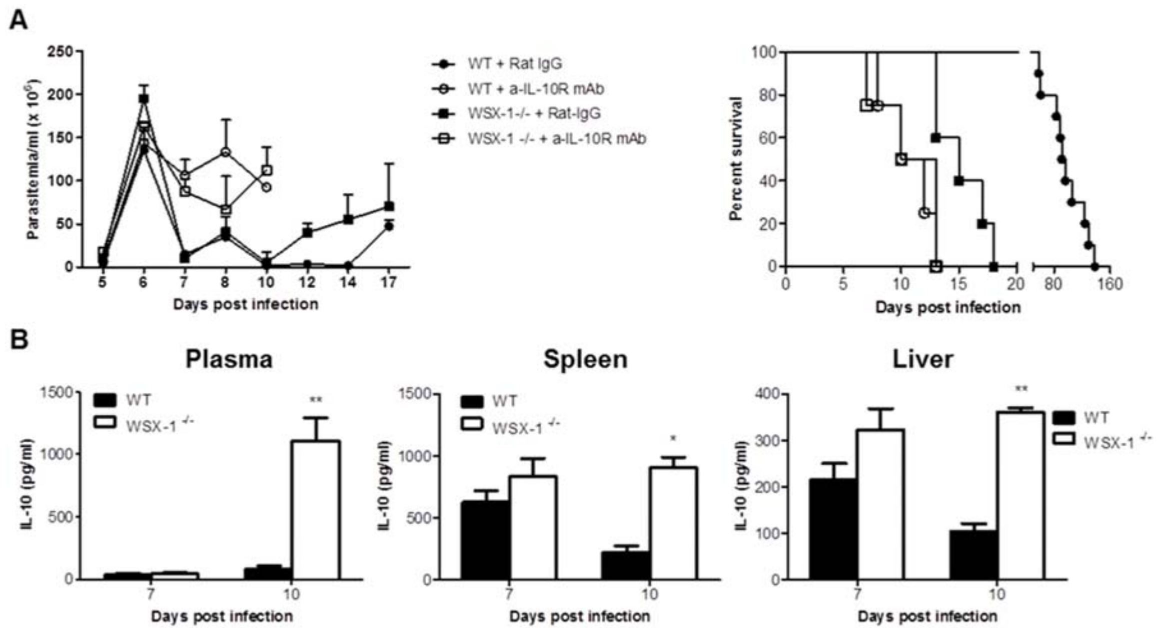

Fig. 2.4 IL-10 production is not impaired in IL-27R$^{-/-}$ (WSX-1$^{-/-}$) mice infected with *T. congolense*.

(A) Parasitemia and survival of IL-27R$^{-/-}$ and wild-type mice (n=4-10) treated with 0.4 mg anti-IL-10R mAb or rat IgG on day 0, 2, 4, and 6 after infection with *T. congolense*, respectively. (B) IL-10 levels in plasma, and supernatant fluids of cultured spleen cells, and liver leukocytes from IL-27R$^{-/-}$ and wild-type mice (n=4) on day 7 and 10 after infection with *T. congolense*. Data are presented as the mean ± SEM. The results presented are representative of 2-3 separate experiments.

5. Enhanced CD4$^+$ T cell responses and elevated secretions of inflammatory cytokines in the liver of IL-27R$^{-/-}$ mice infected with *T. congolense*

Because early mortality of IL-27R$^{-/-}$ mice infected with African trypanosomes was associated with severe liver pathology without impaired secretion of IL-10 as shown above and because IL-27 has been shown to mainly regulate T cell, particularly CD4$^+$ T cell activation during infection with intracellular pathogens (156-160), we next characterized CD4$^+$ T cell responses in the liver of IL-27R$^{-/-}$ mice during infection with *T. congolense*. We found that the frequency and the absolute number of activated hepatic CD4$^+$ T cells (CD44^{hi}CD62L^{low}) were significantly higher in IL-27R$^{-/-}$ mice infected with *T. congolense*, compared to infected wild-type mice ($p<0.01$, Fig. 2.5A). The production of IFN-γ, IL-12p40, and TNF-α by cultured liver leukocytes from infected IL-27R$^{-/-}$ mice was significantly higher than production of these cytokines by liver leukocytes from infected wild-type mice ($p<0.001$, <0.01 or <0.05, Fig. 2.5B). In particular, the production of IFN-γ was enhanced by 4-8 folds in the liver leukocyte cultures of infected IL-27R$^{-/-}$ mice (Fig. 2.5B). Thus, we further evaluated the activation of liver CD4$^+$ T cells by examining their secretions of IFN-γ using single cell analysis. A small and similar percentage and absolute number of CD4$^+$ T cells from uninfected wild-type and IL-27R$^{-/-}$ mice secreted IFN-γ after 12 h

stimulation with Cell Stimulation Cocktail (containing PMA, ionomycin, and protein transport inhibitors). In contrast, by day 7 and 10 post infection significantly higher percentage and absolute number of IFN-γ-producing $CD4^+$ T cells were detected in $IL\text{-}27R^{-/-}$ mice as compared to wild-type cohorts (Fig. 2.5C). Collectively, these data suggested that the early mortality of $IL\text{-}27R^{-/-}$ mice infected with African trypanosomes was associated with exacerbated Th1-mediated immune responses with overactivation of $CD4^+$ T cells.

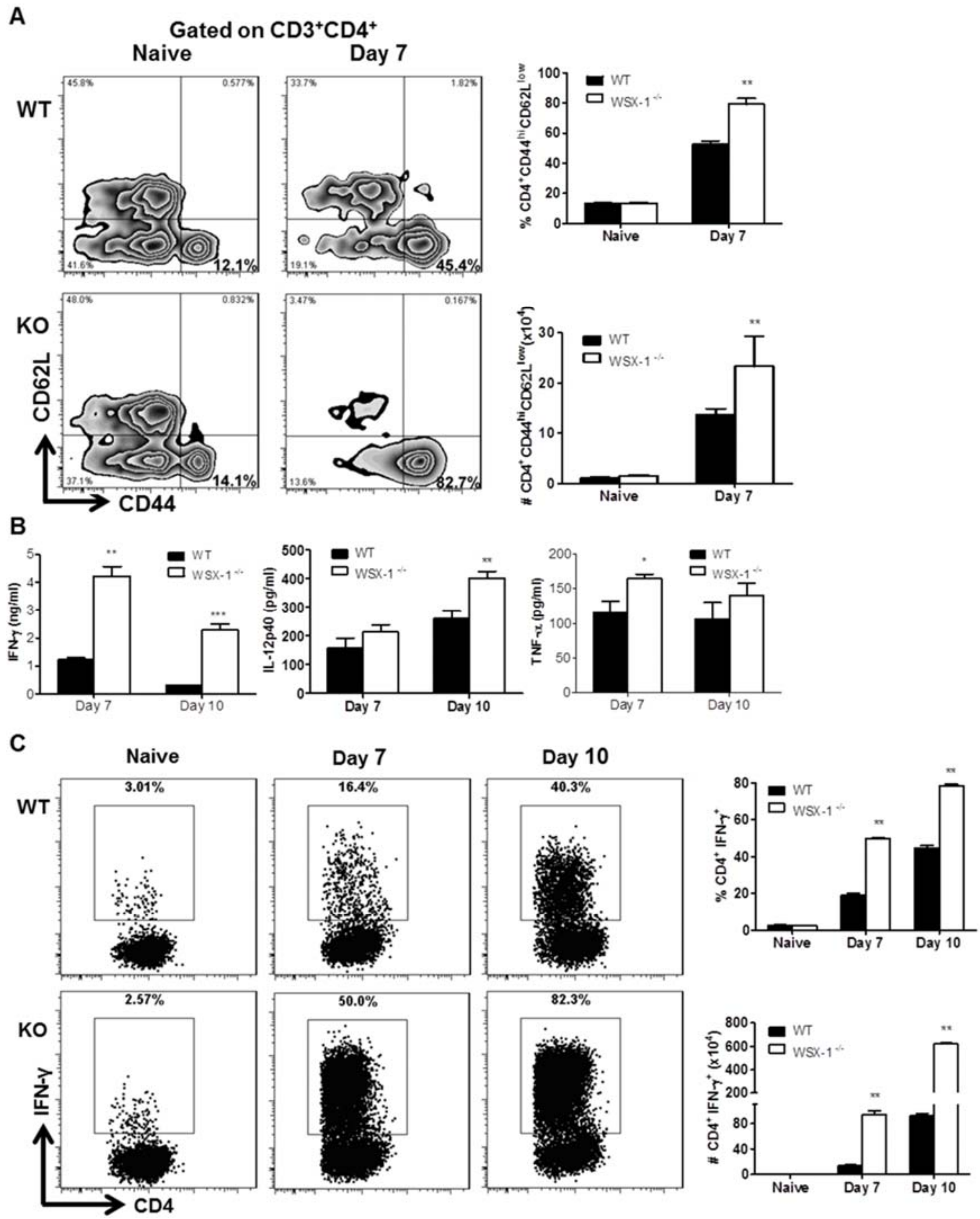
A
Gated on CD3+CD4+
Naive
Day 7
WT
KO
CD62L
CD44
12.1%
45.4%
14.1%
82.7%
WT
WSX-1 -/-
% CD4+CD44hiCD62L low
# CD4+CD44hiCD62Llow (x10^4)
Naive
Day 7
B
IFN-γ (ng/ml)
IL-12p40 (pg/ml)
TNF-α (pg/ml)
Day 7
Day 10
C
Naive
Day 7
Day 10
3.01%
16.4%
40.3%
2.57%
50.0%
82.3%
IFN-γ
CD4
% CD4+ IFN-γ+
# CD4+ IFN-γ+ (x10^4)

Fig. 2.5 Enhanced activation of $CD4^+$ T cells and elevated production of inflammatory cytokines in the liver of $IL\text{-}27R^{-/-}$ ($WSX\text{-}1^{-/-}$) mice infected with *T. congolense*.

(A) The frequency (left and upper right) and the absolute number (lower right) of activated $CD4^+$ T cells ($CD44^{hi}CD62L^{low}$) derived from the liver of $IL\text{-}27R^{-/-}$ and wild-type mice (n=3) on day 0 and 7 after infection with *T. congolense*. (B) Production of IFN-γ, IL-12p40, and TNF-α in the supernatant fluids of cultured liver leukocytes purified from $IL\text{-}27R^{-/-}$ and wild-type mice (n=4) on day 7 and 10 after infection with *T. congolense*. (C) The frequency (left and upper right) and the absolute number (lower right) of IFN-γ-producing $CD4^+$ T cells derived from the liver of $IL\text{-}27R^{-/-}$ and wild-type mice (n=3) on day 0, 7 and 10 after infection with *T. congolense* following 12 h *in vitro* restimulation with Cell Stimulation Cocktail (containing PMA, ionomycin, and protein transport inhibitors). Data are presented as the mean ± SEM. The results presented are representative of 2-3 separate experiments.

6. $CD4^+$, but not $CD8^+$, T cells mediate the early mortality of IL-$27R^{-/-}$ mice infected with *T. congolense*

As shown above, $CD4^+$ T cells were excessively activated in the liver of IL-$27R^{-/-}$ mice infected with African trypanosomes, raising the possibility that the early mortality of infected IL-$27R^{-/-}$ mice was a consequence of a $CD4^+$ T cell-dependent immune-mediated pathology. To test this, IL-$27R^{-/-}$ mice infected with *T. congolense* were treated with depleting anti-mouse CD4 mAb, anti-mouse CD8 mAb, or rat IgG as control; and the course of infection, immune responses, and severity of liver damage were assessed. As shown in Figure S2.1, administration of the antibodies efficiently depleted $CD4^+$ T cells or $CD8^+$ T cells in the spleen and liver of the infected mice. Infected mice from all three groups could effectively control the first wave of parasitemia, although depletion of $CD4^+$ T cells resulted in a significantly higher parasitemia at some time points of infection ($p<0.01$ or <0.05, Fig. 2.6A). Strikingly, infected IL-$27R^{-/-}$ mice treated with anti-CD4 mAb had two fold increase of survival compared to infected IL-$27R^{-/-}$ mice treated with rat IgG ($p<0.01$, Fig. 2.6A). In contrast, depletion of $CD8^+$ T cells did not affect the survival of infected IL-$27R^{-/-}$ mice (Fig. 2.6A). These results demonstrated that IL-27 signaling had a crucial

role in dampening $CD4^+$ T cell activation in experimental *T. congolense* infection in mice, allowing for prolonged survival.

We next evaluated the effect of $CD4^+$ T cells on weight loss and liver pathology of IL-27R$^{-/-}$ mice infected with *T. congolense*. Infected IL-27R$^{-/-}$ mice treated with anti-CD4 mAb had significantly less weight loss at the later stage of infection, compared to infected IL-27R$^{-/-}$ mice treated with rat IgG or anti-CD8 mAb ($p<0.01$; S2.2A Fig). Importantly, infected IL-27R$^{-/-}$ mice treated with rat IgG or anti-CD8 mAb exhibited many large areas with loss of hepatocyte cellular architecture in the liver, whereas these pathological changes were hardly seen in the liver of infected IL-27R$^{-/-}$ mice treated with anti-CD4 mAb (S2.2B Fig). In addition, depletion of $CD4^+$, but not $CD8^+$, T cells significantly reduced the serum activities of ALT in IL-27R$^{-/-}$ mice infected with *T. congolense* ($p<0.05$, Fig. 2.6B). These data suggested that $CD4^+$ T cells played a central role in the development of liver pathology in experimental *T. congolense* infection, and that IL-27 was crucial for dampening this $CD4^+$ T cell-mediated pathology.

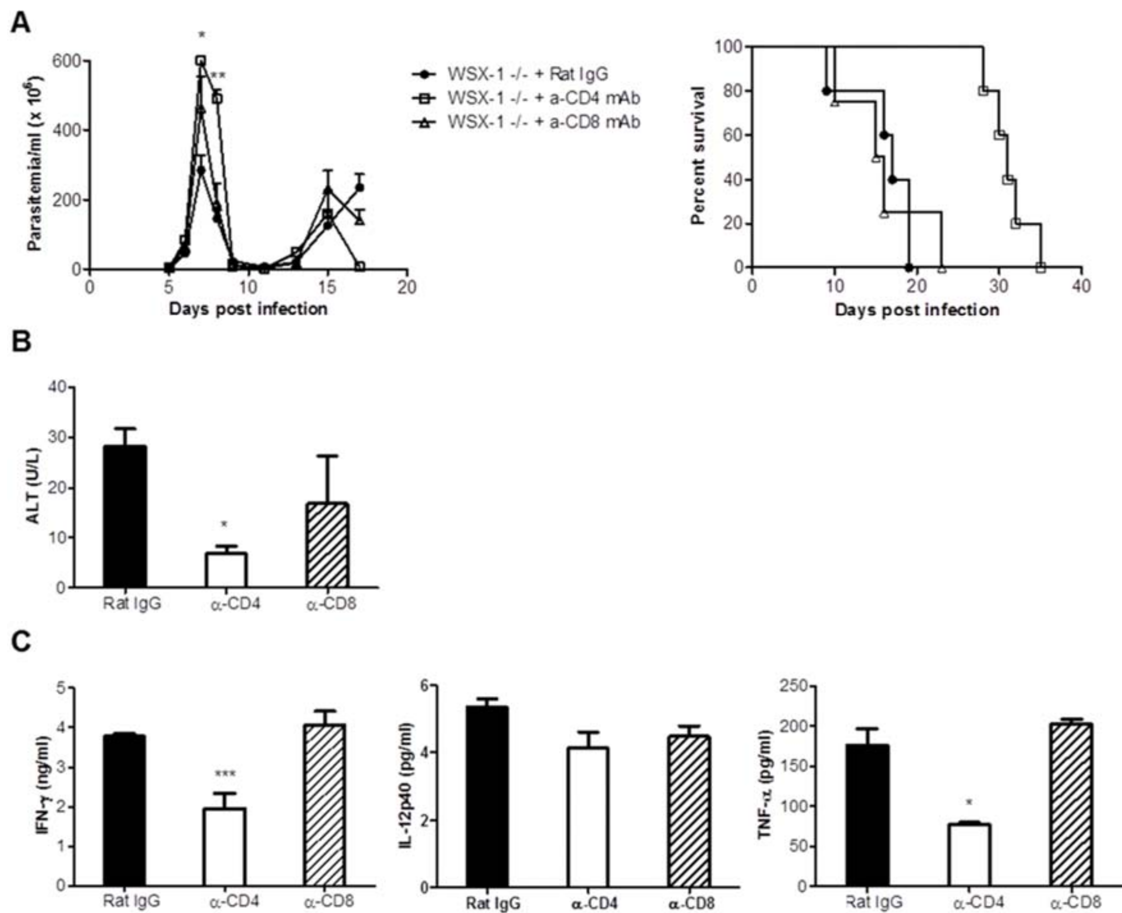

Fig. 2.6 Depletion of CD4$^+$, but not CD8$^+$, T cells significantly reduces the production of inflammatory cytokines and the serum activities of ALT, and enhances the survival of IL-27R$^{-/-}$ (WSX-1$^{-/-}$) mice infected with *T. congolense*.

IL-27R$^{-/-}$ mice were infected with *T. congolense*, and treated with 0.5 mg rat anti-mouse CD4 mAb, rat anti-mouse CD8 mAb, or rat IgG on day 0, 2, 4, and 6 after infection, respectively. (A) Parasitemia and survival of the IL-27R$^{-/-}$ mice (n=4-5) infected with *T. congolense*. (B) Serum ALT activities were assessed in IL-27R$^{-/-}$ mice (n=4) on day 7 after infection with *T. congolense*. (C) Plasma levels of IFN-γ, IL-12p40, and TNF-α of IL-27R$^{-/-}$ mice (n=4) on day 7 after infection with *T. congolense*. Data are presented as the mean ± SEM. The results presented are representative of 2 separate experiments.

We further characterized the contributions of $CD4^+$ T cells to secretion of cytokines in IL-27R$^{-/-}$ mice infected with *T. congolense*. Depletion of $CD4^+$, but not $CD8^+$, T cells significantly reduced plasma levels of IFN-γ and TNF-α in infected IL-27R$^{-/-}$ mice ($p<0.001$ or <0.05), although the reduction of IL-12p40 did not reach statistical significance (Fig. 2.6C). In addition, depletion of $CD4^+$, but not $CD8^+$, T cells also resulted in significantly less secretion of IFN-γ by spleen cells from infected IL-27R$^{-/-}$ mice ($p<0.05$, S2.2C Fig). Interestingly, depletion of $CD4^+$ T cells almost abrogated the production of IL-10 by spleen cells in infected IL-27R$^{-/-}$ mice ($p<0.01$, S2.2C Fig), suggesting that IL-10 was predominantly produced by $CD4^+$ T cells. Importantly, the observation that the enhanced survival of infected IL-27R$^{-/-}$ mice treated with anti-CD4 mAb was correlated with very little secretion of IL-10 further suggested that IL-27 signaling inhibited hyperactivation of Th1 cells in an IL-10 independent manner as shown above in Figure 2.4.

