2016-03-02

Cytosolic and Endosomal DNA-Sensing Pathways Differentially Regulate Inflammatory Arthritis, Autoantibody Production, and Bone Remodeling: A Dissertation

Rebecca A. Baum
University of Massachusetts Medical School

CYTOSOLIC AND ENDOSOMAL DNA-SENSING PATHWAYS DIFFERENTIALLY REGULATE INFLAMMATORY ARTHRITIS, AUTOANTIBODY PRODUCTION, AND BONE REMODELING

A Dissertation Presented

By

Rebecca Ann Baum

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
In partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

March 2, 2016

MD/PhD Program

CYTOSOLIC AND ENDOSOMAL DNA-SENSING PATHWAYS DIFFERENTIALLY REGULATE INFLAMMATORY ARTHRITIS, AUTOANTIBODY PRODUCTION, AND BONE REMODELING

A Dissertation Presented
By
Rebecca Ann Baum

The signatures of the Dissertation Defense Committee signify completion and approval as to the style and content of the Dissertation

_____
Ellen M. Gravallese, M.D., Thesis Advisor

_____
Katherine A. Fitzgerald, Ph.D., Member of Committee

_____
Evelyn Kurt-Jones, Ph.D., Member of Committee

_____
Jie Song, Ph.D., Member of Committee

_____
Vicki Rosen, Ph.D., Member of Committee

The signature of the Chair of the Committee signifies that the written dissertation meets the requirements of the Dissertation Committee

_____
Paul Odgren, Ph.D., Chair of Committee

The signature of the Dean of the Graduate School of Biomedical Sciences signifies that the student has met all graduation requirements of the school

_____
Anthony Carruthers, Ph.D.,
Dean of the Graduate School of Biomedical Sciences

MD/PhD Program
March 2, 2016

Dedication

To Joyce

For all of the times that I have wanted to help you

Acknowledgements

First, I would like to thank my mentor Dr. Ellen Gravallese. She allowed me to pursue a project that greatly interested me, guided me in number of fruitful collaborations, and always made my meetings with her a priority. I have appreciated her time and guidance throughout this graduate process. I would also like to thank the past and present members of the Gravallese laboratory for their help and kindness including Drs. Yukiko Maeda, Anita Shaw, Zheni Stavre, Melissa Matzelle as well as Catherine Manning, Sierra Williams, and Gail Germain. These individuals taught me a variety of laboratory techniques, maintained the mouse colonies, and/or handled administrative matters. I especially can't thank Anita, Yuki, and Cathy enough for listening to me and supporting me. I admire and respect you all a great deal, not only as scientists, but also as wives/mothers who balance so much in your lives.

The research in this dissertation would not have been possible without the contributions of numerous people. I primarily need to thank our collaborators Drs. Katherine Fitzgerald and Ann Marshak-Rothstein and the members of their laboratories who contributed to the work described herein, including Drs. Kerstin Nündel, Shruti Sharma, Susan Carpenter, Sudesh Pawaria, Krishna Moody, Patricia Busto, as well as Tara Robidoux. These projects required expertise from multiple fields, and the sheer number of animal studies and techniques required would not have been possible without your help, support, and experience. I would also like to acknowledge the contributions of several other noteworthy individuals including Stacey Russell for the generation of our beautiful micro-CT images, Julia Charles for her osteoclast differentiation protocol, and

Paul Odgren for his osteoclast resorption protocol. I would also like to thank Drs. David Burr and Jason Organ as well as Keith Condon at IUPUI for their extensive histomorphometry knowledge. My TRAC members Drs. Paul Odgren, Evelyn-Kurt Jones, Jie Song, and Katherine Fitzgerald have given of their time, support, and valuable insights throughout the course of my graduate studies, for which I am truly grateful. I would also like to thank Dr. Vicki Rosen for taking the time out of her schedule to participate in my dissertation committee.

My path to biomedical research began at the NIH with two of my favorite scientists and most supportive mentors, Drs. Paul Plotz and Nina Raben, to whom I owe a great deal. Their guidance and counsel played a decisive role in my plans to pursue a MD-PhD degree. Paul and Nina taught me much about science, but they also genuinely cared about me and their kindness has always stayed with me. Paul has also been a great example to me of not only a gifted physician-scientist, but also of a person who keeps his family, the arts, and other interests alive in his life. I am grateful for his example of balance and culture. I have found throughout my graduate studies that the fields of immunology and rheumatology are fascinating but also quite complex. To describe this complexity, Paul often quotes Ralph Waldo Emerson's words, "Our life is an apprenticeship to the truth, that around every circle another can be drawn; that there is no end in nature, but every end is a beginning; that there is always another dawn risen on mid-noon, and under every deep a lower deep opens."

Most importantly, I would like to thank my family. Their love and choices have motivated me throughout my life. I am continually blessed by their complexity, kindness,

and resilience. Dad, Mom, Nicole, and Andrew – you listen to me, help me become better, and are always by my side. Your examples have taught me to work hard, have courage, be independent, and keep perspective. Every day I'm so thankful that I have you in my life. For the past thirty years, my family has dealt with severe autoimmune diseases, and so I learned early about the frustrations of illness and the limitations of medicine. There have been many occasions when I have felt helpless and heartbroken, desiring to do more than just wish disease away. To those who suffer from rheumatic diseases, I sincerely hope that one day I can help you. Lastly, I would like to thank my sweet husband Kambiz. You have been my friend and support ever since I moved out East. I never realized how calm and happy life could be until I married you. Dooset daram.

Abstract

Autoimmune diseases such as rheumatoid arthritis (RA) are associated with debilitating chronic inflammation, autoantibody production, articular bone erosions and systemic bone loss. The underlying mechanisms and cell types that initiate these diseases are not fully understood, and current therapies mainly address downstream mechanisms and do not fully halt disease progression in all patients. Moreover, previous studies have largely focused on the role of adaptive immunity in driving these diseases, and less attention has been given to the contribution of innate immune pathways such as DNA sensor signaling pathways in initiating and/or perpetuating autoimmunity and erosive inflammatory arthritis.

Detection of microbial nucleic acids by DNA sensors such as endosomal toll-like receptors (TLRs) and cytosolic sensors is an early form of antiviral defense. Upon detection of nucleic acid, TLRs dependent on Unc93B and cytosolic sensors dependent on the adaptor stimulator of interferon genes (STING) orchestrate production of type 1 interferons and pro-inflammatory cytokines to resolve infection. Additionally, the cytosolic DNA sensor absent in melanoma 2 (AIM2), which is not dependent on STING, also recognizes microbial DNA and coordinates the cleavage of pro-IL-1β. Previous studies have largely focused on the role of these DNA sensors in macrophages and dendritic cells in the context of antiviral immunity. In recent years, however, the inappropriate recognition of host nucleic acids by these sensors has been associated with several autoimmune diseases including RA.

This dissertation aims to delineate the mechanisms by which DNA sensors contribute to inflammatory arthritis and bone remodeling in the context of a murine model of autoimmunity. In DNase II deficient mice, excessive accrual of undegraded, endogenous DNA leads to robust production of type 1 interferons (IFNs) and pro-inflammatory cytokines. The high levels of type 1 IFNs result in anemia and embryonic lethality; therefore, the gene for the type 1 IFN receptor (IFNaR) has also been deleted so that the mice survive. DNase $II^{-/-}$ $IFNaR^{-/-}$ double knockout (**DKO**) mice develop erosive inflammatory arthritis, anti-nuclear antibodies, and splenomegaly not seen in the DNase $II^{+/-}$ $IFNaR^{-/-}$ (**Het**) control group. To evaluate whether cytosolic or endosomal DNA sensors contribute to the clinical manifestations of DKO mice, genes involved in TLR or cytosolic sensor signaling were deleted on the DKO background. Genetically altered mice include STING/DNaseII/IFNaR TKO (STING TKO), AIM2/DNase II/IFNaR TKO (AIM2 TKO), and Unc93b/DNase II/IFNaR TKO (Unc93 TKO) mice.

Our hypothesis was that the STING, AIM2, and/or Unc93 pathways would contribute to the autoimmune manifestations in DNase II deficient mice. Rigorous examination of inflammation in these lines revealed important roles for both the STING and AIM2 pathways in arthritis. Despite the substantial effects of the STING and AIM2 pathways on arthritis, STING TKO and AIM2 TKO mice still exhibited prominent autoantibody production. Interestingly, inflammation persisted in Unc93 TKO mice while autoantibody production to nucleic acids was abrogated. Collectively, these data indicate that innate immune pathways contribute to the initiation/perpetuation of inflammatory arthritis and demonstrate that cytosolic and endosomal pathways play distinct roles in the

manifestations of autoimmunity. Moreover, they reveal a previously undescribed role for AIM2 as a sensor of endogenous nucleic acids in inflammatory arthritis. Thus, therapeutics that target the STING and AIM2 pathways may be beneficial for the treatment of inflammatory joint diseases.

While the role of hematopoietic cells in driving autoimmunity has been well established, the contribution of stromal elements to disease pathogenesis is less well understood. Therefore, we generated bone marrow chimeras to delineate the contribution of hematopoietic and non-hematopoietic cells to the various autoimmune manifestations in DKO mice. These studies revealed that both donor hematopoietic and host radioresistant cells are required for inflammation in the joint as well as for other features of autoimmunity in DKO mice, including splenomegaly, extramedullary hematopoiesis, and autoantibody production. This data demonstrates that stromal host cells play a major role in DNA-driven autoimmunity. Moreover, these results suggest that targeting not only hematopoietic but also stromal elements may be advantageous in the setting of inflammatory arthritis.

In the final chapter of this thesis, a role for innate immune sensor pathways in bone is described. The majority of inflammatory arthritides have been shown to lead to systemic loss of bone. Surprisingly, however, we found that DKO mice accumulate trabecular bone in the long bones over time as well as ectopic bone in the spleens, both sites of robust DNA accrual. Moreover, deficiency of the STING pathway abrogated this bone accumulation. Collectively, these data demonstrate that DNA accrual promotes dysregulated bone remodeling through innate immune sensing pathways. These findings

are the first to reveal a role for the STING pathway in bone and may unveil novel targets for the treatment of diseases associated with bone disorders.

Table of Contents

List of Figures

List of Abbreviations

ACP5	Acid phosphatase 5 (also known as TRAP)
ACPA	Anti-citrullinated protein antibody
ACVR1	Activin A receptor type I
AIA	Antigen induced arthritis
AIM2	Absent in melanoma-2
AIM TKO	$AIM2^{-/-}$ $DNaseII^{-/-}$ $IFNaR^{-/-}$ triple knockout
Alpl	Alkaline phosphatase
ANA	Anti-nuclear antibody
AS	Ankylosing spondylitis
B.Ar.	Bone area
BFR	Bone formation rate
BM	Bone marrow
BMD	Bone mineral density
BMDM	Bone marrow derived macrophage
BMP	Bone morphogenetic protein
BS	Bone surface
BV/TV	Bone volume per total volume
CFU	Colony forming unit
cGAS	Cyclic GMP-AMP Synthase
CIA	Collagen induced arthritis
Col1A1	Collagen type 1 alpha 1
CTSK	Cathepsin K
CTX-1	C-terminal telopeptide 1
DAI	DNA-dependent activator of IRFs
DAMP	Danger associated molecular pattern
DKK	Dickkopf
DKO	$DNase^{-/-}$ $IFNAR^{-/-}$ double knockout
Dmp1	Dentin matrix acidic phosphoprotein 1
DNA	Deoxyribonucleic acid
ds	Double-stranded
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FGF	Fibroblast growth factor
FLS	Fibroblast-like synoviocyte
FOP	Fibrodysplasia ossificans progressiva
GC	Germinal center
GDF3	Growth differentiation factor 3
GFP	Green fluorescent protein

GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPI	Glucose-6-phosphate isomerase
H&E	Hematoxylin & eosin
Het	$DNase^{+/-}$ $IFNaR^{-/-}$
HMBS	Hydroxymethylbilane synthase
HO	Heterotopic ossification
hTNFtg	Human TNF transgenic
Ibsp	Integrin-binding sialoprotein
IFI16	Interferon-inducible protein 16
IFN	Interferon
IFNaR	Type I IFN receptor
IGF	Insulin-like growth factor 1
IgG	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
IRF	Interferon regulatory factor
LPS	Lipopolysaccharide
LRP	Lipoprotein receptor-related protein
MAR	Mineral apposition rate
MCP	Metacarpophalangeal
M-CSF	Macrophage colony-stimulating factor
Micro-CT/μCT	Micro-computed tomography
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
MSC	Mesenchymal stem cell
MTX	Methotrexate
NET	Neutrophil extracellular trap
NFκB	Nuclear factor kappa B
O.Ar.	Osteoid area
Ob.S	Osteoblast surface
OCN	Osteocalcin (also known as BGLAP)
OC.S	Osteoclast surface
ODN	Oligodeoxynucleotide
Omd	Osteomodulin
OPG	Osteoprotegerin
OPN	Osteopontin
PAMP	Pathogen associated molecular pattern
Phex	Phosphate regulating endopeptidase
PRR	Pattern recognition receptor
PTH	Parathyroid hormone
PYHIN	Pyrin and HIN domain family
qPCR	Quantitated reverse transcriptase PCR
RA	Rheumatoid arthritis
RANKL	Receptor activator of nuclear factor (NF)-κB ligand

RASF	Rheumatoid arthritis synovial fibroblast
RF	Rheumatoid factor
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
Runx2	Runt-related transcription factor 2
SEM	Standard error of the mean
sFRP	Secreted frizzled-related protein
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
Spl	Spleen
Spp1	Secreted phosphoprotein 1 (also known as OPN)
SS	Single stranded
STA	Serum transfer arthritis
STING	Stimulator of interferon genes
STING TKO	$STING^{-/-}$ $DNase^{-/-}$ $IFNaR^{-/-}$ triple knockout
TGF-β	Transforming growth factor β
TKO	Triple knockout
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAP	Tartrate-resistant acid phosphatase
Unc93 TKO	$Unc93b1^{-/-}$ $DNase^{-/-}$ $IFNaR^{-/-}$ triple knockout
VdR	Vitamin D receptor
Wnt	Wingless
Wt	Wild type

Copyrighted Material

The work presented in this dissertation has appeared in the following publications or manuscripts:

Chapter I

Baum R., Gravallese EM. Impact of inflammation on the osteoblast in rheumatic diseases. Curr Osteoporos Rep 2014; 12: 9-16.

Baum R, Gravallese EM. Bone as a Target Organ in Rheumatic Disease: Impact on Osteoclasts and Osteoblasts. Clin Rev Allergy Immunol. 2015; epub ahead of print.

Chapter II

Baum R, Sharma S, Carpenter S, Li QZ, Busto P, Fitzgerald KA, Marshak-Rothstein A, Gravallese EM. AIM2 and endosomal TLRs differentially regulate arthritis and autoantibody production in DNase II-deficient mice. J Immunol. 2015; 194:873-877.

Chapter III

Baum R[*], Nündel K[*], Pawaria S, Sharma S, Busto P, Fitzgerald KA, Gravallese EM[§], Marshak-Rothstein A[§]. Synergy between Hematopoietic and Radioresistant Stromal Cells Is Required for Autoimmune Manifestations of DNase II-/-IFNaR-/- Mice. J Immunol. 2016; 196:1348-54.

[*]Co-first authors
[§]Co-senior authors

Chapter IV

Baum R, Sharma S, Organ JM, Jakobs C, Hornung V, Burr DB, Marshak-Rothstein A, Fitzgerald KA, Gravallese EM. STING regulates bone formation induced by accrual of DNA. Submitted to Arthritis and Rheumatology 2016.

Preface

The work presented in this thesis was done in collaboration with the following individuals:

Chapter II:

Western in Figure 2.2B – Shruti Sharma, University of Massachusetts Medical School

Autoantigen array in Figure 2.4A – Quan-Zhen Li, UT Southwestern Medical Center

Chapter III:

FACS in Figures 3.3 and 3.4 – Kerstin Nündel and Sudesh Pawaria, University of Massachusetts Medical School

ANA stains in Figures 3.4E and 3.5E – Patricia Busto, University of Massachusetts Medical School

Chapter IV:

Histomorphometry in Figure 4.4 - David Burr, Jason Organ, and Keith Condon, Indiana University

Appendix:

Osteoclast differentiation assay in Figure A3 - Tingting Huang, University of Massachusetts Medical School

CHAPTER I

Introduction

Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by synovial inflammation and hyperplasia, autoantibody production, articular bone erosions and systemic bone loss. This disease affects nearly 1% of the population worldwide and presents with symmetrical joint pain and tenderness, but also systemic and cardiovascular complications that result in reduced life expectancy (1). The clinical progression of RA is extremely variable ranging from a mild slowly progressing disease to a rapidly developing disease accompanied by severe joint destruction and permanent disability (2).

The pathophysiology of RA involves several cell types and factors within the inflamed synovial joint (3). Local hypoxic conditions within the RA joint stimulate neoangiogenesis and activate endothelial cells to express adhesion molecules and chemokines which, in turn, recruit adaptive and innate immune cells into the synovial lining including macrophages, T cells, B cells, neutrophils, dendritic cells, and mast cells. Secretion of the pro-inflammatory cytokines tumor necrosis factor (TNF), interleukin-1 (IL-1), interleukin-6 (IL-6), and interleukin-17 (IL-17) by these cell types lead to further leukocyte infiltration and local inflammation. The presence of these pro-inflammatory cytokines also drives osteoclast differentiation (4-6) and inhibits osteoblast maturation (7), leading to bone destruction and visible joint damage (8).

In addition, apart from hematopoietic cells, stromal cells such as synoviocytes, fibroblasts, and chondrocytes also contribute to arthritic joint destruction (9). Activation of local synovial fibroblasts derived from mesenchymal precursor cells results in profound synovial hyperplasia. This inflamed “pannus” tissue releases various factors

including matrix metalloproteinases (MMPs) that induce chondrocyte cell death and cartilage destruction. The precise role of activated synoviocytes in arthritic joints is still under investigation. A schematic of the RA joint can be seen in **Figure 1.1**.

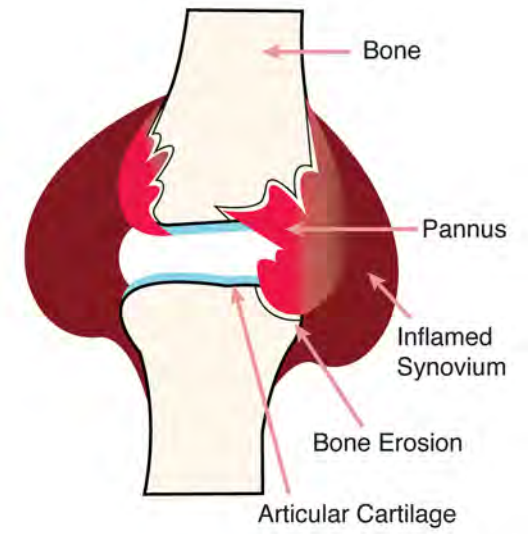


Figure 1.1 Inflamed RA joint. In RA the synovial membrane becomes swollen and inflamed by infiltrating leukocytes and synovial hyperplasia. Eventually this inflammatory "pannus" tissue invades and destroys cartilage and articular/subchondral bone, resulting in joint destruction and deformity.

The first recorded description of inflammatory arthritis was documented in Ebers Papyrus, the medical papyri of ancient Egypt, around 1500 B.C. Samples collected from Egyptian mummies also suggest the presence of RA during this time period (10). Later, around 400 B.C., the Greek physician Hippocrates, likely described a patient with RA in these words, "In the arthritis which generally shows itself about the age of thirty-five there is frequently no great interval between the affection of the hands and feet; both these becoming similar in nature, slender, with little flesh… For the most part their arthritis passeth from the feet to the hands, next the elbows and knees, after these the hip joint. It is incredible how fast the mischief spreads" (11). Although RA has possibly been

present for over 3500 years, the etiology of this disease remains unknown and a cure has yet to be found.

Immense progress has been made during the last two decades in the treatment of RA including the development of biologic therapies and small molecule inhibitors that alleviate symptoms and diminish radiographic progression (12). However, despite these therapeutic advances, the majority of contemporary therapies target downstream pathways, as opposed to initiating events, and a variety of unmet needs remain in the patient population (13). For example, current treatment regimes and biologic therapies are often costly and difficult to administer in the form of injections or infusions and may result in serious side effects. Additionally, although these therapies reduce the rate of bone erosions in RA, they typically do not result in healing of the erosions and the repair of damaged joints (14, 15). Perhaps of greatest consequence, is the finding that current therapeutic regimes rarely switch off the disease to result in permanent remission. Moreover, these therapies are not effective in all patients. In fact, randomized placebo-controlled clinical trials of numerous therapies consistently show a subset of 15-30% of patients who do not respond to therapy and others who become resistant to therapy over time (16).

RA patients not only have variable responses to therapy but also present clinically with diverse phenotypes (2). This heterogeneous nature of RA likely reflects a combination of unidentified genetic and environmental factors that initiate this disease. While much progress has been made in understanding the role of adaptive immunity in RA, the impact of the innate immune system in this disease is just beginning to be

appreciated. Moreover, further exploring the interplay between the hematopoietic and stromal cell types present in inflamed joints may also shed some light on the etiology of this disease. Elucidation of the mechanisms that initiate and perpetuate RA may eventually explain why current therapies are not effective in all patients and answer how we can permanently induce remission of this disease at the molecular level.

Adaptive Immune Mediators

The genetics associated with RA and the production of autoantibodies in this disease have long suggested a critical role for adaptive immunity in the rheumatic process. The well-known HLA-DRB1 locus represents the largest genetic risk factor for RA. In fact, it is estimated to account for nearly 60% of the disease risk and implicates a role for T cells in early disease pathogenesis. The Th1 subset of CD4+ T cells are abundant in the RA synovium and have been shown to activate macrophages, dendritic cells, and synovial fibroblasts and to promote B cell proliferation and autoantibody production (17, 18). Another subset of Th1 cells, Th17 cells, have also been identified in RA synovial tissue and implicated in contributing to this disease (19). Th17 cells express the pro-inflammatory cytokine IL-17A along with other cytokines including IL-17F, IL-6, IL-23, and IL-22 (20). In addition, T regulatory cells, which suppress pathogenic T cells and contribute to immunologic homeostasis (21), are impaired in RA patients. Interestingly, TNF was shown to inhibit the suppressive function of T regulatory cells and CD4+CD25hi T regulatory cells isolated from RA patients were found to be less active than the same population of cells isolated from healthy controls (22). Moreover, treatment with anti-TNF therapy restored the suppressive function of these cells. This

finding suggests an imbalance between T regulatory cells that suppress inflammation and Th17 cells that promote inflammation in RA. Therapeutic targeting of T cells has had variable efficacy in RA. T cell depletion therapies such as cyclosporine and anti-CD3 antibodies have had meager success in treating RA (23); however, inhibition of T cell activation by the drug Abatacept has been efficacious in treating arthritis (24).

Autoreactive B cells have also been identified as an important component of the rheumatoid synovium. B cells in RA serve as antigen presenting cells, promote activation of T cells, and also produce autoantibodies. It is recognized that B cells exist in inflamed joints within lymphoid aggregates or germinal follicle-like structures in both the synovial lining and bone marrow. B cells isolated from these structures demonstrate affinity maturation and intraclonal diversity, suggesting that germinal center reactions can take place in the synovium, outside of lymphoid tissue (25). These ectopic lymphoid structures have also been shown to support autoantibody production (26). Specific disease-associated autoantibodies are often found in the sera and joints of RA patients, including rheumatoid factor (RF), an autoantibody against the Fc portion of immunoglobulin and anti-citrullinated protein antibodies (ACPAs) (27, 28). These autoantibodies are often used as diagnostic markers for the identification and prognosis of RA; for example, RF can be detected in 60-80% of patients while ACPAs are detectable in nearly 70% of patients and are 98% specific for RA (29).

Interestingly, ACPAs can be detected in serum years before the onset of RA (30). During this initial phase of autoimmunity, patients are asymptomatic and often unaware of their potential for developing RA. In addition, ACPAs are a strong predictor of joint

erosion (31). It has now been demonstrated that even during the pre-clinical phase of disease, prior to synovitis, systemic bone loss occurs in ACPA-positive patients, raising very interesting questions regarding the mechanistic role of ACPAs in bone resorption (32). Antibodies to other autoantigens can also be found in RA including anti-nuclear antibodies (ANAs) that are found in 20% of patients (33). The important role for B cells in RA is further demonstrated by the efficacy of anti-CD20 B cell depletion therapies in this disease (34).

Innate Immune Mediators

In addition to adaptive effector cells, innate immune cells including neutrophils, mast cells, and macrophages also play pivotal roles in the pathogenesis of RA. Neutrophils are largely found in RA synovial fluid, as opposed to the synovial membrane, and produce a spectrum of cytokines, prostaglandins, proteases, and reactive oxygen intermediates (35, 36). Moreover, neutrophils from patients with RA form neutrophil extracellular traps (NETs) that contain citrullinated proteins. Thus neutrophils may be a source of autoantigens in this disease (37). Despite their small numbers in the RA joint, mast cells are increased in arthritic synovium compared to controls and have been observed to be a source of cytokine, chemokine, histamine, and eicosanoid production (38). Surprisingly, a large percentage of the IL-17A in RA synovium is derived from mast cells, suggesting that innate immune cells, in addition to T cells, contribute to the IL-17A response (39). Mast cells secrete proteases, including tryptase, which induces neutrophils and synovial fibroblasts to release cytokines. Additionally,

mast cell-derived tryptase has been shown to inhibit the apoptosis of arthritic synovial fibroblasts (40). This mechanism may contribute to the process of synovial hyperplasia in RA.

Macrophages are abundant in the RA synovium and likely play a central role in the pathogenesis of this disease. Interestingly, the number of macrophages in the synovium correlates positively with radiographic disease progression and negatively with therapeutic success (41). Growth factors that enhance macrophage maturation such as granulocyte macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) are increased in RA synovial fluid and blockade of these factors consistently reduces the severity of arthritis in animal models (42-44). Macrophages are involved in phagocytosis, antigen presentation, and in the production of chemokines and pro-inflammatory cytokines including TNF, IL-1, IL-6, IL-12, IL-18, type I interferons (IFNs), and IL-23 (45). These inflammatory cytokines, especially TNF and IL-6, have been shown to play critical roles in disease pathogenesis. For example, therapeutic blockade of the TNF and IL-6 pathways have shown dramatic benefits in the clinic and are some of the most promising therapies that currently exist for the treatment of RA (46, 47). Moreover, both etanercept and infliximab, anti-TNF therapies, have been shown to induce cell-specific apoptosis in monocytes/macrophages but not in lymphocytes, again placing macrophages at the center of RA pathogenesis (48).

It has been well established that toll-like receptors (TLRs) in macrophages recognize numerous pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) from bacterial and viral ligands (49). Upon

detection of these ligands, macrophages are activated to produce pro-inflammatory cytokines as a means of resolving the infection. Interestingly, a spectrum of infectious agents have been linked to RA including Epstein-Barr virus (EBV), E. coli, porphyromonas gingivalis, and proteus mirabilis; however, the exact significance of these pathogens in RA remains unknown (50-54). In recent years, cytosolic nucleic acid sensors have been identified in the cytosol of macrophages and other cell types and have been found to detect microbial DNA and RNA. It is of interest to determine whether TLRs and cytosolic sensors play a role in macrophage activation and in the initiation and perpetuation of inflammation in arthritic joints. Understanding the molecular basis for inflammation in response to microbial and endogenous ligands has broad implications for the treatment of autoimmune, autoinflammatory, and even infectious diseases.

Role of DNA-sensing pathways autoimmunity

Autoimmune diseases including RA are classically thought of as diseases involving autoreactive T cells and B cells. In recent years, however, components of the innate immune system have been identified as key players in driving the overproduction of type I IFNs and pro-inflammatory cytokines TNF, IL-6, and IL-1 in these diseases. The pattern recognition receptors (PRRs) and ligands responsible for triggering and sustaining the cytokine production in the setting of RA have not been fully evaluated. In the past, attention in this area has largely focused on the role of microbial nucleic acids and nucleic acid sensing TLRs in the pathogenesis of autoinflammatory disease (55). The recent discovery of PRRs in the cytosol of cells, namely cytosolic DNA and RNA sensors, has suggested that additional receptors may be involved (56). Recent data also

indicate that endogenous (host) ligands, as opposed to microbial ligands, may also trigger these PRRs and contribute to autoimmunity (57). By identifying the role of innate immune receptors and their ligands in inflammatory arthritis, potential targets may be discovered for the development of novel therapeutics intended to inhibit the early signaling events in RA.

Endosomal Toll-like Receptors

TLRs were the first innate immune PRRs identified in the context of host defense. These receptors have evolved to recognize several PAMPs expressed by microbes such as lipopolysaccharide (LPS), flagellin, dsRNA, and CpG DNA. Once activated by ligand binding, TLRs form homo- or hetero-dimers and initiate a downstream signaling cascade, which ultimately leads to the nuclear translocation of key transcription factors such as interferon regulatory factors (IRFs) that induce the production of type I IFNs, and nuclear factor kappa B (NF-kB) that drives the production of pro-inflammatory cytokines (49). The production of these factors is intended to resolve infection.

Ten human TLRs have been identified, the majority of which are expressed on the cell surface. TLRs 3, 7, 8, and 9, however, are trafficked to endosomal compartments via the adaptor Unc93b where they recognize foreign nucleic acid (58). TLR3 recognizes dsRNA, whereas TLR7 and TLR8 detect ssRNA. TLR9 responds to unmethylated CpG dsDNA motifs present in bacterial and viral genomes. These TLRs are likely localized to endosomes to prevent aberrant activation by host nucleic acids.

There is a growing body of evidence that indicates that endosomal TLRs not only recognize microbial ligands, but also aberrantly detect endogenous DNA and RNA

released from stressed or dying cells. As a result, TLRs may play an important role in the initiation and progression of chronic inflammatory diseases, including systemic lupus erythematosus (SLE) and RA. In fact, endosomal TLR signaling has been implicated in the pathogenesis of SLE through a variety of murine and clinical studies. Deficiency of TLR7, TLR9, Unc93b1, or IRF5 in autoimmune-prone mice decreases manifestations of SLE including inflammatory cytokine and autoantibody production as well as nephritis (59-62). Furthermore, endosomal TLRs 7 and 9 are often essential to the activation of B cells and the production of autoantibodies in SLE (63). TLR7 promotes the production of autoantibodies reactive to RNA or RNA-binding autoantigens, whereas TLR9 promotes the production of autoantibodies reactive to DNA or nucleosomes. In the Yaa mouse model, overexpression of TLR7 results in increased production of autoantibodies reactive to RNA as well as in the development of lupus nephritis (64). In human genetic studies, polymorphisms in IRF5, a transcription factor downstream of TLR7 and TLR9, was linked with SLE (65). Additionally, increased TLR7 copy number is a risk factor for the onset of juvenile SLE (66).

Activation of endosomal TLRs may also contribute to the pro-inflammatory cytokine and autoantibody production in RA. In the murine model of collagen-induced arthritis (CIA), TLR7 deficiency was shown to decrease clinical paw swelling and histological joint inflammation in comparison to wild type (Wt) mice (67). In agreement with this data, knockdown of TLR7 using lentiviral delivery of shRNA suppresses paw swelling, radiographic progression, and IL-1/IL-6 expression in synovial tissue in the CIA model (68). Moreover, abundant expression of TLR-7 and TLR-3 has been shown in

synovial tissue from RA patients (69). Interestingly, patients deficient in Unc93b1, whose cells cannot respond to TLR3, TLR7, TLR8, or TLR9 ligands, do not develop autoreactive antibodies in their serum (70).

Polymorphisms in IRF5, a transcription factor downstream of TLR signaling, are associated with an elevated risk of developing RA (71) and in the modulation of the erosive nature of this disease (72). Moreover, in the K/BxN serum-transfer arthritis model, IRF5-deficient, TLR3-deficient, and TLR7-deficient mice demonstrated reduced arthritis severity compared to Wt mice (73). These findings indicate that microbial or endogenous RNA ligands may be present in inflamed arthritic joints. The presence of dsDNA from EBV was recently identified in monocytes and neutrophils isolated from the synovial fluid in a significant portion of RA patients (74). EBV has long been suspected to contribute to RA pathogenesis (75). In this same study, overexpression of TLR9 was shown in monocytes derived from RA synovial fluid. Interestingly, compared to healthy controls, monocytes from RA patients demonstrated an increased capacity to produce pro-inflammatory cytokines after stimulation with TLR9 agonists such as EBV. Thus, various TLR ligands may be present in the RA joint, activating endosomal TLRs and promoting the breakthrough of tolerance and inflammation.

Additional support for a role of TLRs in driving autoimmunity is demonstrated by the efficacy of antimalarial drugs such as hydroxychloroquine and chloroquine that have been used for decades to treat RA and SLE. Hydroxychloroquine blocks endosomal TLR activation by inhibiting endosomal acidification. Additionally, antimalarials have recently been shown to modify nucleic acid ligands so that they no longer bind and activate their

TLR receptors (76). Understanding the contribution of endosomal TLR ligands and pathways may allow for the development of therapeutics to target initiating events in inflammatory diseases and RA pathogenesis.