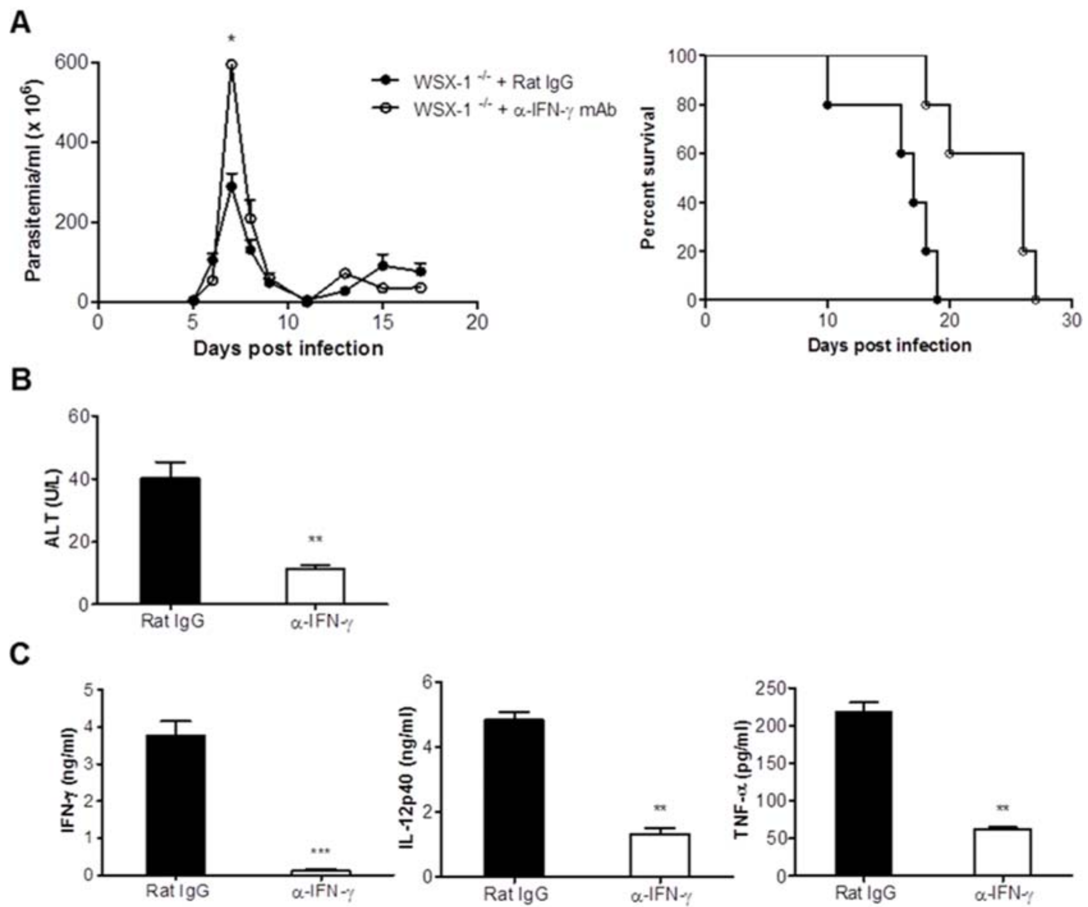

Fig. 2.7 Neutralization of IFN-γ significantly reduces the production of inflammatory cytokines and the serum activities of ALT, and prevents the early mortality of IL-27R$^{-/-}$ (WSX-1$^{-/-}$) mice infected with *T. congolense*.

IL-27R$^{-/-}$ mice were infected with *T. congolense*, and treated with 0.4 mg rat anti-mouse IFN-γ mAb or rat IgG on day 0, 2, 4, 6, 8, 10, 12, and 14 after infection, respectively. (A) Parasitemia and survival of infected IL-27R$^{-/-}$ mice (n=5). (B) Serum ALT activities were assessed in IL-27R$^{-/-}$ mice (n=4) on day 7 after infection. (C) Plasma levels of IFN-γ, IL-12p40, and TNF-α of IL-27R$^{-/-}$ mice (n=4) on day 7 after infection. Data are presented as the mean ± SEM. The results presented are representative of 2 separate experiments.

7. Neutralization of IFN-γ prevents the early mortality of IL-27R$^{-/-}$ mice infected with *T. congolense*

Having demonstrating that IL-27 is crucial for dampening trypanosomiasis-associated CD4^{+} T cell activation, needed for prolonged survival, we next addressed the mechanism of CD4^{+} T cell-mediated mortality of infected IL-27R$^{-/-}$ mice. Because the production of IFN-γ, and the frequency and the absolute number of IFN-γ-producing cells were enhanced in infected IL-27R$^{-/-}$ mice compared to infected wild-type mice (Fig. 2.2 and Fig. 2.5), and also because depletion of CD4^{+} T cells dramatically reduced the IFN-γ production (Fig. 2.6; S2.2 Fig), we examined whether the early mortality of infected IL-27R$^{-/-}$ mice was directly attributed to the overproduction of IFN-γ. IL-27R$^{-/-}$ mice infected with *T. congolense* were treated with neutralizing anti-IFN-γ mAb or rat IgG as a control. Although administration of anti-IFN-γ mAb led to doubled parasitemia in infected IL-27R$^{-/-}$ mice at the peak on day 7 after infection ($P<0.05$), the infected IL-27R$^{-/-}$ mice treated with anti-IFN-γ mAb efficiently controlled the first wave of parasitemia as infected control mice did (Fig 2.7A). Importantly, administration of anti-IFN-γ mAb significantly enhanced the survival of infected IL-27R$^{-/-}$ mice ($p<0.01$; Fig. 2.7A), demonstrating that high levels

of IFN-γ accelerated the mortality of IL-27R$^{-/-}$ mice infected with African trypanosomes.

We next assessed the effects of IFN-γ neutralization on weight loss and liver pathology of IL-27R$^{-/-}$ mice infected with *T. congolense*. Infected IL-27R$^{-/-}$ mice treated with anti IFN-γ mAb had significantly less weight loss than infected IL-27R$^{-/-}$ mice treated with rat-IgG on the late stage of infection ($p<0.01$, S2.3A Fig). Importantly, infected IL-27R$^{-/-}$ mice treated with anti-IFN-γ did not exhibit areas with loss of hepatocyte cellular architecture in the liver whereas these pathological changes were observed in the liver of infected IL-27R$^{-/-}$ mice treated with rat IgG (S2.3B Fig). Moreover, neutralization of IFN-γ significantly reduced the serum activities of ALT in infected IL-27R$^{-/-}$ mice ($p<0.01$, Fig. 2.7B). These data suggested that IFN-γ played a critical role in the development of liver pathology in IL-27R$^{-/-}$ mice infected with African trypanosomes.

We finally examined cytokine responses of infected IL-27R$^{-/-}$ mice treated with anti-IFN-γ mAb. IFN-γ was almost undetectable in the plasma of IL-27R$^{-/-}$ mice treated with anti-IFN-γ, suggesting the neutralization was successful ($p<0.01$, Fig.

2.7C). Plasma levels of IL-12p40 and TNF-α were dramatically reduced in infected IL-27R$^{-/-}$ mice treated with anti-IFN-γ mAb, compared to infected IL-27R$^{-/-}$ mice treated with rat IgG ($p<0.01$, Fig. 2.7C). Neutralization of IFN-γ also significantly reduced the production of IL-12p40 and TNF-α by cultured spleen cells ($p<0.01$, or <0.05, S2.3C Fig). Thus, the results indicated that IFN-γ was critically involved in the enhanced inflammatory responses in IL-27R$^{-/-}$ mice infected with African trypanosomes.

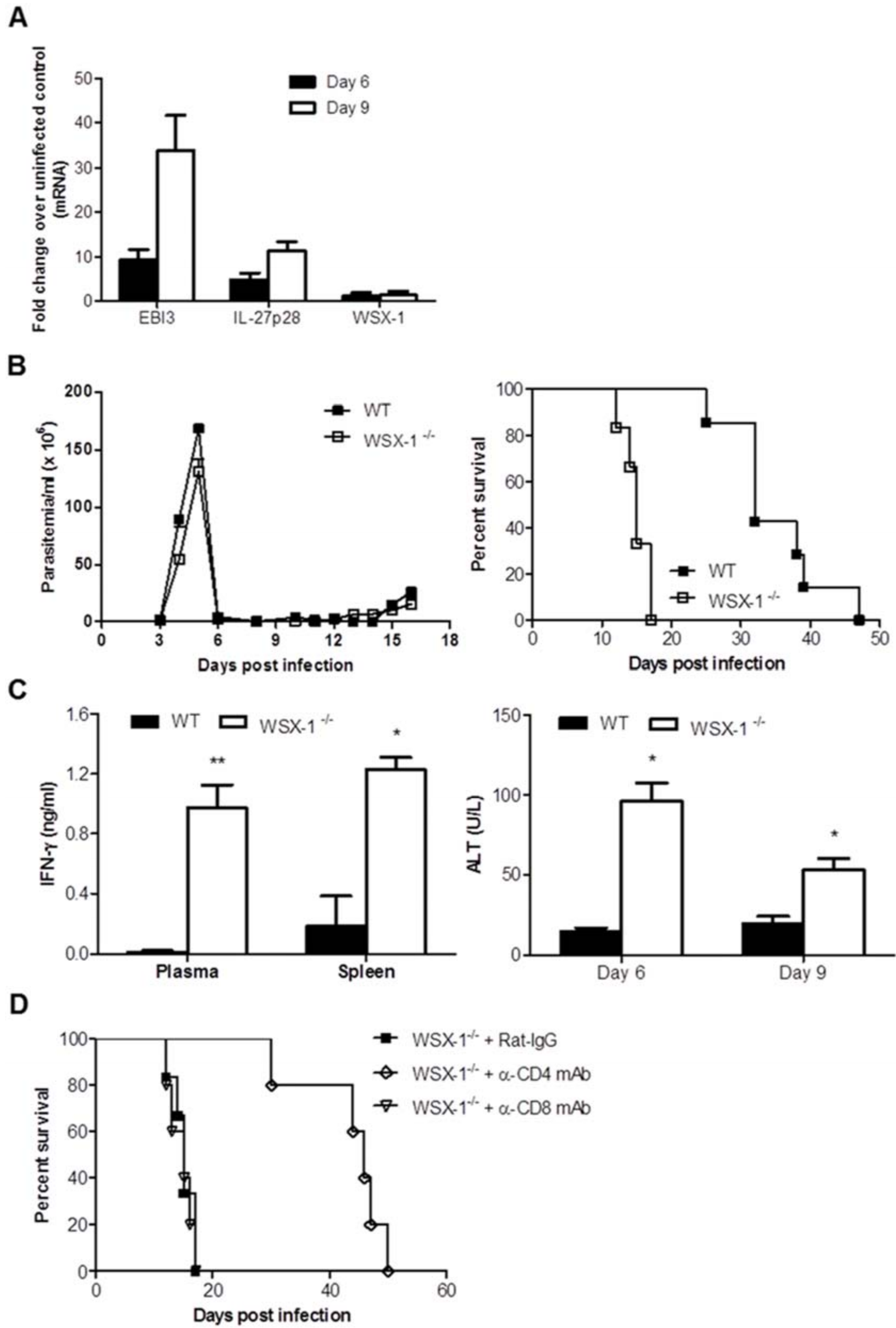
A
Fold change over uninfected control (mRNA)
Day 6
Day 9
EBI3
IL-27p28
WSX-1
B
Parasitemia/ml (x 10^6)
WT
WSX-1 -/-
Days post infection
Percent survival
WT
WSX-1 -/-
Days post infection
C
IFN-γ (ng/ml)
WT
WSX-1 -/-
**
*
Plasma
Spleen
ALT (U/L)
WT
WSX-1 -/-
*
*
Day 6
Day 9
D
Percent survival
WSX-1-/- + Rat-IgG
WSX-1-/- + α-CD4 mAb
WSX-1-/- + α-CD8 mAb
Days post infection

Fig. 2.8 IL-27 signaling plays a crucial role in dampening Th1 mediated immune responses, allowing prolonged survival of mice infected with *T. brucei.*

(A) mRNA expression levels of IL-27p28, EBI3 and WSX-1 in the liver of wild-type mice infected with *T. brucei* on day 6 and 9 versus day 0 (uninfected). (B) Parasitemia and survival of IL-27R$^{-/-}$ (WSX-1$^{-/-}$) and wild-type mice (n=6-7) infected with *T. brucei*. (C) Production of IFN-γ detected on day 6 in the plasma and supernatant fluids of cultured spleen cells and serum activities of ALT examined on day 6 and 9 in IL-27R$^{-/-}$ and wild-type mice after infection with *T. brucei*. (D) Survival of IL-27R$^{-/-}$ mice (n=5-6) infected with *T. brucei*, following administration of 0.5 mg rat anti-mouse CD4 mAb, rat anti-mouse CD8 mAb, or rat IgG on day 0, 2, 4, and 6 after infection, respectively. Data are presented as the mean ± SEM. The results presented are representative of 2-3 separate experiments.

8. Essential role of IL-27 signaling in preventing lethal effect of CD4$^+$ T cells in mice infected with *T. brucei.*

We finally characterized the role of IL-27 signaling in regulation of immune responses during *T. brucei* infection. In contrast to *T. congolense*, *T. brucei* species have the ability to penetrate the walls of capillaries, invade interstitial tissues, including the brain tissues, thus serving as a model of human African trypanosomiasis (166, 167). *T. brucei* infection also upregulated the mRNA expressions of IL-27p28 and EBI3, but not IL-27R in the liver of mice (Fig. 2.8A). IL-27R$^{-/-}$ mice infected with *T. brucei* efficiently controlled the first wave of parasitemia as infected wild-type did, but survived significantly shorter than infected wild-type mice (15 days vs. 32 days, $p<0.01$, Fig. 2.8B), demonstrating an essential role of IL-27 signaling in prevention of the early mortality of mice infected with *T. brucei*. IL-27R$^{-/-}$ mice infected with *T. brucei* also showed enhanced IFN-γ production in plasma and supernatant fluids of spleen cultures, as well as enhanced serum activities of ALT, compared to infected wild-type mice ($p<0.01$ or <0.05, Fig. 2.8C). Importantly, depletion of CD4$^+$, but not CD8$^+$, T cells enhanced the survival of IL-27R$^{-/-}$ mice infected with *T. brucei* by 3 folds ($p<0.01$, Fig. 2.8D). Thus, IL-27 signaling is also

required for survival of mice via preventing excessive Th1 immune responses during *T. brucei* infection.

Discussion

Successful clearance of African trypanosomes in the bloodstream requires induction of inflammatory immune responses; however, failure to control this inflammation leads to immune-mediated pathology (83, 168). IL-10 signaling has been previously suggested to be involved in maintaining this immunological balance in African trypanosomiasis (131, 139). In the current study, we have identified IL-27 signaling as a novel pathway to maintain this immunological balance in African trypanosomiasis. Our data are the first to demonstrate the essential role of IL-27 signaling in regulating immune responses to extracellular protozoan infections. More importantly, we provided direct evidence, that infection-associated IL-27 signaling served to extend the survival of the infected host by dampening $CD4^+$ T cell activation and their secretion of IFN-γ.

Indeed, the early mortality of infected mice lacking IL-27 signaling (IL-27R$^{-/-}$ mice) was correlated with exaggerated inflammatory responses and liver immunopathology. The disease similarity of infected mice lacking IL-27 and IL-10 signaling raised the possibility that regulatory function of IL-27 is mediated via the induction of IL-10 secretion, as IL-27 has the capability of promoting CD4^{+} T cells to secrete IL-10 (163-165). However, the fact that blocking IL-10R further shortened the survival of infected IL-27R$^{-/-}$ mice and the fact that infected mice lacking IL-10 signaling and infected mice lacking IL-27 signaling had distinct survival suggested that IL-27 functions through a mechanism independent of IL-10. In addition, compared to infected wild-type mice, infected IL-27R$^{-/-}$ mice produced similar or even higher amounts of IL-10, depending on the time points examined. Furthermore, the enhanced survival of infected IL-27R$^{-/-}$ mice following depletion of CD4^{+} T cells was correlated with dramatically reduced secretion of IL-10. These data suggested that a defect of IL-10 signaling is unlikely to contribute to the early mortality of IL-27R$^{-/-}$ mice. Thus, we suggest that IL-27 suppresses the liver pathology and prevents the early mortality of mice infected with African trypanosomes through IL-10-independent mechanisms, possibly by direct modulation of T cell function.

It has been previously demonstrated that IL-10 inhibits accumulation and activation of M1-type myeloid cells, in particular, TIP-DCs ($CD11b^+Ly6C^+CD11c^+$TNF and iNOS producing DCs) in the liver during infection with African trypanosomes (141, 145, 146). Accordingly, African trypanosomes-infected CCR2 deficient mice and MIF (macrophage migrating inhibitory factor) deficient mice exhibited significantly reduced accumulation of TIP-DCs, which was correlated with remarked diminished liver pathology, and significantly prolonged survival (145, 162). Thus, IL-10 signaling suppresses liver pathology, mainly through downregulation of M1-type myeloid cells (124, 168). In contrast, IL-27$R^{-/-}$ mice infected with African trypanosomes displayed more activation of T cells, in particular, $CD4^+$ T cells. Moreover, depletion of $CD4^+$ T cells prevented liver pathology and early mortality of infected IL-27$R^{-/-}$ mice. Obviously, IL-27 signaling functions through limiting activation of $CD4^+$ T cells in African trypanosomiasis. Thus, although both IL-10 signaling and IL-27 signaling are crucial for limiting the inflammatory complications associated to African trypanosome in particular in preventing liver pathology, the two signal pathways involve distinct mechanisms.

Dampening accumulation of highly activated $CD4^+$ T cells by IL-27 signaling has also been recently observed in infection with other microorganisms, particularly intracellular protozoan and bacterial pathogens (156, 158-160, 169). Our data demonstrate that the same mechanism exists during infections with extracellular protozoan parasites such as African trypanosomes. However, the precise mechanism of $CD4^+$ T cell-mediated early mortality in previous models was not fully elucidated (156, 160). One of the most important properties of $CD4^+$ T cells is that they secrete a large amount of IFN-γ upon activation. IFN-γ is required to eliminate intracellular parasites, but also has potential to induce immunopathology (170, 171). Indeed, early mortality of IL-27R$^{-/-}$ mice infected with *Toxoplasma gondii*, or *Plasmodium berghei* is associated with significantly enhanced production of IFN-γ (156, 160), suggesting that IFN-γ might be a critical molecule for $CD4^+$ T cell-mediated mortality in the absence of IL-27 signaling. Surprisingly, neutralization of IFN-γ did not prolong the survival, and had no effect on the liver pathology of IL-27R$^{-/-}$ mice infected with *T. gondii* or *P. berghei* at all (156, 172). Thus, although $CD4^+$ T cell-mediated mortality coincides with significantly elevated secretion of IFN-γ, it still remains inconclusive whether IFN-γ is the direct mediator of $CD4^+$ T cell-dependent mortality in these infections. In contrast, neutralization of IFN-γ significantly enhanced the survival IL-

27R$^{-/-}$ mice infected with African trypanosomes accompanied by a major amelioration of liver pathology, providing direct evidence that IFN-γ directly mediated the mortality of infected IL-27R$^{-/-}$ mice. In addition, enhanced survival of infected IL-27R$^{-/-}$ mice depleted of CD4^{+} T cells was correlated with a dramatically reduced production of IFN-γ. Obviously, either removing of CD4^{+} T cells or neutralization of IFN-γ got rid of the lethal effect of IFN-γ, leading to the prolonged survival of infected IL-27R$^{-/-}$ mice. Thus, another important finding of this study is that, in the absence of IL-27 signaling, CD4^{+} T cells mediated mortality directly through their secretion of IFN-γ, at least, during infection with extracellular protozoan parasites African trypanosomes.

It is important to point out that our results in no way exclude the protective role of CD4^{+} T cells and IFN-γ during infection with the parasites. Indeed, early studies have shown that there was a correlation between high IFN-γ levels in serum, low parasitemia, and host resistance during infection with African trypanosomes (137). Subsequent studies demonstrated that VSG-specific CD4^{+} T cells mediated protection via secretion of IFN-γ (132, 173); and splenic DCs were the primary cells responsible for activating naïve VSG-specific CD4^{+} T cell responses (135, 136). The

protective role of $CD4^+$ T cells and IFN-γ in African trypanosomiasis has been recently confirmed by independent groups (133, 134, 138). In support of previous findings, we showed that either depletion of $CD4^+$ T cells or neutralization of IFN-γ resulted in a significantly elevated peak parasitemia level in IL-27R$^{-/-}$ mice infected with *T. congolense*, confirming the protective role of $CD4^+$ T cells and IFN-γ during the infection. It is likely that IFN-γ promotes M1-type myeloid cells to produce IL-12, TNF-α and iNOS, which has been shown to be critically involved in lysis or damage of African trypanosomes (134, 140, 142, 144, 174). On the other hand, excessive production of IL-12, TNF-α and iNOS driven by IFN-γ could also mediate immunopathology of mice infected with African trypanosomes (141, 143, 145, 146, 175). Further, IL-12 and TNF-α could stimulate T cells to produce more IFN-γ (83, 140). Thus, IL-10 is required to down-regulate the production of IL-12, TNF-α and iNOS possibly by direct modulation of M1-type myeloid cells (131, 141, 145, 146). In the present study, we identified IL-27 signaling as a novel pathway to down-regulate the secretion of IFN-γ by direct modulation of $CD4^+$ T cells. Obviously, in the absence of IL-27 signaling, excessive secretions of IFN-γ by $CD4^+$ T cells also mediate liver pathology and mortality, although IL-10 signaling still fully functions and the infected mice produce even more IL-10, in African trypanosomiasis. Thus,

both IL-10 signaling and IL-27 signaling are required for survival of mice infected with the parasites via preventing aberrant inflammatory responses, although they function in a distinct manner in African trypanosomiasis.