Cytosolic DNA Sensors

In recent years, additional PRRs have been identified, apart from TLRs, which are located in the cytosol and also detect and respond to nucleic acid. These cytosolic receptors include RNA sensors belonging to the RIG-I family (i.e. RIG-I and Mda5) and numerous DNA sensors (77). Several receptors for intracellular DNA are upstream of the endoplasmic reticulum (ER)-associated protein stimulator of interferon genes (STING), including cyclic GMP-AMP synthase (cGAS), IFN-inducible protein 16 (IFI16, and its mouse orthologue, Ifi204/p204), DAI, and DDX41 (78-83). Upon activation with a DNA ligand, these sensors promote the dimerization of STING, which then leads to the activation and nuclear translocation of IRF3 and NF-kB and the subsequent transcription of type I IFNs and pro-inflammatory cytokines, respectively. Another cytosolic DNA receptor, which is not dependent on STING, has recently been identified as absent in melanoma-2 (AIM2). AIM2 also recognizes DNA, but instead coordinates the assembly of an inflammasome complex that activates caspase-1 and cleaves pro-IL-1β and pro-IL-18 into their active forms (84). The endosomal TLR and cytosolic sensor pathways that are described in this thesis are displayed in **Figure 1.2**.

These DNA sensors are crucial in macrophages and dendritic cells for detecting the presence of viruses and activating a potent antiviral response. In recent years,

however, the inappropriate activation of these receptors by endogenous (host) ligands has been associated with autoinflammatory conditions (85). Several sources of endogenous immunostimulatory nucleic acids have been identified. Self DNA released from stressed, damaged, or dying cells can be displayed on the surface of apoptotic cells and subsequently endocytosed, triggering PRRs (86, 87). Mitochondrial DNA is another major source of host nucleic acid. Typically, stressed and damaged mitochondria are engulfed by autophagosomes and trafficked to lysosomes for recycling. However, defects in autophagy or lysosomal integrity (i.e. lysosomal storage diseases) can result in accrual of mitochondrial DNA in the host and trigger PRRs (88, 89).

Recently, oxidized DNA has been identified as a DAMP that activates cytosolic DNA sensors. Oxidative damage results in structural changes to DNA, decreasing its susceptibility to degradation by the cytosolic exonuclease Trex1 (56). As a consequence, oxidized DNA accumulates in the cytosol and acts as a much more potent agonist of STING-dependent DNA sensors than unmodified DNA. In humans, oxidative damage to DNA accrues with aging and results largely from sun exposure and reactive oxygen species. An additional source of autologous nucleic acids includes transcribed and reverse-transcribed retrotransposons (57). Endogenous retroelements represent remnants of non-infectious viruses that constitute over 40% of the mammalian genome. Retrotransposons have been linked to multiple examples of autoinflammatory diseases.

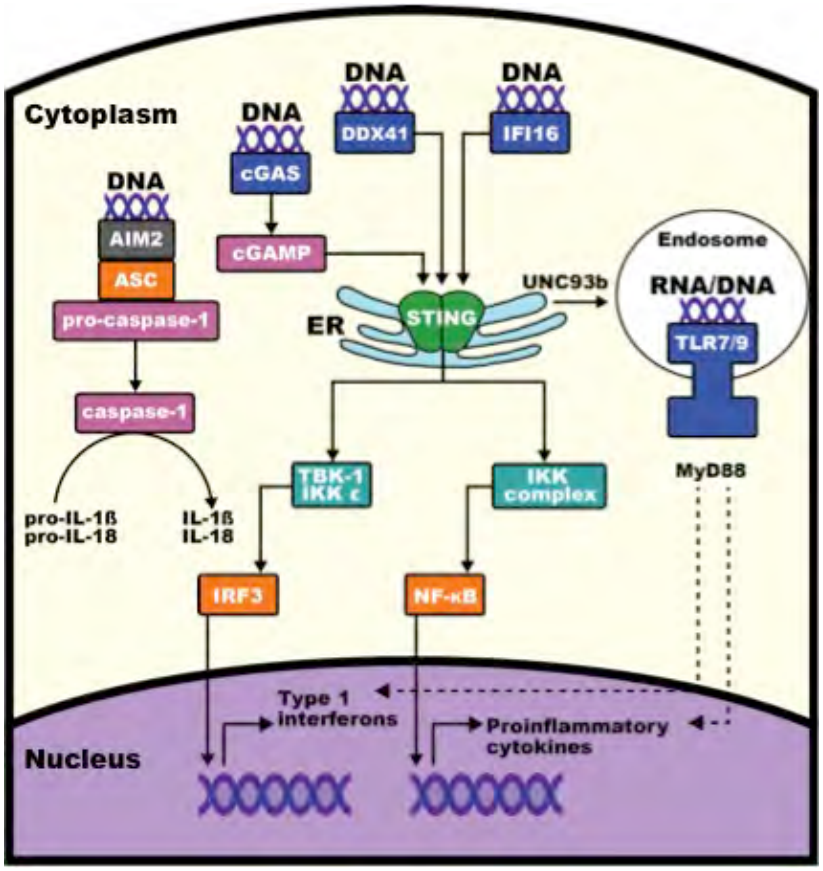


Figure 1.2 Endosomal TLR and cytosolic DNA sensor pathways. Cytosolic DNA is detected by a number of sensors that signal through the adaptor STING including cGAS, DDX41, and IFI16, leading to the production of type 1 IFNs and pro-inflammatory cytokines. DNA can also be detected by STING-independent cytosolic sensors such as AIM2. AIM2 leads to the formation of a caspase-1 activating inflammasome that cleaves pro-IL-1β and pro-IL-18 into their active forms. Another pathway for DNA detection is through endosomal TLRs, including TLR7 and 9. These TLRs traffic from the endoplasmic reticulum (ER) to endosomes via Unc93b where they sense nucleic acid and also lead to the production of IFNs and pro-inflammatory cytokines.

Additionally, inactivation of intracellular DNases leads to endogenous DNA accrual and results in autoinflammatory syndromes. Perhaps the best described example of this is deficiency of the exonuclease DNase III (Trex1). In mice, deficiency of Trex1 leads to accrual of DNA in the cytosol, which triggers STING-dependent cytosolic sensors and production of type I IFNs and pro-inflammatory cytokines, ultimately leading to systemic inflammation and lethal myocarditis (90). In humans, loss-of-function mutations in *Trex1* are associated with Aicardi-Goutieres syndrome, an early onset neuro-inflammatory disease, chilblain lupus, and SLE (91-96). In addition, gain-of-function mutations in STING have also been shown to result in an autoimmune syndrome now called STING-associated vasculopathy with onset of infancy (SAVI) (97). In these patients, gain-of-function mutations in *Tmem173* cause the spontaneous dimerization and constitutive activation of STING and lead to vasculopathy, skin and pulmonary inflammation, and arthritis. These clinical features are a result of enhanced production of IFNs and pro-inflammatory cytokines.

Finally, inactivation of another DNase, DNase II, also leads to endogenous DNA accrual and autoimmune manifestations. The enzyme DNase II is a ubiquitously expressed lysosomal nuclease that degrades double-stranded DNA engulfed from either apoptotic cell debris or the extrusion of reticulocyte nuclei (98). In non-phagocytic cells, DNase II degrades damaged DNA that leaks out of nuclear pores and is delivered to lysosomes via autophagosomes (88). In humans, single nucleotide polymorphisms (SNPs) in the promoter region of the *DNase II* gene that result in reduced DNase II activity confer a significant increase in RA disease susceptibility (99). In mice deficient

in DNase II, endogenous DNA accumulates in phagolysosomes and in the cytosol of multiple cell types including macrophages, fibroblasts and dendritic cells and leads to the production of pro-inflammatory cytokines including TNF, IL-1β, and IL-6, as well as type 1 IFNs (100). Excessive type I IFN production leads to severe anemia and embryonic lethality. These DNase $II^{-/-}$ mice are rescued by intercrossing them with mice that fail to express the type I IFN receptor (IFNaR). DNase $II^{-/-}$ $IFNaR^{-/-}$ double deficient (**DKO**) mice survive, but develop a distal, erosive inflammatory arthritis by 2-3 months of age that resembles RA, which is absent in $DNaseII^{+/-}$ $IFNaR^{-/-}$ (**Het**) controls (100). Importantly, these DKO mice also develop additional manifestations of autoimmunity including robust autoantibody production and splenomegaly. In this dissertation we reveal the PRRs that detect the accumulated DNA and drive the autoimmune manifestations in DKO mice.

Synovial Fibroblasts: Key players in RA

While the role of hematopoietic cells in driving RA has been well established, the contribution of non-hematopoietic stromal elements to disease pathogenesis is just beginning to be appreciated. The synovium is a key site of pathology in RA. In healthy joints, the thin synovial layer is only 2-3 cells thick; however, in RA joints, synovial fibroblasts undergo robust hyperplasia and result in a thickened synovium nearly 10-15 cells thick (101). This hyper-proliferative synovial tissue constitutes a large percentage of the “pannus” tissue that invades and destroys articular cartilage and bone. In fact, activated fibroblasts secrete MMPs that destroy cartilage and receptor activator of NF-κB

ligand (RANKL) that promotes osteoclast differentiation leading to bone erosions. The increased fibroblast population in RA is largely due to inhibition of pro-apoptotic pathways (101). In addition, a variety of oncogenes including c-fos, ras, and myc are overexpressed in RA synovial fibroblasts (RASFs) (102, 103). Mutations in the p53 tumor suppressor gene were also identified in RASFs and likely contribute to fibroblast survival (104).

For years, macrophages in the inflamed synovium have been known to detect innate immune stimuli via PRRs and respond by producing a broad repertoire of chemokines and cytokines. These pro-inflammatory factors have been shown to activate RASFs. Recently, increasing evidence has suggested that RASFs themselves respond to innate stimuli and produce a variety of inflammatory mediators that recruit and prolong the survival of leukocytes. Synovial fibroblasts were shown to express TLRs 1-6, and in synovial tissues from RA patients, TLR-3 and TLR-4 were highly expressed compared to levels in osteoarthritis (105). In addition, stimulation of RASFs with TLR3 ligands leads to the production of IFN-β, IL-6, CCL5, and CXCL10. Moreover, incubation of RASFs with necrotic synovial fluid from RA patients upregulated these cytokines through TLR-3 (106). In a recent study, IL-17 was shown to increase TLR-3 expression via STAT3 in RASFs (107). TLR-2 has also shown to play a role in RASFs. Synovial tissues from RA joints demonstrated increased TLR2 expression compared to controls (108), and stimulation of RASFs by TLR-2 ligands upregulated numerous chemokines and cytokines including granulocyte chemotactic protein (GCP)-2, RANTES, monocyte chemoattractant protein (MCP)-2, and IL-8 (109). These findings suggest that microbial

or endogenous TLR ligands may contribute to the initiation of inflammatory arthritis through activation of synovial fibroblasts.

RASFs have also been shown to drive the chronicity of inflammation by recruiting and prolonging the survival of leukocytes in the inflamed synovium. Thus, the stroma may play a key role in driving the persistence of chronic inflammatory diseases (110). Resolution of inflammation requires apoptosis of the infiltrated immune cells; however, RASFs inhibit this process by producing leukocyte survival factors. For example, RASFs have been shown to mediate CD4 T cell survival by producing type 1 IFNs and augment the survival of neutrophils through production of GM-CSF (111).

Additionally, evidence suggests that RASFs recruit and retain B cells in the rheumatoid joint. In RA, B cells migrate and accumulate in the synovium and often form ectopic germinal centers in diseased joints. These B cells have been shown to experience prolonged survival and demonstrate clonal expansion. RASFs have been shown to constitutively express the chemokines stromal cell-derived factor-1 (SDF-1) and CD106 (VCAM-1), which support B cell recruitment/migration (112). Furthermore, immunohistochemistry (IHC) staining of synovial tissue in RA compared to normal or osteoarthritic tissue, revealed increased expression of the B cell survival factor, B cell-activating factor of the TNF family (BAFF) (113). This same study found that IFN-γ and TNF induced BAFF mRNA expression in fibroblast-like synoviocytes (FLS). Furthermore, B cells co-cultured with IFN-γ/TNF-treated FLS lived longer than B cells cultured alone. RASFs have also been reported to constitutively express IL-15 on their surface, which was subsequently shown to protect B cells from apoptosis. Consistent with

this finding, peripheral blood B cells in RA patients demonstrated upregulation of IL-15R and increased survival in co-cultures with RASFs (114). Collectively, these findings demonstrate that RASFs recruit leukocytes in the rheumatoid joint and prolong their survival in inflammatory environments.

The initiation and persistence of inflammation within arthritic joints likely result from a complex interaction between leukocytes and mesenchymal stromal cells. While the interplay between these elements is still not fully understood, stromal cells have emerged as key players in the pathogenesis of RA. Stromal cells contribute to the initiation of inflammation through activation of their PRRs and the subsequent production of inflammatory mediators. Additionally, stromal cells contribute to the chronicity of inflammation by promoting leukocyte migration and survival. Current therapies for RA and other autoinflammatory diseases largely target hematopoietic cells and the factors produced by these cells. Moreover, these therapies are not effective in all patients. The development of therapeutics that target the stromal microenvironment may increase the number of patients that respond to therapy and prove beneficial for the treatment of RA.

Pathogenesis of Bone Loss in RA

Chronic inflammation in RA promotes focal articular bone erosions within inflamed joints as well as systemic osteopenia/osteoporosis involving the axial and appendicular skeleton. Destruction of articular bone is a critical measure for the assessment of therapeutic interventions and is predictive of long-term disability and

increased mortality in these patients. Furthermore, if left untreated, bone loss can lead to joint deformity and fractures (115).

Disruption of the bone remodeling process is seen in several rheumatic diseases. Under normal physiologic conditions, there is a balance between the action of osteoclasts, cells that resorb bone, and osteoblasts, cells that form bone. The coupling of these two cell types is tightly coordinated to avoid a net loss or gain of bone. In RA, local and systemic production of pro-inflammatory cytokines disrupts the balance between bone resorption and bone formation, resulting in abnormal bone loss. While their effects on bone are complex, cytokines expressed in the inflamed synovium and pannus such as TNF, IL-1, IL-6, and others, mediate the erosive process by enhancing osteoclast differentiation and activity. In addition, inflammation also targets the osteoblast in RA, inhibiting osteoblast maturation and limiting the repair of erosions (8).

Osteoclast: Increased Bone Resorption in RA

Since the late 1990s, the osteoclast has become widely recognized as the cell responsible for bone erosions in RA. It was first noted that cells expressing the osteoclast marker tartrate resistant acid phosphatase 5b (TRAP5b) were present in subchondral bone in surgical samples from RA patients (116). Large, multinucleated cells were noted not only in subchondral bone, but also at the pannus-bone interface in these surgical samples. These cells expressed osteoclast-specific markers including TRAP5b, cathepsin K, and calcitonin receptor, placing osteoclasts squarely at the site of bone erosion (117).

Subsequent studies also showed that osteoclasts were present at similar sites within erosions in animal models of arthritis (118-121).

Osteoclasts were demonstrated to be essential for bone erosion in the K/BxN serum transfer model of arthritis by demonstrating that mice deficient in RANKL, a factor required for osteoclast differentiation, develop arthritis as well as cartilage destruction, but are protected from bone erosions in the absence of osteoclasts (121). Inflamed synovial tissues provide several cellular sources of RANKL, including lymphocytes and FLSs. RANKL synergizes with pro-inflammatory cytokines to promote the differentiation of osteoclasts from mononuclear precursors in synovial tissues. These findings were confirmed in a follow up study in which mice lacking c-fos, a transcription factor essential for osteoclastogenesis, were crossed with transgenic mice expressing human TNF (hTNFtg) (122). These mice are osteoclast deficient and develop a TNF-dependent inflammatory arthritis closely resembling RA. Despite the presence of inflammation, the arthritic joints of these mice were also protected from bone erosion and destruction. Furthermore, blocking the RANK-RANKL interaction using osteoprotegerin (OPG), the decoy receptor for RANKL, protected against systemic bone loss in hTNFtg mice (123), as well as arthritic bone loss in other animal models of arthritis (124, 125).

Inhibition of osteoclast activity in patients with RA has also demonstrated some efficacy in inhibiting the progression of bone erosion. The bisphosphonate zolendronic acid has been shown to reduce development of new erosions in the joints of both arthritic mice (126, 127) and in patients with RA (128). Furthermore, neutralization of RANKL with the monoclonal antibody denosumab attenuated bone erosion and systemic bone loss

in RA patients (129-131). Collectively, these studies have confirmed that osteoclasts mediate bone erosions in patients with RA, and provide a target cell type for the prevention of articular bone loss.

Osteoclastogenesis is enhanced in the RA joint by several factors. Well known are the effects of pro-inflammatory cytokines on osteoclastogenesis, and recently the production of autoantibodies have also been linked enhanced osteoclast differentiation. Cytokines including TNF, IL-1 and IL-17 promote the differentiation of osteoclast precursors to osteoclasts through several mechanisms, including upregulating the expression of RANKL in osteoblasts (132-134). IL-17 (6) and TNF (135) induce RANKL expression in FLSs. TNF also directly promotes the differentiation of osteoclast precursor cells, as well as expands the pool of these precursor cells (136). In addition, IL-1 directly enhances the ability of osteoclasts to resorb bone (4) and is a mediator of TNF-induced osteoclastogenesis (132). The development of biologic agents that inhibit pro-inflammatory cytokines such as anti-TNF agents (46), IL-6R antagonists (47), and small molecule agents that block JAK/STAT signaling (137-139), among others, can retard or arrest radiographic progression in RA and has revolutionized the treatment of this disease.

In addition, clinical studies have helped to validate the impact of pro-inflammatory cytokines on osteoclastogenesis in RA. On MRI scanning, there is a strong relationship between the presence of “bone marrow edema” and the subsequent progression of bone erosions in RA patients (140-142). The phrase “bone marrow edema” was coined to reflect the decreased fat and increased water content seen by MRI

in the marrow of arthritic joints. Histologic analysis of areas of bone edema in animal models of inflammatory arthritis and of joints from patients with RA has revealed that this MRI finding represents the replacement of bone marrow fat by inflammatory infiltrates, comprised largely of mature B cells, activated T cells, and macrophages (140). Thus, “bone marrow edema” is better referred to as “osteitis.” This influx of inflammation into the marrow space introduces cell types that express RANKL, as well as pro-inflammatory cytokines that further induce osteoclastogenesis and promote the progression of articular bone erosions.

Intriguing new data has linked the production of autoantibodies to the process of osteoclast differentiation. Clinical studies have demonstrated that high titers of ACPAs are associated with radiographic progression in RA patients (143, 144). Furthermore, ACPAs and RF together have been shown to have an additive effect on erosion size and number in patients with RA (145). These findings are further supported by the surprising discovery that ACPA-positive healthy individuals show signs of bone loss compared to ACPA-negative individuals (32). Micro-CT analysis was performed on joints of age and gender-matched ACPA-positive individuals who showed no signs of synovitis, and ACPA-negative healthy individuals. Although no evidence of bone erosion was seen in ACPA-positive individuals, bone volume per total volume and bone mineral density (BMD) were both significantly reduced in ACPA-positive individuals compared to ACPA-negative controls. Since ACPAs can be detected in serum years before the onset of RA (146, 147), it appears likely that bone damage in RA precedes the clinical onset of disease through mechanisms independent of inflammation.

Since autoantibodies have been shown to promote inflammatory-mediated as well as inflammatory-independent bone loss in RA (148), there has been a great deal of interest in possible mechanisms. Stimulation of mononuclear cells or macrophages with immune complexes and ACPAs from RA patients results in the production of high levels of TNF by these cells, contributing to osteoclastogenesis (149-151). Recent studies have shown that autoantibodies not only bind macrophages, but also bind osteoclast precursors. ACPAs bind citrullinated vimentin on the surface of osteoclast precursors, inducing these precursors to produce TNF, thus promoting their differentiation to mature osteoclasts (152).

The effect of immunoglobulin (IgG) sialylation on the interaction of immune complexes with osteoclasts has also been investigated (153). Modification of IgGs by attachment of sialic acid residues to the Fc portions is known to mediate the anti-inflammatory effects of intravenous IgG (154). Harre and colleagues have demonstrated that only non-sialylated immune complexes stimulate osteoclastogenesis in vitro and in vivo. Furthermore, administration of a sialic acid precursor results in elevated sialylation levels of IgG and decreased bone erosions in mice with collagen-induced arthritis (153). Together, these studies emphasize the importance of autoantibodies in mediating bone loss in RA, and suggest a new mechanism by which osteoclastogenesis in RA may be promoted.

Limited repair of bone erosions in the RA joint

Clinical studies have shown that therapies that reduce joint inflammation can slow or halt the progression of osteoclast-mediated bone resorption in patients with RA. Despite treatment, however, repair of existing erosions is unusual (155). These persisting erosions are associated with cartilage loss, as subchondral bone, which provides the scaffold for articular cartilage, is typically eroded. With erosion of subchondral bone, articular cartilage is lost. Persistent erosions have been shown to be associated with functional decline, and are associated with joint instability and likely also changes in mechanical forces across joints, which may further impact articular cartilage.

Dohn and colleagues investigated the frequency and extent of erosion repair in patients with RA given combination therapy with anti-TNF and methotrexate (MTX) (14). After 12 months of therapy, high resolution computed tomography (CT) of the wrist and hand joints demonstrated that although erosion progression was halted, repair of erosions was rare. Subsequently, it was demonstrated that although biologic therapy significantly decreased synovitis scores in a cohort of RA patients, all patients had remaining synovitis after 12 months of treatment as determined by MRI (15). This same study showed that erosion repair occurred in only 6% of patients treated with adalimumab, suggesting that residual inflammation may impair osteoblast function and healing of erosions.

Similar findings were published in a study that examined erosion repair in RA patients treated with TNF inhibitors and MTX compared to matched patients treated with MTX alone (156). The width and depth of erosions in joints were measured by high-

resolution micro-CT at baseline and after one year of treatment. Repair of erosions was shown to be very limited and all erosions could still be identified following treatment. Similar findings were demonstrated in RA patients treated with IL-6R blockade (157). Collectively, these results suggest that despite treatment, residual inflammation persists in joints, likely impairing osteoblast function and healing of erosions.

Osteoblast Differentiation Pathways

Despite the focus on osteoclasts at known sites of bone loss in RA, it has recently been shown that inflammation not only induces osteoclastogenesis, but also inhibits osteoblast differentiation and function (7). This inhibition contributes to the development of arthritic bone loss in RA, as well as to the markedly diminished capacity of existing erosions to heal through new bone formation. For this reason, it is important to understand the pathways that promote or inhibit osteoblast differentiation. Unlike osteoclasts, osteoblasts derive from mesenchymal precursor cells and are responsible for the synthesis and mineralization of bone, as well as the modulation of osteoclast differentiation. Several factors are known to regulate the stages of differentiation from mesenchymal stem cells (MSCs) to mature osteoblasts (**Figure 1.3**). These include growth factors and hormones such as insulin-like growth factor (IGF) and parathyroid hormone (PTH) that aid in the transition of MSCs to mesenchymal stromal cells (158). Upregulation of the pro-osteogenic transcription factor runt-related transcription factor 2 (Runx2) commits stromal cells toward an osteoprogenitor cell fate, while expression of the transcription factor osterix further promotes differentiation of the cell into a mature

osteoblast. Mature osteoblasts produce type I collagen as well as non-collagenous proteins involved in bone mineralization, including osteocalcin and bone sialoprotein. This newly formed bone matrix eventually surrounds mature osteoblasts, embedding these cells within the bone matrix as terminally differentiated osteocytes.

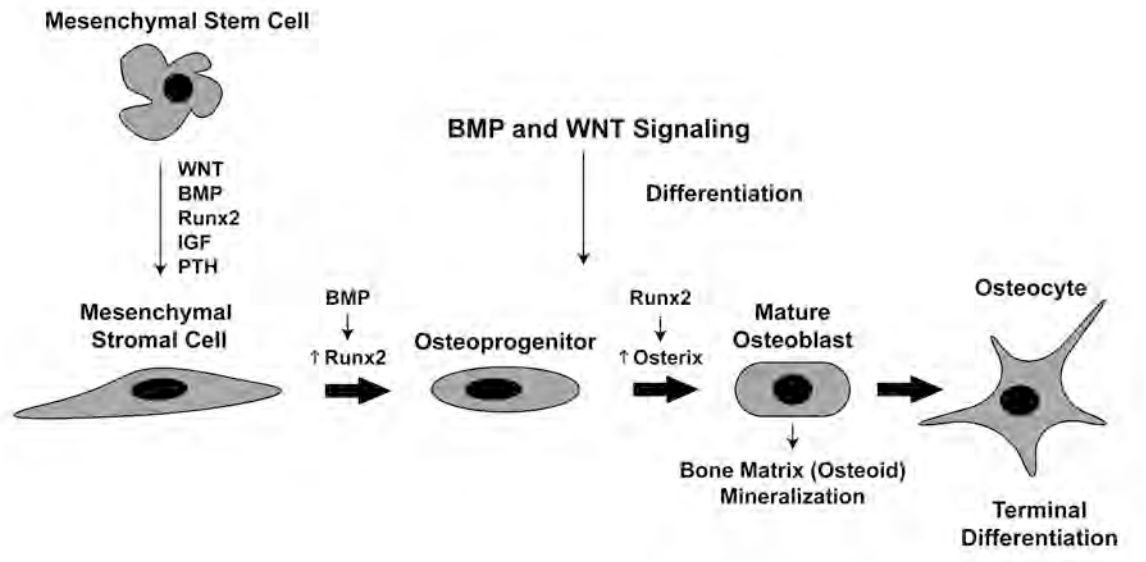


Figure 1.3. Stages of Osteoblast Differentiation. Osteoblasts are derived from mesenchymal stem cells. Wingless (WNT) and bone morphogenic protein (BMP) pathways promote their differentiation to mesenchymal stromal cells, in conjuction with Runx2, insulin-like growth factor (IGF) and parathyroid hormone (PTH). Activation of BMP signaling promotes Runx2 expression in stromal cells, leading to further differentiation to osteoprogenitors. Runx2 subsequently induces expression of the transcription factor osterix, with further differentiation to a mature osteoblast. These ultimately become embedded in bone matrix as terminally differentiated osteocytes.

In addition to their role in bone formation, osteoblasts also regulate osteoclast differentiation through the production of several factors including M-CSF, RANKL, and OPG. M-CSF acts to expand the pool of osteoclast precursor cells and promotes their survival (159). RANKL, an essential factor for osteoclastogenesis, interacts with the RANK receptor on the osteoclast precursor to promote osteoclast differentiation (160). To protect against excessive bone resorption, osteoblasts also produce OPG, a soluble decoy receptor that binds RANKL to inhibit osteoclast differentiation.

The wingless (Wnt) (161) and bone morphogenetic protein (BMP) (162) pathways known to regulate skeletal development and organogenesis are also critical pathways regulating osteoblast differentiation. Wnt signaling includes the canonical Wnt/β-catenin pathway and two noncanonical pathways, the Wnt-calcium and the Wnt-planar cell polarity pathways (163). In the canonical Wnt pathway, secreted Wnts, such as Wnt1 and Wnt3a, bind and activate a complex that includes the low-density lipoprotein receptor related proteins (LRP)5 and LRP6. These receptors complex with Frizzled co-receptors in the plasma membrane to promote the stabilization of cytosolic β-catenin, allowing its translocation to the nucleus to induce transcription of genes that promote osteoblast differentiation and bone formation.

Several antagonists to the Wnt pathway, including Dickkopf (DKK) and secreted frizzled-related protein (SFRP) family members, as well as sclerostin, have been identified. DKK-1 crosslinks LRP5/6, leading to the suppression of Wnt signaling in osteoblast precursors. Inhibition of DKK-1 expression has been linked to high bone mass (164) while overexpression of DKK-1 results in osteopenia in mice (165). SFRPs inhibit

Wnt signaling by binding directly to Wnt proteins. Deletion of the SFRP1 gene results in increased bone volume in mice (166), while its overexpression has been linked to decreased bone density (167). Interestingly, inflamed synovial tissue has been found to be a source of DKK-1, which inhibits osteoblast-mediated bone formation in arthritic joints (168). In contrast, the expression of DKK-1 is diminished in animal models and patients with ankylosing spondylitis (AS) (169). The expression of SFRPs is also upregulated in arthritic synovial tissue (170) and likely contributes to the inhibition of osteoblast differentiation in inflammatory arthritis. Sclerostin, a glycoprotein secreted predominantly, if not exclusively, by osteocytes, also inhibits canonical Wnt signaling by binding to the LRP5/6 receptor (171). The effects of sclerostin on bone were originally brought to light when loss-of-function mutations in or near the sclerostin-encoding gene *SOST* were identified in patients with van Buchem's disease (172-174) and sclerosteosis (175, 176), diseases associated with high bone mass.

Mesenchymal stem cells also require activation of BMP signaling to commit to the osteoblast lineage. BMPs belong to the transforming growth factor beta (TGF-β) superfamily and are secreted mainly by osteoblasts, chondrocytes, and endothelial cells (162). Pro-osteogenic BMPs, such as BMPs 2, 4, and 7, bind membrane-bound receptors and result in phosphorylation of intracellular SMADs 1/5/8. These factors complex with SMAD4 and translocate to the nucleus to promote the transcription of BMP-responsive genes. A variety of secreted molecules, such as noggin and sclerostin itself, have been identified that sequester BMP ligands and inhibit their interaction with their receptor (177). Dysregulation of BMP signaling has been associated with several skeletal

disorders including heterotopic ossification, osteoporosis, and low and high bone mass diseases.

Osteoblast: Inhibition of function in RA

Many studies now suggest that pro-inflammatory cytokines not only provoke osteoclastogenesis, but additionally contribute to bone loss by inhibiting osteoblast differentiation. For example, TNF is a potent inhibitor of osteoblast differentiation in cultured cells. Treatment of calvarial osteoblasts or the MC3T3 osteoblast-lineage cell line with TNF inhibited differentiation, as shown by reductions in both mineralizing nodules and osteocalcin secretion (178). TNF also induces degradation of Runx2, a critical transcription factor for osteoblast differentiation (179). In addition, high dose TNF treatment of osteoblast precursor cells induces their apoptosis (180). Exposure of osteoblast cultures to IL-1 has also been shown to inhibit mineralizing nodule formation, as well as collagen protein synthesis and cellular replication (181) and IL-1 impairs the recruitment and migration of osteoblasts toward chemotactic factors (182). Furthermore, the interaction of IL-6 with sIL-6R on osteoblasts upregulates prostaglandin E2 synthesis and reduces the ratio of OPG/RANKL, thus promoting osteoclast differentiation (183). Thus, in vitro studies demonstrate that pro-inflammatory cytokines influence the osteoblast by impairing its differentiation and/or function, and in some cases by promoting osteoblasts to induce osteoclast differentiation.

Arthritic inflammation has also been shown to inhibit osteoblast differentiation and function in vivo. Arthritis was induced in mice using the K/BxN model and dynamic

histomorphometry was adapted to erosion sites to evaluate bone formation rates (BFRs) (7). BFRs were significantly reduced at bone surfaces adjacent to inflammation compared to bone surfaces adjacent to normal marrow, demonstrating that inflammation inhibits osteoblast activity. Furthermore, in and around sites of articular erosion, there was a complete absence of cells expressing late-stage osteoblast lineage markers (mature osteoblasts). These findings demonstrate that synovial inflammation inhibits the capacity of osteoblasts to mature and form mineralized bone.

Studies in the hTNFtg model of RA have shown that cells within inflamed synovial tissues secrete the Wnt signaling pathway antagonist DKK-1, impairing osteoblast-mediated bone formation (168). Furthermore, TNF was shown to upregulate DKK-1 expression in synovial fibroblasts, as well as in osteoblasts. Blockade of DKK-1 when given at the onset of inflammation, led to an absence of joint erosions in typical sites, despite the presence of arthritic inflammation. Histomorphometry performed on the periosteal surface of bone showed increased BFRs with DKK-1 blockade, as well as an increase in osteoblast numbers and osteoid deposition in the arthritic mice treated with the DKK-1 neutralizing antibody compared to controls. Clinical studies have validated the relevance of DKK-1 in arthritic joint remodeling. Serum levels of DKK-1 were significantly increased in patients with RA compared to healthy controls, and DKK-1 was expressed in the inflamed synovium from patients with RA compared to controls (168). These studies illustrate the impact of the Wnt antagonist DKK-1 on osteoblast inhibition in the setting of inflammatory arthritis.

The Gravallese laboratory identified several Wnt signaling antagonists whose expression was upregulated in arthritic synovium using the K/BxN model, including members of the DKK family as well as SFRP1 and 2 (7, 170). In addition, they induced arthritic inflammation and subsequently allowed the inflammation to resolve. Dynamic histomorphometry and micro-CT showed that upon resolution of inflammation, mature osteoblasts populated the eroded bone, and bone formation was induced at these sites, followed by repair of erosions. Notably, as synovial inflammation almost completely resolved, synovial expression of the Wnt antagonists sFRP1 and sFRP2 was downregulated and expression of the Wnt agonist Wnt10b was induced compared to arthritic synovium. These findings demonstrated that significant resolution of inflammation is necessary to promote Wnt signaling and erosion repair in the arthritic joint. Thus, cells within inflamed synovial tissues secrete factors that antagonize the Wnt signaling pathway and inhibit osteoblast differentiation and osteoblast-mediated bone formation. These effects are manifested clinically in the arthritic joint, where persistent inflammation likely contributes to the limited healing of erosions. **Figure 1.4** provides a summary of pathways involved in the regulation of osteoclasts and osteoblasts in RA.