In conclusion, we have described an essential role for IL-27 signaling in preventing early mortality of mice infected with African trypanosomes through dampening IFN-γ secretion by $CD4^+$ T cells, thus identifying, in addition to previously described IL-10 signaling, a novel pathway for maintenance of immunological balance during infection with extracellular protozoan parasites African trypanosomes. These data contribute significantly to our understanding of both immunopathogenesis of African trypanosomiasis and mechanisms underlying IL-27 immunoregulation during infection with extracellular protozoan and bacterial pathogens.

Materials and Methods

Ethics statement

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of

Health. The animal protocols involving mice were approved by the University of Maryland Institutional Animal Care and Use Committee (IACUC) under protocol R-12-60.

Mice and parasites

Eight- to ten-week-old C57BL/6NCrJ (C57BL/6) mice and five- to six-week-old outbred Swiss white mice (CD1) were purchased from the National Cancer Institute (Frederick, MD). B6N.129P2-Il27ra^{tm1Mak} (IL-27R$^{-/-}$, or WSX-1$^{-/-}$) mice were purchased from the Jackson Laboratory and bred in-house. All animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee and Institutional Bio-safety Committee of the University of Maryland, College Park.

T. congolense, Trans Mara strain, variant antigenic type (VAT) TC13 was used in this study. The origin of this parasite strain has been previously described (176). *T. brucei* AnTat1.1E was obtained from the Institute of Tropical Medicine (Antwerp, Belgium). Frozen stabilates of parasites were used for infecting CD1 mice immunosuppressed with cyclophosphamide, and passages were made every third day

as described previously (176). The parasites were purified from the blood of infected CD1 mice by DEAE-cellulose chromatography (177) and used for infecting mice.

Antibodies

Purified rat anti-mouse IL-10 receptor (IL-10R) mAb (Clone 1B1.3a), purified rat anti-mouse CD4 mAb (Clone GK1.5), purified rat anti-mouse CD8 (Clone 53-6.72), and purified rat anti-mouse IFN-γ mAb (Clone XMG1.2) were purchased from BioXCell (West Lebanon, NH). Purified anti-mouse CD16/CD32 (FcγIII/IIR, Clone 2.4G2) were purchased from BD Biosciences. APC-Cy7–anti-mouse CD3e (145-2C11), PE-anti-mouse IFN-γ (XMG1.2), PE-Cy7-anti-mouse CD4 (GK1.5), PE-Cy7-anti-mouse CD4 (RM 4-4), FITC-anti-mouse CD8 (53-6.72), FITC-anti-mouse CD8 (YTS156.7.7), APC-anti-mouse CD44 (IM7), PE-anti-mouse CD62L (MEL-14), and matching controls were purchased from eBioscience or Biolegend.

Infections, treatment of mice with mAbs, estimation of parasitemia and survival time of mice

Mice were infected *i.p.* with 10^3 *T. congolense* TC13 (131) or 5×10^3 *T. brucei* AnTat1.1E (162). Some groups of infected mice were injected *i.p.* with rat anti-mouse

IL-10R mAb (1B1.3a; 0.4 mg on day 0, 2, 4, and 6 after infection, respectively), anti-mouse CD4 mAb (GK1.5; 0.5 mg on day 0, 2, 4, and 6 after infection, respectively), anti-mouse CD8 mAb (53-6.72; 0.5 mg on day 0, 2, 4, and 6 after infection, respectively), anti-mouse IFN-γ mAb (XMG1.2; 0.4 mg on day 0, 2, 4, 6, 8, 10, 12, and 14 after infection, respectively), or rat IgG (as a control). Parasitemia was counted at ×40 magnification by phase-contrast microscopy. The survival time was defined as the number of days after infection that the infected mice remained alive.

Detection of IL-27/WSX-1 mRNA levels

For analysis of mRNA expression, total RNA was extracted from the homogenates of the liver of uninfected wild-type C57BL/6 mice or mice infected with *T. congolense* or *T. brucei*, following the manufacturer's recommendation (Life Technologies). IL-27p28, EBI3, and WSX-1 mRNA levels were quantified by real-time quantitative RT-PCR. The cDNA expression for each sample was standardized using the house keeping gene β-actin. Cycling conditions were as follows: initialization 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Primer pair used were: IL-27p28: 5'-CTGGTACAAGCTGGTTCCTG-3', 5'-CTCCAGGGAGTGAAGGAGCT-3; EBI3:

5’-CAGAGTGCAATGCCATGCTTCTC-3’, 5’-CTGTGAGGTCCTGAGCTGAC-3’; WSX-1: 5’-CAAGAAGAGGTCCCGTGCTG-3’, 5’-TTGAGCCCAGTCCACCACAT-3’.

Splenocyte or liver leukocyte cultures for measurement of cytokine synthesis

Splenocytes were collected from mice. Cells were cultured at a concentration of 5 × 10^6 cells/ml (200 μl/well) in 96-well tissue culture plates in a humidified incubator containing 5% CO_2. The culture supernatant fluids were collected after 48 h and centrifuged at 1,500g for 10 min, and the supernatant fluids were stored for cytokine assays at -20°C until used.

Liver leukocytes were isolated as described previously (178). Briefly, the liver was perfused with PBS until it became pale. Thereafter, the gallbladder was removed and the liver excised carefully from the abdomen. The liver was minced into small pieces with surgical scissors and forced gently through a 70 um cell strainer using a sterile syringe plunger. The preparation obtained was suspended in 50 ml RPMI-1640 medium containing 10% FCS. The cell suspension was centrifuged at 30g with the off-brake setting for 10 min at 4 °C. The obtained supernatant was centrifuged at

300g with the high-brake setting for 10 min at 4 °C. The pellet was resuspended in 10 ml 37.5% Percoll in HBSS containing 100 U/ml heparin and then centrifuged at 850g with the off-brake setting for 30 min at 23 °C. This new pellet was resuspended in 2 ml ACK buffer (erythrocyte lysing buffer), and incubated at room temperature for 5 min, then supplemented with 8 ml RPMI-1640 medium containing 10% FCS, followed by centrifugation at 300g with the high-brake setting for 10 min at 8 °C. Cells were collected and cultured at a concentration of 5×10^6 cells/ml (200 µl/well) in 96-well tissue culture plates in a humidified incubator containing 5% CO_2. The culture supernatant fluids were collected after 48 h and centrifuged at 1,500g for 10 min, and the supernatant fluids were stored for cytokine assays at -20°C until used.

Cytokine assays

Recombinant murine cytokines and Abs to these cytokines for use in ELISA were purchased from BD Biosciences or R&D Systems. The levels of cytokines in culture supernatant fluids or plasma were determined by routine sandwich ELISA using Immuno-4 plates (Dynax Technologies), according to the manufacturer's protocols.

Flow cytometry

To assess the activation of T cells, intrahepatic leukocytes were isolated as described above. The cells were incubated (15 min, 4°C) with purified anti-mouse CD16/CD32 ([FcγIII/II Receptor], clone: 2.4G2) to block nonspecific binding of Abs to FcRs, washed with staining buffer (eBioscience), resuspended in staining buffer, and stained with mAbs specific for various cell surface markers, or the relevant isotype-matched control Abs. For intracellular IFN-γ staining, spleen cells or intrahepatic leukocytes were diluted to 5×10^6 cells/ml and cultured (200 μl/well) in a 96-well plate in the presence of 1x Cell Stimulation Cocktail (containing PMA, ionomycin, and protein transport inhibitors, eBioscience) for 12 h. The cells were then harvested and washed twice in staining buffer. The cells were incubated (15 min, 4°C) with purified anti-mouse CD16/CD32, washed with staining buffer, followed by staining with mAbs specific for cell surface markers. The cells were fixed and permeabilized using Intracellular Fixation & Permeabilization Buffer Set (eBiosciences). Intracellular staining was then performed using mAbs specific for IFN-γ. Samples were resuspended in staining buffer, tested by FACSAria II, and analyzed using FlowJo software.

Aminotransferase determination and histopathological examination

Liver alanine transaminase (ALT) activities were determined using EnzyChrom Alanine Transaminase Assay Kit (BioAssay Systems) according to the manufacturer's instructions. For histopathological examination, the liver was taken from mice on day 10 after infection and fixed with 10% formalin in PBS. Sections were stained with Hematoxylin and Eosin.

Statistical analysis

Data are represented as the mean ± SEM. Significance of differences was determined by ANOVA or a log-rank test for curve comparison using the GraphPad Prism 5.0 software. Values of $p \leq 0.05$ are considered statistically significant.

Acknowledgements

We thank Kenneth Class (MPRI Flow Cytometry Core Facility, University of Maryland) for his assistance with FACS analyses.

Supporting Information

Supplemental figure 1

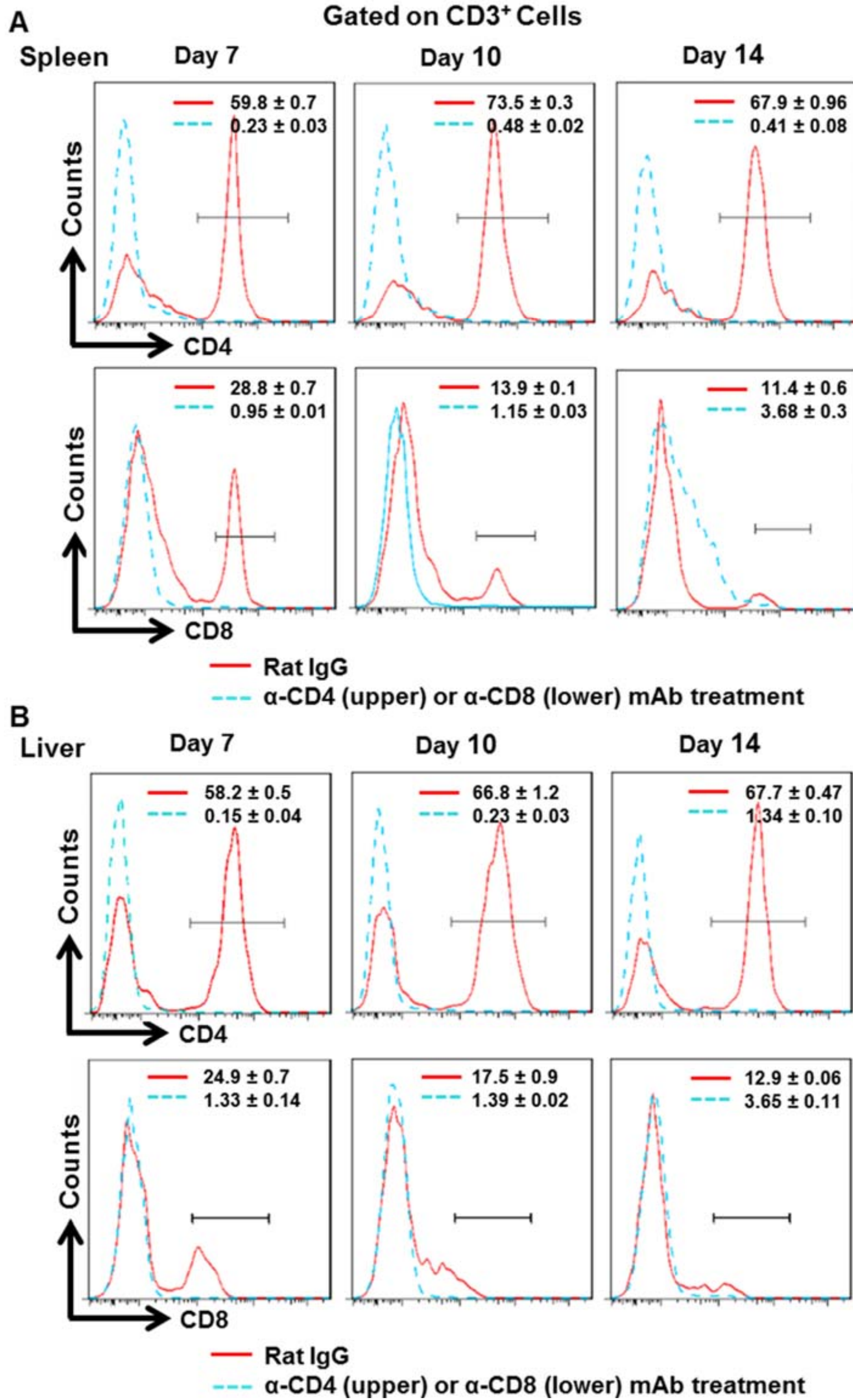

Fig. S2.1 Efficacy of depletion of $CD4^+$ and $CD8^+$ T cells by antibodies in the spleens and livers of mice infected with *T. congolense*.

Representative flow cytometry histograms showing the depletion of $CD4^+$ and $CD8^+$ T cells in the spleens (A) and livers (B) of IL-27R$^{-/-}$ (WSX-1$^{-/-}$) mice on day 7, 10, and 14 after infection. The infected mice were administrated with 0.5 mg rat anti-mouse CD4 mAb, rat anti-mouse CD8 mAb, or rat IgG on day 0, 2, 4, and 6 after infection, respectively. The results presented are representative of 2 separate experiments.

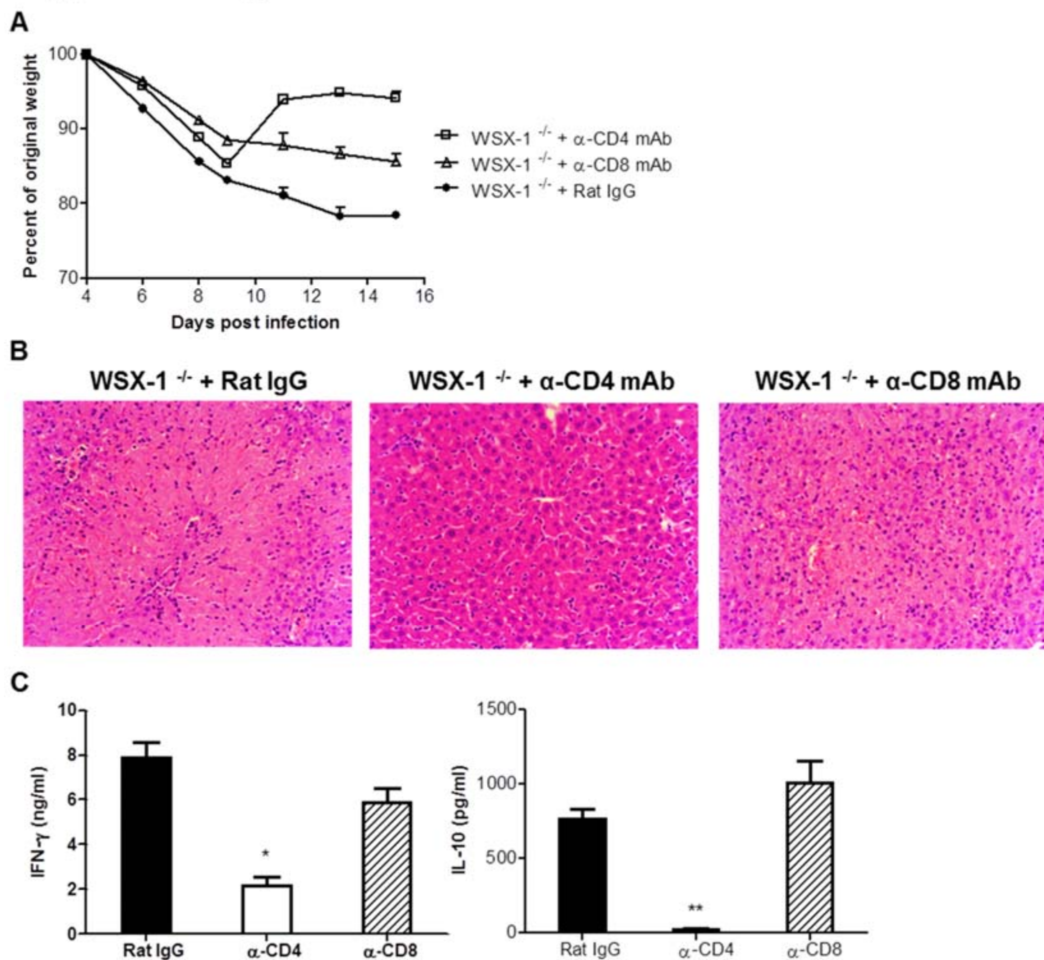

Fig. S2.2 Depletion of $CD4^+$ T cells ameliorates the weight loss, prevents the development of liver pathology, reduces the production of IFN-γ by spleen cells, and abolishes the secretion of IL-10 by spleen cells in $IL\text{-}27R^{-/-}$ ($WSX\text{-}1^{-/-}$) mice infected with *T. congolense*.

$IL\text{-}27R^{-/-}$ mice were infected with 10^3 *T. congolense* TC13 and treated with 0.5 mg rat anti-mouse CD4 mAb, rat anti-mouse CD8 mAb, or rat IgG on day 0, 2, 4, and 6 after infection, respectively. (A) The weight loss of infected mice (n=4-5) was determined at indicated days after infection. (B) Hematoxylin and eosin staining was performed on the liver sections at day 10 after infection to detect pathological changes (original magnification ×20). (C) Production of IFN-γ and IL-10 in supernatant fluids of cultured spleen cells collected from mice (n=4) at day 7 after infection. Data are presented as the mean ± SEM. The results presented are representative of 2 separate experiments.

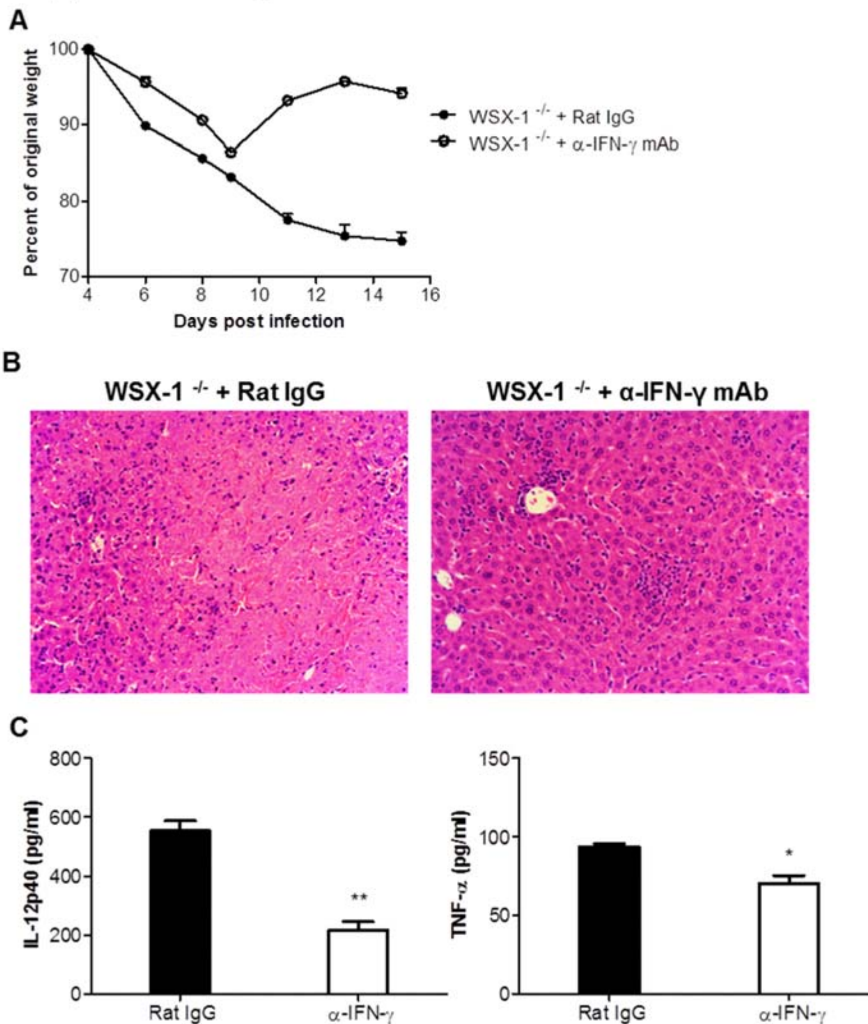

Fig. S2.3 Neutralization of IFN-γ ameliorates the weight loss, prevents the development of liver pathology, and reduces the production of IL-12p40 and TNF-α by spleen cells in IL-27R$^{-/-}$ (WSX-1$^{-/-}$) mice infected with *T. congolense*.