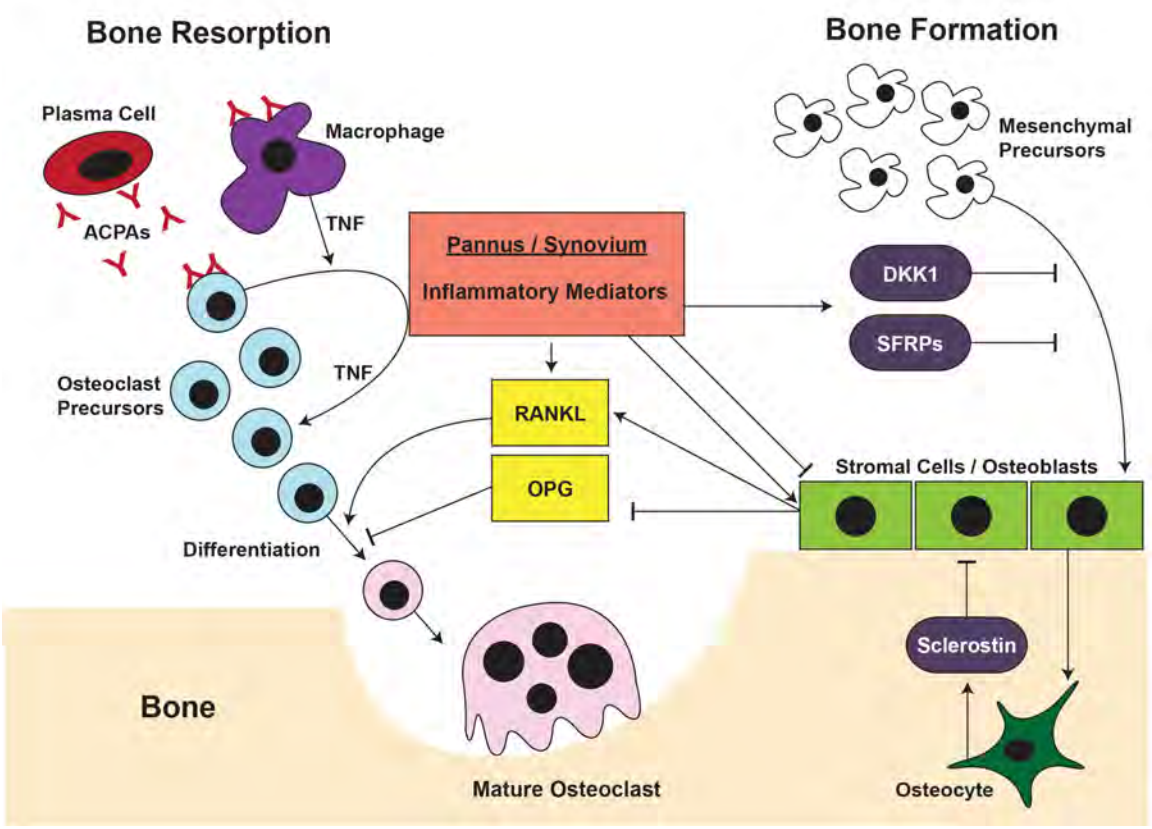


Figure 1.4 Cell types and factors regulating bone in rheumatic disease.

Resorption: The inflamed synovium/pannus produces several inflammatory mediators that enhance osteoclastogenesis and inhibit osteoblast maturation in the joint, leading to the development and persistence of articular bone erosions. These mediators promote the differentiation of osteoclast precursors to mature osteoclasts, in part by the upregulation of receptor activator of NF-kB ligand (RANKL). Anti-citrullinated protein antibodies (ACPAs) can also promote osteoclastogenesis by binding to macrophages and/or osteoclast precursor cells and inducing TNF production, thus enhancing cellular expansion and differentiation.

Formation: Bone formation occurs through the action of mature osteoblasts that produce organic bone matrix and orchestrate bone mineralization. These derive from mesenchymal precursors, whose differentiation is inhibited by antagonists of the Wnt signaling pathway, including Dickkopf (DKK) and secreted frizzled-related protein (SFRP) family members, and sclerostin, derived from osteocytes embedded in bone matrix. Inflammatory mediators also induce the production of RANKL, and inhibit the production of osteoprotegerin (OPG) by stromal cells/osteoblasts.

Role of the Innate Immune System in Heterotopic Ossification

Dysregulation of the immune system and of the TGF-β/BMP signaling pathways can also result in excess bone formation, as in the disorder heterotopic ossification (HO). Acquired and hereditary HO is a debilitating condition associated with formation of lamellar bone at extra-skeletal sites. The progression of this disease is episodic and the mechanism is unknown. Viral or traumatic events are thought to initiate flare-ups of the innate immune system in HO and precede the development of new ectopic bone lesions (184). Acquired HO occurs in cases of various types of soft tissue traumatic events, including combat-related trauma, amputations, total joint replacement surgery, elbow and acetabular fracture repair, traumatic brain injury, spinal cord injuries, encephalitis, and severe burns (185). HO was recognized during World War I when it was found that soldiers with blast-related injuries frequently acquired ectopic bone lesions. Today, HO remains a major concern and cause of morbidity in soldiers serving in Iraq and Afghanistan and has been reported in 63% of traumatic amputations in this population (186). Acquired HO also commonly occurs in orthopedic trauma after fixation of acetabular or elbow fractures (187). Presently, there is no cure for this disease and treatments aim to limit inflammation. Non-steroidal anti-inflammatory medications such as indomethacin and celecoxib are mildly effective as prophylaxis against HO (188). Surgical excision is at times used to remove the heterotopic lesions; however, surgical trauma itself can induce reoccurrence of ectopic bone (189).

Hereditary syndromes of HO are rare, but include life-threatening disorders such as fibrodysplasia ossificans progressiva (FOP). Heterotopic bone formation in FOP

causes severe physical immobility, including loss of joint mobility, restricted movement of the jaw, and limited expansion of the rib cage and diaphragm. Eventually, this condition will encase patients in a “second skeleton” of ectopic bone, leaving them immobile and with a life expectancy of around 40 years (190). Heterozygous, missense gain-of-function mutations in activin A receptor type I (ACVRI), a BMP type I receptor, are known to contribute to FOP. These mutations lead to a conformational change in the receptor and enhanced BMP signaling (191). Interestingly, transgenic mice with constitutively activated ACVR1 do not develop ectopic bone formation without an inflammatory stimulus. Only transgenic mice infected with adenovirus and demonstrating subsequent inflammation will form heterotopic lesions (192). Thus, ACVR1 mutations are necessary but perhaps not sufficient to induce bone formation. Accordingly, despite the presence of ACVR1 mutations, FOP patients exhibit variability in the severity and progression of their disease, and form bone episodically, rather than continuously, following a viral infection or tissue trauma (184, 193). These FOP flare-ups precede the development of new ectopic bone lesions and strongly implicate a role for innate immune triggers and inflammatory pathways in the pathophysiology of heterotopic bone formation.

It has been postulated that HO may be considered an auto-inflammatory disease. Trauma and viral-induced release of DAMPs and PAMPs are thought to stimulate TLRs and other PRRs of the innate immune system, leading to the production of bone-inducing factors like BMPs, activins, and TGF-β family members (184). Indeed, inhibition of BMP and TGF-β signaling pathways has been shown to ameliorate osteogenic

differentiation in models of FOP (194, 195). Furthermore, the requirement for inflammation in the process of HO is demonstrated by the infiltration of macrophages, lymphocytes, and mast cells in affected sites during early phases of flare-ups (196, 197), and also by the improvement of symptoms in patients after treatment with high-dose corticosteroids (189). Thus, while the cells postulated to give rise to the ectopic bone formation are of mesenchymal-endothelial origin (198), hematopoietic cells likely contribute to FOP progression as well (199). Understanding the interplay between innate immune triggers and ectopic bone formation will be critical for identifying effective therapeutic targets and treatments for debilitating conditions such as HO.

Introduction of Dissertation Aims

Despite emerging evidence implicating innate immune pathways in the generation of autoimmune diseases, a number of questions remain concerning the contribution of distinct DNA sensors to inflammatory arthritis and bone remodeling. The aim of this dissertation is to elucidate the cytosolic and endosomal DNA receptors that may initiate or perpetuate inflammatory arthritis and dysregulated bone remodeling. To address this aim the research in this dissertation is divided into three objectives:

1) *Investigate the distinct contributions of STING-dependent cytosolic sensors, AIM2, and endosomal TLRs dependent on Unc93b1 to the generation of pro-inflammatory cytokines, inflammatory arthritis and autoantibody production.* Using the DNase $II^{-/-}$ $IFNaR^{-/-}$ double knockout (DKO) model of DNA accrual, we reveal central roles for the STING and AIM2 pathways in initiating and perpetuating the erosive inflammatory arthritis in DKO mice. Furthermore, we show that anti-nuclear autoantibody production in DKO mice depends on a third type of DNA sensor, endosomal TLRs. Collectively, these data support distinct roles for cytosolic and endosomal nucleic acid sensing pathways in disease manifestations, and suggest that therapeutics that target the STING and AIM2 pathways may be beneficial for the treatment of inflammatory joint diseases.

2) *Define the cell types responsible for the inflammatory arthritis in DKO mice.* Despite the emerging importance of DNA sensor pathways, little is known about their role in cell types other than hematopoietic cells. By generating and analyzing bone marrow

chimeras, we reveal that DNase II deficiency in both donor hematopoietic and host radioresistant cells is required for inflammation in the joint as well as for other features of autoimmunity in DKO mice. These data demonstrate that stromal host cells play a major role in DNA-driven autoimmunity and suggest that targeting not only hematopoietic but also stromal elements may be advantageous in the setting of inflammatory arthritis.

3) *Explore a role for DNA-sensing pathways in bone*. While the effect of DNase II deficiency in macrophages has been well studied, the actual range of affected cell types, including cells involved in bone erosion and formation, has not been investigated. By rigorously analyzing the bone phenotype in DKO mice, we identified a novel role for cytosolic DNA sensing pathways in bone. We found that DKO mice accumulate trabecular bone in the long bones and form ectopic bone in the spleen, both sites of robust DNA accrual. Moreover, STING deficiency significantly inhibits this bone accumulation. These findings are the first to reveal a role for the STING pathway in bone and may unveil novel targets for the treatment of diseases associated with disorders of bone remodeling.

CHAPTER II

AIM2 and endosomal TLRs differentially regulate arthritis and autoantibody production in DNase II deficient mice

The work presented in this chapter is contained in the publication:

Baum R, Sharma S, Carpenter S, Li Q, Busto P, Fitzgerald K, Rothstein A, Gravallese E. AIM2 and Endosomal TLRs differentially regulate arthritis and autoantibody production in DNase II-deficient mice. J Immunol; 2015 194 (3) 873-7.

Summary

Innate immune PRRs sense nucleic acids from microbes and orchestrate cytokine production to resolve infection. Inappropriate recognition of host nucleic acids also results in autoimmune disease. Here we utilize a model of inflammation resulting from accrual of self DNA (DNase $II^{-/-}$ $IFNaR^{-/-}$) to understand the role of PRR sensing pathways in arthritis and autoantibody production. Using mice deficient in DNase II/IFNaR together with deficiency in either STING or AIM2 (97), we reveal central roles for the STING and AIM2 pathway in arthritis. AIM2 TKO mice show limited inflammasome activation and, like STING TKO mice, have reduced inflammation in joints. Surprisingly, autoantibody production is maintained in AIM2 and STING TKO mice, while DNase $II^{-/-}$ $IFNaR^{-/-}$ mice also deficient in Unc93b, a chaperone required for TLR7/9 endosomal localization, fail to produce autoantibodies to nucleic acids. Collectively, these data support distinct roles for cytosolic and endosomal nucleic acid sensing pathways in disease manifestations.

Introduction

Innate immune responses play a critical role in the initiation and perpetuation of several autoimmune disorders in which the sensing of self nucleic acids has become a common theme (200). While attention in this area has focused on the endosomal nucleic acid sensing TLRs, cytosolic DNA sensing receptors can also detect endogenous ligands and promote inflammatory and autoimmune responses (97). DNase II is a lysosomal endonuclease that plays a critical role in the phagosomal degradation of apoptotic debris. In DNase II deficient mice, undigested DNA is sensed by PRRs to induce fatal levels of type I IFNs. Deletion of the type I IFN receptor (IFNaR) rescues the embryonic lethality induced by DNase II deficiency (201). However, DNase $II^{-/-}$ $IFNaR^{-/-}$ double knock out (DKO) mice, eventually succumb to autoimmune disease associated with polyarthritis, autoantibody production and elevated levels of the proinflammatory cytokines TNF, IL-1ß, and IL-6 (100, 202).

In this model, DNA from the phagolysosomal compartment gains access to cytosolic nucleic acid sensing receptors. Cytosolic sensing of DNA results in the subsequent engagement of the adaptor protein STING and the downstream transcription factor IRF3, leading to the excessive production of type I IFN. In addition to their roles in type I IFN production, STING-dependent pathways play an important role in the IFN-independent inflammatory arthritis that develops in adult DKO mice (203). However, the contribution of additional cytosolic or endosomal nucleic acid sensors to the systemic disease characteristic of DKO mice has not yet been explored. In addition to controlling

transcription of interferon responses and NF-kB-driven inflammation, cytosolic DNA is also recognized by AIM2 (204). AIM2 works independently of STING to form a caspase-1 activating inflammasome that controls the proteolytic maturation of IL-1ß and IL-18 and an inflammatory form of cell death called pyroptosis. Here we set out to define the contribution of the STING and AIM2 pathways in the development of arthritis in DKO mice by generating triple knockout (TKO) mice for comparative analysis to DKO mice. Rigorous examination of inflammation and clinical disease in these lines reveals important roles for both the STING and AIM2 pathways in arthritis. Furthermore, we define an additional contribution of endosomal nucleic acid sensors in regulating autoantibody production. Collectively these observations highlight the importance of multiple PRR pathways in controlling autoimmunity. Moreover, they unveil a previously undescribed role for AIM2 as a sensor of endogenous nucleic acids in autoimmunity.

Materials and Methods

Mouse Strains: C57BL/6 *DNase II*$^{+/-}$ embryos were kindly provided by Dr. S. Nagata through the RIKEN Institute, and mice were crossed to IFNaR$^{-/-}$ C57BL/6 mice to produce DKO and DNase II$^{+/-}$ IFNaR$^{-/-}$ heterozygous (Het) mice. DKO mice were bred with STING-deficient mice on a B6/129 background (205) or AIM2-deficient mice (84) to yield STING or AIM2 TKO mice. AIM2-deficient mice on a B6/129 background were generated through the use of a gene-trap embryonic stem cell line and deletion of AIM2 was confirmed by RT-PCR and immunoblot analysis (84). DKO mice were also bred to Unc93b-deficient mice on a B6 background, (Jackson Laboratories), yielding Unc93b TKO mice. All animal procedures were approved by and performed in accordance with the Institutional Animal Care and Use Committee at the University of Massachusetts Medical School.

Clinical and Histologic Inflammation Scores: Clinical arthritis was measured using a previously described scoring system (121). Histologic inflammation was assessed in paraffin-embedded left hind limbs. Blocks from 10 month-old female mice (n=5-8/genotype) were sectioned at 5 μm, deparaffinized, and stained with H&E. 50 sections were cut from each block and sections 10, 20, 30, 40, and 50 were scored using a modification of a previously described system (121) on a scale from 0-4.

K/BxN serum transfer arthritis: KRN T cell-transgenic mice (provided by Drs. Benoist and Mathis, Harvard Medical School and the Institut de Genetique et de Biologie Moleculaire et Cellulaire, Illkirch, France) (206) were crossed with NOD mice (Jackson Laboratory). Arthritogenic serum was obtained from progeny (121) and transferred to 11 week-old male STING-deficient (STING KO) or 8 week-old male AIM2-deficient (AIM2 KO) mice and controls by intraperitoneal injection of 150ml on days 0, 2, and 7. Clinical inflammation scores and ankle thickness measurements were taken every other day. Histologic inflammation (n=8/genotype) was scored as previously described (121).

Quantitative RT-PCR: Ankle joints from 10-12 month-old mice (n=4-6/genotype) were homogenized in liquid nitrogen using a mortar and pestle. Total RNA was isolated and 500ng was amplified as previously described (7). Gene expression was normalized to expression of the housekeeping gene hydroxymethylbilane synthase (HMBS). All primers were obtained from Qiagen. Data are expressed as the fold-increase in gene expression compared to normalized Het controls, using the $2^{-\Delta\Delta CT}$ method.

ELISA and Western Blots: Serum levels of IL-18 were determined by ELISA according to the manufacturer's instructions (Medical & Biological Laboratories). Hind ankles were homogenized and sonicated in RIPA buffer with protease inhibitors for Western blots. Equal amounts of protein were loaded and blots were probed with primary antibody for IL-18 (Biovision, 5180R). Chemiluminescence reagent (ThermoScientific) was used for

detection. Equivalent loading and transfer of protein was demonstrated by Ponceau S staining.

Autoantigen arrays: Serum samples pretreated with DNAse I were diluted 1:100 and incubated with autoantigen arrays bearing 125 antigens. The autoantibodies binding to the antigens on the arrays were assayed with fluorescent-labeled secondary antibodies (cy3-labeled anti-mouse IgG and cy5-labeled anti-mouse IgM) and the images were generated using Axon 4300A Scanner and analyzed with Genepix Pro 7.0 software (Molecular Devices). Net fluorescence intensities (NFI) were defined as the background subtracted averaged signal intensity normalized to internal controls. The NFI of each autoantibody was used to generate heatmaps using Cluster and Treeview software (207). These data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE63503. (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSExxx).

Statistical analysis: Statistical significance of differences in mean values was analyzed with the unpaired, two-tailed Student’s t test or ANOVA for multiple comparisons. Statistical significance is represented by the following notation in the figures: $p<0.05$ = *, $p<0.01$ = **, $p<0.001$ = ***.

Results and Discussion

To investigate the contribution of the AIM2 inflammasome to disease in DKO mice, we initially compared the spontaneous arthritis in DKO mice with that of STING TKO and AIM2 TKO mice and controls by clinical joint scoring and histologic evaluation. As reported previously (100), inflammation was significant in the distal joints and paws in DKO mice, but not in the Het control group. STING deficiency completely abrogated clinical arthritis (**Figure 2.1 A and B**), although we noted minimal but detectable inflammation in STING TKO mice upon histologic evaluation (**Figure 2.1 C and D**). This is consistent with previously reported findings in DNaseII/STING DKO mice (203). AIM2 TKO mice also demonstrated a significant attenuation of arthritis, as assessed by both clinical and histologic scoring. These results reveal a role for a second, STING-independent, cytosolic DNA sensor in arthritis resulting from the accumulation of DNA in DNase II deficient mice. Of note, unlike DNase $II^{-/-}$ $STING^{-/-}$ mice that are rescued from lethality, our DNase $II^{-/-}AIM2^{-/-}$ mice were embryonic lethal, indicating that AIM2 is not a pathway responsible for type I IFN signatures (data not shown).

To better understand how both STING and AIM2 contribute to the development of arthritis, we collected joint tissue from 10-12 month-old mice and compared RNA expression levels for the pro-inflammatory cytokine TNF, as well as expression levels of matrix metalloproteinase 3 (MMP3) as a surrogate marker of inflammation (**Figure 2.2**). STING TKO joints had significantly reduced levels of TNF compared with DKO joints, whereas AIM2 TKO joints showed a trend toward decreased TNF expression (**Figure 2.2A**) as well as IL-6 expression (not shown). Expression of MMP3 mRNA was

significantly decreased in both STING TKO and AIM2 TKO mice compared with DKO mice, consistent with reduced inflammation.

We then determined protein levels of IL-1ß and IL-18 in joint extracts as a marker of AIM2 inflammasome activity. Although we were unable to detect IL-1ß protein, IL-18 protein expression was markedly diminished in the joints of AIM2 TKO mice compared with DKO mice (**Figure 2.2B**). In addition, consistent with defects in AIM2 inflammasome activation, we found that the AIM2 TKO mice had reduced systemic levels of IL-18 (**Figure 2.2C**). IL-18 itself, a product of several cell types including macrophages and synovial fibroblasts, has been implicated as an important pro-inflammatory cytokine in autoimmune diseases including rheumatoid arthritis (RA). Inflammation and cartilage destruction are significantly reduced in mice deficient in IL-18 in the collagen-induced arthritis model of RA (208). As noted above, there is also a trend toward decreased TNF expression in the joints of AIM2 TKO compared to DKO mice. This trend could be explained by the presence of known feedback loops among cytokines in inflammatory arthritis.

Our data point to a distinct and prominent role for the AIM2 inflammasome in arthritis pathogenesis. While AIM2 has previously been shown to act in a non-redundant fashion in response to intracellular bacteria and DNA viruses (84), these findings demonstrate that AIM2 also recognizes endogenous DNA, and that the recognition of cytosolic DNA by AIM2 contributes to the pathogenesis of clinical disease.

Arthritis is only one manifestation of the autoimmune disease that results from DNase II deficiency, as these DKO mice also show splenomegaly and autoantibody production (100). Interestingly, we found that although STING deficiency ameliorates the arthritis in DKO mice, it does not largely reverse splenic enlargement, whereas, AIM2 deficiency reduces the size of the spleen to a greater extent (**Figure 2.3 A and B**). H&E images from splenic sections reveal that the normal splenic architecture, shown by the presence of organized follicles in the Het controls, is disrupted in the DKO and STING TKO spleens, but not in the AIM2 TKO sections (**Figure 2.3C**).

Since B cells express both STING and AIM2 (209), it was of interest to determine whether STING and/or AIM2 are required for autoantibody production. As shown by an autoantigen microarray, DKO mice make autoantibodies reactive to an extensive panel of autoantigens by 10 months of age, but not at 3 months of age at a time when arthritis is not present (**Figure 2.4A**). Surprisingly, despite the significant effect of STING and AIM2 deficiency on arthritis and a prior report that STING is required for the production of anti-DNA autoantibodies (203), the STING TKO and AIM2 TKO mice demonstrate robust autoantibody production. Thus the STING and AIM2 pathways are not required for this process. In murine models of SLE, the production of anti-nuclear antibodies (ANAs) depends on the endosomal TLRs 7 and 9 that detect either RNA or DNA. We therefore evaluated autoantibody production in DKO mice that also failed to express Unc93b**,** a chaperone protein required for TLR7 and TLR9 endosomal localization. Despite the presence of arthritis in Unc93b TKO mice (**Figure 2.4B**), autoantibody production to nucleic acid is abrogated (**Figure 2.4A**), in stark contrast to the DKO mice.

STING TKO and AIM2 TKO mice developed significantly less joint inflammation than DKO mice, despite the presence of high titer autoantibody production. To clarify whether STING or AIM2 play a role in antibody-mediated joint inflammation, we generated arthritis in STING-deficient and AIM2-deficient mice and their respective controls by transfer of arthritogenic serum from K/BxN mice (206). Arthritis in this model is mediated by the deposition of immune complexes within the joint, leading to fixation of complement and ensuing pathology (210). Importantly, we found no differences between the arthritic inflammation generated in STING-deficient (**Figure 2.5 A and B**), AIM2-deficient (**Figure 2.5C**), and control mice. Thus, antibody-induced inflammation proceeds independently of cytosolic nucleic acid sensors. Furthermore, STING and AIM2 appear to regulate inflammation strictly in settings where accrual of cytosolic DNA is a key pathogenic event.

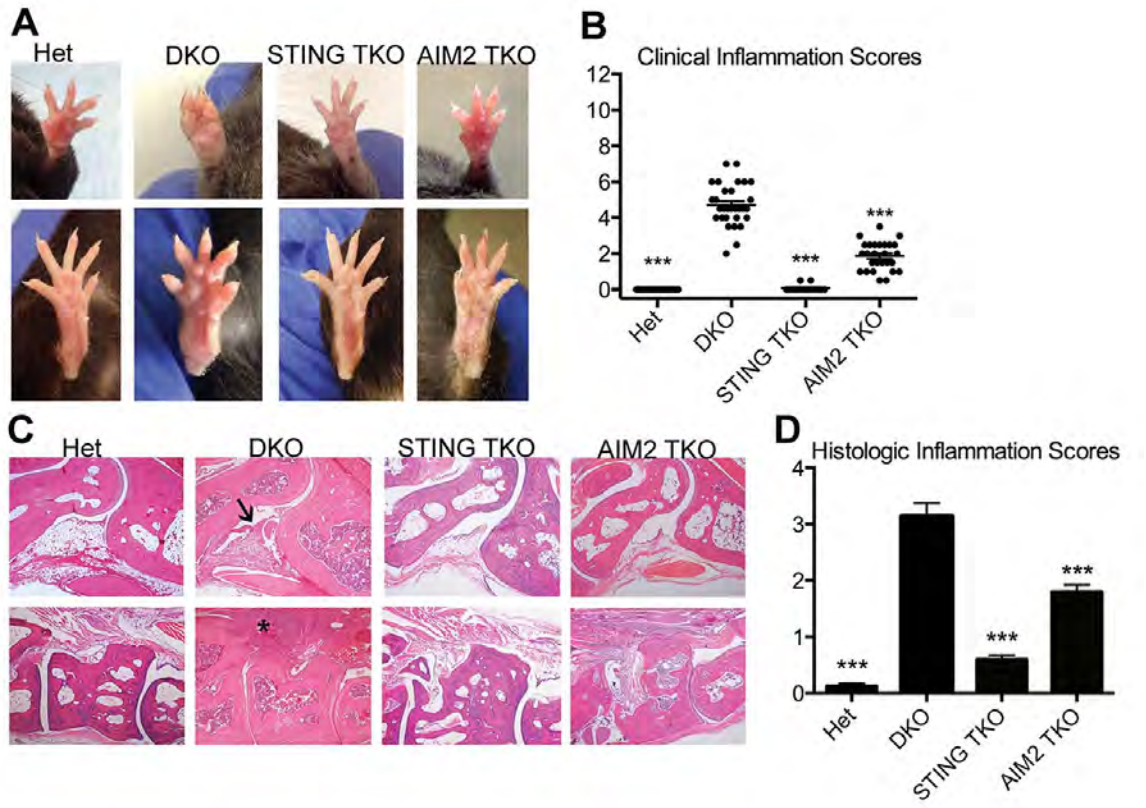


Figure 2.1 ***Arthritis in DKO mice is regulated by distinct DNA sensing pathways***.

A) Representative images of clinical arthritis in forepaws (top) and hindpaws (bottom) from 10 month-old female mice demonstrating significant swelling in DKO mice, absence of swelling in STING TKO mice, and an intermediate arthritic phenotype in AIM2 TKO mice. **B)** Clinical inflammation scores (n=14-24/genotype) showing a statistically different mean inflammation score in STING TKO and AIM2 TKO compared with DKO mice. 10 month-old mice: Het (14 female, 5 male), DKO (11 female, 14 male), STING TKO (9 female, 6 male), AIM2 TKO (9 female, 13 male). **C)** Representative image of histologic inflammation at the ankle (upper panel) and midfoot (lower panel) and **D)** Quantitation of histologic inflammation (n=7 mice/genotype), confirming differences in inflammation in STING TKO and AIM2 TKO

mice compared with DKO mice. Histological analysis performed on 10 month-old female mice. Values are the mean ± SEM; *** = $p<0.001$ compared to DKO. Arrow and (*) designate two sites of inflammation.

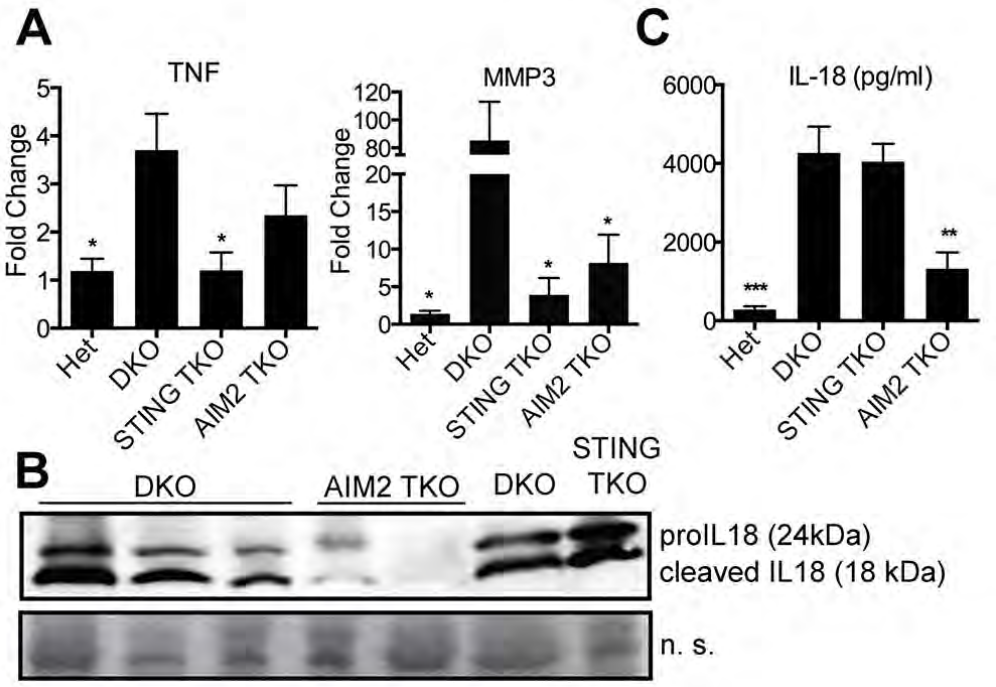


Figure 2.2 ***AIM2 TKO mice demonstrate a significant decrease in IL-18 expression.***

A) Joint cytokine mRNA levels show a significant decrease in TNF in STING TKO mice compared with DKO mice, whereas a trend toward a decrease in TNF is seen in AIM2 TKO mice. MMP3 levels are significantly decreased in both STING TKO and AIM2 TKO mice compared with DKO mice, consistent with attenuation of clinical arthritis (n=4-6/genotype). 10 month-old mice were used: Het (4 female, 2 male), DKO (2 female, 4 male), STING TKO (3 female, 2 male), AIM2 TKO (2 female, 3 male). **B)** Western blot confirms a decrease in IL-18 protein expression in the joints of AIM2 TKO mice compared with DKO and STING TKO mice (n=1-4/genotype). n.s. designates non-specific protein staining (Ponceau S.). 10 month-old male mice. **C)** Serum ELISA assay demonstrates that IL-18 levels are significantly decreased in AIM2 TKO mice compared

with DKO and STING TKO mice (n=6/genotype, 3 males, 3 females; 10 month-old mice). Values are the mean ± SEM; * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$ compared to DKO.

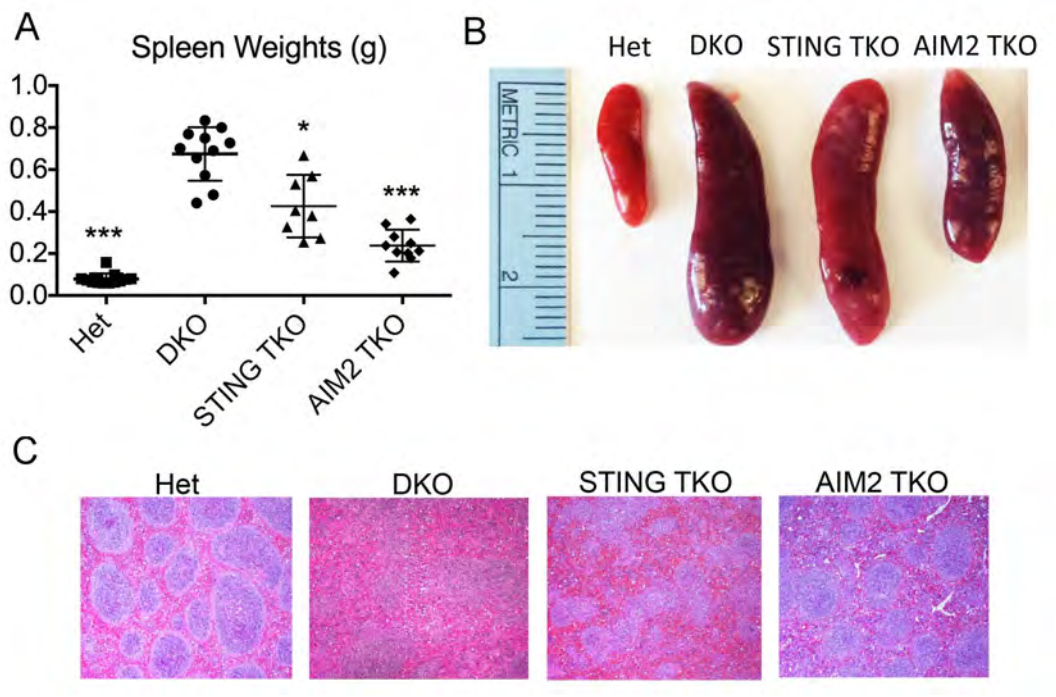
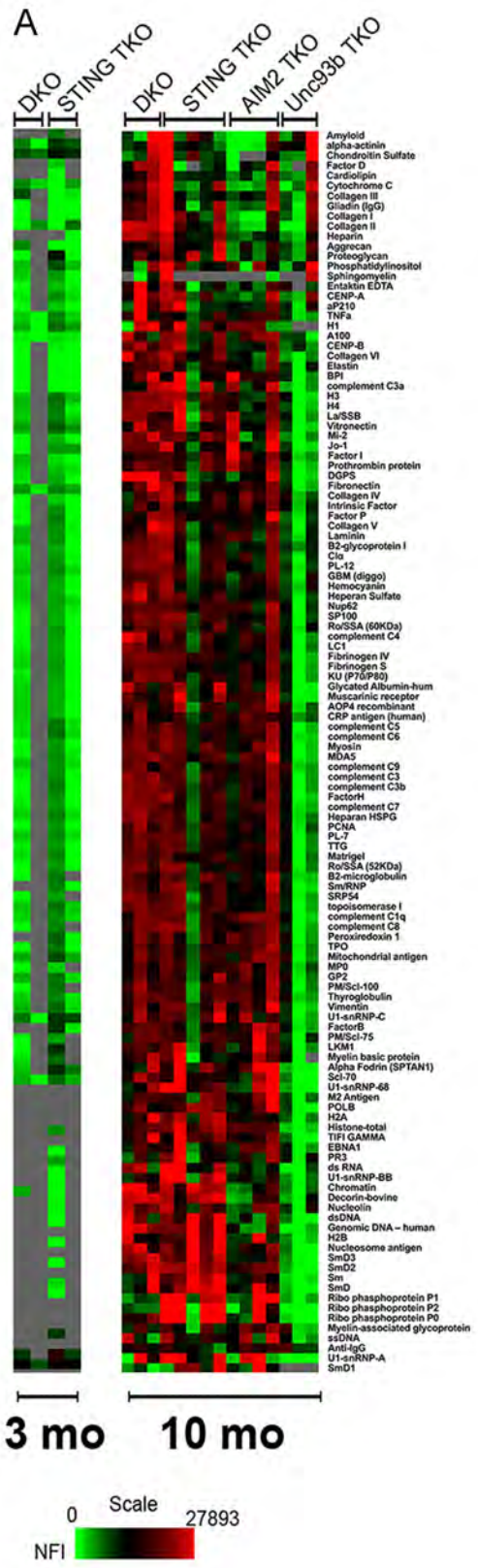


Figure 2.3 AIM2 regulates splenomegaly in DKO mice. **A**) Spleen weights (n=8-12 mice/genotype) **B**) Representative image of spleens **C**) H&E stain of splenic sections. 10 month-old male and female mice. Values are the mean ± SEM; * = $p<0.05$, *** = $p<0.001$ compared to DKO.