IL-27R$^{-/-}$ mice were infected with 10^3 *T. congolense* TC13 and treated with 0.4 mg rat anti-mouse IFN-γ mAb or rat IgG on day 0, 2, 4, 6, 8, 10, 12, and 14 after infection, respectively. (A) The weight loss of infected mice (n=5) was determined at indicated days after infection. (B) Hematoxylin and eosin staining was performed on the liver sections at day 10 after infection to detect pathological changes (original magnification ×20). (C) Production of IL-12p40 and TNF-α in supernatant fluids of cultured spleen cells collected from mice (n=4) at day 7 after infection. Data are presented as the mean ± SEM. The results presented are representative of 2 separate experiments.

CHAPTER 3: IL-27 PERTURBS THE DIFFERENTIATION PROGRAM OF LY6C$^+$ MONOCYTES INTO TIP-DCS THROUGH SUPPRESSING CD4$^+$ CELL TO SECRETE IFN-Γ

Abstract

Ly6C$^+$ monocytes are recruited to sites of infections, where they differentiate into CD11b$^+$Ly6C$^+$CD11C$^+$ TNF/iNOS producing DCs (Tip-DCs). Tip-DCs play a central role in control and clearance of invading pathogens, but also contribute to the pathogenesis in infections, including African trypanosomiasis. However, less is known about how the differentiation of inflammatory Tip-DCs from Ly6C$^+$ monocytes is regulated. In this regard, IL-27 has been recently identified as a negative regulator of inflammation in African trypanosome infection. Here we report that IL-27R deficient mice have remarkably higher incidence of Ly6C$^+$ monocytes recruitment to the liver, where Ly6C$^+$ monocytes preferentially differentiates into Tip-DCs. This is coincided with reduced counts of Ly6C$^-$ monocytes and macrophages in the liver. Depletion of CD4$^+$ T cells or neutralization of IFN-γ in infected IL-27R deficient mice diminishes the recruitment of Ly6C$^+$ monocytes, and

their differentiation and maturation into Tip-DCs. This is accompanied by enhanced counts of Ly6C$^-$ monocytes and macrophages in infected IL-27R deficient mice. Adoptive transfer experiments show that, 1) IFN-γ directly drives Ly6C$^+$ monocytes to differentiate into Tip-DCs in infected IL-27R deficient mice. 2) IFN-γ switches the differentiation program of Ly6C$^+$ monocytes from Ly6C$^-$ macrophages to Tip-DCs through inducing cell death of the anti-inflammatory Ly6C$^-$ monocytes in infected IL-27R deficient mice. Thus, we identify IL-27 signaling as a novel immunoregulator to prevent Ly6C$^+$ monocytes from differentiation into Tip-DCs through suppressing CD4$^+$ T cells to secrete IFN-γ. The findings from this study would be beneficial to the development of new therapeutics aiming at enhancing antimicrobial immune defense or dampening detrimental inflammation through manipulating the Tip-DC development.

Author Summary

The goal of this chapter is to elucidate the mechanisms involved in regulation of the development of Tip-DCs during parasitic infections. The development of Tip-DCs includes three stages: 1) CCR2-dependent emigration of Ly6C$^+$ monocytes from the bone marrow, 2) their migration to infected tissues, and 3) their differentiation

into Tip-DCs. A critical gap in our understanding remains: how is the development of Tip-DCs regulated? This question will be addressed by the proposed studies in this chapter using a model of infection with African trypanosomes. Here we report that absence of IL-27 signaling results in enhanced accumulation of Tip-DCs in the liver of infected mice. We further demonstrate that Tip-DC development is negatively regulated by IL-27 via suppressing $CD4^+$ T cells to produce IFN-γ.

Introduction

Monocytes are myeloid leukocytes that are derived from the bone marrow and circulate in the bloodstream (179). Monocytes consist of two major subsets based on chemokine receptor and Ly6C expression in mice, and CD14 and CD16 expression in humans (180, 181). One subset of murine monocytes is $Ly6C^-$ monocytes expressesing low level of the chemokine receptor CCR2, but high level of the chemokine receptor CX3CR1 (182). This subset patrols the luminal surface of blood vessels, thus is also referred to as patrolling monocytes or $Ly6C^-$ monocytes (183). The function of this subset is poorly documented, but it may contribute to tissue repair (184). The other subset is $Ly6C^+$ monocytes which expresse high level of CCR2, but low level of CX3CR1. This subset is recruited to sites of inflammation,

thus is known as inflammatory monocytes or Ly6C$^+$ monocytes (182). During infection, Ly6C$^+$ monocytes emigrate from the bone marrow, and then migrate to infected tissues where they can differentiate into Tip-DCs (185). Tip-DCs make essential contributions to immune defense against microbial pathogens including *Listeria monocytogenes* (185, 186), *Brucella melitensis* (187), *Mycobacterium tuberculosis* (188), *Toxoplasma gondii* (189, 190), *Plasmodium chabaudi* (191), *Leishmania major* (192), and *Cryptococcus neoformans* (193-195), but they can also lead to immunopathology during infection with *Trypanosoma brucei* (145, 161) and influenza virus (196). It is known that Ly6C$^+$ monocytes use CCR2 signaling to emigrate from bone marrow into blood (186, 197). During infection with *L. monocytogenes*, adhesion molecules CD11b, CD44, and ICAM-1 are involved in recruitment of Ly6C$^+$ monocytes to the liver (198). However, less is known about how Ly6C$^+$ monocytes differentiate into Tip-DCs is regulated in the infected tissues.

Interleukin (IL)-27 is mainly produced by antigen presenting cells, such as macrophages and dendritic cells (DCs) (199). The IL-27 receptor is expressed on numerous subsets of leukocytes including CD4$^+$ T cells, CD8$^+$ T cells, NK cells, monocytes, macrophages and DCs (199). As a major anti-inflammatory cytokine

(152), IL-27 has been shown to inhibit immunopathology via down-regulation of Th1 response during infections, particularly with intracellular protozoan parasites including *T. gondii* and *T. cruzi* (50, 51, 158, 160, 200) and bacteria such as *M. tuberculosis* (169). Recently, we have found that IL-27 directly inhibits $CD4^+$ T cells to produce IFN-γ, preventing liver pathology during infection with African trypanosomes (Chapter 2) (201). In some infectious or autoimmune conditions, IL-27 promotes Th1, Th2, Th17 and Tr1 cell subsets to secrete IL-10 (163, 164, 202). Although both IL-27 and IL-10 play crucial roles in maintaining immune balance, it appears that they use different mechanisms, i.e. IL-27 down-regulates $CD4^+$ T cells whereas IL-10 suppresses M1-type myeloid cells (203). Compared to effects on T cells, the role of IL-27 on myeloid cells such as macrophages and DCs is less clear (152). In particular, it is unknown whether and how IL-27 signaling affects development of $Ly6C^+$ monocytes into Tip-DCs.

African trypanosomiasis is a vector-borne fatal disease of humans and animals. African trypanosomes survive in the blood stream. We and others have shown that the liver is the major site of African trypanosomes clearance (127-129, 131). Pro-inflammatory cytokines such as IL-12, TNF-α, as well as iNOS produced by myeloid

cells are critical for host resistance to African trypanosomes (104, 115, 141, 143, 144). In particular, TNF-α and NO mediate direct lysis and killing of the parasites (104, 142, 144, 174). IFN-γ, mainly secreted by $CD4^+$ T cells (104, 132, 133), has been shown to mediate protection during African trypanosomiasis (104, 132, 137-139). However, we have shown that excessive production of IFN-γ mediates immunopathology in mice infected with African trypanosomes (131, 147). More recently, using IL-27 receptor-deficient ($IL\text{-}27R^{-/-}$) mice we have shown that IL-27 is crucial for inhibition of liver pathology and survival during African trypanosomiasis through down-regulation of IFN-γ secretion by $CD4^+$ T cells (201). However, the question remains as to how IFN-γ promotes the pathology. In this regard, Tip-DCs have recently been identified as a subset of pathogenic cells causing liver pathology in mice infected with African trypanosomes (145), raising the question of whether there is a link between IFN-γ mediated pathology and Tip-DC mediated pathology. With this in mind, we would in this chapter examine how IFN-γ signaling regulates the development of Tip-DCs in the IL-27 deficient mice infected with African trypanosomes.

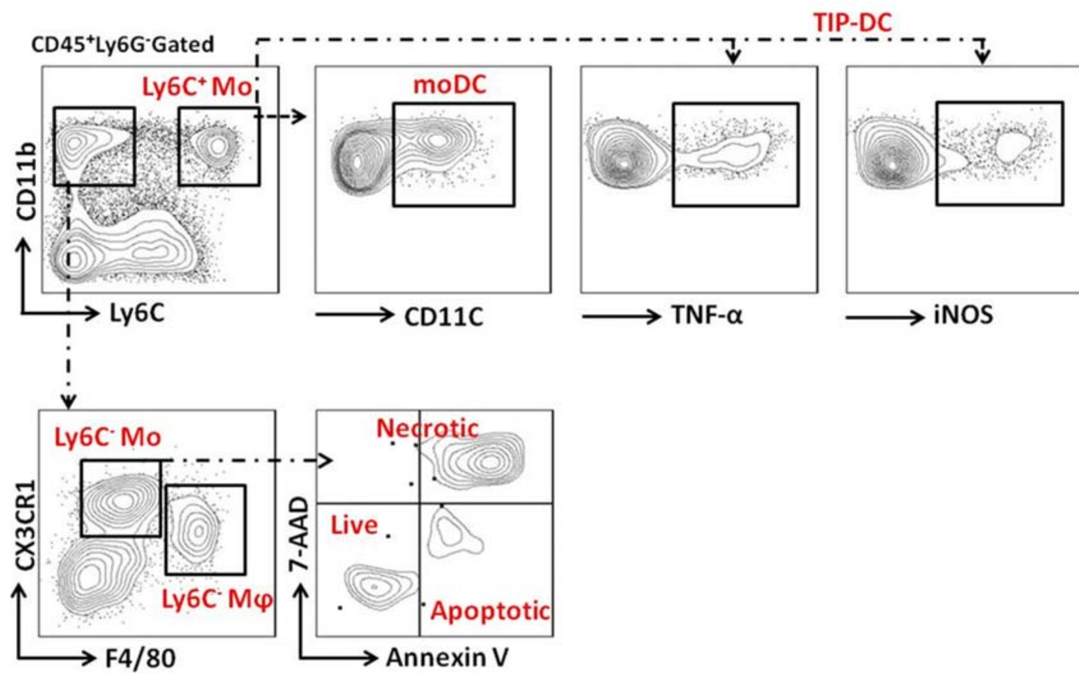

Fig. 3.1 Gating Strategy for Ly6C^{+} monocytes, moDCs, Tip-DCs, Ly6C^{-} monocytes (Ly6C^{-}CX3CR1^{hi}), macrophages (Ly6C^{-}F4/80^{+}), and apoptotic cells (Annexin V^{+}). Intrahepatic CD45^{+}Ly6G^{-} cells were gated.

Results

1. IL-27 limits the accumulation of intrahepatic $Ly6C^+$ monocytes via suppressing CCL2/7 production.

$Ly6C^+$ monocytes are generated through hematopoiesis and emigrate from the bone marrow in a CCR2 dependent fashion (185, 204). CCL2 and CLL7, ligands of CCR2, are induced in the inflamed tissue and secreted to the circulation, which facilitates the emigration of $Ly6C^+$ monocytes (179, 186). We infected mice with *T. congolense* and developed a gating strategy to evaluate the recruitment of $Ly6C^+$ monocytes and their differentiation and maturation into Tip-DCs in the liver as shown in Figure 3.1. Compared to WT mice, the systematic levels of CCL2 and CCL7 were exceedingly up-regulated approximately 2-4 times in IL-27R deficient mice at both day 7 and day 10 post infection (Fig. 3.2A), indicating a potential responsiveness of CCR2 signaling in monocyte trafficking. The percentage of $Ly6C^+$ monocytes among the liver monocytic cells ($CD45^+Ly6G^-$) (Fig. 3.2B) as well as the percentage within total intrahepatic leukocyte population were profoundly augmented in infected IL-$27R^{-/-}$ mice (Fig. 3.2C), suggesting that IL-27 signaling likely limits the $Ly6C^+$ monocytes rather than other subpopulations of myeloid cells. In addition, infected IL-

27R$^{-/-}$ mice had higher absolute numbers of Ly6C^{+} monocytes per liver than the WT mice on day 7 and day 10 after infection (Fig. 3.2D). Collectively, these results suggest that IL-27 signaling limits Ly6C^{+} monocyte recruitment to the liver during *T. congolense* infection.

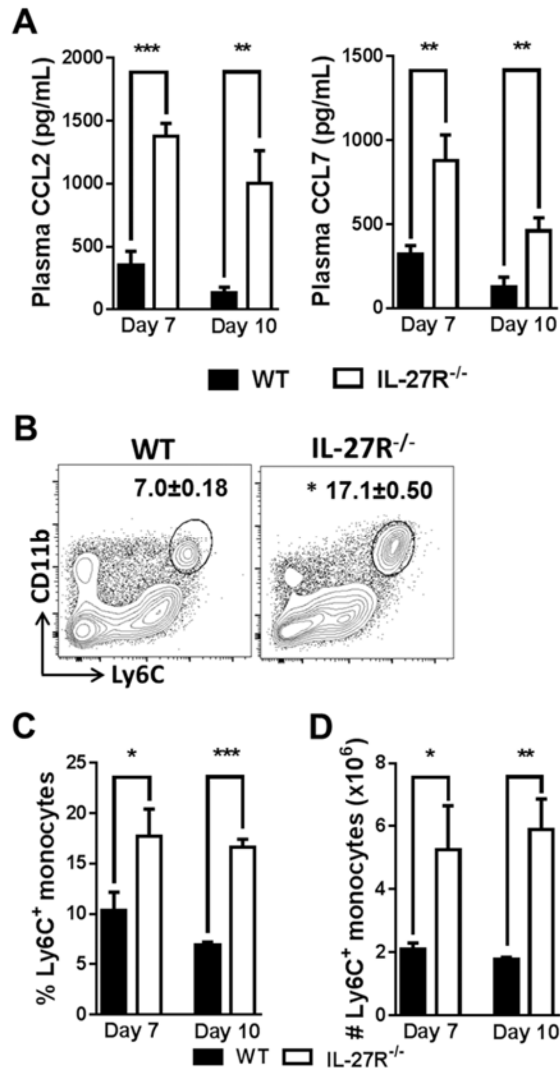

Fig. 3.2 IL-27R$^{-/-}$ mice display enhanced recruitment of liver Ly6C^{+} monocytes during *T. congolense* infection.

IL-27R$^{-/-}$ and WT mice were i.p. infected with 1x10^{3} *T. congolense*. Plasma samples were prepared for ELISA. Liver leukocytes were purified by density gradient centrifugation on day 7 and 10 post infection and leukocytes were gated as Fig. 3.1. (A) The plasma levels of CCL2 and CCL7 were determined by ELISA. (B) Representative FACS plots of Ly6C^{+} monocytes showing the frequency of Ly6C^{+} monocytes among all liver monocytic cells were illustrated. (C&D) The frequency (C) of Ly6C^{+} monocytes within total liver leukocytes and the absolute number (D) of Ly6C^{+} monocytes were determined. Data are representative of 2 independent experiments.

*p<0.05**p<0.01***p<0.001 (Student's t-test)

2. IL-27 signaling limits Ly6C$^+$ monocytes from differentiating into Tip-DCs.

Ly6C$^+$ monocytes can give rise to monocyte-derived DCs (moDCs) which can mature into Tip-DCs upon being induced to up-regulate the expression of MHC II or to produce effector molecules TNF-α/iNOS (196). Tip-DCs mediate liver pathology during infection with African trypanosomes (145). As described in Chapter 2, IL-27 signaling prevents liver pathology during African trypanosome infection (201). Interestingly, there is an inverse correlation between IL-27 signaling function and Tip-DC function. This observation leads to the hypothesis that IL-27 signaling negatively regulates the differentiation of Ly6C$^+$ monocytes into Tip-DCs during infection. The results showed that the percentage of moDCs among either liver CD11b$^+$Ly6C$^+$ cells or total liver immune cells was tremendously increased in infected IL-27R$^{-/-}$ mice (Fig. 3.3A&B). The absolute number of moDCs was also significantly higher in the liver of infected IL-27R$^{-/-}$ mice compared to infected WT mice (Fig. 3.3C). In addition, IL-27R deficient moDCs were more matured as indicated by the greatly enhanced expression of MHC II (Fig. 3.3D). Furthermore, infected IL-27R$^{-/-}$ mice had much higher percentage of intrahepatic Tip-DCs (Fig. 3.3E-H) and absolute number of these cells than infected WT mice. Taken together, these results demonstrate that IL-27 signaling inhibits Tip-DC development.

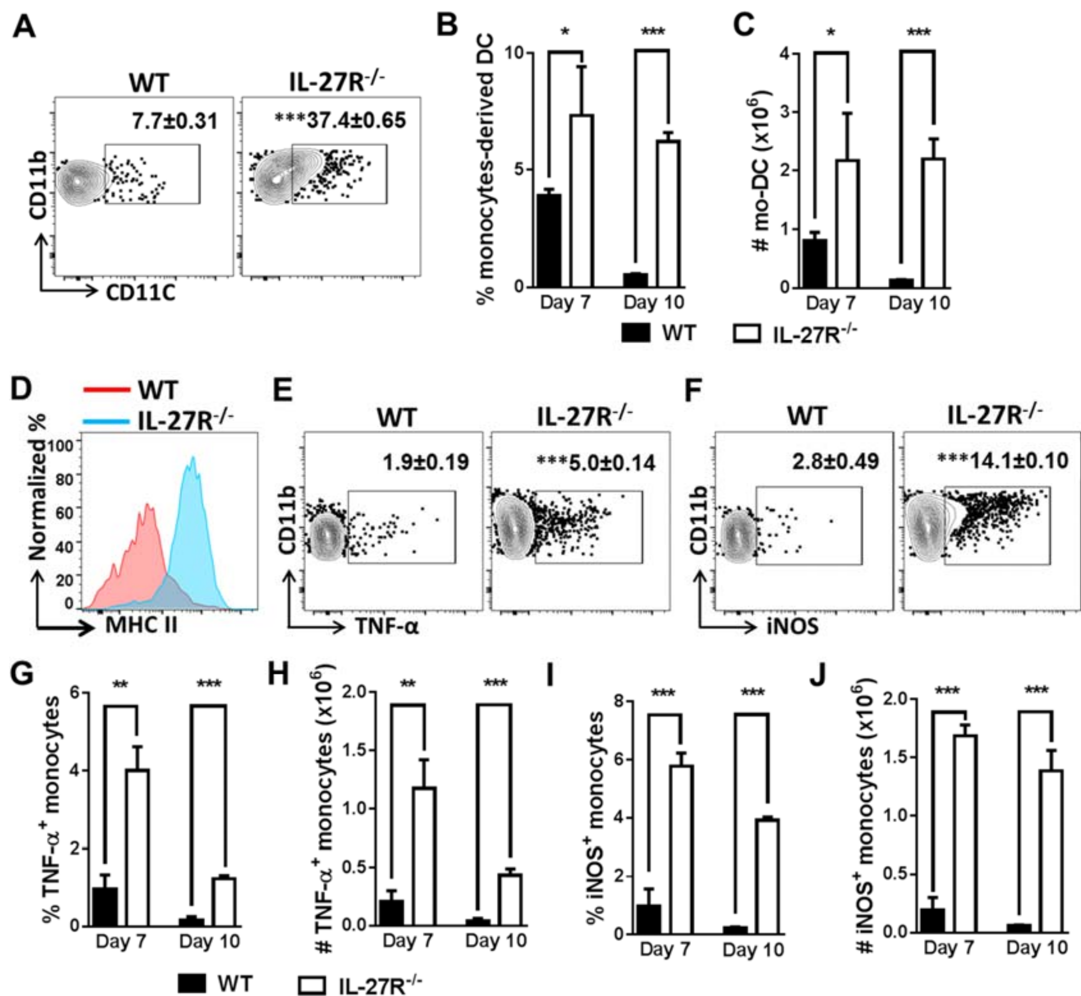

Fig. 3.3 IL-27R$^{-/-}$ mice exhibit higher frequency and absolute number of liver Tip-DCs during *T. congolense* infection.