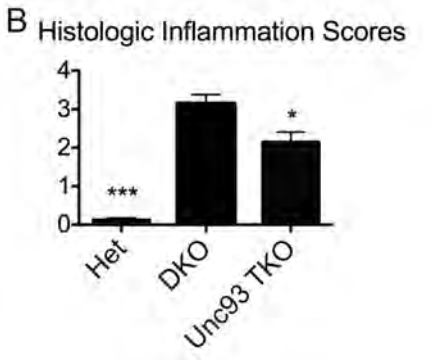


Figure 2.4 ***The Unc93b pathway uniquely regulates autoantibody production to nucleic acid.*** **A**) Heat map of an array of 125 autoantigens. Autoantibody production is minimal in DKO and STING TKO mice at 3 months of age (left panel), prior to the onset of arthritis. Despite the significant decrease in arthritic inflammation in STING TKO and AIM2 TKO mice at 10 months, heat maps demonstrate autoantibody production, as in DKO mice. Marked attenuation of autoantibody production is seen in Unc93b TKO mice. **B**) Histologic inflammation scores for 10 month-old female mice (n=6 mice/genotype). Values are the mean ± SEM; * = $p<0.05$, *** = $p<0.001$ compared to DKO.

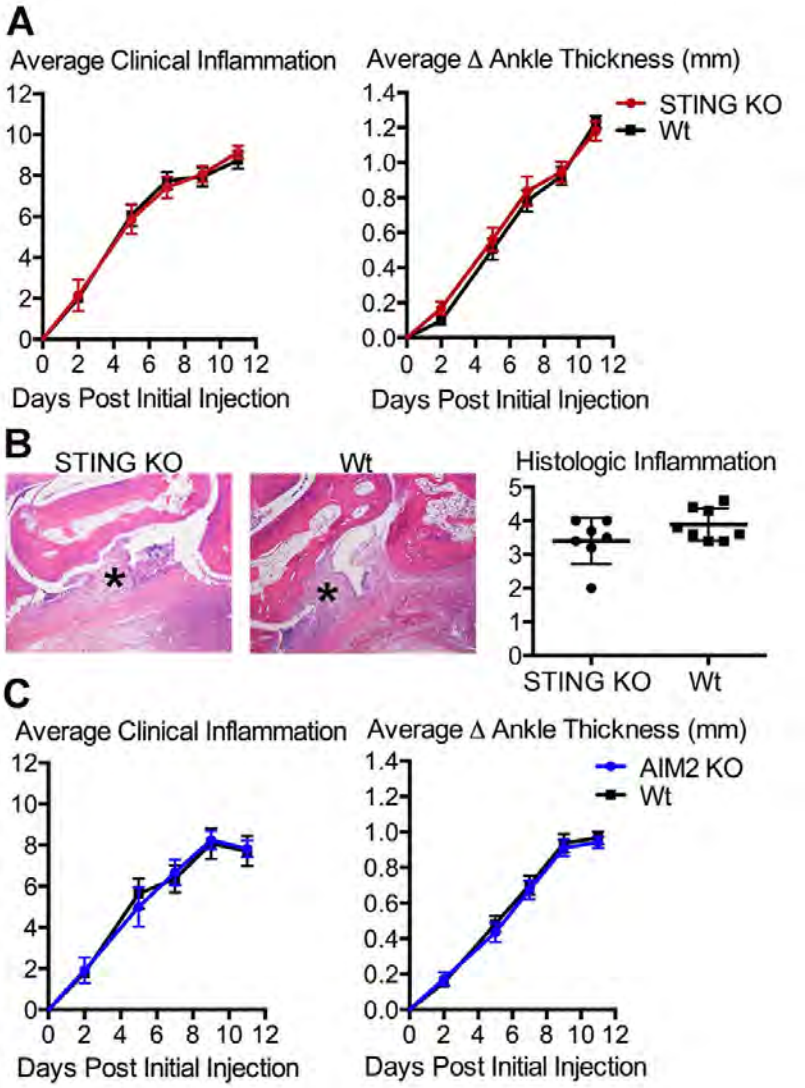


Figure 2.5 ***STING and AIM2 do not regulate arthritic inflammation in an immune complex-mediated model.*** **A)** Clinical inflammation scores and measurements of change in ankle thickness demonstrate no difference in inflammation in STING deficient (KO) mice compared with controls. 11 week-old male mice (n=9-10 mice/genotype). **B)** H&E images and histologic scoring confirm equivalent inflammation. **C)** Clinical inflammation

scores and measurements of change in ankle thickness demonstrate no difference in inflammation in AIM2 deficient (KO) mice compared with controls (Wt). 8 week-old male mice (n=8 mice/genotype). Values are the mean ± SEM. (*) designates synovial inflammation.

There is mounting evidence for a role of cytosolic sensing of DNA during human autoinflammatory conditions. STING has been implicated in a number of type I IFN-driven diseases. For example, patients inheriting mutations in Trex1, and therefore presumably unable to appropriately degrade cytosolic retroelements (90) develop the neuroinflammatory condition Aicardi-Goutieres syndrome (91), while gain of function mutations in STING can lead to pulmonary and vascular inflammation (97). Prior studies of the arthritic phenotype in DKO mice extended the scope of STING-mediated pathologies to the production of type I IFN-independent proinflammatory cytokines (202, 203). We now demonstrate that additional cytosolic and endosomal receptors also contribute to the autoimmune features of DKO mice. AIM2 responds to endogenous ligands in this setting and contributes to the arthritic phenotype through inflammasome activation. Thus, deficiency of either STING or AIM2 attenuates arthritis. DKO mice also make anti-nuclear antibodies, but production depends on yet a third type of nucleic acid sensor, endosomal TLRs. This study highlights the complex relationships between multiple innate pathways engaged during autoimmunity, and demonstrates that distinct DNA sensor pathways play unique roles in the development of the various manifestations of autoimmune disease.

Chapter III

Synergy between Hematopoietic and Radioresistant Stromal Cells is Required for Autoimmune Manifestations of DNase II$^{-/-}$ IFNaR$^{-/-}$ Mice

The work presented in this chapter is contained within the manuscript:

Baum R[‡], Nündel K[‡], Pawaria S, Sharma S, Busto P, Fitzgerald K, Gravallese E[§], Rothstein A[§]. Synergy between hematopoietic and radioresistant stromal cells is required for autoimmune manifestations of DNase $^{-/-}$ IFNaR $^{-/-}$ Mice. J Immunol. 2016; 196(3):1348-54. [‡]Co-first authors; [§]Co-senior authors

Summary

Detection of endogenous nucleic acids by cytosolic receptors, dependent on STING, and endosomal sensors, dependent on Unc93b1, can provoke inflammatory responses that contribute to a variety of autoimmune and autoinflammatory diseases. In DNase II deficient mice, the excessive accrual of undegraded DNA leads to both a STING-dependent inflammatory arthritis and additional Unc93b1-dependent autoimmune manifestations, including splenomegaly, extramedullary hematopoiesis, and autoantibody production. Here we utilize bone marrow chimeras to show that clinical and histological inflammation in the joint depends upon DNase II deficiency in both donor hematopoietic cells and host radioresistant cells. Additional features of autoimmunity in these mice, known to depend on Unc93b1 and therefore endosomal TLRs, also require DNase II deficiency in both donor and host compartments, but only require functional TLRs in the hematopoietic cells. Collectively, our data demonstrate a major role of both stromal and hematopoietic cells in all aspects of DNA-driven autoimmunity. These findings further point to the importance of cytosolic nucleic acid sensors in creating an inflammatory environment that facilitates the development of Unc93b1-dependent autoimmunity.

Introduction

Cytosolic DNA sensors were first identified in the context of host defense but, similar to endosomal nucleic acid detecting TLRs, these sensors can also detect endogenous ligands and thereby promote sterile inflammation. A number of cytosolic DNA receptors have now been identified, including cGAS and Ifi16, among others (77, 78). These sensors converge on the ER-associated protein STING to activate downstream pathways leading to the expression of both IFN-stimulated genes and proinflammatory cytokines (211). Importantly, gain of function mutations in STING have recently been linked to a clinical syndrome called SAVI, associated with upregulation of type I IFN, severe vasculopathy, arthritis, pulmonary fibrosis, and in some cases autoantibody production (92-95, 97, 212). In addition, loss of function mutations in a variety of cellular nucleases can lead to the accumulation of self-DNA and also contribute to inflammatory disease. For example, loss of function mutations in Trex1 (a cytosolic DNase), SAMHD1 (a cytosolic RNase), RNaseH2A, and ADAR1 have been linked to both the neuroinflammatory disease Aicardi-Goutieres syndrome (91, 213-215), and different forms of lupus (92). Trex1 deficiency can also lead to systemic inflammation in mice, initially evident as myocarditis with subsequent progression to other organs (90, 216). Furthermore, SNPs in the promoter region of the DNase II gene have been identified as risk factors for rheumatoid arthritis (99).

Mice lacking the phagolysosomal nuclease, DNase II, are embryonic lethal due to excessive type I IFN production downstream of STING-dependent pathways. These mice can be rescued by intercrossing with mice that lack the type I IFN receptor (IFNaR).

DNase $II^{-/-}$ $IFNaR^{-/-}$ double knockout (**DKO**) mice survive to adulthood but then develop an inflammatory arthritis not seen in the DNase $II^{+/-}$ $IFNaR^{-/-}$ (**Het**) control group. The development of DKO arthritis is STING-dependent (203, 217). DKO mice also produce anti-nuclear antibodies (ANAs) and develop splenomegaly and extramedullary hematopoiesis, and these aspects of disease turn out to require a functional form of Unc93b1, and by inference, signaling by endosomal TLRs (61, 217). Thus, both cytosolic and endosomal nucleic acid sensing receptors contribute to the clinical manifestations of DKO mice.

Previous studies involving radiation chimeras have indicated that radioresistant $Trex1^{-/-}$endocardial cells are sufficient for lymphocyte activation and the development of myocarditis (218), while the arthritic phenotype of DNase $II^{-/-}$ mice was found to depend entirely on hematopoietic cells (202). However, the DKO chimeric mice in the latter study were evaluated at a relatively early stage in the disease process, and the Unc93b1-associated manifestations were not examined. As considerable data now demonstrate a proinflammatory role for STING in non-hematopoietic cells, we reasoned that it was important to re-evaluate the contribution of hematopoietic cells and non-hematopoietic cells to the various DKO disease parameters. Our data reveal a major contribution of both bone marrow-derived hematopoietic and radioresistant host cells to all aspects of the DKO phenotype. Moreover, our data further point to a critical interplay between endosomal and cytosolic nucleic acid receptors in the development of systemic autoimmunity.

Materials and Methods

Mouse Strains: *DNase II*$^{+/-}$ C57BL/6 embryos were kindly provided by Dr. S. Nagata (Osaka Medical School) through the RIKEN Institute, and mice were intercrossed with *Ifnar*$^{-/-}$ C57BL/6 or *Igh*$^{a/a}$ C57BL/6 mice to produce DNase II$^{-/-}$ IFNaR$^{-/-}$ double knockout (DKO), DNase II$^{+/-}$ IFNaR$^{-/-}$ heterozygous (Het), and Igh^{a} DKO mice. C57BL/6 mice expressing GFP under the MHC I class promoter were kindly provided by Dr. R. Gerstein (UMMS), and were crossed to *Ifnar*$^{-/-}$ C57BL/6 mice to generate DNase II$^{+/-}$ IFNaR$^{-/-}$ GFP donor mice for bone marrow chimera studies. *Unc93b1*$^{3d/3d}$ mice on a C57BL/6J background were kindly provided by Bruce Beutler (UTSW) (219). STING-deficient mice were generated on a 129SvEvxC57BL/6J background by Dr. G. Barber (UMiami) (220), backcrossed to C57BL/6J mice and kindly provided by Dr. D. Stetson (UWashington), and then further backcrossed to C57/BL6J at UMMS. The Unc93b1$^{3d/3d}$ and STING-deficient mice were then crossed to the DKO strain to yield Unc93 TKO and STING TKO lines, respectively, as described previously (217). All animal procedures were approved by and performed in accordance with the Institutional Animal Care and Use Committee at the University of Massachusetts Medical School.

Generation of Bone Marrow Chimeras: Lethally irradiated (850R) 8-12 week-old recipients were reconstituted by i.v. injection of 10^7 total bone marrow cells from 8-10 week-old mice. For the Het/DKO chimeras, female Het (Igh^{b}) or DKO (Igh^{b}) hosts were reconstituted with cells from female Het (GFP) or DKO (Igh^{a}) mice. For the Unc93 TKO/DKO chimeras, the Unc93 TKO mice were Igh^{b} and the DKO mice were Igh^{a}. Clinical monitoring for the development of arthritis was performed until the mice were

euthanized for analysis at 10 months post transplant (Het/DKO chimeras) or 4 months post transplant (Unc93 TKO/DKO chimeras). The extent of reconstitution was determined by flow cytometry for GFP^+ or IgD allotype markers in total peripheral blood or mature B cells, respectively, and confirmed by FACS analysis of the spleen at the time of euthanasia.

Clinical and Histologic Inflammation Scores: Clinical arthritis was measured using a previously described scoring system (121). Histologic inflammation was assessed in decalcified, paraffin-embedded left hind limbs. Blocks were sectioned at 5 μm, deparaffinized, and stained with H&E. 40 sections were cut from each block and sections 10, 20, 30, and 40 were scored using a modification of a previously described system (121) on a scale from 0-4. Cellular infiltrates in the distal tibias were scored on a scale from 0-3 (0=no infiltrate, 1= slight infiltrate, 2= moderate infiltrate, 3=severe infiltrate).

Antinuclear Antibodies: Mouse sera diluted 1:50 was incubated on HEp-2 antigen substrate slides (MBL BION), and bound Abs were detected with DyLight 488–coupled detecting reagents. ANA fluorescent intensity was scored on a scale from 0-4 per the manufacturer's instructions.

MMP-3 Quantification: MMP-3 protein levels were measured in the sera of mice per the manufacturer's (R&D) instructions.

Flow Cytometry: Spleen and bone marrow cell suspensions were stained with the following antibodies: Ter119, CD11b, Ly6G, Ly6C, B220, CD95 and GL-7 (eBioscience or BD Biosciences). Multicolor flow cytometry was performed using an LSR II with DIVA software (BD Biosciences), and analysis was conducted with FlowJo software

(TreeStar, Ashland, OR).

Statistical analysis: Data are reported as mean ± SEM. Statistical significance was analyzed with the unpaired, two-tailed Student's t test using Prism software (GraphPad). Statistical significance is represented by the following notation in the figures: $p<0.05$=*, $p<0.01$=**, $p<0.001$=***.

Results

DNase II deficiency in both hematopoietic and radioresistant cells is required for the development of inflammatory arthritis and bone marrow (BM) hypercellularity in DKO mice. To investigate the relative contribution of hematopoietic vs. radioresistant host cells to the various autoimmune manifestations of DKO mice, lethally irradiated (850R) Het (DNase $II^{+/-}$ $IFNaR^{-/-}$**)** or DKO (DNase $II^{-/-}$ $IFNaR^{-/-}$**)** mice were reconstituted with Het or DKO stem cells to generate four experimental groups: Het→Het, DKO→DKO, Het→DKO and DKO→Het. We then assessed arthritis severity by clinical and histologic evaluation. As expected, Het→Het chimeras showed no evidence of clinical arthritis while DKO→DKO mice showed significant inflammation in the distal joints and paws (**Figure 3.1A**). Furthermore, serum levels of matrix metalloproteinase 3 (MMP3), a surrogate marker for inflammation, reflected the arthritis scores and further confirmed the absence of inflammation in the Het→DKO and DKO→Het chimeras (**Figure 3.1B**). Histologic scoring of ankle joints (**Figure 3.1C**) also confirmed the presence of arthritis only in DKO→DKO mice. Remarkably, neither the Het→DKO nor the DKO→Het mice developed any clinical or histological evidence of arthritis over a 10-month period (**Figure 3.1A-C**). Therefore both DKO donor hematopoietic cells and DKO host radioresistant cells are required for the development of arthritis.

Histological examination of the tibiae of DKO→DKO mice also revealed a dense accumulation of cells within the marrow space **(Figure 3.1D)**, also evident in marrow cavities of the ankle sections **(Figure 3.1C)**. This infiltrate included a high proportion of neutrophils as well as engorged erythropoietic island macrophages. This infiltrate was not

detected in Het mice, STING$^{-/-}$ DNase II$^{-/-}$ IFNaR$^{-/-}$ triple knockout (**STING TKO**) mice, or Unc93b1$^{\mathbf{3d/3d}}$ DNase II$^{-/-}$ IFNaR$^{-/-}$ triple knockout (**Unc93 TKO**) mice (**Figure 3.1E)**. Furthermore, as evident from clinical inflammation scores, arthritis still develops in the Unc93b1 TKO mice (**Figure 3.1F**) whereas arthritis is abrogated in STING TKO mice (217). Altogether our data indicate that the arthritic phenotype is completely dependent on STING and not Unc93b1 (203, 217), BM hypercellularity is dependent on both STING and Unc93b1, and both the arthritic and BM hypercellularity depend on a combination of hematopoietic and radioresistant cell types. These outcomes are unlikely to be due to residual host hematopoietic cells as complete hematopoietic repopulation of all groups by donor stem cells was verified through the use of transgene or congenic markers (**Figure 3.1G**).

DNase II deficiency in both hematopoietic and radioresistant cells contributes to the development of additional TLR-dependent features of autoimmunity. DKO mice develop massive splenomegaly, clearly apparent by as early as 2 wks of age, which depends upon functional Unc93b1 and not STING (61). Since endosomal TLRs are preferentially expressed in hematopoietic cells, we expected TLR-mediated splenomegaly to track with the Unc93b1-sufficient DKO hematopoietic compartment. As expected, the DKO→DKO chimeras developed splenomegaly, and the Het→DKO did not. However, surprisingly, the DKO→Het mice also failed to develop splenomegaly (**Figure 3.2A**). Along with splenomegaly, normal splenic architecture, associated with the presence of organized follicles, is disrupted in DKO but not in Het mice. From H&E

staining of splenic sections, it was clear that a similar loss of defined T and B cell regions occurred in the DKO→DKO spleens but not in the Het→DKO or DKO→Het spleens (**Figure 3.2B**). These data demonstrate that a host component is required for the TLR-dependent splenic abnormalities characteristic of DKO mice.

Other Unc93b1-dependent abnormalities of DKO mice include disruption of bone marrow (BM) erythropoiesis and ensuing extramedullary hematopoiesis in the spleen (61). To assess the contribution of the radioresistant host and hematopoietic elements to these aspects of hematopoiesis, the chimeric mice were evaluated for the frequency of BM and spleen cells expressing the RBC lineage marker Ter119. Similar to the DKO strain, the percentage and overall number of $Ter119^+$ cells in the DKO→DKO chimeras was decreased in the BM and dramatically increased in the spleen. In contrast, the frequency of $Ter119^+$ cells in the BM of the Het→DKO and DKO→Het chimeras was not significantly different from the Het→Het control, and the frequency of $Ter119^+$ cells in the spleen of the Het→DKO and DKO→Het chimeras was only slightly increased relative to the Het→Het controls (**Figure 3.3A**). These data point to a requirement for both hematopoietic and non-hematopoietic elements in the overall disruption of erythropoiesis in DKO mice.

DKO mice also develop an increased frequency of $CD11b^+$ myeloid cells, and especially $Ly6C^+$ $Ly6G^{hi}$ granulocytes, in both the BM and spleen. Comparable increases were only found in the DKO→DKO chimeras (**Figure 3.3B,C)**. However, both the Het→DKO and DKO→Het chimeras tended toward a greater frequency of granulocytes in the spleen, consistent with the notion that hematopoietic and radioresistant DKO cells

independently provoke modest inflammatory responses, while more severe inflammation depends on donor and host cell synergy.

In addition, B cell differentiation is compromised in DKO mice, as evidenced by a markedly reduced frequency of immature and mature B cells in both the BM and spleen (19). Here again the DKO phenotype was recapitulated by the DKO→DKO chimeras, as shown by the overall percent of $B220^+$ cells, but B cell development appeared relatively normal in Het→DKO and DKO→Het mice (**Figure 3.4A,B**). Normal B cell development was restored in the Unc93b1 TKO mice (19). Despite the reduced frequency of mature $B220^+$ lymphocytes, the frequency of $CD95^+$ germinal center (GC) cells, an indication of autoreactive B cell activation, was increased in the DKO→DKO chimeras, and not in the other chimera groups (**Figure 3.4C**).

As further evidence of B cell activation, DKO mice produce high titers of autoantibodies. Endogenous dsDNA associated with cell debris cannot activate TLR9 in DKO mice, because in the absence of DNase II the DNA is not degraded sufficiently to generate a functional TLR9 ligand (61, 221). Therefore sera from DKO mice normally show HEp2 immunofluorescent staining patterns consistent with BCR/TLR7 driven autoreactive B cell activation (e.g. speckled nuclear or cytoplasmic). As expected, Het→Het chimeras failed to make anti-nuclear antibodies and all the DKO→DKO mice developed high ANA titers with speckled nuclear or cytoplasmic staining patterns (**Figure 3.4D,E**). Remarkably, 4 of 7 Het→DKO mice also developed ANA titers, despite the fact that they failed to exhibit most other indications of systemic autoimmunity. However, in contrast to the speckled nuclear staining pattern characteristic

of the DKO mouse sera and the DKO→DKO chimeric sera, the ANA^+ Het→DKO chimeric sera showed homogeneous nuclear staining patterns (**Figure 3.4E**). This homogeneous nuclear pattern most likely reflects the expression of functional DNase II by the Het-derived B cells, and therefore the ability of these B cells to degrade dsDNA and generate endogenous TLR9 ligands. The Het B cells in the ANA^+ Het→DKO chimeras are presumably responding to the excessive DNA accrual that occurs in radioresistant DKO host cells, and becoming activated through a TLR9-dependent mechanism. One (out of 5) ANA^+ DKO→Het chimera sera showed a modest speckled nuclear pattern, again indicative of the inability of DNase $II^{-/-}$ B cells to respond to endogenous DNA ligands. The limited number of ANA^+ Het→DKO and DKO→Het mice represent one exception to the overall requirement for DNase II deficiency in both the recipient and host for clinical manifestations of disease. In all cases the B cells are presumably responding to an external source of nucleic acid-associated ligand. Overall the defects in B cell development (reduced number of $B220^+$ cells) and increased numbers of GC B cells are only apparent in DKO→DKO chimeras.

Expression of functional Unc93b1 in hematopoietic cells is sufficient for the development of Unc93b1-dependent clinical manifestations. One possible explanation for the failure of DKO→Het chimeras to develop Unc93b1-dependent clinical manifestations was a requirement for a TLR-expressing host component. To test this possibility, we used Unc93 TKO mice to generate DKO→Unc93 TKO and Unc93 TKO→DKO chimeras and compared them to DKO→Het chimeras. Since synovial

inflammation is Unc93b1-independent, it was not surprising that both the DKO→Unc93 TKO and Unc93 TKO→DKO chimeras developed arthritis as determined by clinical examination (**Figure 3.5A**) and joint histology (**Figure 3.5B**). Furthermore, as expected, the Unc93 TKO→DKO chimeras had less severe BM inflammation (**Figure 3.5C**), failed to develop splenomegaly (**Figure 3.5D)**, and failed to make ANAs **(Figure 3.5E**). Thus, the Unc93b1-dependent clinical manifestations require Unc93b1 expression in hematopoietic cells. However, the DKO→Unc93 TKO chimeras were essentially indistinguishable from DKO→DKO chimeras by all criteria evaluated. Thus, the hematopoietic expression of endosomal TLRs is necessary but not sufficient for SLE-like clinical manifestations, and there must be an additional TLR-independent host component(s) that promotes the onset of TLR-dependent autoimmunity.

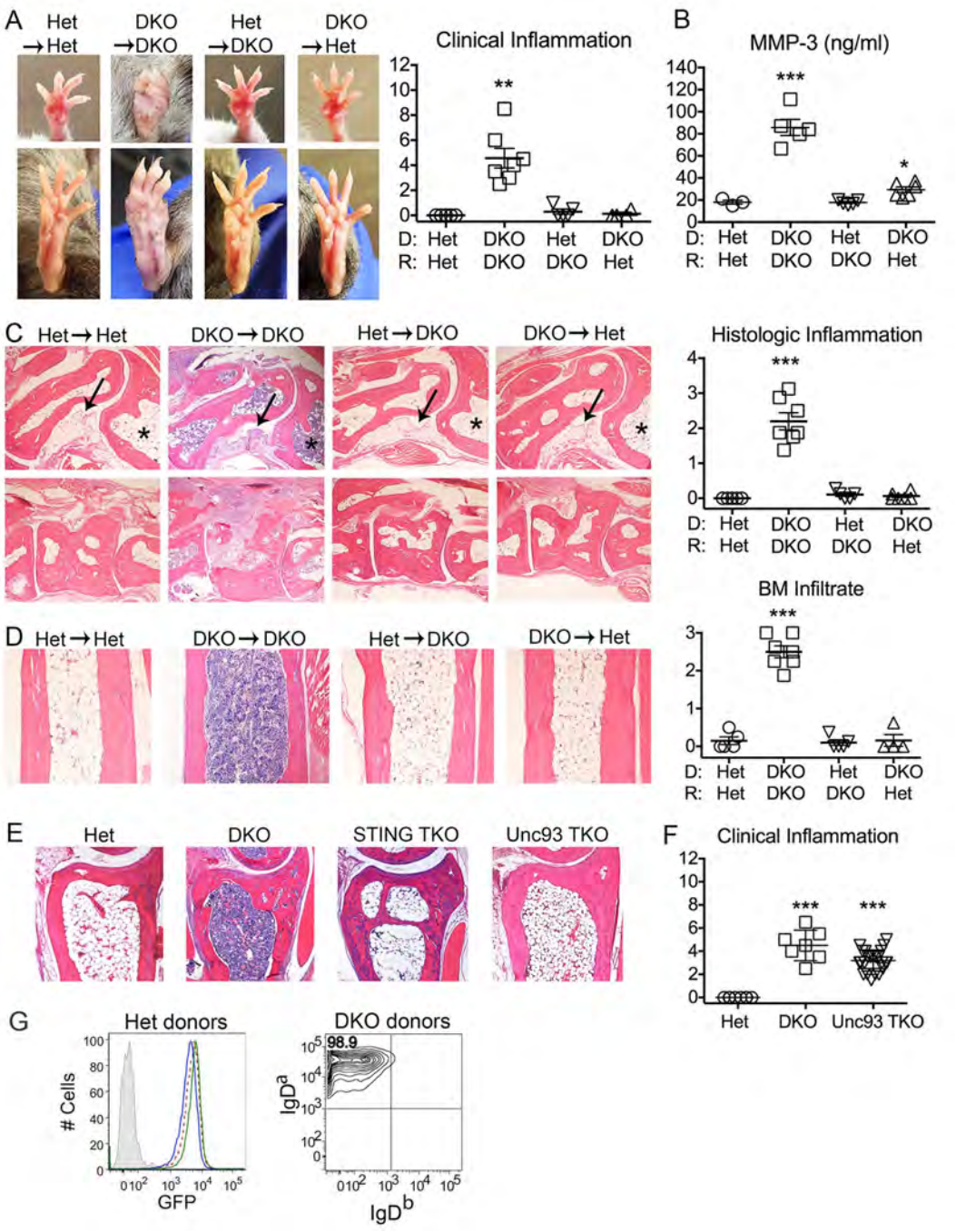

A
Het →Het
DKO →DKO
Het →DKO
DKO →Het
Clinical Inflammation
D: Het DKO Het DKO
R: Het DKO DKO Het
B
MMP-3 (ng/ml)
C
Het→Het
DKO→DKO
Het→DKO
DKO→Het
Histologic Inflammation
D
BM Infiltrate
E
Het
DKO
STING TKO
Unc93 TKO
F
Clinical Inflammation
Het
DKO
Unc93 TKO
G
Het donors
DKO donors
Cells
GFP
98.9
IgDa
IgDb

Figure 3.1 Arthritis in DKO mice depends on DNase II deficiency in both donor-derived hematopoietic and radioresistant host cells. Donor and recipient strains indicated by donor→recipient or by D (donor) and R (recipient). **A**) Representative images of arthritis in forepaws (top) and hindpaws (bottom) of chimeric mice and summary of clinical inflammation scores. **B**) Serum MMP-3 protein levels. **C**) Representative histologic images of inflammation in the ankle (upper panel) and midfoot (lower panel) and quantitation of histologic inflammation. Arrow designates synovium and asterisk marks bone marrow cavity. Final magnification 4x. **D/E**) Representative images of bone marrow cellularity in the distal tibiae and quantitation of degree of cellularity (4 sections/tibia were analyzed). Final magnification 4x. All analyses were performed on 10 month-old female chimeric mice (n=4-7 mice/genotype). **F**) Clinical inflammation scores of 6-12 month-old male and female mice (n=8-16 mice/genotype). Values are the mean ± SEM; *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$ compared to Het→Het. **G**) Engraftment in bone marrow chimeras. Reconstituted mice were analyzed by flow cytometry 5 months after bone marrow transplant for the %GFP^+ cells in total peripheral blood mononuclear cells or for the %IgD donor allotype in mature B cells. Representative data are shown (n=4-7 mice/genotype). Left: Het (GFP+)→Het (GFP-): blue line; Het (GFP+)→DKO (GFP-): green line; GFP positive control: dashed red line; GFP negative control: grey fill. Right: Representative image of DKO donors, including DKO (IgD^a)→DKO (IgD^b) and DKO (IgD^a)→Het (IgD^b).

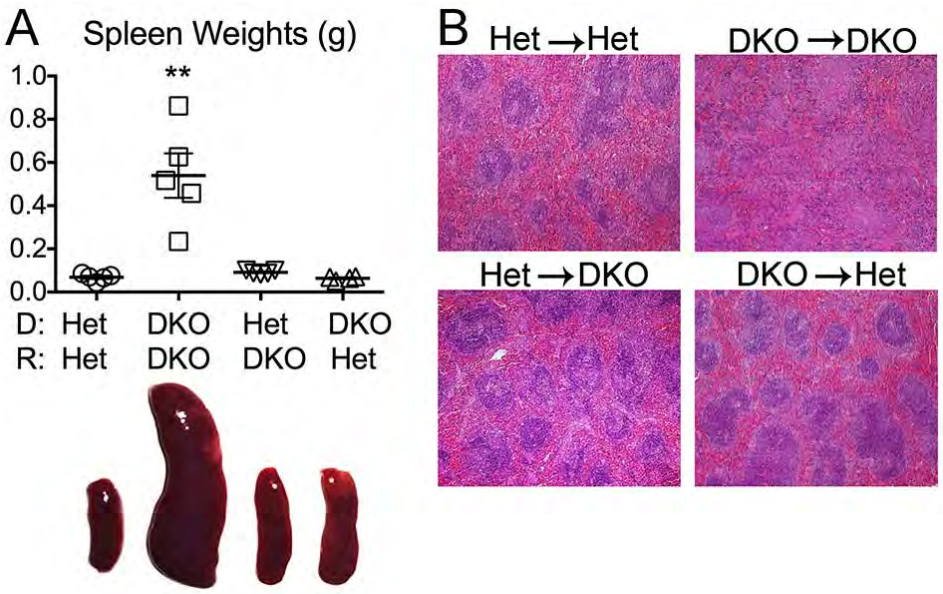


Figure 3.2 Splenomegaly and disrupted splenic architecture depend on DNase II deficiency in both donor-derived hematopoietic and radioresistant host cells. A) Splenic weights (upper panel) and representative images of spleens (lower panel). **B**) Splenic histology in 10 month-old female chimeric mice (n=4-7 mice/genotype; H&E-stained, final magnification 4x). Data is pooled from 3 individual experiments. Values are the mean ± SEM; **=$p<0.01$ compared to Het→Het.

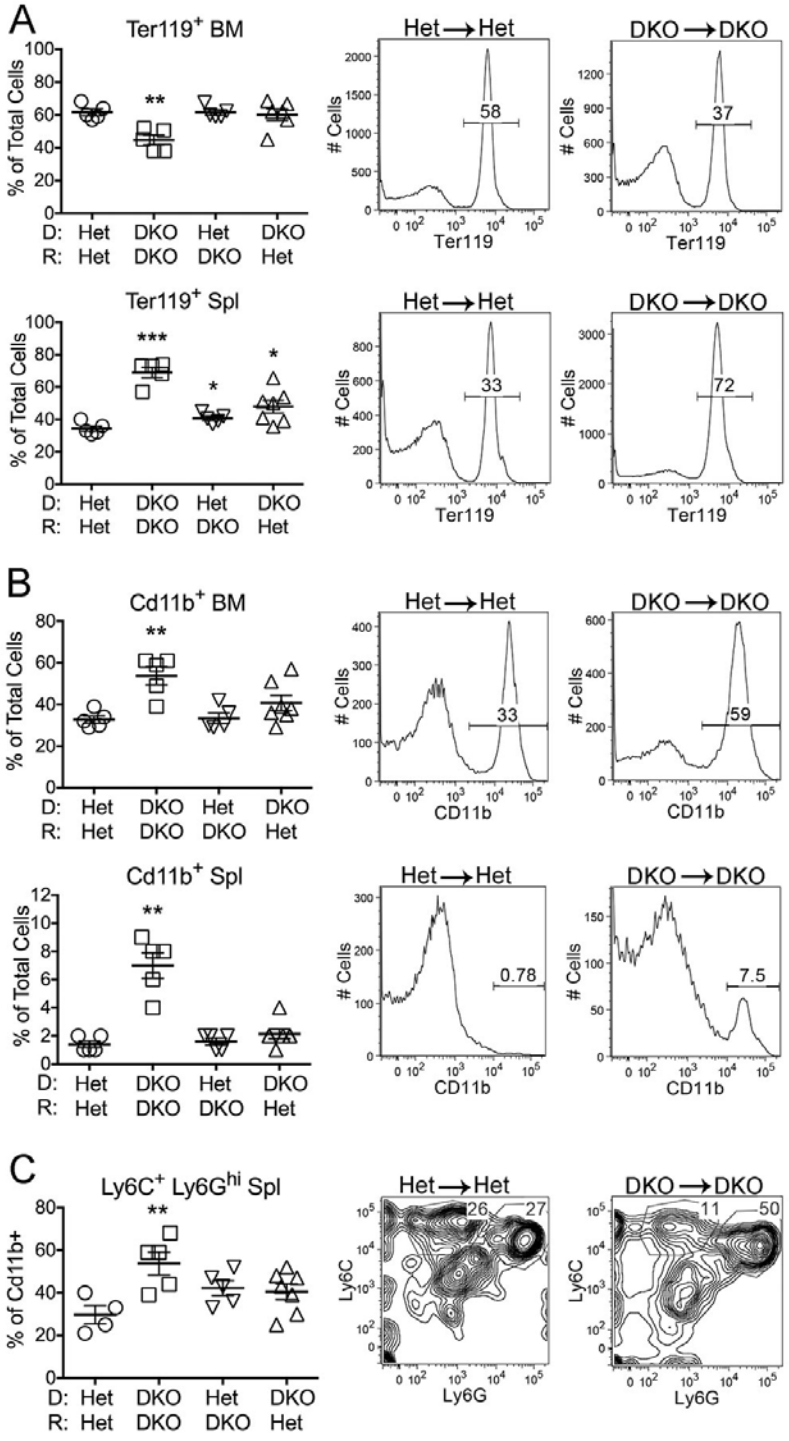

A
Ter119+ BM
% of Total Cells
D: Het DKO Het DKO
R: Het DKO DKO Het
Het → Het
DKO → DKO
Cells
58
37
Ter119
Ter119+ Spl
33
72
B
Cd11b+ BM
59
CD11b
Cd11b+ Spl
0.78
7.5
C
Ly6C+ Ly6Ghi Spl
% of Cd11b+
26
27
11
50
Ly6C
Ly6G

Figure 3.3 Extramedullary hematopoiesis and myeloid cell expansion depend on DNase II deficiency in both donor-derived hematopoietic and radioresistant host cells. A) Percentage of total bone marrow (BM) and spleen (Spl) cell suspensions expressing the erythroid lineage marker Ter119. **B**) Percentage of total bone marrow and spleen cell suspensions expressing the myeloid lineage marker CD11b. **C**) Percentage of $CD11b^{+}$ cells expressing the granulocyte phenotype $Ly6C^{+}$ $Ly6G^{hi}$. Representative FACS plots for Het→Het and DKO→DKO are shown to the right of the compiled data figures in A, B, and C. The experiment was repeated 3 times. Values are the mean ± SEM; *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$ compared to Het→Het.

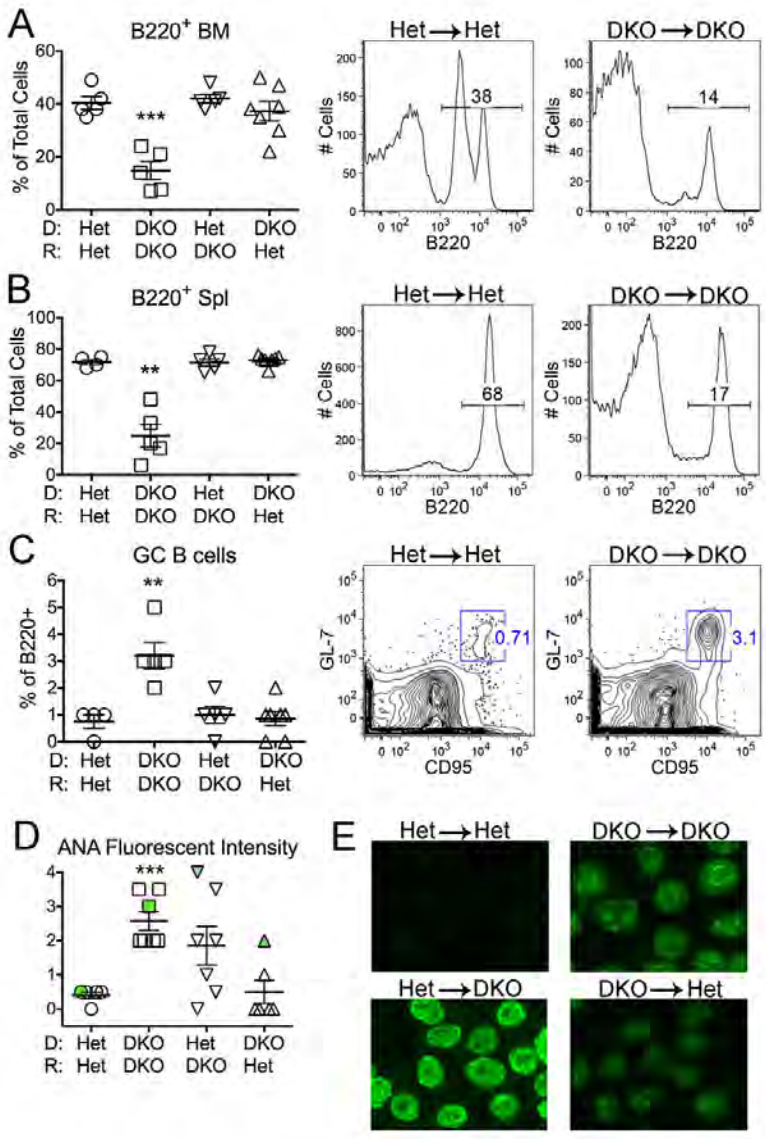


Figure 3.4 Defective B cell development and autoantibody production depend on DNase II deficiency in both donor-derived hematopoietic and radioresistant host cells. Percentage of B220$^+$ cells in **A**) bone marrow (BM) and **B**) total spleen (Spl) by FACS analysis. **C)** Percentage of B220$^+$ germinal center (GC) B cells in spleens. Representative FACS plots for Het→Het and DKO→DKO are shown to the right of the

compiled data figures in A, B, and C. **D**) Quantitation of anti-nuclear antibody (ANA) fluorescent intensity. The samples marked in green correspond to the ANA patterns shown in panel E. **E**) ANA staining patterns from sera by immunofluorescence (original magnification x 200). Het→Het chimeras: negative; DKO→DKO: speckled nuclear; Het→DKO: homogeneous nuclear; DKO→Het: speckled nuclear, 1/5 mice). All analyses performed on 10 month-old female chimeric mice (n=5-7 mice/genotype). Values are the mean ± SEM; **=$p<0.01$, ***=$p<0.001$ compared to Het→Het.

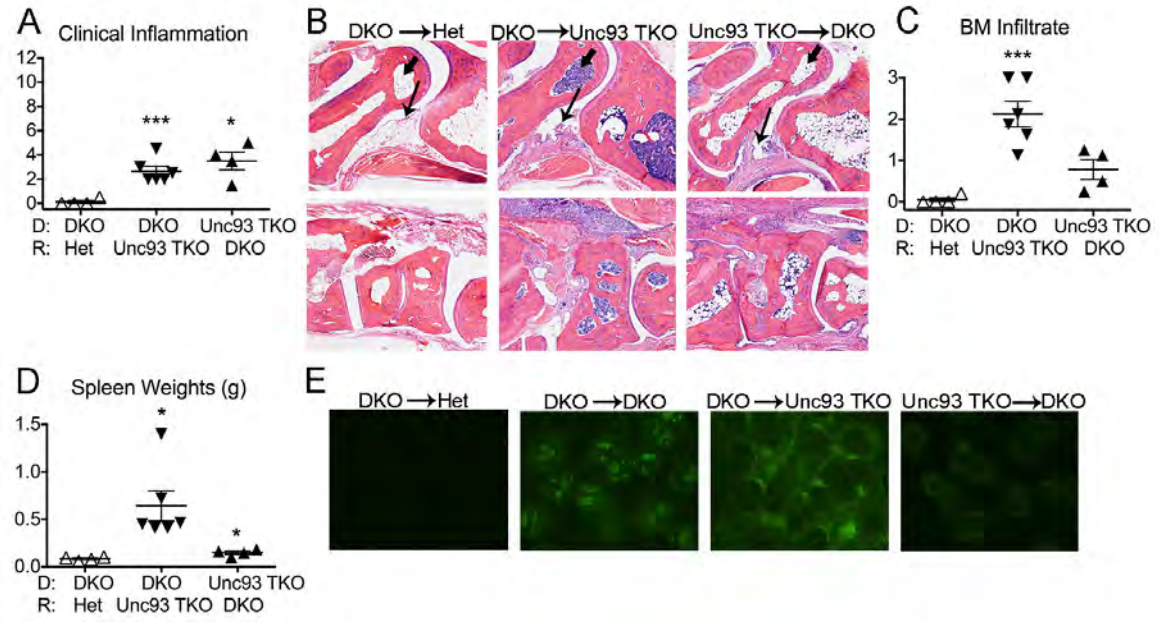


Figure 3.5 Expression of Unc93b1 in hematopoietic cells is required for the development of Unc93b1-dependent clinical manifestations. A) Clinical inflammation scores. **B**) Representative histologic images of inflammation in the ankle (upper panel) and midfoot (lower panel). Arrow designates synovium and arrowhead marks cellular infiltrate in the talus. Final magnification 4x. **C**) Quantitation of degree of bone marrow (BM) cellularity. **D**) Spleen weights. **E**) Representative ANA staining patterns of chimeric sera (original magnification x 200). All analyses performed on female chimeric mice. Values are the mean ± SEM; *=$p<0.05$, ***=$p<0.001$ compared to DKO→Het.

Discussion

The main message that emerges from the current study, together with previous reports, is that the accrual of excessive undegraded DNA in multiple cell types of DKO mice promotes the activation of both cytosolic and endosomal nucleic acid sensors, leading to a type I IFN-independent disease spectrum that incorporates features of both inflammatory arthritis and SLE. Cytosolic sensors are responding to DNA and the endosomal sensors are most likely responding to RNA-associated cell debris internalized by a cell surface receptor. Our Het/DKO chimera data further show that both hematopoietic and radioresistant host cells are required for all the various clinical manifestations.

The importance of radioresistant cells in autoinflammation has been previously reported by Stetson and colleagues in their analysis of mice lacking Trex-1, a model of STING-dependent autoinflammation. By using a Trex1$^{-/-}$ IFN-reporter line, they identified cardiac endothelial cells as the initial site of IFN-driven inflammation. As a result, these mice first develop myocarditis although additional tissues subsequently became inflamed. In contrast to the DKO mice described in the current report, Trex1 deficiency in radioresistant host cells was sufficient to activate WT bone marrow derived cells and trigger a systemic response where organ damage depended on activated T and B lymphocytes. However, a role for hematopoietic cells in the DKO model is not surprising since DNase II is a lysosomal DNase required for the degradation of cell debris/DNA phagocytosed by myeloid lineage phagocytes.

Another difference between the Trex1$^{-/-}$ and DNase II$^{-/-}$ IFNaR$^{-/-}$ DKO models is the

dependency on type I IFNs. IFNaR$^{-/-}$ x Trex1$^{-/-}$ mice fail to develop clinical manifestations of disease (218), while both STING-dependent and Unc93-dependent aspects of our DKO mice are type I IFN independent due to the absence of a functional type I IFN receptor. The strong autoantibody response, presumably dependent on TLR7, is particularly unexpected, especially since we have previously shown that TLR7-dependent B cell responses are highly type I IFN dependent (222). Exactly how cytokine production by DKO mice circumvents a need for type I IFNs as far as RNA-dependent B cell activation and other Unc93-dependent outcomes is not clear. Experiments are in progress to determine whether IFN-inducible genes downstream of RNA-sensing TLRs will exacerbate the Unc93-dependent SLE-like aspects of the DKO phenotype in IFNaR-sufficient DNase II$^{-/-}$ STING$^{-/-}$ mice (203).

The nature of the radioresistant cell(s) activated in DKO mice remains to be determined. Previous studies have documented an important role for STING dependent pathways in fibroblast lineage cells (205, 212). Therefore it is possible that STING promotes arthritis through its capacity to activate synovial fibroblasts. These cells have long been known to play a key role in the pathogenesis of RA (101) and are a source of proinflammatory cytokines and other factors that promote and perpetuate chronic inflammation and joint damage. Studies in early RA have demonstrated changes in the stromal compartments of synovium as early as the first months of disease (223). With disease progression, there is expansion of the synovial fibroblast compartment and these cells secrete proteinases that destroy cartilage, as well as factors that promote chronicity of inflammation through recruitment and retention of inflammatory cells (110).

Therefore, it is likely that synovial fibroblasts play a critical role in the initiation and perpetuation of disease in our model. Although we assume that the radioresistant cell is a stromal component, we cannot at this stage rule out a role for embryonally-derived macrophages. These are radioresistant tissue resident macrophages that develop from precursors, which seed peripheral tissues during fetal development, and could reside in synovial tissues as well (224).

The distinct overall phenotype of the DKO→DKO chimera group compared to the Het→DKO and DKO→Het chimeras demonstrated an absolute requirement for DKO radioresistant cells in the development of all the Unc93b1 clinical manifestations. Because the DKO→Unc93 TKO mice completely recapitulated the phenotype of the DKO→DKO chimeras, the host component is most likely to be due to inflammation driven by one or more cytosolic sensors, and not an endosomal cytosolic sensor. Both STING TKO and Unc93 TKO mice failed to develop the hypercellularity we observed in the long bone marrow, and therefore we propose that at a minimum, bone marrow hypocellularity depends on a STING triggered host cell, perhaps located in the hematopoietic niche of the marrow, and a TLR responsive hematopoietic cell. In both BM hypercellularity and splenomegaly, the relevant TLR is expected to be an RNA sensor (TLR7, TLR8, TLR3 or perhaps even TLR13), since endogenous DNA is not sufficiently degraded in DKO mice to activate TLR9 (61, 221). The BM infiltrate includes a high percentage of neutrophils and a similar accumulation of neutrophils in the BM of pristane-injected mice has been shown to be TLR7 dependent. These observations

may be related to the activated neutrophils recently identified in the marrow of SLE patients (225).

In summary, the combined data from the Het/DKO and Unc93 TKO/DKO chimeras demonstrate a critical interplay between cytosolic and endosomal sensors in the development of all the clinical manifestations of DKO mice and suggest that cytosolic sensors may play a more general role in promoting SLE and related autoimmune disorders. Cytosolic DNA sensors appear to be particularly responsive to inherent cell stress such as mitochondrial depolarization and the subsequent release of mitochondrial DNA into the cytosol (226, 227), or to the excessive accumulation of damaged DNA resulting from defective autophagosome formation (88). We previously demonstrated a role for the inflammasome-associated DNA sensor AIM2 in the development of arthritis in DKO mice. Whether RNA-sensing cytosolic sensors such as MDA-5 or RIG-I also play a role remains to be determined. From a broader perspective, cytosolic sensors may play an active role in detecting environmental insults that can trigger the onset and/or perpetuation of systemic autoimmune or autoinflammatory conditions. A better understanding of the in vivo networks that promote the distinct features of systemic autoimmune diseases will provide a platform for the design of therapeutics that address the unmet needs of patient populations.

Chapter IV

STING regulates bone formation induced by accrual of DNA

The work presented in this chapter is contained within the manuscript:

Baum R, Sharma S, Organ J, Jakobs C, Hornung V, Burr D, Rothstein A, Fitzgerald K, Gravallese E. STING regulates bone formation induced by accrual of DNA. Submitted to Arthritis & Rheumatology.

Summary

Cytosolic DNA sensors detect microbial nucleic acids and induce production of type I IFNs and pro-inflammatory cytokines through the adaptor STING to resolve infection. Endogenous DNA also engages the STING pathway, contributing to autoimmune disease. We have identified a novel role for cytosolic DNA sensing pathways in bone by analyzing the DNase II/IFNaR double deficient (DKO) model of autoimmunity. In the absence of DNase II, self-DNA accumulates, leading to STING-dependent polyarthritis and articular bone erosion. Here we show that DKO mice paradoxically accumulate trabecular bone in long bones over time. CFU assays and bone histomorphometry demonstrate a predominant role for osteoblasts in this phenotype. Surprisingly, we also found that DNA accrual promotes ectopic bone formation in the spleen of these mice, a site of extramedullary erythropoiesis. Moreover, STING deficiency significantly inhibits bone accrual in this model. Collectively, our data demonstrate that DNA promotes trabecular bone formation, a process that requires an intact STING pathway. These data may be directly relevant to the innate immune mechanisms leading to heterotopic bone formation, in which viral infection or trauma result in ectopic bone in soft tissues. Furthermore, identification of pathways linking innate immunity and bone should reveal novel targets for treatment of dysregulated bone remodeling in autoimmune disease.

Introduction

Innate immune PRRs, including cytosolic DNA sensors, detect nucleic acids from microbial organisms and orchestrate immune events and the production of cytokines to clear infection (77). Upon detection of DNA, cytosolic sensors including cGAS, IFI16/Ifi204 and others, signal through the adaptor STING, an endoplasmic reticulum-associated molecule, leading to the nuclear translocation of IRF-3 and NF-κB, and production of type I IFNs and pro-inflammatory cytokines, respectively (78).

Recent studies have demonstrated that endogenous host DNA derived from stressed or dying cells can also activate these same cytosolic DNA sensing pathways, contributing to the initiation and perpetuation of autoimmune disease (216, 217, 228). Inactivation of intracellular DNases in animal models leads to endogenous DNA accrual, resulting in autoinflammatory and autoimmune disease. For example, deficiency in the endonuclease DNase III (TREX1) in mice results in systemic inflammation and myocarditis due to accrual of DNA in the cytosol and STING-dependent production of type 1 IFNs and pro-inflammatory cytokines by non-hematopoietic cells (90, 218). In humans, loss-of-function mutations in *TREX1* are associated with the autoimmune disorders Aicardi-Goutieres Syndrome, chilblain lupus, and SLE (91-96). Further evidence for a role for the STING pathway in autoimmunity is provided by the discovery of human gain of function mutations in *Tmem173*, leading to constitutive activation of STING in fibroblasts and endothelial cells. This activation is associated with enhanced production of type I IFNs and pro-inflammatory cytokines, resulting in the clinical

syndrome STING-associated vasculopathy with onset of infancy (SAVI), manifested by vasculopathy, skin lesions, pulmonary fibrosis, and arthritis (97).

Finally, endogenous DNA accrual from inactivation of DNase II, a lysosomal nuclease that degrades double-stranded DNA, has also been associated with autoimmunity. In humans, SNPs in the promoter region of the *DNase II* gene that result in reduced DNase II activity are associated with the development of RA (99). In mice deficient in DNase II, DNA accumulates in phagolysosomes and secondarily in the cytosol of multiple cell types including macrophages, fibroblasts and dendritic cells (88). Macrophages also engulf nuclei from apoptotic cells, leading to DNA accrual and the production of pro-inflammatory cytokines including TNF, IL-1b, and IL-6, as well as type 1 IFNs. Excessive type I IFN production leads to anemia-driven embryonic lethality, from which these mice are rescued when the gene for the type I interferon receptor (IFNaR) is also deleted. DNase $II^{-/-}$ $IFNaR^{-/-}$ double deficient (**DKO**) mice survive, but develop a distal, erosive inflammatory arthritis by 3 months of age, which is absent in $DNaseII^{+/-}$ $IFNaR^{-/-}$ (**Het**) controls (100). This arthritis is entirely abrogated in the setting of STING deficiency (203, 217), and is significantly attenuated by loss of the inflammasome-promoting cytosolic DNA sensor, AIM2 (217, 228). These DKO mice also develop clinical manifestations of SLE through pathways that are independent of STING but rely on Unc93B1, an adaptor protein required for endosomal TLR activity (61, 217).

In the setting of inflammatory arthritis, the production of pro-inflammatory cytokines contributes to both articular and systemic bone loss due to enhanced local and

systemic osteoclast-mediated bone resorption and inhibition of osteoblast-mediated bone formation (7, 121, 229, 230). In DKO mice, we therefore anticipated the development of osteoporosis due to the systemic production of TNF and IL-1b (100, 202), which should induce osteoclastogenesis and bone resorption (4, 5, 132, 231). In addition, type I IFNs inhibit osteoclast differentiation, and IFNaR deficient mice demonstrate enhanced osteoclastogenesis and lose bone systemically (232). However, in the setting of DNA accrual in the DKO model of arthritis, we found an unexpected and dramatic enhancement of bone formation in the long bones and spleens, two sites of erythropoiesis and local DNA accumulation in DKO mice (100). Furthermore, STING deficiency abrogated this bone accrual, revealing a role for cytosolic DNA sensor pathways in bone remodeling. Collectively, these data demonstrate that DNA accrual promotes aberrant bone remodeling and may provide insights into bone disorders occurring in the context of autoimmunity, or in the context of disorders associated with ectopic bone formation.

Materials and Methods

Mouse Strains: C57BL/6 *DNase II*$^{+/-}$ embryos were provided by Dr. S. Nagata (Osaka Medical School) through the RIKEN Institute, and mice were crossed to *Ifnar*$^{-/-}$ C57BL/6 mice to produce *DNase II*$^{-/-}$ *Ifnar*$^{-/-}$ double knockout (DKO) and *DNase II*$^{+/-}$ *Ifnar*$^{-/-}$ heterozygous (Het) mice. DKO mice were intercrossed with STING-deficient mice to yield STKO mice (217). All animals were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were handled according to protocols approved by the Institutional Animal Care and Use Committee at the University of Massachusetts Medical School.

Histopathologic Analyses: Left hind limbs were fixed in 4% paraformaldehyde for 24h, decalcified in 15% EDTA, embedded in paraffin, and sectioned at 5μm. Sections were deparaffinized and stained with either H&E or TRAP (170). Spleens were fixed in 4% paraformaldehyde for 24h, embedded in methylmethacrylate, and sectioned at 5μm. The sections were then stained with H&E, TRAP, Von Kossa, or Goldner's trichrome.

Micro–computed Tomography (micro-CT): Femurs and vertebrae were fixed in 4% paraformaldehyde for 48 hours, transferred to 70% ethanol, then imaged at the Musculoskeletal Imaging Core at UMMS using a Scanco Medical μCT 40 at 70kVp and 114μA with resolutions of 10μm and 6μm, respectively. Analyses included trabecular bone within the entire femur from the proximal to distal growth plates, a 0.5 mm section in the central diaphysis of cortical bone, and trabecular bone within the rostral to caudal growth plates of the L3 lumbar vertebrae. The segmentation parameters included the

values: 0.8 Gauss sigma, 1.0 Gauss support, and a threshold of 220-1000 Hounsfield units (density range of >600mg of HA/cm^3).

Static histomorphometric analysis: Femurs from 10 month-old Het and DKO mice were fixed in 10% neutral buffered formalin for 72 hours and embedded in methylmethacrylate, as described previously (7). The proximal femoral metaphysis was sectioned longitudinally (5μm), mounted to slides with non-fluorescent medium, and stained with McNeal's trichrome for osteoid assessment and TRAP for osteoclast assessment. A region of interest approximately 4mm^2 within the secondary spongiosa (~0.5mm distal to the growth plate) was defined, and osteoid area (O.Ar/BV), bone area (B.Ar.), bone surface (BS), osteoblast surface (Ob.S/BS) and osteoclast surface (Oc.S/BS) were measured using a Nikon Optiphot 2 microscope interfaced to a semiautomatic analysis system (Bioquant OSTEO 7.20.10; Bioquant Image Analysis). Histomorphometric measurements were performed on two sections/sample (separated by ~25μm) and measurements were summed prior to normalization to obtain a single measure per sample. All parameters were measured and defined in accordance with ASBMR standards (233).

ELISAs: Serum levels of Osteocalcin (Biomedical Technologies), TRAP-5b (Immunodiagnostic Systems), and CTX-1 (Immunodiagnostic Systems) were determined by ELISA according to the manufacturer's instructions.

Colony Forming Unit (CFU) Assays: Total bone marrow was flushed from femurs of 2.5 month-old mice and RBCs were lysed. 2 x 10^6 cells/well were plated in 6-well plates in media containing α-minimum essential medium (α-MEM) without ascorbic acid,

20% FBS, and pen/strep. After 2 days in culture, cells were washed and exposed to osteoblast differentiation medium (50ug/ml ascorbic acid and 10mM β-glycerophosphate) for 14 days. Colony formation was assessed by staining for alkaline phosphatase (Sigma).

Calvarial Osteoblast Cultures: Primary osteoblasts were isolated from calvariae of C57BL/6 pups (Charles River). 8x10^4 cells/well (6-well plate) were cultured in α-MEM supplemented with 10% FBS and treated with osteoblast differentiation factors (50mg/ml ascorbic acid and 10mM β-glycerophosphate). On day 4, the cells were transfected with 1mg/ml of poly(dA:dT) using Lipofectamine 2000 (Invitrogen). RNA was isolated from the cells 5 days after transfection and subjected to quantitative polymerase chain reaction (qPCR).

Quantitative RT-PCR: Total RNA was isolated and 500ng was amplified as previously described (7). Gene expression was normalized to expression of the housekeeping gene hydroxymethylbilane synthase (HMBS). All primers were obtained from Qiagen. Data are expressed as the fold increase in gene expression compared to normalized lipofectamine controls, using the $2^{-\Delta\Delta CT}$ method.

Osteoclast Differentiation and Resorption Assays: For osteoclast assays, cell culture experiments were performed in α-MEM containing 10% FBS, 100U penicillin and 100µg/ml streptomycin. Cells were flushed from the bone marrow and differentiated in 40ng/ml of M-CSF (R&D) for 4 days. For osteoclast differentiation, osteoclast precursors were seeded at a density of 6,000 cells/well on 96-well plates and differentiated in medium containing 20ng/ml of M-CSF and 10ng/ml of RANKL (R&D) for 5 days. Half of the medium was replaced with fresh medium/cytokines on day 3. On day 5 of

differentiation, the cells were fixed and stained for TRAP5 using a leukocyte acid phosphatase kit (Sigma). TRAP-stained osteoclasts with 3-10 nuclei were counted.

For osteoclast resorption, osteoclast precursors were seeded at a density of 40,000 cells/well on 24-well hydroxyapatite-coated Osteo Assay plates (Corning) and differentiated in α-MEM containing 40ng/ml of M-CSF and 20ng/ml of RANKL (R&D) for 10 days. Half of the medium was replaced with fresh medium/cytokines every two days. Cells were then removed with 10% bleach and wells were rinsed in water and air-dried overnight. The wells were scanned on a flatbed scanner (Microtek 9800 XL) and the percentage of resorbed area was analyzed using NIH ImageJ software.

Xrays: Organs were imaged for 1 second at 35kV using the Faxitron MX-20 machine.

Nanostring: Total RNA was isolated using the RNeasy kit (Qiagen). Each RNA sample was quantitated via a Nanodrop ND-1000 spectrophotometer (Thermo Scientific), and volumes were adjusted to contain 100ng. RNA was hybridized and quantified with the NanoString nCounter analysis system (NanoString Technologies) per the manufacturer's instructions. Gene-expression data were normalized to internal positive and negative control sets and to three housekeeping genes, i.e., GAPDH, β-glucuronidase (GUSB), and hypoxanthine phosphoribosyltransferase 1 (HPRT1). All values were scaled by a log2(X-min(X)+1) function and a heatmap was generated using the open-source R-based software at UMMS.

Gene Array: Total RNA was purified from spleens using the RNeasy kit (Qiagen). For gene array analysis, cDNA was generated from 200ng of total RNA using the SensationPlus FFPE Amplification and WT Labeling Kit from Affymetrix. The samples

were run on Affymetrix GeneChip Mouse Transcriptome Arrays 1.0 (MTA) (Affymetrix, Santa Clara, CA), and quality control was performed using Expression Console (Affymetrix, Santa Clara, CA). Expression values were RMA normalized and detection p-values for each probe set determined with the detectable above background (DABG) algorithm. Biological replicate average, fold change, and ANOVA p-value were calculated between groups using the transcriptome analysis console (TAC, Affymetrix). Differential expression of mRNAs was identified as those significantly changing at least 1.5 fold with an ANOVA p-value <0.05. All values were scaled by a log2(X-min(X)+1) function and a heatmap was generated using the open-source R-based software at UMMS.

Statistical analysis: Statistical significance was analyzed with the unpaired, two-tailed Student's t test or ANOVA for multiple comparisons. Data are presented as the mean ± SE. Statistical significance is represented by the following notation in the figures: $p<0.05$=*, $p<0.01$=**, and $p<0.001$=***.

Results

Deficiency of DNase II promotes trabecular bone formation.

As reported previously, DNase II/IFNaR double deficient (DKO) mice develop a distal inflammatory polyarthritis accompanied by osteoclast-mediated articular bone erosion, resulting from the local expression of pro-inflammatory cytokines (100). We confirmed the presence of synovitis, pannus formation and osteoclast-mediated articular erosion in the distal joints of DKO mice and the absence of these findings in Het littermate controls (**Figure 4.1A**). However, despite local and systemic production of the pro-inflammatory cytokines TNF, IL-1β and IL-6 in these mice that promotes osteoclastogenesis, there is a surprising and significant accrual of trabecular bone in the tibiae adjacent to inflamed ankle joints, compared to Het controls (**Figure 4.1B**). This trabecular bone formation is preceded by the accumulation and persistence of a marrow infiltrate that we have previously shown to be dependent on the expression of both the STING and endosomal TLR pathways (234).

To quantitate bone accrual in the long bones, we analyzed the femurs of female DKO mice from 2 to 16 months of age by micro-computed tomography (micro-CT). As expected, Het control mice lose trabecular bone over time due to aging, as well as to the absence of type I IFN signaling, which promotes osteoclastogenesis and bone loss (232). In contrast, arthritic DKO mice that are also deficient in type I IFN signaling paradoxically demonstrate a dramatic accrual of trabecular bone that appears by 5-6 months of age and progresses over time, such that by 16 months of age the marrow cavity is largely replaced by bone (**Figure 4.1C**). Micro-CT analyses of femurs from female

mice confirmed a significant increase in trabecular bone volume/total volume (BV/TV), trabecular surface, and trabecular connectivity density in 10 month-old DKO mice compared to controls (**Figure 4.1D**). Further analysis of femurs by micro-CT in female mice revealed a trend toward a decrease in cortical bone volume/total volume and a significant decrease in cortical thickness in DKO compared to Het mice (**Figure 4.2**), demonstrating a loss of cortical bone over time in female DKO mice compared to Het controls. This loss may result from the accrual of trabecular bone, which provides mechanical support and reduces load on cortical bone. Micro-CT analyses of femurs from male DKO mice also revealed a trend toward increased trabecular bone surface and trabecular connectivity density compared to the Het controls as well as a significant loss of cortical bone compared to controls (**Figure 4.3**). Nevertheless, the trabecular bone phenotype was more dramatic in female mice. Thus, despite the expression of pro-inflammatory cytokines in these mice, the effects of aging on bone, and the lack of type I IFN signaling, all of which would be predicted to result in bone loss, DNase II deficiency in fact results in the accrual of excessive trabecular bone in the long bones over time.

Bone accrual results from an increase in osteoblast number and function

To explore the mechanism of trabecular bone accrual in these mice, parameters of osteoblast and osteoclast number and function were determined. Static histomorphometric measurements of trabecular bone in femurs from 10-month old female mice showed a marked increase in the number of osteoblasts (i.e. osteoblast surface, Ob.S) and production of osteoid (osteoid area, O.Ar.) in DKO mice compared with controls (**Figure 4.4A**). The ratio of Ob.S/bone surface (BS) and O.Ar./B.Ar. was not

significantly increased, demonstrating that there are appropriate numbers of osteoblasts for the amount of bone produced (**Figure 4.4A**). A significant increase in bone surface covered by osteoclasts (osteoclast surface, OC.S) was also observed in DKO mice compared to controls, demonstrating a concomitant increase in osteoclast number (**Figure 4.4B**). Again, similar ratios of OC.S/BS exist between Het and DKO mice suggesting that the number of osteoclasts lining bone is the expected number for the amount of bone present. In this model, bone is laid down rapidly as woven bone, precluding measurement of bone formation rates.