IL-27R$^{-/-}$ and WT mice were i.p. infected with 1x10^3 *T. congolense*. Liver leukocytes were purified on day 7 and 10 post infection and gated as Fig 3.1. (A) Representative FACS plots showing the frequency of moDCs within CD11b$^+$Ly6C$^+$ monocytes were illustrated. (B&C) The total percentage and absolute number of moDC among all liver leukocytes were determined (D) Representative FACS plot of MHC II intensity of moDC was illustrated. (E&F) Representative FACS plots of Tip-DC showing the frequency of TNF-α$^+$ and iNOS$^+$ within Ly6C$^+$ monocytes were illustrated.(G-J) The frequency (G&I) of Tip-DCs within total liver leukocytes and the absolute number (H&J) of Tip-DCs were determined. Data are representative of 2 independent experiments.

*p<0.05**p<0.01***p<0.001 (Student's t-test)

3. Depletion of CD4$^+$ T cells or neutralization of IFN-γ limits the development of Tip-DCs in IL-27R deficient mice.

It is conceivable that cytokines orchestrate the fate choice of the immune cells. Excessive secretion of IFN-γ by CD4$^+$ T cells in the absence of IL-27 signaling (201) may drive Ly6C$^+$ monocytes to differentiate into Tip-DCs. Thus, we hypothesize that IL-27 signaling negatively regulates the differentiation of Ly6C$^+$ monocytes into Tip-DCs during infection via inhibiting CD4$^+$ T cell activation and IFN-γ secretion. First, we examined the effects of CD4$^+$ T cells on production of CCL2 and CCL7 in infected IL-27R$^{-/-}$ mice. As shown in Figure 3.4A, the peripheral level of CCL2 and CCL7 were dramatically decreased in IL-27R$^{-/-}$ mice with depletion of CD4$^+$ T cells. This was associated with diminished recruitment of Ly6C$^+$ monocytes to the liver as indicated by the lower frequency and absolute number of Ly6C$^+$ monocytes on both Day 7 and Day 10 post infection (Fig. 3.4B, C&D). Taken together, over-activation of CD4$^+$ T cells in infected IL-27R$^{-/-}$ mice promotes the accumulation of Ly6C$^+$ monocytes in the liver associated with higher production of CCL2 and CCL7.

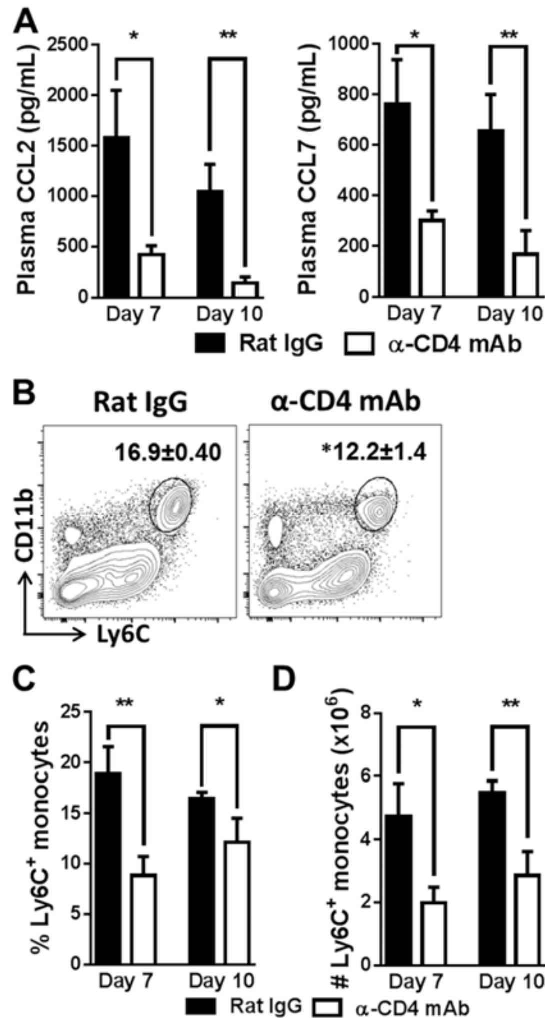

Fig. 3.4 Depletion of CD4$^+$ T cells diminishes the plasma levels of CCL2 and CCL7 and reduces the accumulation of Ly6C$^+$ monocytes in the liver of IL27R$^{-/-}$ mice during *T. congolense* infection.

IL-27R$^{-/-}$ mice were i.p. infected with 1x10^3 *T. congolense* and treated with anti-CD4 mAb or control mAbs. Plasma samples were prepared for ELISA. Liver leukocytes were purified by density gradient centrifugation on day 7 and 10 post infection and leukocytes were gated as Fig. 3.1. (A) The plasma level of CCL2 and CCL7 was determined by ELISA. (B) Representative FACS plots of Ly6C$^+$ monocytes showing the frequency of Ly6C$^+$ monocytes among all liver monocytic cells were illustrated. (C&D) The frequency (C) of Ly6C$^+$ monocytes within total liver leukocytes and the absolute number (D) of Ly6C$^+$ monocytes were determined. Data are representative of 2 independent experiments.

*$p<0.05$**$p<0.01$ (Student's t-test)

Next we examined the role of $CD4^+$ T cells in differentiation of $Ly6C^+$ monocytes into Tip-DCs in infected IL-$27R^{-/-}$ mice. As shown in Figure 3.5A, depletion of $CD4^+$ T cells remarkably restricted the generation of moDCs (CD11c expressing $CD11b^+Ly6C^+$ cells), indicating that IL-27 limits development of moDCs from $Ly6C^+$ monocytes by antagonizing activation of $CD4^+$ T cells. In addition, depletion of $CD4^+$ T cells significantly reduced the frequency and absolute number of moDCs in the liver of infected IL-$27R^{-/-}$ mice (Fig. 3.5B & C). Moreover, depletion of $CD4^+$ T cells resulted in reduction of MHC-II expression on moDCs in infected IL-$27R^{-/-}$ mice, suggesting that IL-27 prohibits the maturation of moDC via inhibiting $CD4^+$ T cell activation. Furthermore, as compared to the control group, Tip-DC development in IL-$27R^{-/-}$ mice depleted of $CD4^+$ T cells was immensely impaired as indicated by significantly lower frequency and absolute number of TNF-α^+/ $iNOS^+$ cells (Fig. 3.5E-J).

Collectively, these data demonstrate that IL-27 inhibits accumulation of $Ly6C^+$ monocytes in the liver and their differentiation into Tip-DCs through suppressing $CD4^+$ T cell activation.

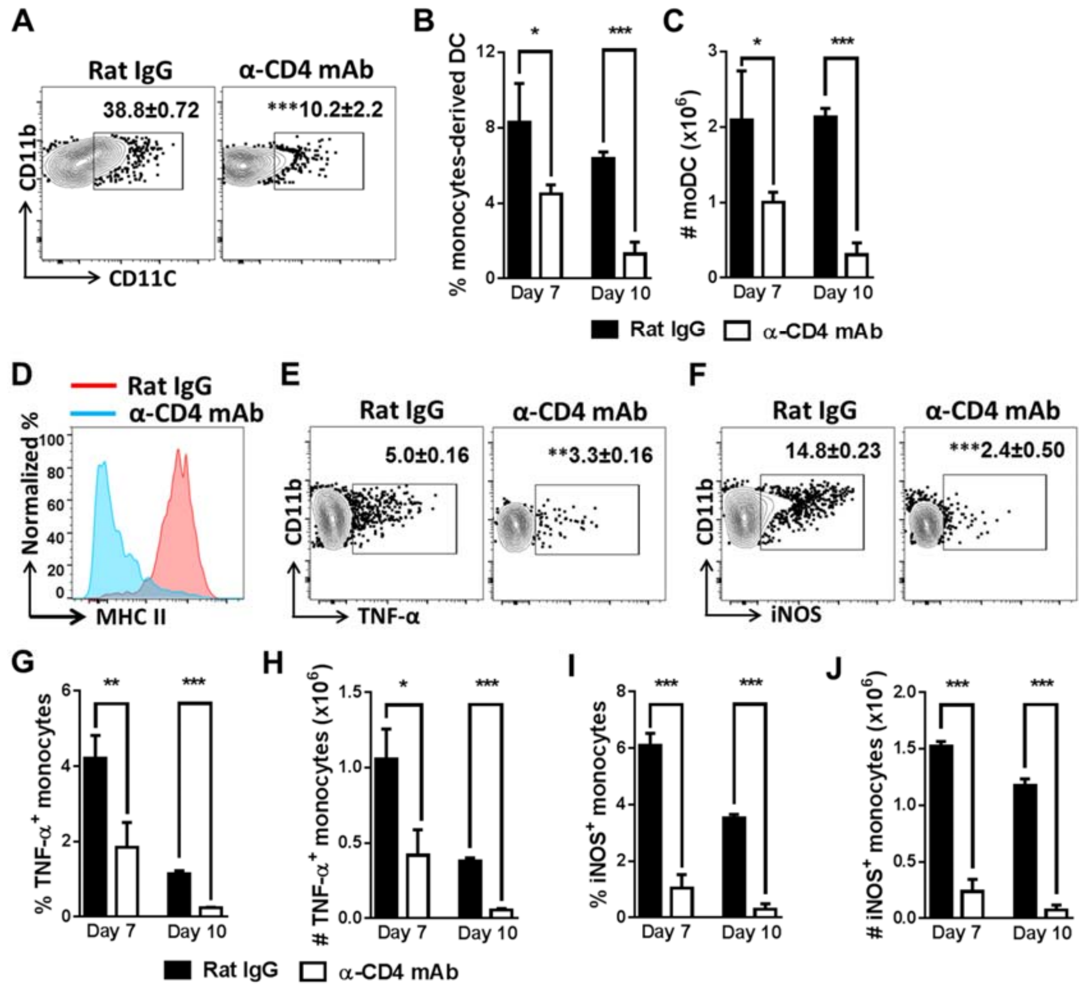

Fig. 3.5 Depletion of CD4$^+$ T cells impairs Tip-DC development and accumulation in the liver of IL-27R$^{-/-}$ mice during *T. congolense* infection.

IL-27R$^{-/-}$ mice were i.p. infected with 1x10^3 T. *congolense* and treated with anti-CD4 mAb and control mAb. Liver leukocytes were purified on day 7 and 10 post infection and gated as Fig 3.1. (A) Representative FACS plots of moDC showing the frequency of moDC within Ly6C$^+$ monocytes were illustrated. (B&C) The total percentage among all liver leukocytes and absolute number of moDC were determined. (D) Representative FACS plot of MHC II intensity of moDC were illustrated. (E&F) Representative FACS plots of Tip-DC showing the frequency of TNF-α$^+$ and iNOS$^+$ within Ly6C$^+$ monocytes were illustrated.(G-J) The frequency (G&I) of Tip-DCs within total liver leukocytes and the absolute number (H&J) of Tip-DCs were determined. Data are representative of 2 independent experiments.

*p<0.05**p<0.01***p<0.001 (Student's t-test)

As IL-27 inhibits $CD4^+$ T cells to secrete IFN-γ, preventing immunopathology in mice infected with African trypanosomes as shown in Chapter 2. Next we examined the role of IFN-γ in Tip-DC development in infected $IL\text{-}27R^{-/-}$ mice. To this end, $IL\text{-}27R^{-/-}$ mice were infected with trypanosomes and treated with anti-IFN-γ mAb to neutralize IFN-γ. Neutralization of IFN-γ significantly diminished the production of CCL2 and CCL7 (Fig.3.6 A), associated with dramatically decreased frequency and absolute number of $Ly6C^+$ monocytes in the liver as compared to the control group (Fig. 3.6B&C), suggesting that IL-27 inhibits secretions of CCL2 and CCL7, and reduces $Ly6C^+$ monocyte accumulation in the liver in an IFN-γ-dependent manner.

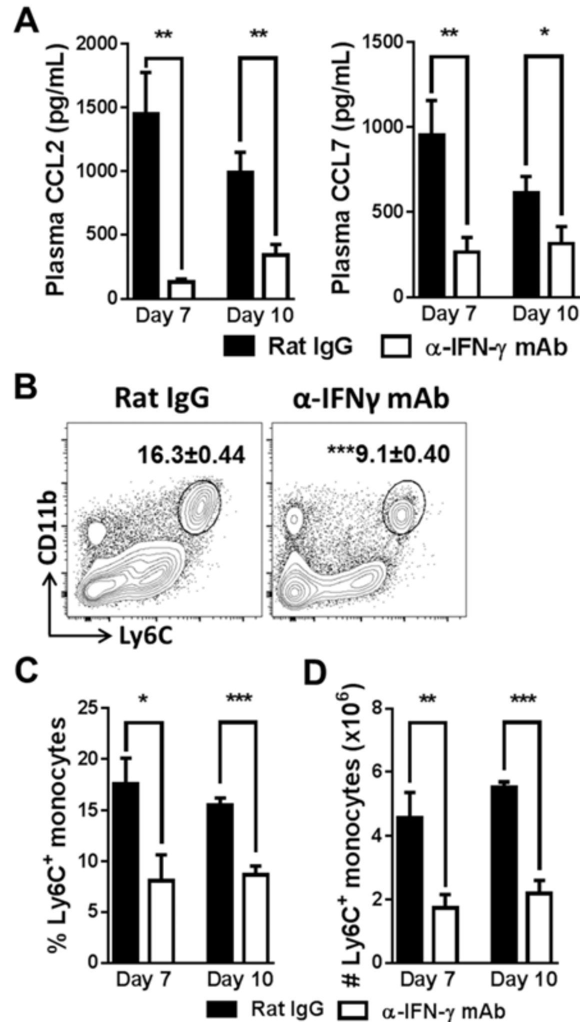

Fig. 3.6 Neutralization of IFN-γ reduces the plasma levels of CCL2 and CCL7 and diminishes accumulation of Ly6C$^+$ monocytes in the liver of IL27R$^{-/-}$ mice during *T. congolense* infection.

IL-27R$^{-/-}$ mice were i.p. infected with 1x10^3 *T. congolense* and treated with anti-IFN-γ-mAb or control mAb. Plasma samples were prepared for ELISA. Liver leukocytes were purified by density gradient centrifugation on day 7 and 10 post infection and leukocytes were gated as Fig. 3.1. (A) The plasma level of CCL2 and CCL7 was determined by ELISA. (B) Representative FACS plots of Ly6C$^+$ monocytes showing the frequency of Ly6C$^+$ monocytes among all liver monocytic cells were illustrated. (C&D) The frequency (C) of Ly6C$^+$ monocytes within total liver leukocytes and the absolute number (D) of Ly6C$^+$ monocytes were determined. Data are representative of 2 independent experiments.

*p<0.05**p<0.01***p<0.001 (Student's t-test)

Neutralization of IFN-γ also reduces the frequency of moDCs within $Ly6C^+$ population as well as the absolute number of moDCs in the liver of infected $IL\text{-}27R^{-/-}$ mice (Fig. 3.7A, B & C), indicating that IL-27 limits moDC development and accumulation by suppressing IFN-γ production. In addition, Infected $IL\text{-}27R^{-/-}$ mice treated with anti-IFN-γ mAb showed reduced expression level of MHC II on moDCs (Fig. 3.7D), suggesting that IL-27 inhibits moDC maturation via inhibiting IFN-γ secretion. Furthermore, neutralization of IFN-γ in infected $IL\text{-}27R^{-/-}$ mice reduced the frequency and the absolute number of moDCs producing TNF-α or iNOS (Fig. 3.7 E-J). Together, these results demonstrate that IL-27 inhibits differentiation and maturation of $Ly6C^+$ monocytes into Tip-DCs through inhibiting IFN-γ secretion.

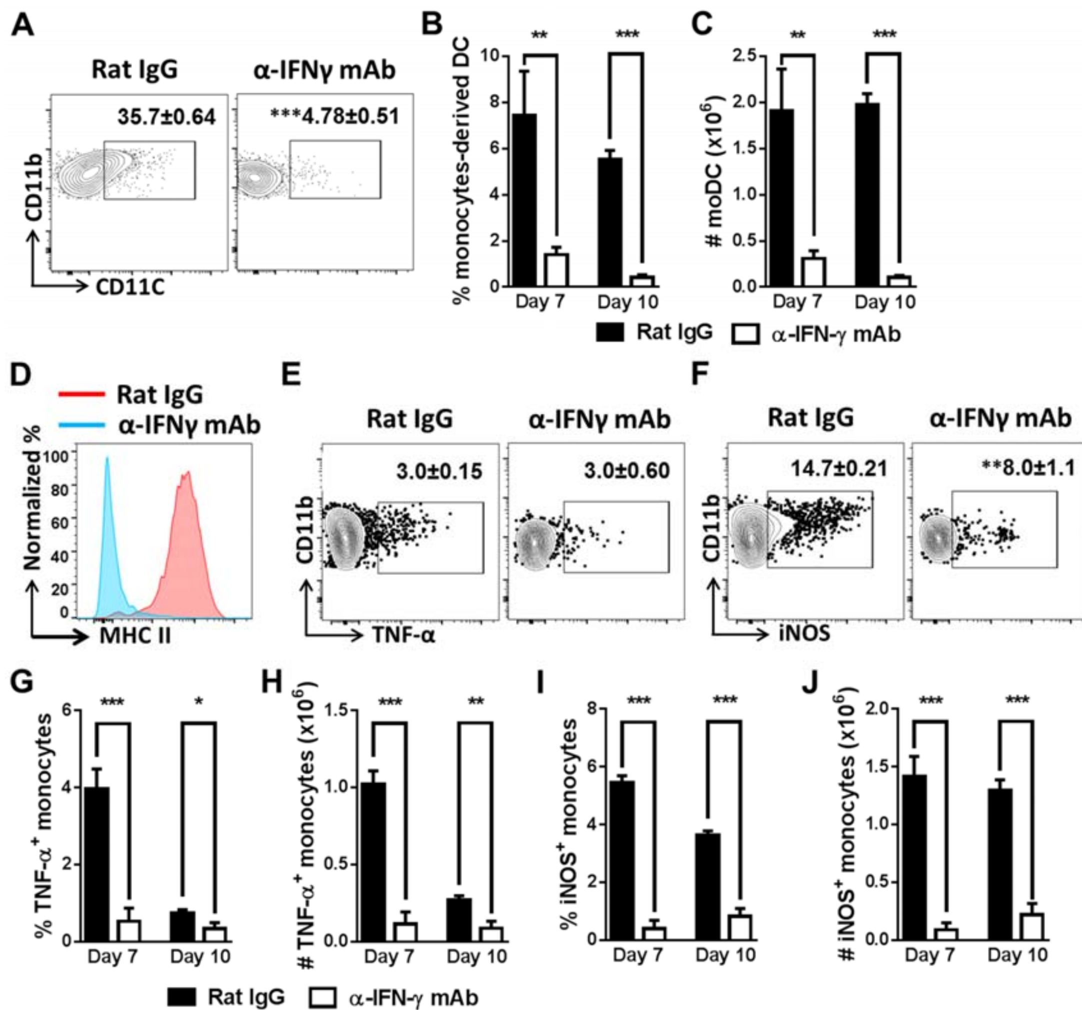

Fig. 3.7 Neutralization of IFN-γ perturbs Tip-DC development and accumulation in the liver of IL-27R$^{-/-}$ mice during *T. congolense* infection.