To determine whether this bone phenotype is a result of increased osteoblast number, colony forming unit (CFU) assays were performed. Cells from DKO bone marrow demonstrated an increase in CFUs compared with Het control mice, consistent with an increased number of mesenchymal precursors in the marrow of these mice (**Figure 4.4C**). Serum markers of bone remodeling including osteocalcin (bone formation), CTX-1 (bone resorption) and Trap5b (osteoclast number) in 10 month-old female mice demonstrated that serum levels of osteocalcin trended higher in DKO mice compared to controls (**Figure 4.4D**), consistent with an increase in osteoblast activity. Levels of CTX-1 and Trap5b were significantly elevated compared to controls (**Figure 4.4E**), indicating increased osteoclast activity and number. To determine whether there is an intrinsic alteration in either differentiation or function of osteoclasts that could be contributing to trabecular bone accrual, osteoclast differentiation and resorption assays were performed. These studies showed a trend toward increased osteoclast differentiation in DKO compared to Het mice (**Figure 4.4F**) and no significant difference in resorption

on hydroxyapatite-coated plates (**Figure 4.4G**) between DKO mice and controls. Overall, these data demonstrate that the trabecular bone accrual in DKO mice results from an increase in osteoblast number, with a concomitant increase in osteoclast differentiation and activity. The balance between osteoblast and osteoclast activity over time thus appears to skew in favor of bone formation in the setting of DNA accrual, and does not result from a decrease in osteoclast number or function.

Ectopic Bone Forms in DKO spleens with aging

Imaging of 10 month-old female Het and DKO mice was performed to evaluate the entire skeleton. Unexpectedly, X-ray images revealed multiple radiopacities in the left upper abdomen of DKO mice, suggesting areas of splenic calcification. Splenic enlargement was present in DKO mice compared to Het controls, as previously reported, and calcified nodules in the spleen measured up to 3mm (**Figures 4.5A and B**). Histologic staining demonstrated woven bone within splenic white pulp (**Figure 4.5C**), with osteoblasts (**Figure 4.5D**) and osteoclasts (**Figure 4.5E**) lining the surface of the bone. Additional stains showed that the bone is mineralized (**Figure 4.5F**) and robust osteoid production by osteoblasts is present at bone surfaces (**Figure 4.5G**). Analysis of spleens from younger mice revealed that ectopic bone formation in the spleen begins at approximately 9 months of age in female mice and accrues over time (data not shown). Bone was not identified in lymph nodes, liver, kidney, heart, intestine or brain (**Figure 4.5H**), revealing that ectopic bone formation is unique to the spleen. While many mouse models of autoimmunity do show splenic enlargement, the presence of ectopic bone is

extremely rare (235), and suggests that osteoblasts are generated from mesenchymal precursor cells recruited to the spleen or present locally within the spleens of DKO mice.

To further explore mechanism, we utilized a customized Nanostring code set containing 150 genes involved in innate immune and bone pathways to identify differences in gene expression in the spleens of DKO and Het control mice. This analysis revealed significant upregulation of genes associated with bone remodeling in DKO mice compared to controls, and confirmed the spleen as a site of active bone formation (**Figure 4.5I**). The *Col1A1* gene that produces a component of type 1 collagen, a major constituent of bone, was highly upregulated in DKO mice compared to controls as were the *Bglap* and *Spp1* genes encoding osteocalcin and osteopontin, respectively, both noncollagenous proteins found in bone. Moreover, the genes encoding alkaline phosphatase, as well as DMP1, a critical factor for bone mineralization produced by osteocytes, were also upregulated in DKO compared to Het controls. Upregulation of the matrix metalloproteinase (MMP) genes *MMP-9* and *MMP-13* in DKO compared with Het spleens reflect remodeling of bone extracellular matrix. Additionally, the *CTSK* and *ACP5* genes, encoding cathepsin K and Trap5b, were also significantly upregulated in DKO compared to Het spleens, demonstrating enhanced osteoclast numbers, consistent with bone remodeling.

Histologic analysis strongly suggests that the process of bone formation in DKO mice is similar in the long bones and spleen (**Figure 4.5J**), with complete lack of evidence for endochondral ossification at either site; rather, the production of organic bone matrix, with subsequent mineralization of that matrix, is noted at both sites (**Figure**

4.5J). In DKO mice, the long bones and spleen are sites of erythropoiesis (100, 202). To determine whether bone accrual was present in bones in which erythropoiesis typically does not occur in mice, such as the vertebrae (236), vertebral bodies were subjected to micro-CT analysis, revealing a significant decrease in BV/TV, trabecular surface, and trabecular connectivity density in DKO mice compared to Het controls (**Figure 4.6**). These data suggest that local factors that promote the differentiation of mesenchymal precursor cells to osteoblasts may be released at sites of erythropoiesis (long bones and spleen) (98, 100).

DNA accrual promotes enhanced osteoblast differentiation and function in the spleen

The finding of bone in the spleen of DKO mice provides additional evidence that bone accrual in this model is driven by the excessive differentiation and activity of osteoblasts, leading to the production and mineralization of bone matrix. This could result from an increase in mesenchymal osteoblast precursors due to factors produced during the process of erythropoiesis that promote osteoblast differentiation, or from enhanced osteoblast differentiation due to DNA accumulation within the cytosol of mesenchymal precursor cells themselves. To test the effect of DNA on differentiation, calvarial osteoblasts were transfected with the dsDNA mimetic poly(dA:dT) and differentiation was determined by expression of alkaline phosphatase. Interestingly, transfection with DNA upregulated the expression of p204 in calvarial osteoblasts, a cytosolic DNA sensor previously shown to also act as a transcriptional coactivator for bone formation (237-239). However, alkaline phosphatase levels were significantly reduced in cells transfected with dsDNA (**Figure 4.7**), suggesting that accumulation of dsDNA in osteoblast precursors

inhibits, rather than promotes, differentiation. These results support the alternative hypothesis that there is an increase in osteoblast precursor cells and/or that extrinsic factors expressed by other cell types drive the differentiation of mesenchymal precursors to the osteoblast lineage in this model.

To identify factors promoting osteoblast differentiation and bone formation, gene expression in 10 month-old DKO spleens was examined by array analysis. This analysis revealed upregulated expression of numerous genes regulating osteoblast differentiation and bone formation (**Figure 4.8**). Among the most highly upregulated osteoblast-related genes in whole spleen were two genes in the transforming growth factor beta (TGFβ) family, *Tgfbi* and *Tgfbr1*. TGF-β signaling promotes the expansion of osteoblast progenitors and contributes to the early differentiation of osteoblasts (177). Moreover, the gene encoding the BMP-signaling transducer *Smad1*, which regulates expression of the osteoblast-specific transcription factors Runx2 and Osterix, as well as the *Runx2* gene itself, were upregulated in DKO mice compared to controls. The *Bmp1* gene that encodes a type I collagen C-propeptidase required for mature collagen maturation in bone was also upregulated in DKO mice compared to Het controls. We did not detect upregulation of other BMP receptor ligands known to promote osteoblast differentiation, including BMP2, 4, 6 or 7. In addition, we found an upregulation of the *Gdf3* gene, a member of the TGF-β superfamily and an inhibitor of BMP signaling, which would be expected to inhibit bone formation and may represent a compensatory mechanism to inhibit local osteoblast differentiation.

Regulation of several other genes supports the interpretation of splenic tissue as a site at which mesenchymal precursor cells are differentiating to osteoblasts. The *Vdr* gene, encoding the vitamin D receptor, is upregulated in DKO compared to Het mice. The vitamin D receptor has been shown to induce expression of factors that enhance osteoblast differentiation and inhibit apoptosis of osteoblasts. Recently, transgenic overexpression of *Vdr* in mature osteoblasts was shown to increase trabecular bone volume (240). The *Serpinf1* gene, encoding pigment epithelium-derived factor (PEDF), was also upregulated in DKO compared to Het spleens. PEDF modulates human and murine mesenchymal stem cell (MSC) differentiation by promoting osteogenesis and inhibiting adipogenesis (241). In addition, the *Serpinh1* gene, encoding heat shock protein 47 (HSP47), is upregulated in DKO spleens compared to controls. Hsp47 is localized in the endoplasmic reticulum and acts as a molecular chaperone for the maturation of collagen (242). Finally, expression of the *Pth1r* gene encoding the PTHR was upregulated, which would promote osteoblast differentiation in the presence of PTH (243).

The STING pathway is required for bone formation

In order to further define mechanism, we examined the role of the STING pathway in bone formation in DKO mice. In this model, cytosolic sensors detect host DNA and signal through STING to induce production of pro-inflammatory cytokines including TNF, IL-6, and IL1β. We and others have previously shown that arthritis in DKO mice is abrogated by deletion of STING in STING$^{-/-}$DNase II$^{-/-}$IFNaR$^{-/-}$ triple knockout (STKO) and in STING$^{-/-}$DNase II$^{-/-}$ mice (203, 217). In order to determine

whether the osteogenic factors identified in DKO mice were upregulated in a STING-dependent fashion, we performed gene array analysis in STING deficient DKO mouse (STKO) spleens. Expression of pro-osteogenic genes including *Tgfbi, Tgfbr1, Smad1, Runx2, Vdr, Serpinf1, and Pth1r* is not induced in the setting of STING deficiency (**Figure 4.8**).

Importantly, in vivo, STING deficiency in DKO mice ameliorates not only arthritis, but also bone accrual in both the long bones (**Figure 4.9A**) and spleens (**Figure 4.9B**). Trabecular BV/TV and trabecular bone surface are dramatically decreased in STKO compared to DKO mice. Moreover, the expression of genes associated with osteoblast activity (*Col1A1, Spp1, Col1A2, Bglap, Omd, Alpl, Ibsp, Phex, Dmp1*), matrix bone remodeling (*MMP13*), and osteoclasts (*CTSK, ACP5*) are significantly decreased in STKO compared to DKO spleens (**Figure 4.9C**). These data demonstrate that local bone formation in the setting of DNA accrual is dependent upon an intact STING pathway, and reveal a novel mechanism for aberrant bone remodeling.

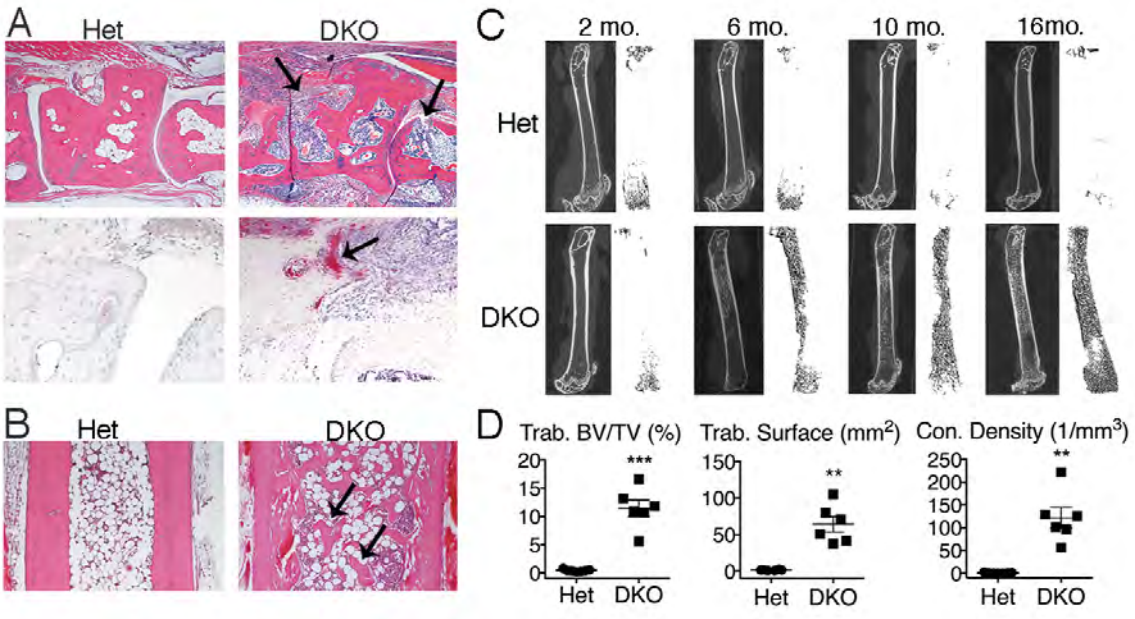


Figure 4.1 Trabecular bone accrual occurs over time in long bones of DKO mice.

A) Midfoot, 10 month-old mice (female, n=4 mice/genotype): Representative H&E (upper panels) and TRAP (lower panels) stained images of inflammation and articular bone erosions (arrows). Magnification 4x and 20x, respectively. **B)** Tibiae, 10 month-old mice (female, n=6 mice/genotype). Representative images of H&E stained sections. Arrows indicate trabecular bone. Magnification 4x. **C)** Micro-CT images of femurs at 2, 6, 10, and 16 months (female, n=3-7 mice/genotype/age). Left images: cortical and trabecular bone. Right images: 3D reconstruction of trabecular bone. **D)** Quantitation of micro-CT data from 10 month-old mice (female, n=6-7 mice/genotype) for trabecular bone volume per total volume (BV/TV), trabecular bone surface, and trabecular connectivity (con.) density. Data are representative of 3 individual experiments. Values are the mean ± SEM compared to Het: **=p<0.01, ***=p<0.001.

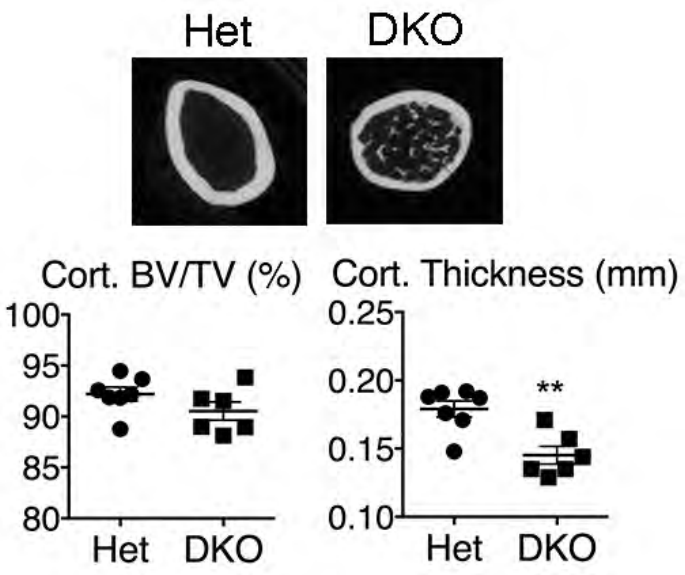


Figure 4.2 Cortical bone loss in DKO femurs. Transverse micro-CT images of femurs and quantitation of cortical bone volume/total volume (BV/TV) and cortical thickness for 10 month-old mice (female, n=6-7 mice/genotype). Data are representative of 2 individual experiments. Values are the mean ± SEM compared to Het: **=$p<0.01$.

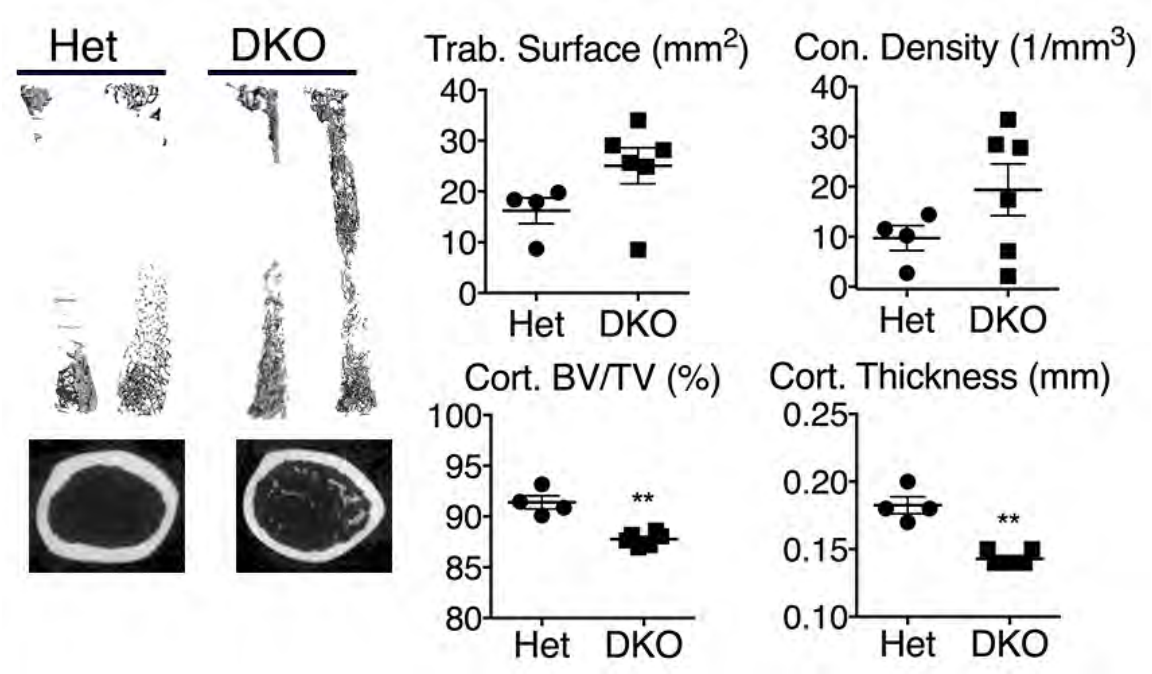


Figure 4.3 Trabecular bone accrual and cortical bone loss in male DKO femurs. Representative micro-CT images of trabecular and cortical bone in femurs, and quantitation of trabecular bone surface, trabecular connectivity density, cortical bone volume/total volume (BV/TV), and cortical thickness (10.5-11.5 month-old mice, male, n=4-6 mice/genotype). Data are representative of 2 individual experiments. Values are the mean ± SEM compared to Het: **=$p<0.01$.

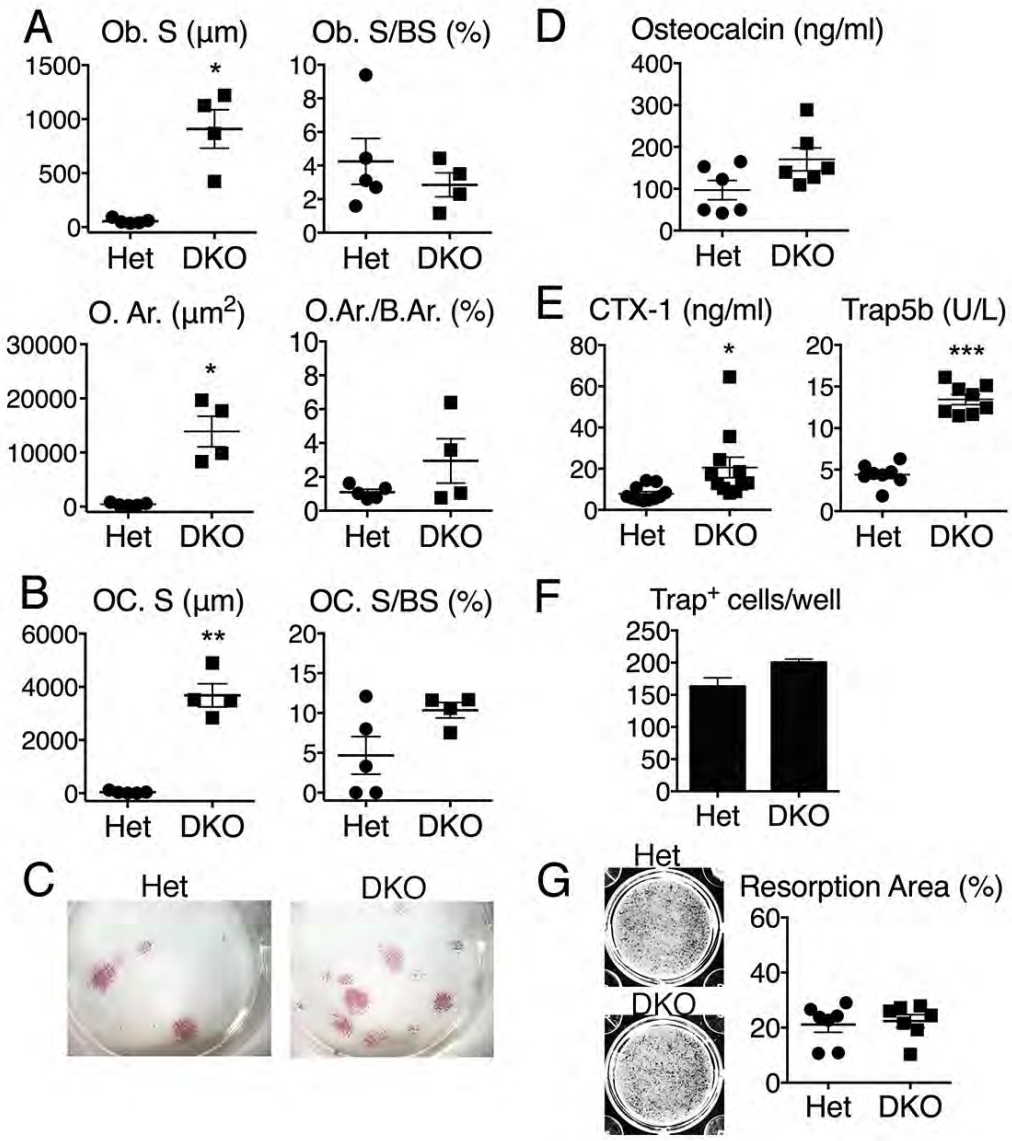
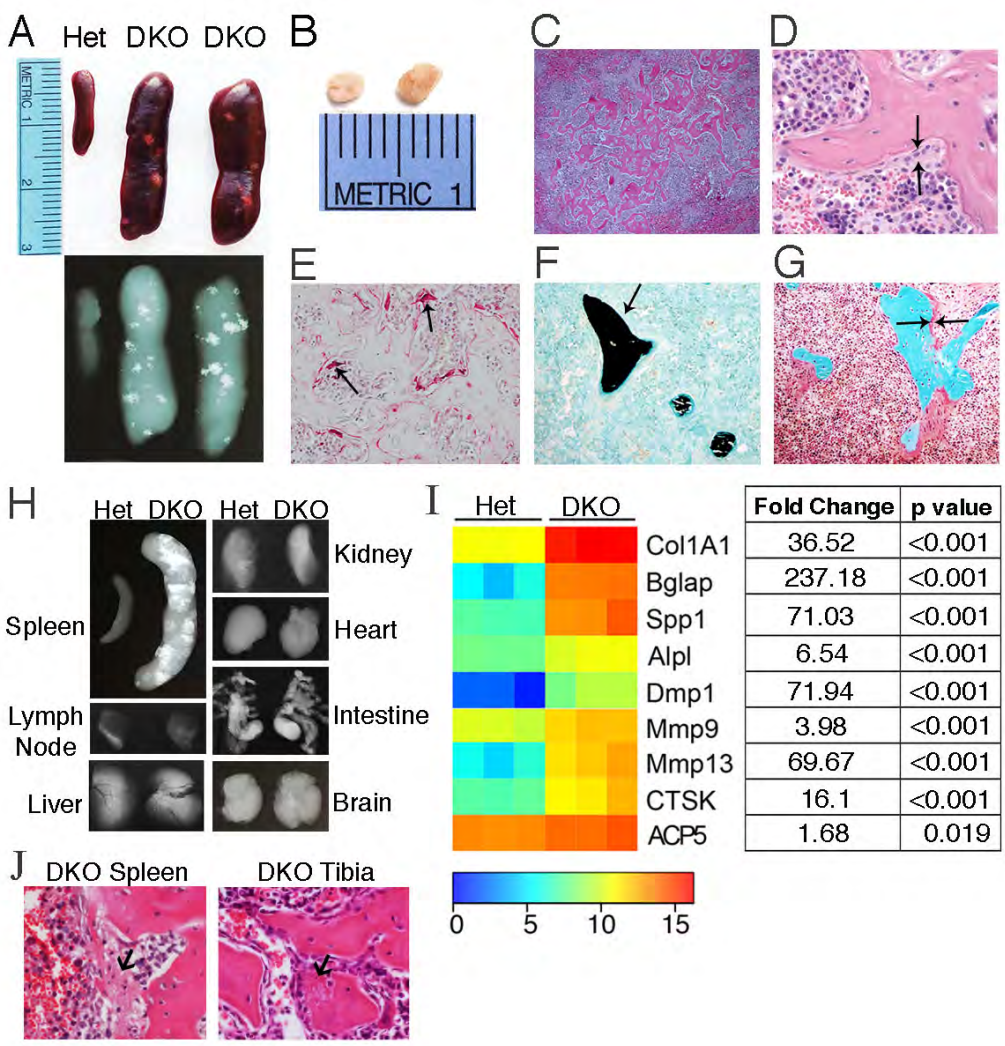


Figure 4.4 Osteoblast and osteoclast parameters in DKO mice. A and B) Static histomorphometry, femurs, 10 months (female, n=4-5 mice/genotype). Abbreviations: osteoblast surface (Ob. S), bone surface (BS), osteoid area (O. Ar.), bone area (B.Ar.), and osteoclast surface (OC. S). **C)** Representative images from osteoblast colony forming unit (CFU) assays, 2.5 month-old mice (male, n=5 mice/genotype). Colonies are stained for alkaline phosphatase (pink). **D and E)** Serum bone turnover markers: Osteocalcin, C-terminal peptides of type I collagen (CTX-1), and Trap5b. 10 month-old mice (female,

n=6-12 mice/genotype). **F)** Number of Trap+ cells in osteoclast differentiation cultures. 2.5 month-old mice (male, n=3 mice/genotype). **G)** Percentage of hydroxyapatite area resorbed by osteoclasts. 2.5 month-old mice (female and male, n=6-7 mice/genotype). Data are representative of 2 individual experiments. Values are the mean ± SEM compared to Het; *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$.



Fold Change	p value
36.52	<0.001
237.18	<0.001
71.03	<0.001
6.54	<0.001
71.94	<0.001
3.98	<0.001
69.67	<0.001
16.1	<0.001
1.68	0.019

Figure 4.5 Ectopic bone forms in DKO spleens. A) Representative images and X-rays of spleens from 10 month-old mice (female, n=6 mice/genotype). B) Islands of bone removed from 10 month-old female DKO spleens. C-G) Representative images of histologic stains performed on DKO spleen sections. 10 month-old mice (female, n=3 mice). C and D) H&E stained sections showing bone formation in white pulp (Magnification 4x & 20x, respectively). Arrows identify osteoblasts lining the surface of bone. E) TRAP stain, osteoclasts (arrows). F) Von Kossa/Fast Green stain shows mineralized bone (black, arrow). G) Goldner's trichrome stain demonstrates osteoid

production (dark pink, arrows). H) Representative X-rays of organs, 22 month-old mice (female, n=3 mice/genotype). I) Heatmap: Nanostring mRNA profiling of key bone remodeling genes, 10 month-old spleens (female, n=3 mice/genotype). Mean intensities of gene expression were transformed by a log2 function. Table lists fold change in DKO vs. Het gene expression and the corresponding p values. J) Representative H&E images of bone in DKO spleen and tibia. Arrows indicate fibrous tissue representing early bone matrix that may become mineralized.

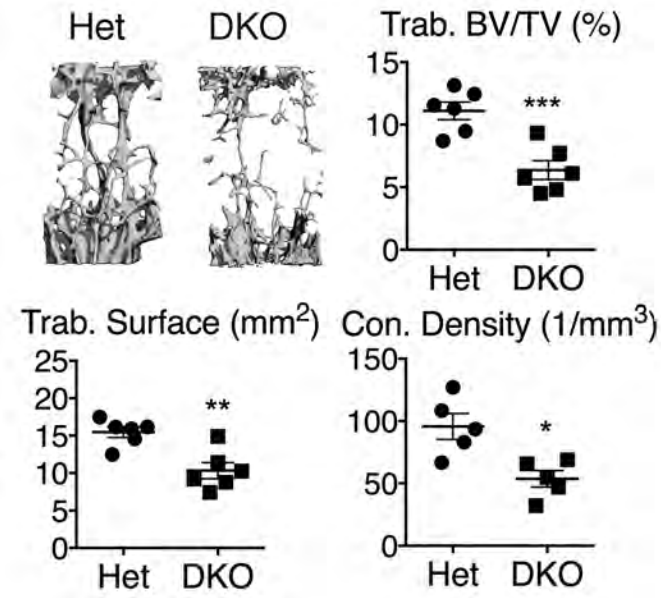


Figure 4.6 Loss of bone in vertebral bodies in DKO mice. Micro-CT images of trabecular vertebral bone and quantitation of trabecular bone volume/total volume (BV/TV), trabecular bone surface, and trabecular connectivity density. Data are representative of 2 individual experiments. Values are the mean ± SEM compared to Het; *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$.

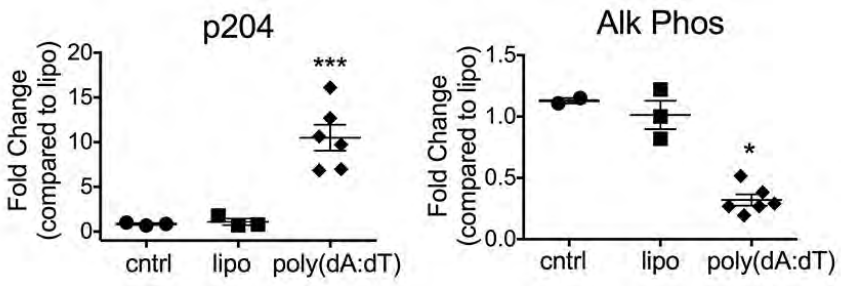
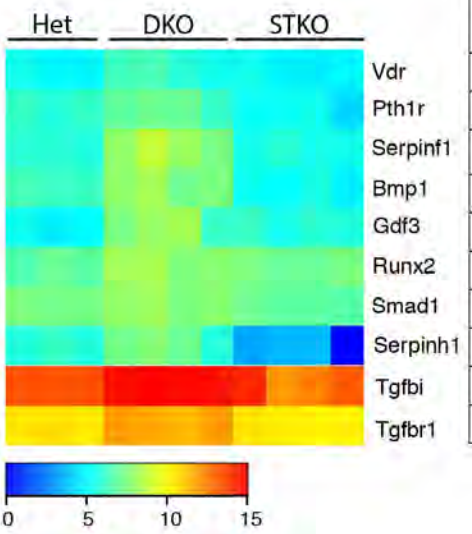
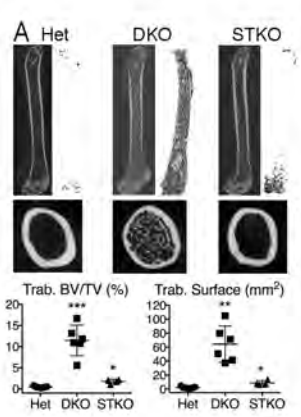


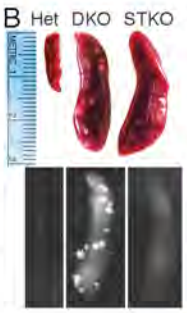
Figure 4.7 Transfection of DNA in osteoblasts induces p204, but inhibits differentiation. Fold change in p204 and alkaline phosphatase gene expression in calvarial osteoblasts stimulated with the DNA ligand poly(dA:dT), lipofectamine, or medium control (cntrl).

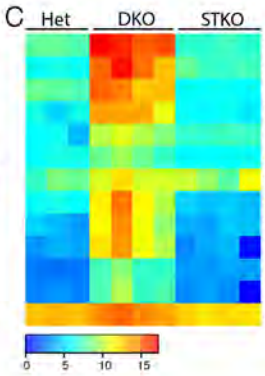


	Fold Change (DKO vs. Het)	p value	Fold Change (STKO vs. DKO)	p value
Vdr	1.65	0.039	-1.69	0.013
Pth1r	1.67	0.042	-2.5	0.002
Serpinf1	3.56	0.002	-4.27	3.4E-4
Bmp1	2.43	0.004	-3.87	1.2E-4
Gdf3	4.5	0.012	-3.3	0.015
Runx2	2.01	0.004	-1.53	0.013
Smad1	1.34	0.058	-1.85	0.002
Serpinh1	2.97	0.057	-34.3	3.8E-4
Tgfbi	2.63	2.6E-5	-3.72	0.009
Tgfbr1	2.45	0.001	-2.78	5.9E-5

Figure 4.8 STING-dependent induction of factors regulating osteoblast differentiation in DKO mice. Heatmap: Mean intensities of expression in gene arrays, spleen, 10 month-old mice (n=3-4 mice/genotype). Signals were transformed by a log2 function. Table lists fold change in DKO vs. Het and STKO vs. Het gene expression and the corresponding p values.







	Fold Change (DKO vs. Het)	p value	Fold Change (STKO vs. DKO)	p value
Col1a1	219.22	3.4E-4	-458.47	2E-6
Spp1	326.21	3.0E-4	-182.34	1.3E-4
Col1a2	53.3	2.7E-4	-168.43	8E-6
Bglap	115.35	3.4E-4	-142.98	3.7E-5
Omd	11.78	0.001	-6.62	8.2E-4
Alpl	3.19	0.021	-3.97	0.005
Mmp9	5.93	0.003	-1.93	0.154
CTSK	34.76	0.005	-64.68	5.6E-4
Mmp13	60.08	0.004	-61.68	9.3E-4
Ibsp	91.83	0.001	-96.17	1.7E-4
Phex	4.98	0.002	-4.24	7.6E-4
Dmp1	5.64	0.007	-5.27	0.002
Acp5	2.33	0.019	-3.63	0.001

Figure 4.9 Bone accrual in DKO mice requires the STING pathway. **A**) Sagittal and transverse micro-CT images of femurs and quantitation of micro-CT data for trabecular bone volume/total volume (BV/TV) and trabecular bone surface. The Het and DKO values are the same as those shown in Figure 1D. 10 month-old mice (female, n=4-7 mice/genotype). Values are the mean ± SEM compared to Het; *=p<0.05, **=p<0.01, ***=p<0.001. **B**) Representative images and X-rays of spleens. All analyses performed on 10 month-old mice (female, n=4-6 mice/genotype). **C**) Heatmap: Mean intensities of expression in gene arrays, spleen, 10 month-old mice (n=3-4 mice/genotype). Signals were transformed by a log2 function. Table lists fold change in DKO vs. Het and STKO vs. Het gene expression and the corresponding p values.