IL-27R$^{-/-}$ mice were i.p. infected with $1x10^3$ *T. congolense* and treated with anti-IFN-γ mAb and control mAbs. Liver leukocytes were purified on day 7 and 10 post infection and gated as Fig. 3.1. (A) Representative FACS plots of moDC showing the frequency of moDC within Ly6C$^+$ monocytes were illustrated. (B&C) The total percentage among all liver leukocytes and absolute number of moDC were determined. (D) Representative FACS plot of MHC II intensity of moDC were illustrated. (E&F) Representative FACS plots of Tip-DC showing the frequency of TNF-α$^+$ and iNOS$^+$ within Ly6C$^+$ monocytes were illustrated.(G-J) The frequency (G&I) of Tip-DCs within total liver leukocytes and the absolute number (H&J) of Tip-DCs were determined. Data are representative of 2 independent experiments.
*p<0.05**p<0.01***p<0.001 (Student's t-test)

Similar to conventional DCs, Tip-DCs express high levels of CD11c and MHC II. Hence, efforts were taken to determine whether Tip-DCs were also capable of priming naive $CD4^+$ T cells similar to their conventional counterparts. Infected IL-$27R^{-/-}$ mice were treated with CCR2 antagonist to retain $Ly6C^+$ in the bone marrow. As shown in Fig.3.8 A&B, administration of CCR2 antagonist led to a roughly 50% decrease of $Ly6C^+$ monocytes in blood. However, the frequency of activated $CD4^+$ T cells ($CD44^+CD62L^-$) as well as the IFN-γ producing capacity remained intact in the spleen (Fig. 3.8C&D). As Tip-DC development is dependent on efficient emigration of $Ly6C^+$ monocytes, the intact $CD4^+$ T cell activation in scenario of impaired $Ly6C^+$ monocytes emigration suggests that Tip-DC is redundant for activation of $CD4^+$ T cells.

In conclusion, in absence of IL-27, excessive secretion of IFN-γ by $CD4^+$ T cells promotes $Ly6C^+$ monocyte accumulation of in the liver and their differentiation into Tip-DCs.

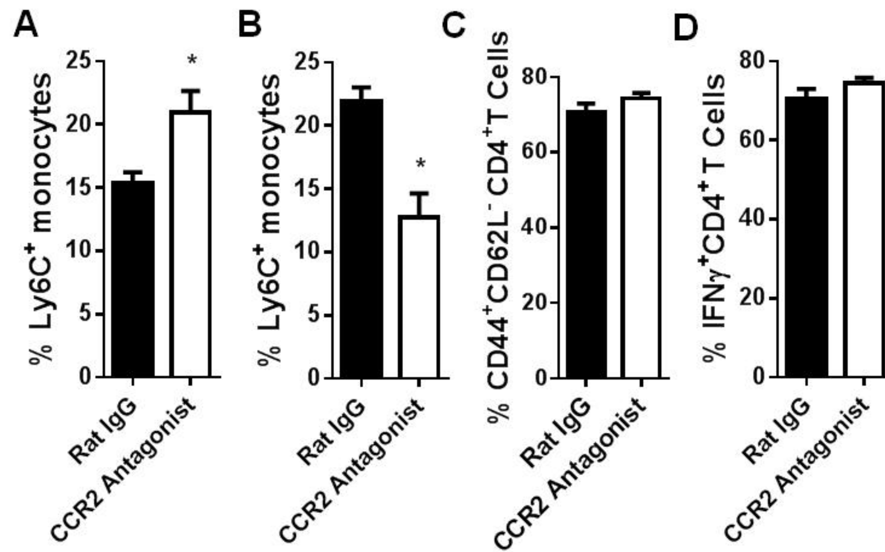

Fig. 3.8 Impaired emigration of Ly6C⁺ monocytes does not affect T cell activation during African trypanosome infection.

WT mice infected with $1x10^3$ *T. congolense* were i.p. injected with CCR2 antagonist (10mg/kg) daily starting on day -1 post infection. The frequency of $Ly6C^+$ monocytes within total leukocytes (A&B) were determined in bone marrow and blood on day 7 post infection to evaluate the efficacy of the CCR2 antagonist. Spleen $CD4^+$ cells were co-stained for CD44 and CD62L to determine T cell activation (C), and co-stained for intracellular IFN-γ (D). Data are representative of 2 independent experiments.

*p<0.05 (Student's t-test).

4. IL-27R signaling limits the cell death of $Ly6C^-$ monocytes and preserves $Ly6C^-$ macrophages during infection.

In addition to $Ly6C^+$ monocytes, blood monocytes consist of the other principal subset: $Ly6C^-$ monocytes phenotypically characterized as $CD11b^+Ly6C^-CX3CR1^{hi}F4/80^{lo}$ (182). It has been recently shown that, instead of becoming Tip-DCs, $Ly6C^+$ monocytes can become anti-inflammatory $Ly6C^-$ macrophages and that $Ly6C^-$ monocytes facilitate this transition (205). Thus, we examined the two anti-inflammatory $Ly6C^-$ populations in the liver of IL-$27R^{-/-}$ mice during *T. congolense* infection. Coinciding with the enhanced Tip-DC development in IL-$27R^{-/-}$ mice, $Ly6C^-$ macrophages ($CD11b^+Ly6C^-CX3CR1^+F4/80^{hi}$) were poorly generated at the day 10 post infection as compared with the WT mice (Fig. 3.9A, C, &E). In addition, the frequency and absolute number of $Ly6C^-$ monocytes were remarkably reduced (Fig. 3.9A, B&D).

$Ly6C^-$ monocytes are more sensitive to stress and depend on multiple survival signals, including CX3CR1, to maintain the sentinel phenotype (204, 206-208). To decipher the total loss of the $Ly6C^-$ monocytes in IL-$27R^{-/-}$ mice, we performed the cell viability assay to determine the live (Annexin V^-7-AAD^-), apoptotic (Annexin

$V^+7\text{-}AAD^-$) and dead ($7\text{-}AAD^+$) events among $Ly6C^-$ monocytes. As shown in Figure 3.9F&G, $Ly6C^-$ monocytes underwent apoptosis resulting in significant higher number of dead cells in infected $IL\text{-}27R^{-/-}$ mice. As compared to $Ly6C^+$ monocytes, $Ly6C^-$ monocytes express higher intensity of CX3CR1 which serves as a survival signal through ligation to its ligand CX3CL1 (208). To shed light on the mechanism of enhanced cell death in $Ly6C^-$ monocytes, we further determined their expression level of CX3CR1. Notably, the surface expression of CX3CR1 on $Ly6C^-$ monocytes from $IL\text{-}27R^{-/-}$ mice was significant lower than that of $Ly6C^-$ monocytes in WT mice (Fig. 3.9H), indicating that IL-27 signaling prevents the cell death of $Ly6C^-$ monocytes through preserving the survival signaling.

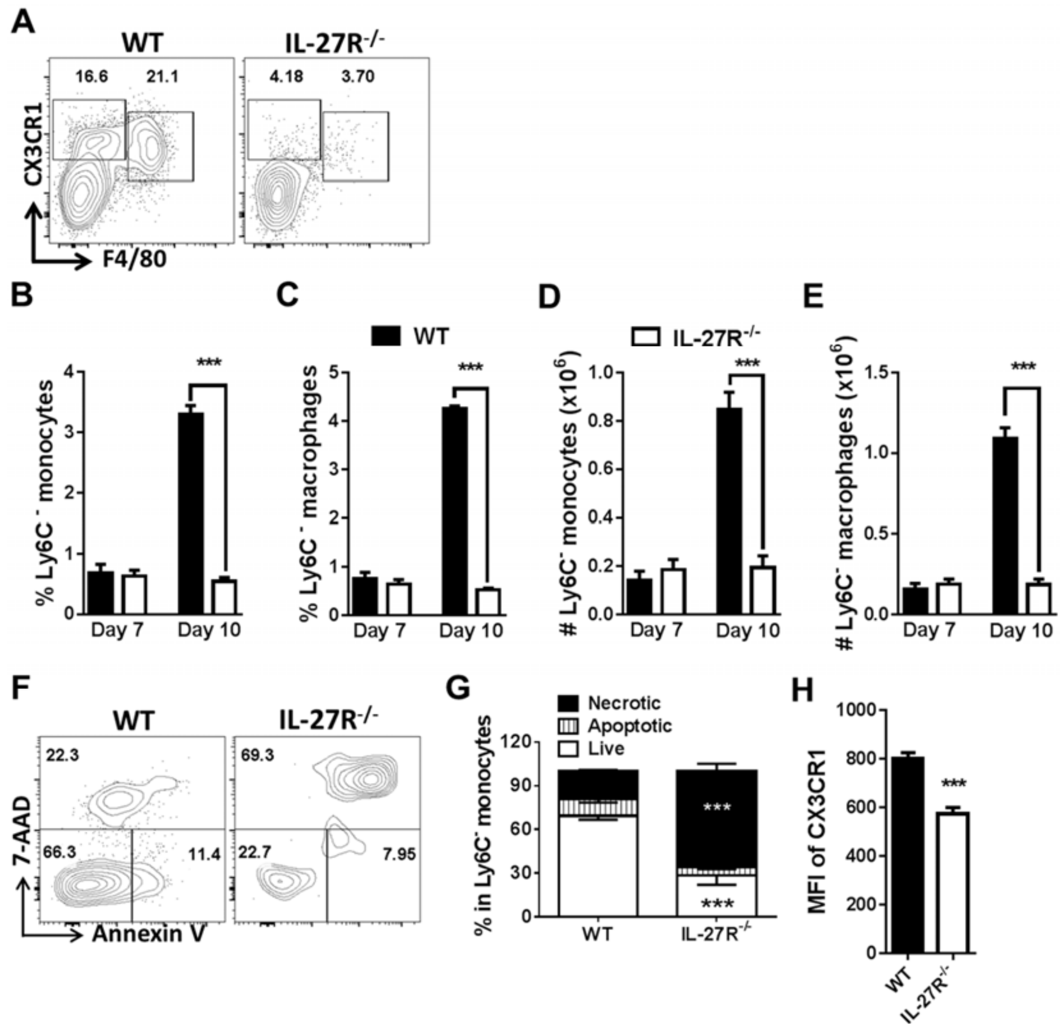

Fig. 3.9 The loss of liver Ly6C$^-$ monocytes in IL-27R$^{-/-}$ mice during *T. congolense* infection is due to increased cell death.

IL-27R$^{-/-}$ and WT mice were i.p. infected with 1x10^3 *T. congolense*. Liver leukocytes were purified on day 7 and 10 post infection and gated as Fig. 3.1. (A) Representative FACS plots of Ly6C$^-$ monocytes (Ly6C$^-$CX3CR1^{hi}F4/80$^-$) and macrophages (Ly6C$^-$Cx3CR1$^+$F4/80^{hi}) showing the respective frequencies within Ly6C$^-$ gate were illustrated. (B&C) The frequency of Ly6C$^-$ monocytes (B) and macrophages (C) within total liver leukocytes were determined. (D&E) The absolute number of Ly6C$^-$ monocytes (D) and macrophages (E) were assessed. (F&G) Representative FACS plots (F) and quantification (G) of viability of Ly6C$^-$ monocytes were exhibited. (H) CX3CR1 expression of Ly6C$^-$ monocytes was evaluated by median fluorescence intensity (MFI). Data are representative of 2 independent experiments.

***p<0.001 (Student's t-test)

5. Ly6C$^-$ monocytes exhibit an anti-inflammatory phenotype and inhibit Tip-DC development in infected IL-27R$^{-/-}$ mice.

As we demonstrated in Figure 3.2& 3.3, Ly6C$^+$ monocytes are biased to mature into Tip-DCs with a MHC II$^+$CD11c$^+$ phenotype in IL-27R$^{-/-}$ mice. In contrast, Ly6C$^-$ monocytes exhibit an anti-inflammatory phenotype as previously described (205). Given the highly inverse correlation of the Ly6C$^+$ and Ly6C$^-$ monocytes in function and cell number, we hypothesized that the loss of Ly6C$^-$ monocytes contributes to the biased development of Ly6C$^+$ monocytes into Tip-DCs in IL-27R$^{-/-}$ mice. Using the strategy in Figure 3.10A, Ly6C$^-$ monocytes were sorted from the liver of infected WT mice 3 weeks after infection and transferred to IL-27R deficient mice at day 3 and 6 post infection. As shown in Figure 3.10B, donor Ly6C$^-$ monocytes were retrieved in the liver of recipient mice and exhibited the original Ly6C negative phenotype, indicating a successful working model with high transfer efficiency and cell phenotype stability. Then, we examined the differentiation program of the native Ly6C$^+$ monocytes in the recipient IL-27R$^{-/-}$ mice. Consequently, the frequency of MHC II$^+$CD11C$^+$ cells within CD11b$^+$Ly6C$^+$ population of recipient IL-27R$^{-/-}$ mice was significantly reduced as compared to that of the no-transfer control (Fig. 3.10C&D), indicating that boosting the Ly6C$^-$ monocytes in IL-27R$^{-/-}$ mice

could inhibit $Ly6C^+$ monocytes from differentiating into Tip-DCs. Collectively, we confirmed an anti-inflammatory effect of $Ly6C^-$ monocytes in limiting $Ly6C^+$ monocytes differentiation into Tip-DCs.

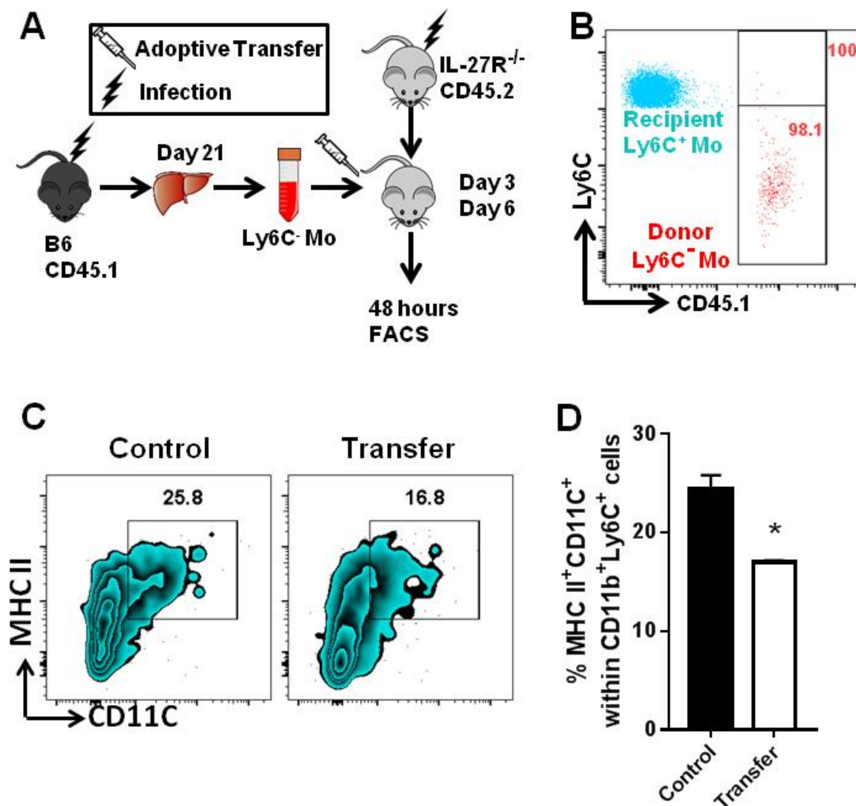

Fig. 3.10 Adoptive transfer of Ly6C$^-$ monocytes reduces the differentiation and maturation of moDCs in recipient IL-27R$^{-/-}$ mice during *T. congolense* infection. Ly6C$^-$ monocytes (1x10^6) were collected from CD45.1 WT mice on day 21 post infection and transferred to CD45.2 IL-27R$^{-/-}$ mice on day 3 and 6 post infection. All parameters were determined in recipient mice at 48 hours post the last transfer. (A) Schematic of experimental design. (B) Integrity of Ly6C negative phenotype of donor Ly6C$^-$ monocytes. (C&D) Representative FACS plots (C) and the frequency (D) of matured moDC (CD11b$^+$Ly6C$^+$MHC II$^+$CD11C$^+$) within liver CD11b$^+$Ly6C$^+$ population in the recipient mice.

*$p<0.05$(Student’s t-test).

As described in Chapter 2, IL-27R deficient mice infected with African trypanosomes exhibited hyper activation and expansion of $CD4^+$ T cells in the spleen (201). In contrast, the liver is the major organ for control and clearance of African trypanosomes by the mononuclear phagocytes, including monocytes and Kupffer cells (101, 102). Upon phagocytosis of the parasites, chemokines are released resulting in the recruitment of T cells to the liver. IL-27R$^{-/-}$ mice receiving Ly6C$^-$ monocytes had impaired accumulation of activated $CD4^+$ T cells ($CD44^+CD62L^-$) in the liver (Fig. 3.11C), lower ratio of $CD4^+/CD8^+$ T cells (Fig. 3.11D) as well as decreased ALT level (Fig. 3.11E), indicating an attenuated inflammatory response in the liver preserved by adoptively transferred Ly6C$^-$ monocytes. As noted, the adoptively transfer of Ly6C$^-$ monocytes did not affect the $CD4^+$ T cells priming and activation in the spleen (Fig. 3.11 A&B).

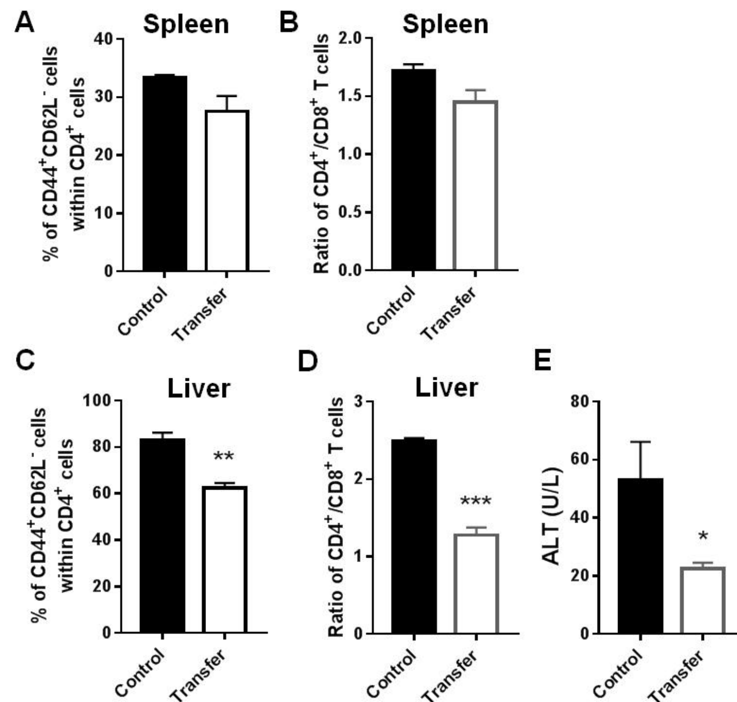

Fig. 3.11 Adoptive transfer of Ly6C$^-$ monocytes diminishes liver inflammation in recipient IL-27R$^{-/-}$ mice during *T. congolense* infection.

Ly6C$^-$ monocytes ($1x10^6$) were collected from CD45.1 WT mice on day 21 post infection and transferred to CD45.2 IL-27R$^{-/-}$ mice on day 3 and 6 post infection as Fig 3.10A. All parameters were determined in recipient mice at 48 hours post the last transfer. (A) The frequency of the activated CD4$^+$ T cells (CD44$^+$CD62L$^-$) in spleen was determined. (B) The ratio of CD4$^+$/CD8$^+$ T cells in the spleen was evaluated. (C) The frequency of the activated CD4$^+$ T cells (CD44$^+$CD62L$^-$) in liver was calculated. (D) The ratio of CD4$^+$/CD8$^+$ T cells in the liver was assessed. (E) Plasma alanine aminotransferase (ALT) level was determined by ELISA.