Discussion

In this study we demonstrate a novel pathway regulating bone homeostasis and show that accrual of undegraded DNA in cytosolic cellular compartments promotes the activation of cytosolic nucleic acid sensors, leading to trabecular bone accrual in the long bones and spleen. This occurs despite the presence of arthritis and osteoclast-mediated articular bone erosion, and the production of pro-inflammatory cytokines that promote bone loss. Bone formation results from an increase in osteoblast number and function and the bone phenotype manifests late, beginning at approximately 5-6 months of age in long bones, while bone formation in the spleen appears by 9-10 months. This late manifestation is likely due to the requirement for accrual of DNA over time, as well as to the time required for the enhancement of bone formation over bone resorption to ultimately favor bone accrual.

One innate immune pathway that is essential for the manifestation of this bone phenotype is the STING pathway as bone accrual in long bones and spleen is significantly inhibited in the absence of STING. Macrophages in the bone marrow and spleen in DKO mice have previously been shown to carry undigested DNA due to engulfment of extruded erythroid nuclei released during the late stages of erythropoiesis, and their inability to break down this DNA. In contrast, macrophages within joint synovium fail to engulf apoptotic cells (202), and osteoclast-mediated bone loss occurs locally within joints. This difference may be due to lack of expression by joint-based macrophages of the required receptors for recognition of apoptotic cells, including Tim4

(244). The failure to degrade DNA leads to entrance of DNA into cytosolic compartments and activation of downstream pathways. STING regulates critical aspects of these processes.

We have shown that other sensors are also activated by nucleic acids in this model, including the DNA-sensing PYHIN protein AIM2 and the RNA-sensing endosomal TLRs (61, 217, 228). DKO mice demonstrate a constellation of systemic autoimmune symptoms including the early onset of splenomegaly and associated extramedullary erythropoiesis. Extramedullary erythropoiesis is associated with markedly decreased numbers of erythroid lineage $Ter119^+$ cells in the BM and increased numbers of $Ter119^+$ cells in the spleen (61). It has been proposed that disrupted erythropoiesis is triggered by the failure of DNase II-deficient erythroid island macrophages in the BM to degrade the reticulocyte nuclei extruded from erythrocyte precursors during late phase erythropoiesis (98). Intriguingly, both splenomegaly and extramedullary erythropoiesis appear to be TLR dependent, as they are absent in Unc93B1-deficient DKO mice (61), and persist in STING-deficient TKO mice (217). Since bone accrual in DKO mice occurs at sites of erythropoiesis, it is likely that Unc93B1-dependent TLR pathways also contribute to bone formation in this model.

Affymetrix data from spleen samples demonstrated a major drive towards osteogenesis and identified a number of osteogenic factors that are upregulated at this site of extramedullary erythropoiesis. Among the most highly regulated of the osteogenic factors in whole spleen were those genes in the transforming growth factor beta (TGF-β) family. Although the role of TGF-β signaling in bone is complex, TGF-β isoforms and

their receptors (including type I receptor (TGFβRI)) are known to expand the pool of mesenchymal osteoblast progenitor cells and promote early differentiation and commitment to the osteoblast lineage (158). Moreover, mice with tissue-specific removal of TGFβRI show reduced trabecular bone in the long bones and decreased proliferation and differentiation of osteoblasts (245). Additionally, the genes encoding the osteogenic BMP-signaling transducer Smad1 and the osteoblast-specific transcription factor Runx2 were upregulated in DKO mice compared to controls.

Bone formation in the long bones and spleen appears to occur by similar mechanisms, given the gene expression profiles and histologic features at these sites. However, bone formation in the spleen requires the presence of a population of mesenchymal osteoblast precursor cells that are either recruited to splenic tissue, or are resident within this site. One possible mesenchymal precursor with osteogenic potential is the pericyte. Microvascular pericytes have long been known to serve as a reservoir for multiple cellular lineages in joints, including osteoblasts (246). It has been shown that vascular pericytes implanted into athymic mice reproducibly form cartilage and bone. Furthermore, these cells can secrete components of bone matrix including bone sialoprotein, which is associated with initiation of bone mineralization and assists in the nucleation of hydroxyapatite (246). Bone forms in DKO spleens in a process similar to what is seen in implanted vascular pericyte cultures. Pericytes may arise from a CD34-expressing progenitor cell within vessel walls (247), and it is of interest that bone in the spleen forms in regions of white pulp, areas rich in vasculature.

These findings are likely related to the dysregulated bone remodeling occurring in autoimmune disease, as well as to several human diseases in which abnormal bone formation occurs in soft tissues. Heterotopic ossification (HO) is a debilitating condition associated with formation of lamellar bone in extra-skeletal sites. Acquired HO occurs in cases of soft tissue trauma including amputation, joint replacement surgery, and traumatic brain and spinal cord injuries (185). The etiology and pathogenesis of acquired HO is unknown and treatments aim to limit the associated inflammation. Hereditary HO is seen in the rare genetic disorder fibrodysplasia ossificans progressiva (FOP). In the case of FOP, gain-of-function mutations in the ACVR1 gene contribute to this disorder, leading to enhanced BMP signaling (248). Despite the presence of ACVR1 mutations, FOP patients exhibit variability in the severity and progression of their disease, and form bone episodically, rather than continuously, following viral infections, immunizations, or tissue trauma (184, 193, 249). These triggers often precede ectopic bone formation and strongly implicate inflammatory innate immune pathways in the pathogenesis of HO. It is likely that viral DNA or DNA released from trauma/damaged cells may overwhelm the activity of DNase II, triggering the activation of innate immune DNA sensors. Further investigation into these mechanisms should provide novel pathways for the prevention and treatment of bone remodeling in autoimmune disease and in heterotopic bone formation.

Chapter V

Discussion

There is increasing evidence for a role of TLR and cytosolic sensing of endogenous DNA in autoinflammatory conditions (250). While these innate immune PRRs were first recognized to detect microbial DNA and orchestrate inflammatory responses to resolve infection, several sources of endogenous DNA have now been shown to inappropriately trigger these sensors, leading to the development of multiple examples of systemic inflammation (90, 97, 100, 251). The objective of this dissertation is to examine the contribution of three separate DNA-sensing pathways, the STING, AIM2, and endosomal TLR pathways, to the initiation and perpetuation of autoinflammatory arthritis and bone remodeling.

Although RA is classically thought of as a disease driven by adaptive immunity, macrophages, dendritic cells, and neutrophils are abundant in arthritic joints, suggesting that innate immune pathways play central roles in the pathogenesis of this disease (252). Interestingly, DNase II deficiency was recently shown to result in DNA accrual in macrophages and neutrophils and result in a phenotype that resembles RA (100). Erosive inflammatory arthritis, autoantibody production, and splenomegaly are present in DNase II/IFNaR deficient (DKO) mice, but not in the Het ($DNase^{+/-}$ $IFNaR^{-/-}$) littermate controls. In Chapter II, we examined the role of innate immune PRRs in driving the autoimmune phenotype of DKO mice. To evaluate whether cytosolic or Unc93-dependent endosomal DNA sensors contribute to the clinical manifestations of DKO mice, genes involved in DNA sensor signaling were deleted on the DKO background. Genetically altered mice include STING/DNase II/IFNaR TKO (STING TKO),

AIM2/DNase II/IFNaR TKO (AIM TKO), and Unc93b/DNase II/IFNaR TKO (Unc93 TKO) mice.

Thorough examination of inflammation in these mouse lines revealed important roles for both the STING and AIM2 pathways in the pathogenesis of arthritis. Clinical joint scoring, histologic evaluation, and cytokine analysis demonstrated a significant attenuation of arthritis in STING TKO and AIM TKO mice compared to DKO mice; however, joint inflammation persisted in Unc93 TKO mice. DKO mice not only develop arthritis, but they also exhibit markedly enlarged spleens as a result of extramedullary hematopoiesis. We and others have shown that splenomegaly is greatly reduced in Unc93 TKO and AIM TKO mice (61). Whereas, STING TKO mice still develop massively enlarged spleens. We also demonstrate that DKO mice produce autoantibodies against an extensive panel of autoantigens, including RNA and histones, as detected by autoantigen arrays. Remarkably, autoantibody production was almost entirely dependent on endosomal TLR nucleic acid sensing receptors, and not on the STING or AIM2 pathways. This evidence strongly suggests that the innate immune system plays a prominent role in the pathophysiology of inflammatory arthritis. Moreover, these data demonstrate that STING-dependent cytosolic sensors, AIM2, and endosomal TLRs dependent on Unc93b play distinct roles in the manifestations of autoimmunity, as each pathway contributes differently to arthritis, autoantibody production, and splenomegaly.

This study was the first to reveal a role for AIM2 as a sensor of endogenous nucleic acids in the pathogenesis of joint inflammation. Upon detection of DNA, AIM2 forms a caspase-1 activating inflammasome that cleaves pro-IL1β and pro-IL18 into their

active forms (84, 253). Although our study showed that AIM2 deficiency decreased joint inflammation in DKO mice, we did not definitely determine whether this attenuation of inflammation was due to decreased levels of IL-1β, IL-18 or both. Administration of IL-1β and/or IL-18 blocking antibodies in DKO mice would reveal whether one or both cytokines contribute to the joint inflammation. Importantly, IL-1 blockade has already been evaluated in patients with RA (254). Treatment with anakinra, a recombinant IL-1 receptor antagonist, is well tolerated and more effective than placebo; however, anti-TNF and anti-IL6 therapies have proven to be superior in the treatment of RA (255, 256). Today anakinra is typically used to treat autoinflammatory diseases including Still's disease and rare hereditary fever syndromes (257-259).

This study also demonstrates that the STING and Unc93b pathways contribute to inflammatory arthritis and autoantibody production, respectively. However, we did not identify the exact cytosolic sensors and endosomal TLRs that detect the nucleic acid in DKO mice, leading to these phenotypes. A number of DNA sensors trigger the STING pathway including cGAS, IFI204, DAI, and DDX41 (79-83). Recently, deletion of cGAS in DNase $II^{-/-}$ mice was shown to rescue the mice from embryonic lethality as well as ameliorate the erosive polyarthritis (260). However, the contribution of other STING-dependent cytosolic sensors to the autoimmune phenotype of DKO mice has yet to be determined. The TLR(s) that detects the nucleic acid in DKO mice and leads to a robust autoantibody response is also unknown. Unc93b is involved in the translocation of TLR3/7/8/9 from the ER to endosomal compartments (261). Thus, any or all of these endosomal TLRs may be contributing to the autoantibody production in this model.

Our data strongly suggest that inhibition of STING and/or AIM2 pathways may be beneficial for the treatment of inflammatory joint diseases. Importantly, certain DNA sequences of TTAGGG repeats, known as suppressive oligodeoxynucleotides (ODN), have been shown to abrogate activation of cytosolic and endosomal nucleic acid receptors (262). These suppressive ODNs function as competitive inhibitors by binding DNA sensors and competing with immune-stimulatory DNA. Additionally, suppressive ODNs have been shown to bind AIM2 and prevent the recruitment of ASC and assembly of the inflammasome (263). It is of interest to determine whether these ODNs would ameliorate joint inflammation in DKO mice. Additionally, systemic administration of suppressive ODNs in other murine models of arthritis would further determine whether this strategy would be of therapeutic value in the treatment of RA.

Although suppressing the STING pathway may be a beneficial therapeutic approach for the treatment inflammatory arthritis, this approach may also exacerbate tumor growth (264). Recent studies have shown that CD8+ T cell priming against tumors was defective in STING-deficient and IRF3-deficient mice (265). Importantly, tumor-derived DNA was found to stimulate the STING pathway in dendritic cells and drive the production of type I interferons, leading to subsequent T cell priming against tumor-associated antigens. Moreover, in a glioma mouse model, tumors were shown to grow more aggressively in STING-deficient mice, while administration of the STING agonist c-di-CMP prolonged the survival of glioma-bearing mice (266). Furthermore, intra-tumor injection of STING agonists have been shown to generate an anti-tumor T cell response and induce profound regression of established tumors (267). Thus, a tenuous balance

between the activation and inhibition of the STING pathway exists. If inappropriately activated, the STING pathway can lead to autoinflammatory disease, and if continuously inhibited this pathway may result in promoting tumor growth.

Chapter III of this dissertation delineates the contribution of hematopoietic and non-hematopoietic cells to the various autoimmune manifestations in DKO mice. The persistence of inflammation within arthritic joints likely results from a complex interaction between leukocytes and stromal cells. Synovial hyperplasia is a hallmark of RA, and synovial fibroblasts from RA patients have been shown to recruit and prolong the survival of leukocytes (268). Although macrophages were identified as a key player in the generation of arthritis in DKO mice, we questioned whether stromal cells also contributed to the arthritic and autoimmune phenotypes. Therefore, we generated a series of adoptive transfer experiments designed to clarify the role of radioresistant stromal cells and bone marrow derived donor cells in sensing DNA and contributing to autoimmunity.

Bone marrow chimeras were made in which neither donor nor recipient cells expressed IFNaR, and we found that the absence of DNase in *both* recipient and donor cells is needed for inflammation in the joints, splenomegaly and extramedullary hematopoiesis in the spleen, granulocytosis in the bone marrow and spleen, altered B cell development, and autoantibody production. The relative contributions of radioresistant stromal cells and sensitive hematopoietic cells to these phenotypes were previously unknown. Through the use of Unc93 TKO bone marrow chimeras, we further showed that endosomal TLR signaling in hematologic donor cells is required for splenomegaly, ANA production, and bone marrow infiltration of inflammatory cells. Nevertheless,

DNase II$^{-/-}$ stromal cells are absolutely required for the expression of these TLR-dependent disorders. These studies add new insights into the cells responsible for DNA sensor signaling during disease and suggest that both hematopoietic and stromal host cells play significant roles in DNA-driven autoimmunity. Moreover, these results suggest that therapeutics should target not only hematopoietic but also stromal elements in the setting of inflammatory arthritis. The design of therapeutics that target stromal compartments may address the unmet needs of RA patient populations.

Radioresistant stromal cells have previously been reported to initiate inflammation in another model of DNA-driven autoimmunity. Stetson and colleagues reported that in Trex1-deficient mice, endogenous retroelements accumulate and trigger STING-dependent cytosolic sensors, resulting in autoimmune myocarditis (90, 216, 269). By using a Trex1$^{-/-}$ IFN-reporter line, they also identified that autoimmunity initiates in non-hematopoietic cells, specifically in cardiac endothelial cells (218). Trex1 deficiency in stromal cells was sufficient to activate Wt T and B lymphocytes and trigger a systemic response. In contrast, our data show that DKO mice require both stromal and hematopoietic cells to drive joint inflammation. Another difference between these two models is that the Trex1-deficient phenotype is dependent on type I IFNs; whereas, both the STING-dependent and Unc93-dependent aspects of our DKO mice are type I IFN independent due to the absence of a functional IFNaR. The chimera data presented in Chapter III were thus created in a system where type I IFN signaling is absent. The immune system can be significantly affected by IFNaR signaling and type I IFNs are critical factors induced in response to DNA sensing (270, 271). Thus, it may be of

interest to generate DKO->WT bone marrow chimeras. In this situation, the radioresistant host cells can respond to the IFN produced by the DKO hematopoietic cells, and the potential contribution of type I IFNs can be explored in this scenario.

The activated hematopoietic cell that contributes to inflammation in this model is likely the macrophage since DNase II is required for the degradation of DNA phagocytosed by myeloid lineage cells (98, 100). However, the radioresistant cell(s) that contributes to inflammation in DKO mice remains to be determined. Synovial fibroblasts play a key role in the pathogenesis of RA. They are a source of pro-inflammatory cytokines and promote the survival and retention of leukocytes in the inflamed joint (101). Therefore, it is likely that synovial fibroblasts play a critical role in the initiation and perpetuation of disease in our model. The generation of DKO->STING TKO and STING TKO->DKO bone marrow chimeras would determine whether or not STING-dependent cytosolic sensors detect DNA and initiate inflammation in radioresistant cells, hematopoietic cells, or both. Although we assume that the radioresistant cell is a stromal component, we cannot rule out a role for embryonally-derived macrophages. These are radioresistant tissue resident macrophages that home to peripheral tissues during fetal development, and could reside in synovial tissues (224).

Lastly, the research in chapter IV demonstrates a novel role for cytosolic DNA sensing pathways in bone. Innate immune PRRs have largely been studied in macrophages and dendritic cells in the context of inflammation (272); however, the impact of these pathways on bone remodeling had not been previously evaluated. Using the DNase II/IFNaR DKO model of inflammatory arthritis, we demonstrate that DNA

accumulation in cytosolic compartments promotes the activation of STING-dependent cytosolic sensors, leading to trabecular bone accrual in the long bones over time and ectopic bone formation in the spleens. This occurs despite the production of pro-inflammatory cytokines in this model, which would induce bone loss. CFU assays and bone histomorphometry demonstrate a predominant role for osteoblasts in this phenotype. Moreover, deficiency of the STING pathway significantly inhibits this bone accumulation. These findings are the first to demonstrate that DNA accrual can promote ectopic bone formation, a novel and likely significant finding. This work also reveals a role for the STING pathway in bone and may unveil new targets for the treatment of bone disorders.

The STING pathway is essential for the manifestation of the bone phenotype in the long bones and spleen of DKO mice. Assuming that there is a DNA trigger, it would be of interest to identify the relevant receptor that is leading to this bone accrual. Cytosolic sensors that activate STING include cGAS, IFI16 (or its mouse orthologue p204), DDX41, and DAI (79-83). Evaluating the bone phenotype of DKO mice intercrossed to mice deficient in one of these receptors would therefore be of interest. Of these receptors, p204 is the only DNA sensor that has been linked to bone. Interestingly, apart from its role as a DNA sensor, p204 has also been shown to act as a transcriptional coactivator for runt-related transcription factor 2 (Runx2), an essential transcription factor for osteoblast differentiation (237, 238). Additionally, p204 has been shown to release Runx2 from inhibitor of differentiation (Id) proteins (239). Specifically, p204 binds Id proteins and transports them from the nucleus to the cytosol. By removing Id

proteins from the nucleus, p204 allows Runx2 to bind its target genes. Thus, p204 is a likely sensor that may be leading to the bone accumulation in the DKO model. Given the in vitro studies mentioned above, p204 may also play a role in normal bone homeostasis. In this regard, it would be interesting to evaluate the bone phenotype in p204-deficient mice. cGAS is another DNA sensor that may contribute to the DKO bone phenotype. Inhibition of cGAS in DNase II-deficient mice has been recently shown to prevent the inflammatory arthritis associated with this model (260). Thus, cGAS detects DNA in DKO mice, triggering inflammatory pathways and potentially bone formation. This work suggests that innate immune pathways regulate bone remodeling in autoimmune settings on the DKO background. These same pathways may also play a role in normal bone homeostasis. It would thus be of interest to analyze the bone phenotypes in STING-deficient, p204-deficient, and cGAS-deficient mice.

The bone phenotype in DKO mice manifests late, beginning at approximately 5-6 months in the long bones, and not appearing in the spleens until nearly 10 months. By 20 months of age, the marrow space in the long bones is almost completely replaced by bone and large islands of bone have formed in nearly every area of the spleen. This late manifestation is likely due to the requirement for accrual of DNA over time. With aging, there are more stressed, damaged, and dying cells that release DNA, which then can be displayed on the surface of apoptotic cells and subsequently endocytosed, triggering PRRs (273). In addition, the replication of endogenous retroelements could provide a source of DNA that accrues with aging (57, 216). Another source of endogenous DNA in DKO mice is from extruded erythroid nuclei. During erythropoiesis, nuclei are expelled

from late-stage erythroid precursor cells (98). These nuclei are then engulfed by phagocytic cells, including macrophages and dendritic cells, and digested by DNase II. In DKO mice, erythropoiesis occurs in both the bone marrow and spleen. In fact, the splenomegaly in these mice is largely due to the expansion of $Ter119^{+}$ erythroid precursor cells. Interestingly, macrophages in DKO mice in the bone marrow and spleen, two sites of erythropoiesis and bone accrual, have previously been shown to carry undigested DNA from engulfment of extruded erythroid nuclei or apoptotic bodies (202). However, macrophages in the joint synovium did not carry the apoptotic cells and osteoclast-mediated bone loss occurs locally within joints. This difference may be due to lack of expression by joint-based macrophages of the required receptors for recognition of apoptotic cells, including Tim4 (244). Thus, aberrant bone formation may occur specifically in the long bones and spleen due to the accrual of DNA over time at sites of erythropoiesis.

The ectopic bone formation in the spleen of DKO mice requires the presence of mesenchymal osteoblast precursor cells that either reside in splenic tissue or are recruited to this site. The local production of osteogenic factors in DKO spleens likely induces the differentiation of the mesenchymal precursors into osteoblasts. One possible mesenchymal precursor within the spleen is the pericyte. Pericytes wrap around endothelial cells of capillaries and have impressive progenitor cell-like features. It has been shown that pericytes are capable of giving rise to multiple cellular lineages including osteoblasts (246). Furthermore, these cells can secrete components of bone matrix including bone sialoprotein, osteocalcin, osteonectin, and osteopontin, all of which

are highly upregulated in the spleens of DKO mice compared to controls. Since pericytes may be isolated from tissue using $CD146^{+}$ $CD34^{-}$ $CD45^{-}$ $CD56^{-}$ markers (274), it may be of interest to isolate and culture pericytes in supernants from DKO spleens to determine whether these cells are driven toward the osteoblast lineage. In any case, future studies investigating the mesenchymal precursor cell in DKO spleens are warranted.

Our study suggests that DNA accrual drives bone formation through STING-dependent cytosolic sensors. It would be valuable to evaluate other models of DNA accrual or models of STING activation for similar bone phenotypes. The Trex1-deficient mouse is a model of DNA accrual induced by deficiency of the cytosolic endonuclease DNase III (i.e. Trex1). In this mouse, DNA activates cGAS and the STING pathway leading to robust type I IFN production and inflammatory myocarditis (90, 260, 269). The bone phenotype of these mice has yet to be evaluated. Since Trex1-deficient mice exhibit reduced postnatal survival, it may be difficult to identify a bone phenotype, if the phenotype manifests late as it does in DKO mice, since Trex1-deficient mice rarely survive past 4 months of age. Recently, gain-of-function mutations in *Tmem173*, the gene encoding STING, were shown to cause the dimerization and constitutive activation of STING, leading to vasculopathy and pulmonary inflammation in a syndrome now called SAVI (97). Since activation of the STING pathway in DKO mice leads to bone accrual, it may be worthwhile to examine the bones of SAVI patients by micro-CT scanning. It is likely, however, that patients with SAVI are being treated with prednisone or other anti-inflammatory therapies that may affect bone remodeling in these subjects. A major difference between the DKO model of autoimmunity and the SAVI and Trex1-/-

syndromes is the dependency on type I IFN signaling. The DKO mice used in our studies, and their Het controls, both lack type I IFN signaling due to the absence of IFNaR. Type 1 IFNs, which signal through the IFNaR, inhibit osteoclastogenesis by reducing the expression of c-Fos, a transcription factor involved in the formation of osteoclasts (232). Accordingly, IFNaR-deficient mice show enhanced osteoclastogenesis and reduced trabeculae in the long bones (with no significant differences in osteoblast activity or number). Thus, the bone phenotype in other models of DNA accrual, in which type I IFN signaling is intact, may differ from the phenotype seen in DKO mice.

The bone phenotype in DKO mice is likely related to human diseases in which abnormal bone formation occurs in soft tissues. Heterotopic ossification (HO) is a debilitating condition associated with formation of lamellar bone in extra-skeletal sites. Acquired HO occurs in cases of soft tissue trauma including amputation, joint replacement surgery, and traumatic brain and spinal cord injuries (185). The etiology and pathogenesis of acquired HO is unknown and treatments aim to limit the associated inflammation. Hereditary HO is seen in the rare genetic disorder fibrodysplasia ossificans progressiva (FOP). In the case of FOP, gain-of-function mutations in the ACVR1 gene contribute to this disorder, leading to enhanced BMP signaling (248). Despite the presence of ACVR1 mutations, FOP patients exhibit variability in the severity and progression of their disease, and form bone episodically, rather than continuously, following viral infections, immunizations, or tissue trauma (184, 193, 249). These triggers often precede ectopic bone formation and strongly implicate a role for inflammatory innate immune pathways in the pathogenesis of HO. Our data suggest that

it is likely that viral DNA or DNA released from trauma/damaged cells may overwhelm the activity of DNase II, triggering the activation of innate immune DNA sensors. In this context, the activation of these receptors may lead to the release of mediators that induce the differentiation of mesenchymal precursors to bone-forming osteoblasts. It would be of interest to analyze models of HO for activation of the STING pathway. If this pathway is indeed activated, the administration of suppressive oligonucleotides, that block cytosolic sensor signaling, should alleviate inflammation and inhibit the formation of new heterotopic lesions. Further investigation into these mechanisms may provide novel pathways for the prevention and treatment of bone remodeling in autoimmune disease and in heterotopic bone formation.

In conclusion, this dissertation demonstrates that the inappropriate activation of cytosolic and endosomal receptors by endogenous DNA contributes to a wide variety of autoimmune manifestations and dysregulated bone remodeling (**Figure 5**). Our findings reveal an important role for the STING and AIM2 pathways in arthritis and for endosomal TLRs in autoantibody production. Furthermore, these data establish that both stromal and hematopoietic cells are required for all aspects of DNA-driven autoimmunity. Lastly, our data reveal a role for the STING pathway in bone and demonstrate that DNA accrual promotes ectopic bone formation through cytosolic DNA sensors, a novel and likely significant finding. The discovery of new pathways relevant to inflammatory arthritis and bone disorders will provide critical insights that may expand the potential targets available for the treatment of these diseases.

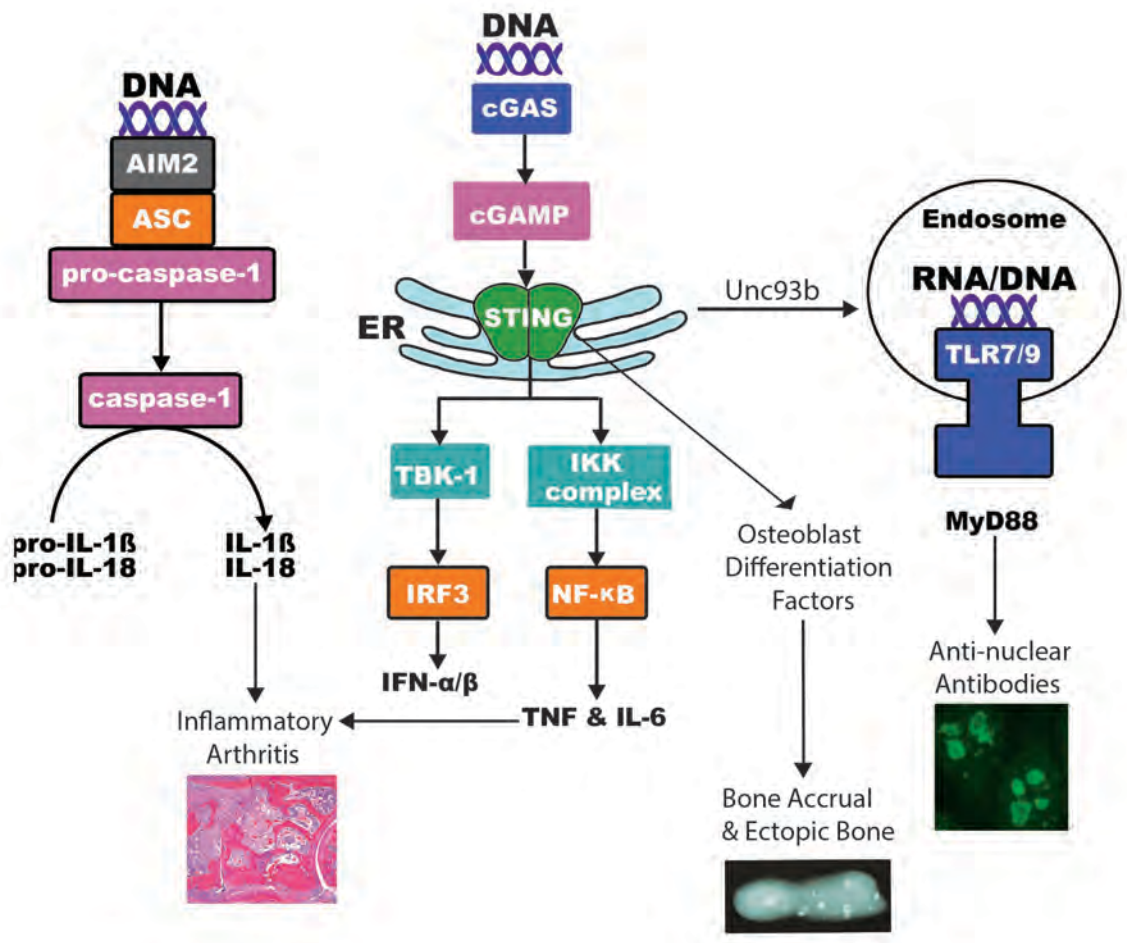


Figure 5. Cytosolic and endosomal DNA-sensing pathways differentially regulate inflammatory arthritis, ANA production, and aberrant bone remodeling in DKO mice. Endogenous DNA is detected by a number of sensors that signal through the adaptor STING including cGAS, leading to the production of type 1 IFNs and pro-inflammatory cytokines. Alternatively, DNA detection by AIM2 leads to the formation of a caspase-1 activating inflammasome that cleaves pro-IL-1β and pro-IL-18 into their active forms. Another pathway for DNA detection is through endosomal TLRs, including TLR7 and 9, which traffic from the endoplasmic reticulum (ER) to endosomes via

Unc93b. In the DKO model of DNA accrual, activation of the STING and AIM2 pathways leads to the production of pro-inflammatory cytokines and an erosive inflammatory arthritis. Alternatively, DNA detection by endosomal TLRs results in production of anti-nuclear antibodies. The STING pathway also leads to the production of osteoblast differentiation factors that promote bone accrual in the long bones and ectopic bone formation in spleens.

APPENDIX

AIM2 and STING pathways differentially regulate bone homeostasis

Introduction

Innate immune PRRs are known to sense nucleic acid from microbial organisms and trigger the production of type 1 IFNs and pro-inflammatory cytokines IL-1, IL-6, and TNF to resolve infection. Various cytosolic DNA receptors signal through an ER-associated protein, STING. Cytosolic sensors that activate STING include IFI16/p204, cGAS, and DDX41 (79, 81-83). Also relevant are STING-independent cytosolic sensors such as AIM2. Upon detection of DNA, AIM2 orchestrates the assembly of an inflammasome complex, resulting in the cleavage of pro-IL-1β and pro-IL-18 (84, 253). Inappropriate activation of cytosolic DNA sensor pathways by endogenous DNA has recently been associated with autoimmune disease (57, 97). Possible endogenous sources of nucleic acid include DNA from stressed, damaged or dying cells, DNA derived from the replication of endogenous retroelements, mitochondrial DNA, and oxidized DNA that is resistant to degradation by cellular DNases (275). Importantly, these sources of endogenous DNA are known to accrue with aging. Despite the emerging importance of cytosolic DNA sensor pathways in autoimmunity, little is known about their role in cell types other than macrophages and dendritic cells. Data from our laboratory was the first to demonstrate an important role for STING-dependent cytosolic sensor pathways in bone in the context of autoimmunity. We now show that the STING and AIM2 pathways differentially regulate bone under normal homeostatic conditions.

The first indication of a role for cytosolic DNA sensors in bone came from our observations in DNase II/IFNaR DKO mice. In this model, DNase II deficiency results in endogenous DNA accrual that activates cytosolic DNA sensors, resulting in the

continuous production of type 1 IFNs and pro-inflammatory cytokines. Because production of type 1 IFNs in these mice leads to anemia-driven embryonic lethality, the gene for the type I interferon receptor (IFNaR) was deleted in this model. DKO mice develop a distal and erosive polyarthritis, resulting from constant production of pro-inflammatory cytokines. We anticipated that these arthritic mice would lose bone systemically since osteopenia/osteoporosis typically accompanies inflammatory arthritis. Surprisingly, we found a striking accrual of bone in the long bones of DKO mice with aging. Trabecular bone accrual was first identified around 5-6 months and by 16 months of age the bone accrual almost completely replaced the marrow space. Interestingly, by 10 months of age, we identified ectopic bone in DKO spleens that continued to accrue by 24 months. Moreover, STING-deficiency abrogated the bone accrual in DKO mice. This study was the first to reveal a role for STING in bone remodeling and to demonstrate that endogenous DNA accrual promotes bone formation over time. Based on this work, we questioned whether innate immune pathways regulate bone remodeling not only in autoimmune settings, but also during normal bone homeostasis. We now demonstrate that the STING and AIM2 pathways are involved in the homeostatic regulation of bone.

Materials and Methods

Micro–computed Tomography (micro-CT): Femurs were fixed in 4% paraformaldehyde for 48 hours, transferred to 70% ethanol, then imaged at the Musculoskeletal Imaging Core at UMMS using a Scanco Medical μCT 40 at 70kVp and 114μA with resolutions of 10μm. Analyses include trabecular bone within the entire femur from the proximal to distal growth plates and a 0.5 mm section in the central diaphysis of cortical bone. The segmentation parameters include the values: 0.8 Gauss sigma, 1.0 Gauss support, and a threshold of 220-1000 Hounsfield units (density range of >600mg of HA/cm^3).