*p<0.05**p<0.01***p<0.001 (Student's t-test)

6. Depletion of $CD4^+$ T cells or neutralization of IFN-γ enhances $Ly6C^-$ monocyte and macrophages in infected $IL\text{-}27R^{-/-}$ mice

Given the anti-inflammatory property of $Ly6C^-$ monocytes in orchestrating Tip-DC development, it is worthwhile to further decipher the mechanism of the remarkable loss of $Ly6C^-$ monocytes in $IL\text{-}27R^{-/-}$ mice. As shown in Figure 3.9, in $IL\text{-}27R^{-/-}$ mice, $Ly6C^-$ monocytes were subject to cell death due to impaired survival signal. We hypothesize that excessive IFN-γ produced by $CD4^+$ T cells in $IL\text{-}27R^{-/-}$ mice promotes the cell death of $Ly6C^-$ monocytes resulting in the accelerated development of $Ly6C^+$ monocytes into inflammatory Tip-DCs. To test our hypothesis, $IL\text{-}27R^{-/-}$ mice infected with *T. congolense* were treated with anti-CD4 mAb, and the frequency and total number of $Ly6C^-$ monocytes and macrophages were assessed. Depletion of $CD4^+$ T cells exceedingly restored the counts of $Ly6C^-$ monocytes and macrophages in infected $IL\text{-}27R^{-/-}$ mice (Figure 3.12A-E), implying that IL-27 promotes the accumulation of $Ly6C^-$ monocytes and macrophages via counteracting $CD4^+$ T cell activation. This is coincided with reduced cell death of $Ly6C^-$ monocytes (Fig. 3.12 F&G). It is worthwhile to mention that depletion of $CD4^+$ T cells resulted in up-regulated expression of CX3CR1 on $Ly6C^-$ monocytes (Fig. 3.12H), supporting that CX3CR1 is required for $Ly6C^-$ monocyte survival (208).

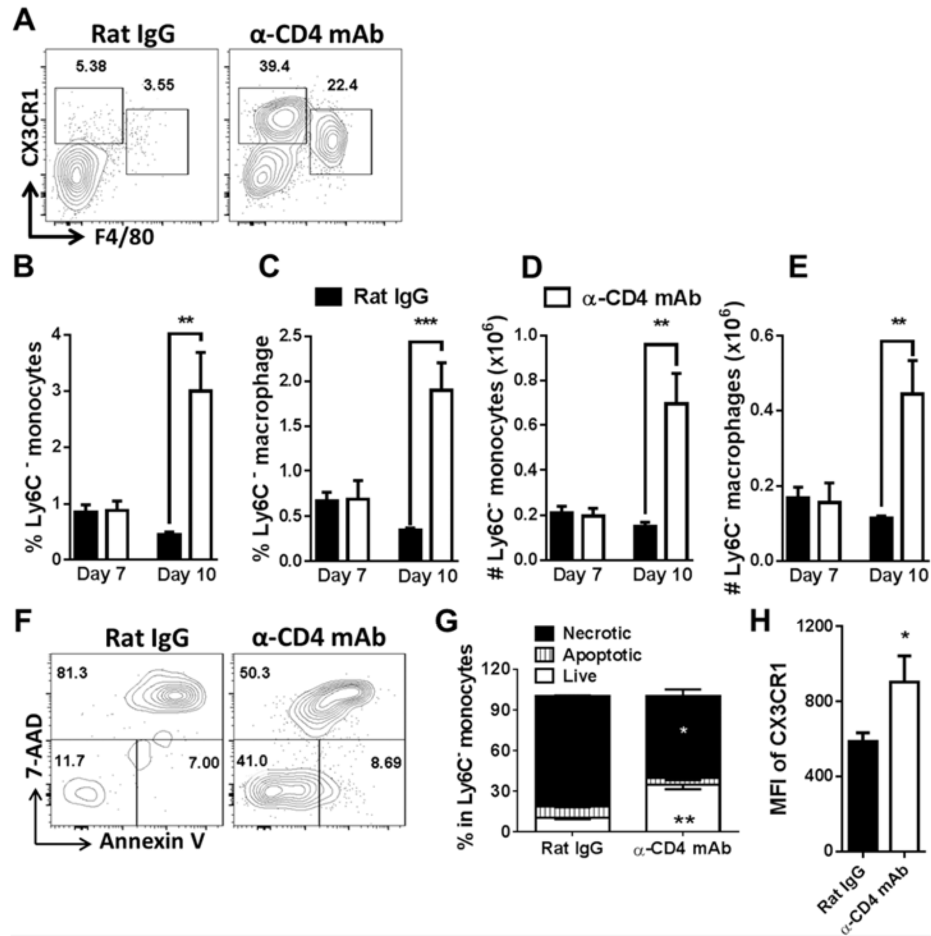

Fig. 3.12 Depletion of CD4^{+} T cells enhances the frequency and absolute number of liver Ly6C^{-} monocytes and macrophages in IL-27R$^{-/-}$ mice during *T. congolense* infection.

IL-27R$^{-/-}$ were i.p. infected with 1x10^{3} *T. congolense* and treated with anti-CD4 mAb or control mAb. Liver leukocytes were purified on day 7 and 10 post infection and gated as Fig. 3.1. (**A**) Representative FACS plots of Ly6C^{-} monocytes (Ly6C^{-}CX3CR1^{hi}F4/80^{-}) and macrophages (Ly6C^{-}Cx3CR1^{+}F4/80^{hi}) showing the respective frequencies within CD11b^{+}Ly6C^{-} gate were illustrated. (**B&C**) The frequency of Ly6C^{-} monocytes (**B**) and macrophages (**C**) within total liver leukocytes were determined. (D&E) The absolute number of Ly6C^{-} monocytes (**D**) and macrophages (**E**) were assessed. (**F&G**) Representative FACS plots (**F**) and quantification (**G**) of viability of Ly6C^{-} monocytes were exhibited. (**H**) CX3CR1 expression of Ly6C^{-} monocytes was evaluated by median fluorescence intensity (MFI). Data are representative of 2 independent experiments.

*p<0.05**p<0.01***p<0.001 (Student's t-test)

Next, we examined the role of IFN-γ in $Ly6C^-$ monocytes in infected $IL\text{-}27R^{-/-}$ mice. As shown in panel A-E of Figure 3.13, the percentage and total number of intrahepatic $Ly6C^-$ monocytes and macrophages were restored by neutralization of IFN-γ in infected $IL\text{-}27R^{-/-}$ mice. This is coincided with reduced death of $Ly6C^-$ monocytes (Fig. 3.13 F&G). Similar to $CD4^+$ T cell depletion, neutralization of IFN-γ also enhanced CX3CR1 expression on $Ly6C^-$ monocytes, a molecule essential for the survival of $Ly6C^-$ monocytes (208).

Overall, via suppressing IFN-γ secretion by $CD4^+$ T cells, IL-27 prevents intrahepatic $Ly6C^-$ monocytes from cell death and facilitates the generation of macrophages in the liver of infected mice.

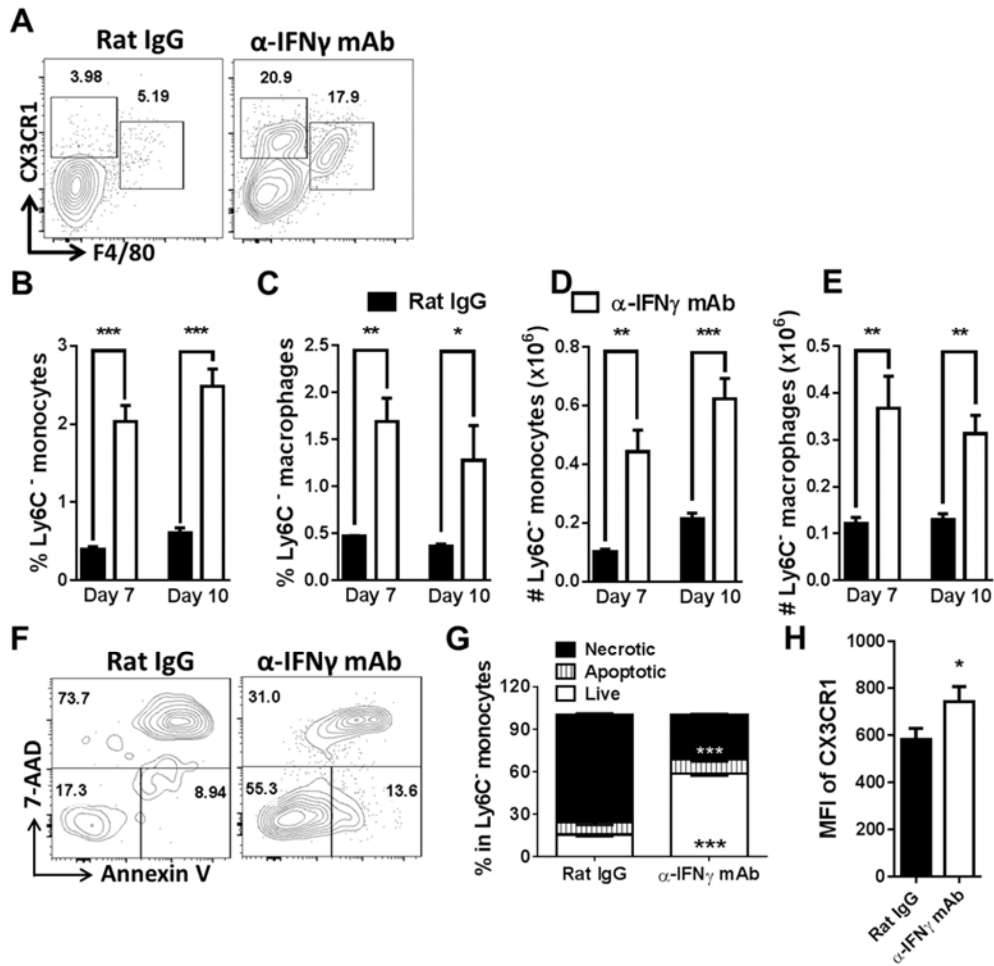

Fig. 3.13 Neutralization of IFN-γ increases the frequency and absolute number of liver Ly6C$^-$ monocytes and macrophages in IL-27R$^{-/-}$ mice during *T. congolense* infection.

IL-27R$^{-/-}$ were i.p. infected with 1x10^3 T. *congolense* and treated with anti-IFN-γmAb or control mAb. Liver leukocytes were purified on day 7 and 10 post infection and gated as Fig. 3.1. (**A**) Representative FACS plots of Ly6C$^-$ monocytes (Ly6C$^-$ CX3CR1^{hi}F4/80$^-$) and macrophages (Ly6C$^-$Cx3CR1$^+$F4/80^{hi}) showing the respective frequencies within CD11b$^+$Ly6C$^-$ gate were illustrated. (**B&C**) The frequency of Ly6C$^-$ monocytes (**B**) and macrophages (**C**) within total liver leukocytes were determined. (D&E) The absolute number of Ly6C$^-$ monocytes (**D**) and macrophages (**E**) were assessed. (**F&G**) Representative FACS plots (**F**) and quantification (**G**) of viability of Ly6C$^-$ monocytes were exhibited. (**H**) CX3CR1 expression of Ly6C$^-$ monocytes was evaluated by median fluorescence intensity (MFI). Data are representative of 2 independent experiments.

*p<0.05**p<0.01***p<0.001 (Student's t-test)

7. Cell intrinsic IFN-γ signaling drives Ly6C^{+} monocytes to differentiate to Tip-DCs in infected IL-27R$^{-/-}$ mice.

The interferon gamma receptor (IFN-γR) consists of two subunits, IFN-γR1 and IFN-γR2 (209). IFN-γR1 is responsible for IFN-γ binding, while both subunits are required for the transduction of the IFN-γ signaling (210). Except for the mature erythrocytes, IFN-γR1 is expressed on most murine cells including monocytes (211). Therefore, it is conceivable that the cell intrinsic IFN-γ signaling could possibly orchestrate the Tip-DC development by directly dictating the differentiation program of Ly6C^{+} monocytes. To test the hypothesis, Ly6C^{+} monocytes were sorted from the bone marrow of naïve IFN-γR$^{+/+}$ and IFN-γR$^{-/-}$ mice by FACS. As illustrated in Figure 3.14A, the cells were labeled with PKH26 (IFN-γR$^{+/+}$) or CellVue (IFN-γR$^{-/-}$) kits, respectively, and injected at a 1:1 ratio via tail vein into recipient infected IL-27R$^{-/-}$ mice on day 7 post infection. The absolute number and the percentage of Tip-DCs within the two distinctly labeled transferred Ly6C^{+} cell populations were determined 48 hours post transfer and the phenotypes between the two transferred populations were compared. The FACS plots in Figure 3.14B and the quantitative data in Figure 3.14D showed that the percentage of MHC II^{+}CD11C^{+} cells within the transferred IFN-γR$^{+/+}$Ly6C^{+} monocytes were significantly higher compared to IFN-

$\gamma R^{-/-}Ly6C^{+}$ monocytes, demonstrating that cell intrinsic IFN-γ signaling of $Ly6C^{+}$ monocytes dictates the differentiation of $Ly6C^{+}$ monocytes into Tip-DCs.

Compared to transferred IFN-$\gamma R^{+/+}Ly6C^{+}$ monocytes, the IFN-$\gamma R^{-/-}Ly6C^{+}$ monocytes strikingly switched to a $Ly6C^{-}$ phenotype in the liver of the recipient IL-$27R^{-/-}$ mice (Fig. 3.14B&C). Furthermore, the resultant $Ly6C^{-}$ population derived from IFN-$\gamma R^{+/+}Ly6C^{+}$ monocytes extensively expressed the macrophage marker F4/80 (Fig. 3.14B&E), supporting the notion that $Ly6C^{+}$ monocytes give rise to $Ly6C^{-}$ macrophages (205). Surprisingly, IFN-$\gamma R^{-/-}Ly6C^{+}$ monocytes gave rise to marginal $Ly6C^{-}$ macrophages but predominantly transfromed to $Ly6C^{-}F4/80^{-}$ cells (Fig. 3.14B&E) , indicating that optimal IFN-γ is a prerequisite for the generation of the $Ly6C^{-}$ macrophages. Taken together, cell intrinsic IFN-γ signaling limits the acquisition of $Ly6C^{-}$ phenotype and drives $Ly6C^{+}$ monocytes to differentiate into Tip-DCs.

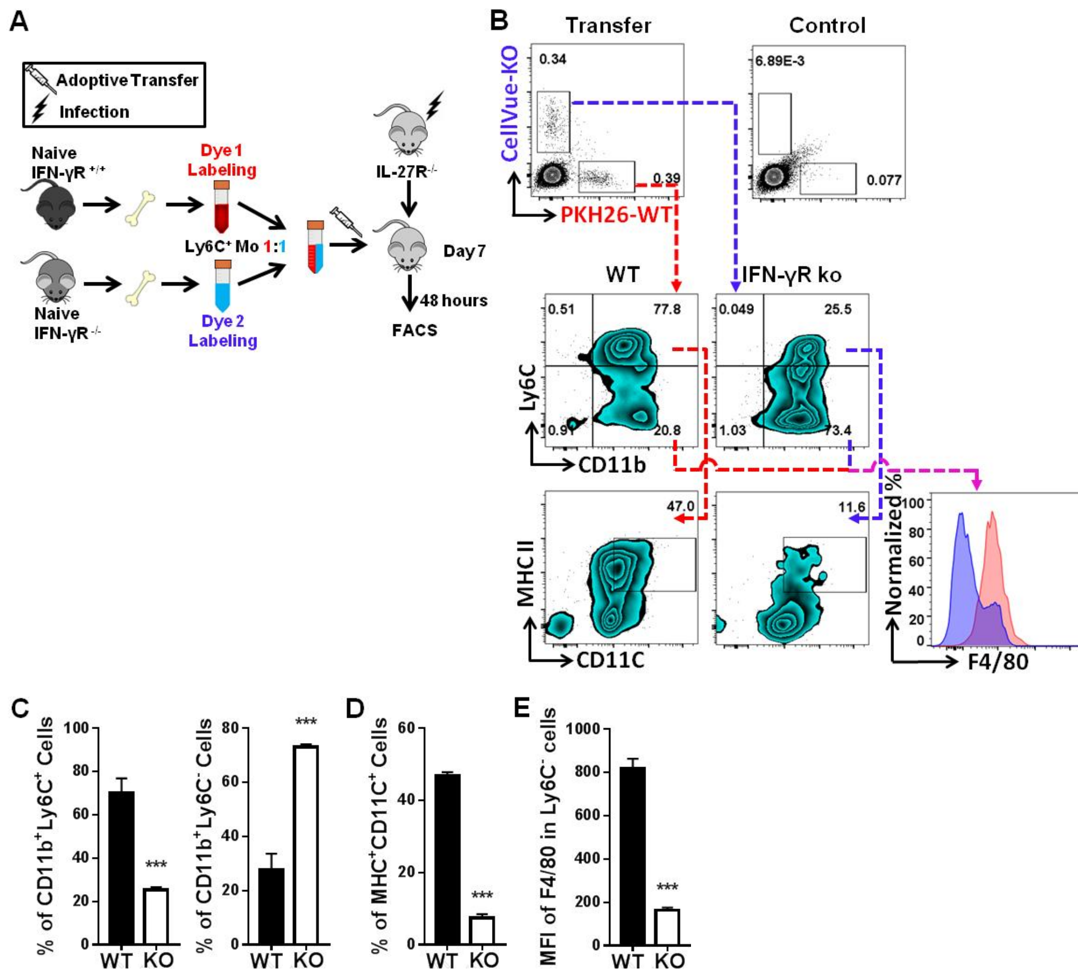

Fig. 3.14 Cell intrinsic IFN-γ signaling inhibit the acquisition of Ly6C⁻ phenotype and drives Ly6C⁺ monocytes to differentiate into Tip-DC.

Ly6C⁺ monocytes ($1x10^6$) were collected from naive IFN-γR$^{+/+}$ or IFN-γR$^{-/-}$ mice, labeled with PKH26 or Cellvue respectively, mixed at 1:1 ratio and transferred to IL-27R$^{-/-}$ mice on day 7 post infection. All parameters were evaluated at the transferred cells at 48 hours post transfer to track the fates of differentiation. (**A**) Schematic of experimental design. (**B**) Representative FACS plots determining the respective differentiation events of the transferred cells. (**C**) The frequencies of the populations with Ly6C⁺ phenotype or Ly6C⁻ phenotype among total corresponding donor cells were calculated. (**D**) The frequencies of matured moDC (CD11b⁺Ly6C⁺MHC II⁺CD11C⁺) within the corresponding CD11b⁺Ly6C⁺ populations were determined. (**E**) The MFI of F4/80 in the Ly6C⁻ populations was assessed. MFI, median fluorescence intensity.

***$p<0.001$ (Student's t-test)

Discussion

As we showed in Chapter 2 (201), IL-27R$^{-/-}$ mice infected with African trypanosomes developed severe liver pathology with a dramatic reduction of survival compared to WT mice. Neutralization of IFN-γ or depletion of CD4^{+} T cells diminished liver pathology, preventing the early mortality of infected IL-27R$^{-/-}$ mice. These results demonstrate that IL-27 limits immunopathology through down-regulation of IFN-γ production by CD4^{+} T cells. At that point, it is not known which subset of leukocytes mediates the pathology, leading to the early mortality of infected IL-27R$^{-/-}$ mice. In this respect, it has recently been shown that Ly6C^{+} monocytes traffic to the liver of mice infected with T. *brucei*, and further differentiate into Tip-DCs, mediating liver pathology (141, 145). In this chapter, we demonstrate that IL-27 signaling is an essential immunoregulator for Tip-DC development through dampening the excessive production of IFN-γ by the hyper-activated CD4^{+} T cells during infection with African trypanosomes.

Ly6C^{+} monocytes are produced by hematopoiesis in the bone marrow and give rise to Tip-DCs in the inflamed tissue (182, 185, 204). The emigration of Ly6C^{+} monocytes from the bone marrow is CCR2 dependent (185). Upon arrival in the

bloodstream, the enhanced expression of adhesion molecules including CD11b and CD44, facilitates the infiltration of $Ly6C^+$ monocytes into the inflamed tissue where they eventually development into matured Tip-DCs (179, 198). Thus, the Tip-DC development consists of $Ly6C^+$ monocyte egression from bone marrow, recruitment to the liver, and its differentiation program.

We found that the percentage of $Ly6C^+$ monocytes ($CD11b^+Ly6C^+$ cells) within the total liver leukocyte population as well as the absolute number of $Ly6C^+$ monocytes per liver of infected IL-27R$^{-/-}$ mice were significantly higher compared to WT mice. This is associated with the enhanced systematic levels of CCL2 and CCL7. The signaling between CCR2 and CCL2/CCL7 is essential for $Ly6C^+$ monocyte egression from the bone marrow (185, 186). Thus, we speculate that the enhanced levels of CCL2 and CCL7 promote emigration of $Ly6C^+$ monocytes from the bone marrow. CCL2 and CCL7 are produced in the liver by Kupffer cells, hepatocytes, and endothelial cells (212-214). All these cells express IFN-γ receptor (215-217). We reason that excessive production of IFN-γ by $CD4^+$ T cells in the absence of IL-27 signaling (201) drives these cells to produce CCL2 and CCL7, resulting in enhanced $Ly6C^+$ monocyte emigration.

In IL-27R$^{-/-}$ mice, Ly6C^{+} monocytes preferentially differentiated into moDCs which had higher expression of MHC II as compared to the counterparts of WT mice during African trypanosome infection. In addition, infected IL-27R$^{-/-}$ mice had significantly higher percentage of Tip-DCs as well as higher absolute number of these cells. Of note, all of these enhanced parameters could be attributed to the over-activation of CD4^{+} T cells and the copious IFN-γ in the absence of IL-27 signaling. However, it's also possible that Tip-DCs drive the priming of CD4^{+} T cells and induce the excessive production of IFN-γ in the IL-27R$^{-/-}$ mice, considering their phenotypic similarity in expression of MHC II and CD11c to conventional DC.

To exclude the possibility that activation of CD4^{+} T cells is driven by Tip-DCs, we treated the infected IL-27R$^{-/-}$ mice with CCR2 antagonist, which should impaire the Tip-DC development in the infected IL-27R$^{-/-}$ mice (4, 58). It was found that CD4^{+} T cells activation is intact in terms of expression of T cell activation markers as well as IFN-γ secretion ability. Indeed, published work has shown that T cell priming is not affected at all in the absence of Tip-DCs (185, 218). In addition, depletion of CD4^{+} T cells or neutralization of IFN-γ in infected IL-27R$^{-/-}$ mice reduced Tip-DC development. Thus, the higher level of IFN-γ detected in the infected

IL-27R$^{-/-}$ mice is the cause, but not the consequence, of the enhanced development of Tip-DCs in the infected IL-27R$^{-/-}$ mice.

Except Ly6C^{+} monocytes, Ly6C^{-} monocytes are the other subset of monocytes (182). In contrast to the inflammatory property of Ly6C^{+} monocytes, Ly6C^{-} monocytes are recognized as a population of sentinel cells with anti-inflammatory properties which can remove foreign particles or cell debris from the blood vessel and repair the endothelial damage by orchestrating the immunity of the vasculature (204, 206). Ly6C^{-} monocytes require multiple survival signals to maintain the phenotype and are susceptible to stress induced cell death (204, 207, 208). In this chapter, we confirmed the anti-inflammatory features of Ly6C^{-} monocytes in IL-27R$^{-/-}$ mice. Indeed, there was a consistent inverse correlation between Ly6C^{+} and Ly6C^{-} monocytes in infected IL-27R$^{-/-}$ mice. In addition, Ly6C^{-} monocytes underwent extensively cell death in the absence of IL-27 signaling. We further demonstrate that CD4^{+} T cell-derived IFN-γ triggered cell death of Ly6C^{-} monocytes in infected IL-27R$^{-/-}$ mice, as depletion of CD4^{+} T cells or neutralization of IFN-γ prevented the death of Ly6C^{-} monocytes, leading to increase of frequency and absolute number of Ly6C^{-} monocytes in the liver of the infected mice.

Obviously, the excessive production of IFN-γ by $CD4^+$ T cells in the absence of IL-27 signaling promoted Tip-DC development in the liver during infection with African trypanosomes. One of the most important questions is how IFN-γ drives the development of Tip-DCs. As discussed above, IFN-γ may enhance CCL2 and CCL7 secretions, leading to massive emigration of $Ly6C^+$ monocytes from the bone marrow. IFN-γ may also stimulate the expression of adhesion molecules such as CD44, CD11b and ICAM-1 on $Ly6C^+$ monocytes and endothelial cells, enhancing the recruitment of $Ly6C^+$ monocytes into the liver of infected IL-$27R^{-/-}$ mice. Our adoptive transfer experiments have shown that IFN-γ signaling on $Ly6C^+$ monocytes directly drove these cells to differentiate into Tip-DCs. Interestingly, $Ly6C^+$ monocyte can also become $Ly6C^-$ macrophages (Fig. 3.14B, E) (205). In this respect, it has been shown that $Ly6C^-$ monocytes are able to promote the switch of $Ly6C^+$ monocytes to $Ly6C^-$ macrophages (205). Thus, it is conceivable that IFN-γ induces death of $Ly6C^-$ monocytes as shown in this chapter, leading to less transition of $Ly6C^+$ monocyte into $Ly6C^-$ macrophages and thus leaving more $Ly6C^+$ monocytes for transition to Tip-DCs. If this is the case, IFN-γ would promote differentiation of $Ly6C^+$ monocytes into Tip-DCs directly and indirectly.

In conclusion, IL-27 negatively regulated the development of Tip-DCs including the recruitment of Ly6C$^+$ monocytes to the liver and their differentiation into Tip-DCs in mice infected with African trypanosomes. Mechanistically, IL-27 inhibited IFN-γ secretions by CD4$^+$ T cells; IFN-γ promoted Tip-DC development. Given the profound functions of Tip-DCs, the knowledge from this study will be fundamental for the development of new therapeutics to enhance antimicrobial immune defense or dampen detrimental inflammation through manipulating the Tip-DC development.

Materials and Methods

Ethics statement

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal protocols involving mice were approved by the University of Maryland Institutional Animal Care and Use Committee (IACUC) under protocol R-15-48.

Mice and parasites

Eight- to ten-week-old C57BL/6NCrJ (C57BL/6) mice and five- to six-week-old outbred Swiss white mice (CD1) were purchased from the Charles River Laboratories. B6N.129P2-Il27ratm1Mak (IL-27R$^{-/-}$, or WSX-1$^{-/-}$) and B6.129S7-Ifngr1tm1Agt/J (IFN-γR$^{-/-}$) mice were purchased from the Jackson Laboratory and bred in-house. All animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee and Institutional Bio-safety Committee of the University of Maryland, College Park.

T. congolense, Trans Mara strain, variant antigenic type (VAT) TC13 was used in this study. The origin of this parasite strain has been previously described (176). Frozen stabilates of parasites were used for infecting CD1 mice immunosuppressed with cyclophosphamide, and passages were made every third day as described previously (176). The parasites were purified from the blood of infected CD1 mice by DEAE-cellulose chromatography (177) and used for infecting mice.

Infections, treatment of mice with mAbs or antagonists, calculation of parasitemia and survival time of mice

Mice were infected i.p. with 10^3 *T. congolense* TC13. Some groups of infected mice were injected i.p. with rat anti-mouse CD4 mAb (GK1.5; 0.5 mg on day 0, 2, 4, and 6 after infection, respectively), anti-mouse IFN-γ mAb (XMG1.2; 0.4 mg on day 0, 2, 4, 6, and 8 after infection, respectively), or rat IgG (as a control). CCR2 antagonist or vehicle was i.p. injected at 10 mg/kg body weight every other day, starting from day -1 to 9 post infection. Parasitemia was counted at x40 magnification by phase-contrast microscopy. The survival time was defined as the number of days after infection that the infected mice remained alive.

Antibodies

Purified rat anti-mouse CD4 mAb (Clone GK1.5), and purified rat anti-mouse IFN-γ mAb (Clone XMG1.2) were purchased from BioXCell (West Lebanon, NH). Antibodies for flow cytometry are listed in the table 1.

Table 1. Antibodies for flow cytometry

Antibody	Clone	Fluerochrome	Supplier
CD3ε	145-2C11	FITC, PE-Cy7	Biolegend
CD4	GK1.5	BV421, PE	Biolegend
CD8a	53-6.7	BV510, APC	Biolegend
CD11b	M1/70	BV510, PE-Cy7, FITC	Biolegend
CD11c	N418	BV421, PE, APC	Biolegend
CD16/32	93	Unconjugated	Biolegend
CD44	IM7	APC, PerCP-Cy5.5	Biolegend
CD45	30-F11	APC/Cy7	Biolegend
CD45-1	A20	APC/Cy7	Biolegend
CD45.2	104	PE/Cy7	Biolegend
CD62L	MEL-14	APC/Cy7, PE	Biolegend
Annexin V		BV421	BD
7AAD			Biolegend
CX3CR1	SA011FAA	Alexa fluor 647, PE, PE/Cy7	Biolegend
F4/80	BM8	Alexa flura 647, PE	Biolegend
Ly6C	HK1.4	FITC, PE	Biolegend
Ly6G	1A8	FITC, PerCP/Cy5.5	Biolegend
IFN-γ	XMG1.2	PE	Biolegend
TNF-α	MP6-XT22	APC	Thermo Fisher Scientific
NOS2	CXNFT	APC	Thermo Fisher Scientific
MHC II	M5/114.15.2	BV510, PE	Biolegend

Cell isolation from Bone marrow, blood, spleen, and liver

Bone marrow cells were isolated from femur and tibia as previously described (219). Briefly, muscle free bones were perfused with ice-cold RPMI-1640 supplemented with 5% heat-inactivated FBS. The obtained cell suspension was passed through a 70 µm cell strainer using a sterile syringe plunger. Thereafter, the cell suspension was centrifuged at 300g for 10 minutes and the pellet was then resuspended in 1% BSA/HBSS solution.

Blood was isolated by heart puncture with Heparin pre-coated syringe, followed by certain cycles of erythrocyte lysis with ACK lysing buffer (Lonza) until pale pellet was obtained. Thereafter, the cell suspension was centrifuged at 300g for 10 minutes and the pellet was then resuspended in 1% BSA/HBSS solution.

Spleen was mined gently with surgical scissors and forced and passed through a 70 µm cell strainer using a sterile syringe plunger, followed by centrifugation and erythrocytes lysis. Thereafter, the cell suspension was centrifuged at 300g for 10 minutes and the pellet was then resuspended in 1% BSA/HBSS solution.

Intrahepatic leukocytes were isolated as described previously (178). Briefly, the liver was perfused with PBS until it became pale. Thereafter, the gallbladder was removed, and the liver was excised carefully from the abdomen. The liver was minced into small pieces with surgical scissors and forced gently through a 70 um cell strainer using a sterile syringe plunger. The preparation obtained was suspended in 50 ml RPMI-1640 medium containing 5% FCS. The cell suspension was centrifuged at 30g with the off-brake setting for 10 min at 4 °C. The obtained supernatant was centrifuged at 300g with the high-brake setting for 10 min at 4 °C. The pellet was resuspended in 10 ml 37.5% Percoll in HBSS containing 100 U/ml heparin and then centrifuged at 850g with the off-brake setting for 30 min at 23 °C. This new pellet was resuspended in 2 ml ACK buffer (erythrocyte lysing buffer), and incubated at room temperature for 5 min, then supplemented with 8 ml RPMI-1640 medium containing 5% FCS, followed by centrifugation at 300g with the high-brake setting for 10 min at 4 °C.

Flow cytometry

For surface staining, the isolated cells were incubated (15 min, 4°C) with purified anti-mouse CD16/CD32 ([FcγIII/II Receptor], clone: 2.4G2) to block

nonspecific binding of Abs to FcRs, followed by staining with mAbs specific for various cell surface markers, or the relevant isotype-matched control Abs, then washed twice in staining buffer (1% BSA/HBSS). For intracellular TNF-α and iNOS staining, cells were diluted to 5×10^6 cells/ml and cultured (200 µl/well) in a 96-well plate in the presence of BFA (protein transport inhibitors, Biolegend) for 5 h. The cells were then harvested and washed twice in staining buffer. The cells were incubated (15 min, 4°C) with purified anti-mouse CD16/CD32, followed by staining with mAbs specific for cell surface markers for 30 mins. The cells were fixed and permeabilized using Intracellular Fixation & Permeabilization Buffer Set (eBiosciences). Intracellular staining was then performed using mAbs specific for TNF-α and iNOS. 20-40 mins later, cells were washed with Intracellular Fixation & Permeabilization Buffer and resuspended in staining buffer. For intracellular IFN-γ staining, cells were diluted to 5×10^6 cells/ml and cultured (200 µl/well) in a 96-well plate in the presence of 1x Cell Stimulation Cocktail (containing PMA, ionomycin, and protein transport inhibitors, eBioscience) for 12 h. The cells were then harvested and stained as TNF-α and iNOS staining. Samples were read by BD Canto II or Aria II, and analyzed using FlowJo software.

Adoptive transfer

Bone marrow Ly6C$^+$ monocytes from naive WT or IFN-γR KO mice were collected through MACS purification and FACS sorting. Briefly, bone marrow cells were pre-purified using MACS CD11b positive selection (Miltenyi). Obtained cells were stained with CD11b, Ly6C, Ly6G, then subjected to FACS sorting for Ly6G$^-$CD11b$^+$Ly6C$^+$ monocytes. WT and IFN-γR KO Ly6C$^+$ monocytes were respectively labeled with PKH26 and CellVue labeling kits (Sigma-Aldrich). 1×10^6 of each labeled cells were then mixed at 1:1 ratio, and i.v. injected into *T. congolense* TC13 infected IL-27R$^{-/-}$ mice on day 7 post infection. 48 hours post transfer, the transferred cells in the recipient IL-27R$^{-/-}$ mice were analyzed. Infected IL-27R$^{-/-}$ mice with no cell transfer were used as background control for gating labeled cells.

Intrahepatic Ly6C$^-$ monocytes from infected WT mice were collected through MACS purification and FACS sorting. Briefly, intrahepatic leukocytes were pre-purified using MACS CD11b positive selection (Miltenyi). Obtained cells were stained with CD11b, Ly6C, Ly6G, CX3CR1 and F4/80, and then subjected to FACS sorting for intrahepatic Ly6C$^-$ monocytes (Ly6G$^-$CD11b$^+$Ly6C$^-$CX3CR1$^+$F4/80$^-$). 1×10^6 of freshly collected Ly6C$^-$ monocytes were i.v. injected into *T. congolense*

TC13 infected IL-27R$^{-/-}$ mice on day 3 and 6 post infection. 48 hours post transfer, the recipients and no-transfer controls were analyzed.

ELISA assays for cytokines

ELISA kits to CCL2 and CCL7 were purchased from Thermo Fisher Scientific. The levels of cytokines in the plasma were determined by routine sandwich ELISA using Immulon 4 HBX plates (Thermo Scientific), according to the manufacturer's protocols.

Aminotransferase determination and histopathological examination

Liver alanine transaminase (ALT) activities were determined using EnzyChrom Alanine Transaminase Assay Kit (BioAssay Systems) according to the manufacturer's instructions.

Statistical analysis

Data are represented as the mean ± SEM. Significance of differences was determined by ANOVA or a log-rank test for curve comparison using the GraphPad Prism 7.0 software. Values of $p<0.05$ are considered statistically significant.

CHAPTER 4: CONCLUSION AND PERSPECTIVE

The murine African trypanosome model of infection has been extensively used to mimic the conditions of human/animal African trypanosomiasis to decipher the mechanisms of pathology. Interleukin (IL)-27 has recently been characterized as a potent immunomodulatory cytokine primarily by orchestrating the adaptive immunity in settings of cancers, autoimmune diseases and infectious diseases. Here in this dissertation, we put forward the immunoregulatory property of IL-27 in the context of extracellular protozoan parasite infection and found that 1) IL-27 signaling is essential for preventing liver pathology and early mortality via inhibiting of IFN-γ secretion by $CD4^+$ T cells during infection with African trypanosomes (Chapter 2). 2) IL-27 negatively regulates the development of Tip-DCs through inhibiting IFN-γ secretion by $CD4^+$ T cells (Chapter 3).

As illuminated in Chapter 2, the expression of IL-27 was dramatically up-regulated upon infections with either *T. brucei* or *T. congolense*. The essential role of IL-27 was demonstrated by the fact that infected IL-$27R^{-/-}$ mice survive shorter than infected wild-type mice. With severe liver pathology, infected IL-$27R^{-/-}$ mice experienced elevated systematic and liver inflammations. The shortened survival and

enhanced pathology of in IL-27R$^{-/-}$ mice were attributed to the aberrant level of IFN-γ produced by the hyper-activated CD4^{+} T cells, as depletion of CD4^{+} T cells or neutralization of IFN-γ ameliorated the liver pathology and extended the survival of IL-27R$^{-/-}$ mice.

IL-10 has been also shown to inhibit immunopathology (108, 116). In current study (Chapter 2), our data indicate that, independent of IL-10 signaling, IL-27 signaling inhibits the immune responses through directly targeting on CD4^{+} T cells. It would be interesting to explore how IL-27 inhibits CD4^{+} T cells. IL-27 has been shown to promote the production of IL-10 (220-222). However, we found that infected IL-27R$^{-/-}$ mice even produced more IL-10. We speculate that the infected hosts produce more IL-10 by CD4^{+} T cells aimed to control the inflammation in case IL-27 signaling is blocked.

In Chapter 3, we documented that IL-27R$^{-/-}$ mice displayed higher counts of intrahepatic Ly6C^{+} monocytes and Tip-DCs. In contrast to inflammatory Ly6C^{+} monocytes, Ly6C^{-} populations, including Ly6C^{-} monocytes and Ly6C^{-} macrophages, have anti-inflammatory properties (205). In the absence of IL-27 signaling, the

number of intrahepatic $Ly6C^-$ monocytes and $Ly6C^-$ macrophages were dramatically reduced during infection. Depletion of $CD4^+$ T cells or neutralization of IFN-γ reduced the recruitment of $Ly6C^+$ monocytes and their differentiation into Tip-DCs in infected $IL\text{-}27R^{-/-}$ mice. This was associated with enhanced accumulation of the two $Ly6C^-$ populations. Adoptively transferred $Ly6C^-$ monocytes attenuated Tip-DC development and inflammation in the liver of $IL\text{-}27R^{-/-}$ mice. Further data of adoptive transfer experiments demonstrated that cell intrinsic IFN-γ signaling on $Ly6C^+$ monocytes promoted their differentiation into Tip-DCs.

Recently, many studies have been focused on transcriptional and epigenetic control of myeloid cells in the bone marrow or blood. It has been shown that transcription factor PU.1 remodels the chromatin structure of IRF8 gene in monocyte-dendritic cell progenitors (MDP) which gives rise to common monocyte progenitors (cMoP) (223). More recently, cMoP has been documented as the direct progenitor for $Ly6C^+$ monocytes and $Ly6C^-$ monocytes (224). IRF8 dependent expression of transcription factor KLF4 is indispensable for development of $Ly6C^+$ monocytes from cMoPs (225-228).

In contrast to Ly6C$^+$ monocyte development, transition of cMoPs to Ly6C$^-$ monocytes is induced by transcription factor KLF2 binding to the enhancer element 2 of the transcription factor NR4A1, upon which enhanced expression of NR4A1 facilitates the phenotype acquisition, function maintenance and survival of Ly6C$^-$ monocytes (207, 229). Most recently, C/EBPβ was shown essential for survival of Ly6C$^-$ monocytes in the peripheral partial through up-regulating the expression of CSF-1R which serves as the hallmark for monocyte lineage (230).

By taking advantage of this unique extracellular protozoan parasitic infection model, we strongly feel enthusiastic and obligated to determine whether the negative correlation in cell counts between Ly6C$^+$ monocytes and Ly6C$^-$ monocytes is dictated by IFN-γ at the transition stage of cMoP in the bone marrow. Meanwhile, it is still unknown whether IFN-γ produced by bone marrow resident NK cells affects the survival of Ly6C$^-$ monocytes, as well as the differentiation program of cMoP and Ly6C$^+$ monocytes before egressing from the bone marrow to the blood. Furthermore, it is important to dissect how IFN-γ dictates the fate choice of the Ly6C$^+$ monocytes at the transcriptional and epigenetic level in peripheral tissues.

Overall, using an extracellular protozoan infection model we have studied the role of IL-27 in regulation of immunopathology. Findings from this dissertation encourage us to further explore the immunoregulatory role of this important cytokine at molecular levels.

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