Osteoclast Differentiation and Resorption Assays: For osteoclast assays, cell culture experiments were performed in α-MEM containing 10% FBS, 100U penicillin and 100μg/ml streptomycin. Cells were flushed from the bone marrow and differentiated in 40ng/ml of M-CSF (R&D) for 4 days. For osteoclast differentiation, osteoclast precursors were seeded at a density of 6,000 cells/well on 96-well plates and differentiated in medium containing 20ng/ml of M-CSF and 10ng/ml of RANKL (R&D) for 5 days. Half of the medium was replaced with fresh medium/cytokines on day 3. On day 5 of differentiation, the cells were fixed and stained for TRAP5 using a leukocyte acid phosphatase kit (Sigma). TRAP-stained osteoclasts with 3-10 nuclei were counted. For osteoclast resorption, osteoclast precursors were seeded at a density of 15,000 cells/well on 24-well hydroxyapatite-coated Osteo Assay plates (Corning) and differentiated in α-

MEM containing 40ng/ml of M-CSF and 20ng/ml of RANKL (R&D) for 10 days. Half of the medium was replaced with fresh medium/cytokines every two days. Cells were then removed with 10% bleach and wells were rinsed in water and air-dried overnight. The wells were scanned on a flatbed scanner (Microtek 9800 XL) and the percentage of resorbed area was analyzed using NIH ImageJ software.

Statistical analysis: Statistical significance was analyzed with the unpaired, two-tailed Student's t test. Data are presented as the mean ± SEM. Statistical significance is represented by the following notation in the figures: $p<0.05$=*, $p<0.01$=**, and $p<0.001$=***.

Results

To investigate the individual roles of STING and AIM2 in bone homeostasis, we analyzed the femurs in STING-/- and AIM2-/- mice by micro-CT scanning. We found that female STING-/- mice show osteopenia at 6 months of age compared with matched controls (**Figure A.1**). This is of considerable significance, as no other phenotype has been identified in STING-/- mice, apart from an abnormal response to viral challenge and treatment with dsDNA. In contrast, femurs from female AIM2-/- mice at 6 months of age demonstrate significant bone accrual (**Figure A.2A**). Quantitation of micro-CT parameters confirms this phenotype, showing a significant increase in trabecular bone volume/total volume (BV/TV), trabecular surface, and cortical thickness in AIM2-/- mice compared to controls (**Figure A.2B**).

Interestingly, this bone phenotype appears earlier in female mice compared to male mice. Femurs from male AIM2-/- mice at 6 months of age show no difference in trabecular or cortical parameters compared to controls (**Figure A.2C&D**). However, over time by 13.5 months of age, the male AIM2-/- mice demonstrate increased trabecular and cortical bone compared to Wt controls (**Figure A.2E&F**). Thus, the bone phenotype is more dramatic in female mice and becomes more apparent with aging. Our preliminary data suggest that the bone phenotype in AIM2-/- mice is due to decreased osteoclast resorption, as shown by a significant difference in resorption on hydroxyapatite-coated plates between AIM2-/- and Wt osteoclasts (**Figure A.3A**). No difference in osteoclast differentiation was detected in AIM2-/- and Wt mice (**Figure A.3B**).

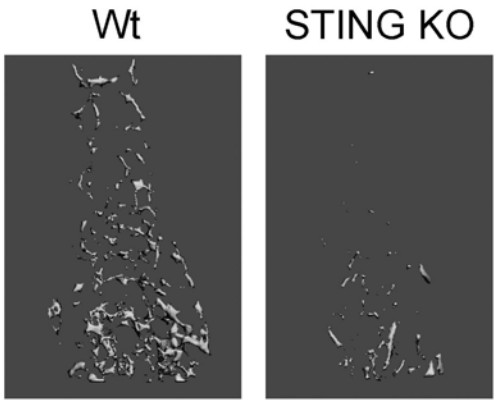


Figure A.1. STING-deficiency leads to trabecular bone loss. Micro-CT images of trabecular bone in femurs from female mice at 6 months of age (n=2 mice/genotype).

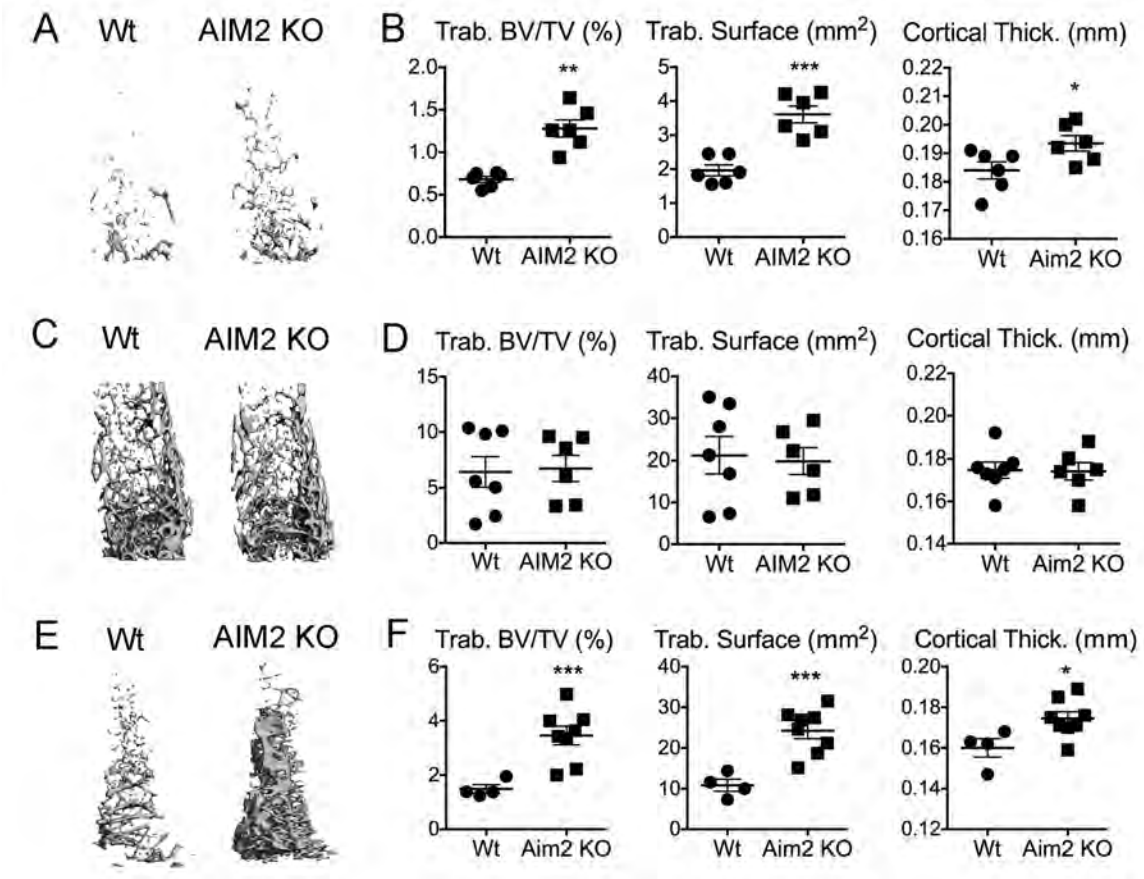


Figure A.2. AIM2-deficiency promotes trabecular and cortical bone accrual with aging. Micro-CT images of trabecular bone in femurs and quantitation of micro-CT data for trabecular bone volume per total volume (BV/TV), trabecular bone surface, and cortical thickness from **A&B**) 6 month-old female mice (n=6 mice/genotype), **C&D**) 6 month-old male mice (n=6-7 mice/genotype), and **E&F**) 13.5 month-old male mice (n=4-8 mice/genotype). Values are the mean ± SEM compared to Wt: *=p<0.05, **=p<0.01, ***=p<0.001.

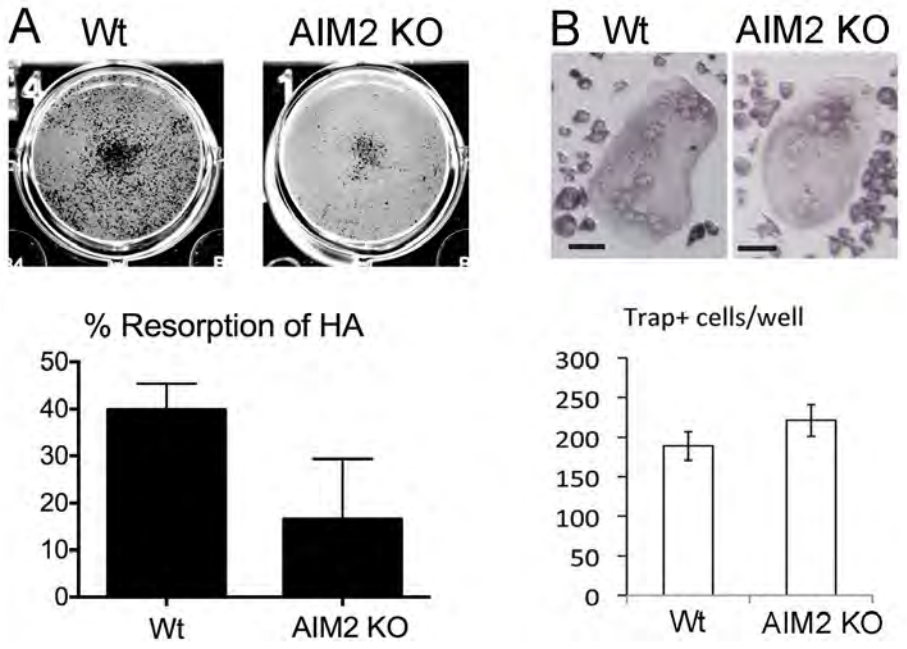


Figure A.3. Decreased osteoclast resorption in AIM2-deficient mice. A) Representative image of resorption well (hydroxyapatite in white) and percentage of hydroxyapatite area resorbed by osteoclasts. **B**) Representative image of multinucleated osteoclasts and number of differentiated Trap+ osteoclasts. 2 month-old female mice (n=2 mice/genotype).

Discussion

These results demonstrate that the STING and AIM2 pathways differentially modulate bone remodeling under homeostatic conditions. This data supports our previous research in which we show that loss of the STING pathway abrogates bone accrual in a model of DNA-driven autoimmune disease. While our preliminary data demonstrate that STING and AIM2 clearly have opposite effects on bone, the cell types and mechanisms involved are unknown. Under homeostatic conditions, it is likely that endogenous DNA accumulates with time and activates innate immune cytosolic receptors. This concept could be highly relevant to bone loss in aging, where DNA from apoptotic cells, oxidized DNA that is resistant to nucleases, and endogenous retroelements accumulate over time (275). It would thus be important to evaluate the bone phenotype of STING-/- mice, AIM2-/- mice, and their littermate controls at 2 months, 6 months, 8 months, and 13 months of age to confirm whether the bone phenotype becomes more prominent with time. It would also be valuable to identify whether apoptotic cells and/or retroelements increase over time in these mice.

Our preliminary data in AIM2-/- mice suggest that the bone phenotype is a reflection of decreased osteoclast resorption. Since STING and AIM2 seem to have differential effects on bone, it is likely that STING-deficiency results in increased osteoclast function. Further studies are needed to determine if osteoclast resorption is indeed affected in these mice and whether osteoblast function is at all disrupted. If the osteoclast is the key cell type involved, it would be important to determine whether cytosolic sensors are expressed in osteoclasts and whether endogenous DNA accrues over

time in these cell types. It is likely that cytosolic sensors are expressed in osteoclasts since these pathways have been extensively studied in their monocyte/macrophage precursors (78).

Since IL-1β is a pro-osteoclastogenic cytokine (132), activation of AIM2 may promote bone resorption through inflammasome-mediated IL-1β production, whereas AIM2 deficiency would limit bone resorption. To demonstrate an inflammasome-dependent role for AIM2, it would be important to perform osteoclast differentiation and functional assays in the presence or absence of an IL-1β blocking antibody. Caspase-1-/- cells can be used as controls, which will abrogate inflammasome function. These studies form a rationale for the generation of AIM2/IL-1 double knockout mice to study the impact of AIM2 on bone homeostasis via IL-1β. To further determine whether osteoclasts are the cells responsible for this bone phenotype, it may be valuable to generate mice in which AIM2 is deleted solely in osteoclasts, using a Cre-Cathepsin K promoter.

Osteoclast precursors derived from STING-/- mice will likely be more active in resorption, and this difference may be augmented upon exposure of the osteoclast precursors to DNA ligands/apoptotic DNA. The STING pathway is known to be a potent producer of type 1 IFNs. Loss of type 1 IFN, a negative regulator of osteoclastogenesis, could explain the osteopenic phenotype in STING-/- mice (232). Generation of STING/IFNaR and/or STING/IRF3 double knockout mice would be valuable for studying the impact of STING on bone homeostasis via type 1 IFNs. It will also be important to evaluate the bone phenotype of cGAS-/- and p204-/- mice, as these cytosolic receptors may be responsible for sensing the endogenous DNA upstream of STING.

For all animal models suggested, micro-CT scanning of aging mice should be performed, followed by static histomorphometry analyses for osteoclast and osteoblast numbers, and analysis of bone turnover markers including Trap5b (osteoclast number), CTX-1 (osteoclast function), and osteocalcin (osteoblast function). It is possible that the phenotypes seen in STING-/- and AIM2-/- mice result from changes in osteoblast differentiation or function. To explore this possibility, calvarial osteoblasts can be isolated from STING-/-, AIM2-/-, and control mice and cultured in osteogenic medium containing ascorbic acid and β-glycerophosphate for 28 days. Changes in osteoblast differentiation can be monitored by protein and mRNA expression for alkaline phosphatase and von Kossa.

Conceptually, this preliminary data explores the novel hypothesis that cytosolic DNA sensing pathways play a role in the homeostatic regulation of bone. We hypothesize that the AIM2 and STING pathways are activated during aging as a result of accumulation of endogenous DNA in cells. We further hypothesize that these pathways differentially regulate osteoclast function. These studies explore an entirely new link between the immune system and bone, and may reveal new anabolic pathways to build bone and novel targets for the treatment of bone loss in aging.

Bibliography

1. Picerno V, *et al.* (2015) One year in review: the pathogenesis of rheumatoid arthritis. *Clinical and experimental rheumatology* 33(4):551-558.
2. Townsend MJ (2014) Molecular and cellular heterogeneity in the Rheumatoid Arthritis synovium: clinical correlates of synovitis. *Best practice & research. Clinical rheumatology* 28(4):539-549.
3. McInnes IB, Buckley CD, & Isaacs JD (2016) Cytokines in rheumatoid arthritis - shaping the immunological landscape. *Nature reviews. Rheumatology* 12(1):63-68.
4. Jimi E, *et al.* (1999) Interleukin 1 induces multinucleation and bone-resorbing activity of osteoclasts in the absence of osteoblasts/stromal cells. *Experimental cell research* 247(1):84-93.
5. Azuma Y, Kaji K, Katogi R, Takeshita S, & Kudo A (2000) Tumor necrosis factor-alpha induces differentiation of and bone resorption by osteoclasts. *The Journal of biological chemistry* 275(7):4858-4864.
6. Lubberts E, *et al.* (2003) IL-17 promotes bone erosion in murine collagen-induced arthritis through loss of the receptor activator of NF-kappa B ligand/osteoprotegerin balance. *Journal of immunology* 170(5):2655-2662.
7. Walsh NC, *et al.* (2009) Osteoblast function is compromised at sites of focal bone erosion in inflammatory arthritis. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 24(9):1572-1585.
8. Baum R & Gravallese EM (2015) Bone as a Target Organ in Rheumatic Disease: Impact on Osteoclasts and Osteoblasts. *Clinical reviews in allergy & immunology*.
9. Patel R, Filer A, Barone F, & Buckley CD (2014) Stroma: fertile soil for inflammation. *Best practice & research. Clinical rheumatology* 28(4):565-576.
10. Joshi VR (2012) Rheumatology, past, present and future. *The Journal of the Association of Physicians of India* 60:21-24.
11. Entezami P, Fox DA, Clapham PJ, & Chung KC (2011) Historical perspective on the etiology of rheumatoid arthritis. *Hand clinics* 27(1):1-10.
12. Zampeli E, Vlachoyiannopoulos PG, & Tzioufas AG (2015) Treatment of rheumatoid arthritis: Unraveling the conundrum. *Journal of autoimmunity* 65:1-18.
13. Chang C (2014) Unmet needs in the treatment of autoimmunity: from aspirin to stem cells. *Autoimmunity reviews* 13(4-5):331-346.
14. Moller Dohn U, *et al.* (2009) Erosive progression is minimal, but erosion healing rare, in patients with rheumatoid arthritis treated with adalimumab. A 1 year investigator-initiated follow-up study using high-resolution computed tomography as the primary outcome measure. *Annals of the rheumatic diseases* 68(10):1585-1590.
15. Dohn UM, *et al.* (2011) No overall progression and occasional repair of erosions despite persistent inflammation in adalimumab-treated rheumatoid arthritis

patients: results from a longitudinal comparative MRI, ultrasonography, CT and radiography study. *Annals of the rheumatic diseases* 70(2):252-258.

16. Nam JL, *et al.* (2014) Efficacy of biological disease-modifying antirheumatic drugs: a systematic literature review informing the 2013 update of the EULAR recommendations for the management of rheumatoid arthritis. *Annals of the rheumatic diseases* 73(3):516-528.
17. Cush JJ & Lipsky PE (1988) Phenotypic analysis of synovial tissue and peripheral blood lymphocytes isolated from patients with rheumatoid arthritis. *Arthritis and rheumatism* 31(10):1230-1238.
18. Cope AP (2002) Studies of T-cell activation in chronic inflammation. *Arthritis research* 4 Suppl 3:S197-211.
19. Chabaud M, *et al.* (1999) Human interleukin-17: A T cell-derived proinflammatory cytokine produced by the rheumatoid synovium. *Arthritis and rheumatism* 42(5):963-970.
20. Yang J, Sundrud MS, Skepner J, & Yamagata T (2014) Targeting Th17 cells in autoimmune diseases. *Trends in pharmacological sciences* 35(10):493-500.
21. Shevach EM (2009) Mechanisms of foxp3+ T regulatory cell-mediated suppression. *Immunity* 30(5):636-645.
22. Valencia X, *et al.* (2006) TNF downmodulates the function of human CD4+CD25hi T-regulatory cells. *Blood* 108(1):253-261.
23. Panayi GS (2006) Even though T-cell-directed trials have been of limited success, is there reason for optimism? *Nature clinical practice. Rheumatology* 2(2):58-59.
24. Kremer JM, *et al.* (2003) Treatment of rheumatoid arthritis by selective inhibition of T-cell activation with fusion protein CTLA4Ig. *The New England journal of medicine* 349(20):1907-1915.
25. Schroder AE, Greiner A, Seyfert C, & Berek C (1996) Differentiation of B cells in the nonlymphoid tissue of the synovial membrane of patients with rheumatoid arthritis. *Proceedings of the National Academy of Sciences of the United States of America* 93(1):221-225.
26. Humby F, *et al.* (2009) Ectopic lymphoid structures support ongoing production of class-switched autoantibodies in rheumatoid synovium. *PLoS medicine* 6(1):e1.
27. Aho K, Heliovaara M, Maatela J, Tuomi T, & Palosuo T (1991) Rheumatoid factors antedating clinical rheumatoid arthritis. *The Journal of rheumatology* 18(9):1282-1284.
28. Rantapaa-Dahlqvist S, *et al.* (2003) Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. *Arthritis and rheumatism* 48(10):2741-2749.
29. Steiner G & Smolen J (2002) Autoantibodies in rheumatoid arthritis and their clinical significance. *Arthritis research* 4 Suppl 2:S1-5.
30. Arkema EV, *et al.* (2013) Anti-citrullinated peptide autoantibodies, human leukocyte antigen shared epitope and risk of future rheumatoid arthritis: a nested case-control study. *Arthritis research & therapy* 15(5):R159.
31. Jilani AA & Mackworth-Young CG (2015) The role of citrullinated protein antibodies in predicting erosive disease in rheumatoid arthritis: a systematic

literature review and meta-analysis. *International journal of rheumatology* 2015:728610.

32. Kleyer A, *et al.* (2014) Bone loss before the clinical onset of rheumatoid arthritis in subjects with anticitrullinated protein antibodies. *Annals of the rheumatic diseases* 73(5):854-860.
33. Yukawa N, *et al.* (2011) Correlation of antinuclear antibody and anti-double-stranded DNA antibody with clinical response to infliximab in patients with rheumatoid arthritis: a retrospective clinical study. *Arthritis research & therapy* 13(6):R213.
34. Edwards JC, *et al.* (2004) Efficacy of B-cell-targeted therapy with rituximab in patients with rheumatoid arthritis. *The New England journal of medicine* 350(25):2572-2581.
35. Mohr W & Menninger H (1980) Polymorphonuclear granulocytes at the pannus-cartilage junction in rheumatoid arthritis. *Arthritis and rheumatism* 23(12):1413-1414.
36. Nemeth T & Mocsai A (2012) The role of neutrophils in autoimmune diseases. *Immunology letters* 143(1):9-19.
37. Wright HL, Moots RJ, & Edwards SW (2014) The multifactorial role of neutrophils in rheumatoid arthritis. *Nature reviews. Rheumatology* 10(10):593-601.
38. Maruotti N, Crivellato E, Cantatore FP, Vacca A, & Ribatti D (2007) Mast cells in rheumatoid arthritis. *Clinical rheumatology* 26(1):1-4.
39. Hueber AJ, *et al.* (2010) Mast cells express IL-17A in rheumatoid arthritis synovium. *Journal of immunology* 184(7):3336-3340.
40. Sawamukai N, *et al.* (2010) Mast cell-derived tryptase inhibits apoptosis of human rheumatoid synovial fibroblasts via rho-mediated signaling. *Arthritis and rheumatism* 62(4):952-959.
41. Haringman JJ, *et al.* (2005) Synovial tissue macrophages: a sensitive biomarker for response to treatment in patients with rheumatoid arthritis. *Annals of the rheumatic diseases* 64(6):834-838.
42. Campbell IK, *et al.* (1998) Protection from collagen-induced arthritis in granulocyte-macrophage colony-stimulating factor-deficient mice. *Journal of immunology* 161(7):3639-3644.
43. Cornish AL, Campbell IK, McKenzie BS, Chatfield S, & Wicks IP (2009) G-CSF and GM-CSF as therapeutic targets in rheumatoid arthritis. *Nature reviews. Rheumatology* 5(10):554-559.
44. Campbell IK, Rich MJ, Bischof RJ, & Hamilton JA (2000) The colony-stimulating factors and collagen-induced arthritis: exacerbation of disease by M-CSF and G-CSF and requirement for endogenous M-CSF. *Journal of leukocyte biology* 68(1):144-150.
45. Li J, Hsu HC, & Mountz JD (2012) Managing macrophages in rheumatoid arthritis by reform or removal. *Current rheumatology reports* 14(5):445-454.
46. Smolen JS, *et al.* (2006) Predictors of joint damage in patients with early rheumatoid arthritis treated with high-dose methotrexate with or without

concomitant infliximab: results from the ASPIRE trial. *Arthritis and rheumatism* 54(3):702-710.

47. Nishimoto N, *et al.* (2007) Study of active controlled monotherapy used for rheumatoid arthritis, an IL-6 inhibitor (SAMURAI): evidence of clinical and radiographic benefit from an x ray reader-blinded randomised controlled trial of tocilizumab. *Annals of the rheumatic diseases* 66(9):1162-1167.
48. Catrina AI, *et al.* (2005) Evidence that anti-tumor necrosis factor therapy with both etanercept and infliximab induces apoptosis in macrophages, but not lymphocytes, in rheumatoid arthritis joints: extended report. *Arthritis and rheumatism* 52(1):61-72.
49. Jimenez-Dalmaroni MJ, Gerswhin ME, & Adamopoulos IE (2016) The critical role of toll-like receptors - From microbial recognition to autoimmunity: A comprehensive review. *Autoimmunity reviews* 15(1):1-8.
50. Albani S, Tuckwell JE, Esparza L, Carson DA, & Roudier J (1992) The susceptibility sequence to rheumatoid arthritis is a cross-reactive B cell epitope shared by the Escherichia coli heat shock protein dnaJ and the histocompatibility leukocyte antigen DRB10401 molecule. *The Journal of clinical investigation* 89(1):327-331.
51. Rashid T & Ebringer A (2007) Rheumatoid arthritis is linked to Proteus--the evidence. *Clinical rheumatology* 26(7):1036-1043.
52. Sawada S & Takei M (2005) Epstein-Barr virus etiology in rheumatoid synovitis. *Autoimmunity reviews* 4(2):106-110.
53. Wegner N, *et al.* (2010) Peptidylarginine deiminase from Porphyromonas gingivalis citrullinates human fibrinogen and alpha-enolase: implications for autoimmunity in rheumatoid arthritis. *Arthritis and rheumatism* 62(9):2662-2672.
54. Hitchon CA & El-Gabalawy HS (2011) Infection and rheumatoid arthritis: still an open question. *Current opinion in rheumatology* 23(4):352-357.
55. Goh FG & Midwood KS (2012) Intrinsic danger: activation of Toll-like receptors in rheumatoid arthritis. *Rheumatology* 51(1):7-23.
56. Gehrke N, *et al.* (2013) Oxidative damage of DNA confers resistance to cytosolic nuclease TREX1 degradation and potentiates STING-dependent immune sensing. *Immunity* 39(3):482-495.
57. Volkman HE & Stetson DB (2014) The enemy within: endogenous retroelements and autoimmune disease. *Nature immunology* 15(5):415-422.
58. Clancy RM, Markham AJ, & Buyon JP (2016) Endosomal Toll-like receptors in clinically overt and silent autoimmunity. *Immunological reviews* 269(1):76-84.
59. Savarese E, *et al.* (2008) Requirement of Toll-like receptor 7 for pristane-induced production of autoantibodies and development of murine lupus nephritis. *Arthritis and rheumatism* 58(4):1107-1115.
60. Kono DH, *et al.* (2009) Endosomal TLR signaling is required for anti-nucleic acid and rheumatoid factor autoantibodies in lupus. *Proceedings of the National Academy of Sciences of the United States of America* 106(29):12061-12066.

61. Pawaria S, *et al.* (2015) Cutting Edge: DNase II deficiency prevents activation of autoreactive B cells by double-stranded DNA endogenous ligands. *Journal of immunology* 194(4):1403-1407.
62. Yasuda K, *et al.* (2007) Murine dendritic cell type I IFN production induced by human IgG-RNA immune complexes is IFN regulatory factor (IRF)5 and IRF7 dependent and is required for IL-6 production. *Journal of immunology* 178(11):6876-6885.
63. Jackson SW, *et al.* (2014) Opposing impact of B cell-intrinsic TLR7 and TLR9 signals on autoantibody repertoire and systemic inflammation. *Journal of immunology* 192(10):4525-4532.
64. Pisitkun P, *et al.* (2006) Autoreactive B cell responses to RNA-related antigens due to TLR7 gene duplication. *Science* 312(5780):1669-1672.
65. Graham RR, *et al.* (2007) Three functional variants of IFN regulatory factor 5 (IRF5) define risk and protective haplotypes for human lupus. *Proceedings of the National Academy of Sciences of the United States of America* 104(16):6758-6763.
66. Garcia-Ortiz H, *et al.* (2010) Association of TLR7 copy number variation with susceptibility to childhood-onset systemic lupus erythematosus in Mexican population. *Annals of the rheumatic diseases* 69(10):1861-1865.
67. Alzabin S, *et al.* (2012) Investigation of the role of endosomal Toll-like receptors in murine collagen-induced arthritis reveals a potential role for TLR7 in disease maintenance. *Arthritis research & therapy* 14(3):R142.
68. Chen SY, *et al.* (2012) Suppression of collagen-induced arthritis by intra-articular lentiviral vector-mediated delivery of Toll-like receptor 7 short hairpin RNA gene. *Gene therapy* 19(7):752-760.
69. Roelofs MF, *et al.* (2005) The expression of toll-like receptors 3 and 7 in rheumatoid arthritis synovium is increased and costimulation of toll-like receptors 3, 4, and 7/8 results in synergistic cytokine production by dendritic cells. *Arthritis and rheumatism* 52(8):2313-2322.
70. Isnardi I, *et al.* (2008) IRAK-4- and MyD88-dependent pathways are essential for the removal of developing autoreactive B cells in humans. *Immunity* 29(5):746-757.
71. Stahl EA, *et al.* (2010) Genome-wide association study meta-analysis identifies seven new rheumatoid arthritis risk loci. *Nature genetics* 42(6):508-514.
72. Dawidowicz K, *et al.* (2011) The interferon regulatory factor 5 gene confers susceptibility to rheumatoid arthritis and influences its erosive phenotype. *Annals of the rheumatic diseases* 70(1):117-121.
73. Duffau P, *et al.* (2015) Promotion of Inflammatory Arthritis by Interferon Regulatory Factor 5 in a Mouse Model. *Arthritis & rheumatology* 67(12):3146-3157.
74. Lacerte P, *et al.* (2016) Overexpression of TLR2 and TLR9 on monocyte subsets of active rheumatoid arthritis patients contributes to enhance responsiveness to TLR agonists. *Arthritis research & therapy* 18(1):10.

75. Balandraud N, Roudier J, & Roudier C (2004) Epstein-Barr virus and rheumatoid arthritis. *Autoimmunity reviews* 3(5):362-367.
76. Kuznik A, *et al.* (2011) Mechanism of endosomal TLR inhibition by antimalarial drugs and imidazoquinolines. *Journal of immunology* 186(8):4794-4804.
77. Sharma S & Fitzgerald KA (2011) Innate immune sensing of DNA. *PLoS pathogens* 7(4):e1001310.
78. Bhat N & Fitzgerald KA (2014) Recognition of cytosolic DNA by cGAS and other STING-dependent sensors. *European journal of immunology* 44(3):634-640.
79. Unterholzner L, *et al.* (2010) IFI16 is an innate immune sensor for intracellular DNA. *Nature immunology* 11(11):997-1004.
80. Takaoka A, *et al.* (2007) DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. *Nature* 448(7152):501-505.
81. Zhang Z, *et al.* (2011) The helicase DDX41 senses intracellular DNA mediated by the adaptor STING in dendritic cells. *Nature immunology* 12(10):959-965.
82. Wu J, *et al.* (2013) Cyclic GMP-AMP is an endogenous second messenger in innate immune signaling by cytosolic DNA. *Science* 339(6121):826-830.
83. Sun L, Wu J, Du F, Chen X, & Chen ZJ (2013) Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. *Science* 339(6121):786-791.
84. Rathinam VA, *et al.* (2010) The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. *Nature immunology* 11(5):395-402.
85. Liu X & Wang C (2015) The emerging roles of the STING adaptor protein in immunity and diseases. *Immunology*.
86. Nagata S (2010) Apoptosis and autoimmune diseases. *Annals of the New York Academy of Sciences* 1209:10-16.
87. Casciola-Rosen LA, Anhalt G, & Rosen A (1994) Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. *The Journal of experimental medicine* 179(4):1317-1330.
88. Lan YY, Londono D, Bouley R, Rooney MS, & Hacohen N (2014) Dnase2a deficiency uncovers lysosomal clearance of damaged nuclear DNA via autophagy. *Cell reports* 9(1):180-192.
89. West AP, *et al.* (2015) Mitochondrial DNA stress primes the antiviral innate immune response. *Nature* 520(7548):553-557.
90. Stetson DB, Ko JS, Heidmann T, & Medzhitov R (2008) Trex1 prevents cell-intrinsic initiation of autoimmunity. *Cell* 134(4):587-598.
91. Crow YJ, *et al.* (2006) Mutations in the gene encoding the 3'-5' DNA exonuclease TREX1 cause Aicardi-Goutieres syndrome at the AGS1 locus. *Nature genetics* 38(8):917-920.
92. Crow YJ & Rehwinkel J (2009) Aicardi-Goutieres syndrome and related phenotypes: linking nucleic acid metabolism with autoimmunity. *Human molecular genetics* 18(R2):R130-136.

93. Lee-Kirsch MA, *et al.* (2007) Mutations in the gene encoding the 3'-5' DNA exonuclease TREX1 are associated with systemic lupus erythematosus. *Nature genetics* 39(9):1065-1067.
94. Ramantani G, *et al.* (2010) Expanding the phenotypic spectrum of lupus erythematosus in Aicardi-Goutieres syndrome. *Arthritis and rheumatism* 62(5):1469-1477.
95. Namjou B, *et al.* (2011) Evaluation of the TREX1 gene in a large multi-ancestral lupus cohort. *Genes and immunity* 12(4):270-279.
96. Crow YJ & Manel N (2015) Aicardi-Goutieres syndrome and the type I interferonopathies. *Nature reviews. Immunology* 15(7):429-440.
97. Liu Y, *et al.* (2014) Activated STING in a vascular and pulmonary syndrome. *The New England journal of medicine* 371(6):507-518.
98. Nagata S (2007) Autoimmune diseases caused by defects in clearing dead cells and nuclei expelled from erythroid precursors. *Immunological reviews* 220:237-250.
99. Kimura-Kataoka K, *et al.* (2012) Genetic and expression analysis of SNPs in the human deoxyribonuclease II: SNPs in the promoter region reduce its in vivo activity through decreased promoter activity. *Electrophoresis* 33(18):2852-2858.
100. Kawane K, *et al.* (2006) Chronic polyarthritis caused by mammalian DNA that escapes from degradation in macrophages. *Nature* 443(7114):998-1002.
101. Turner JD & Filer A (2015) The role of the synovial fibroblast in rheumatoid arthritis pathogenesis. *Current opinion in rheumatology* 27(2):175-182.
102. Pap T, *et al.* (2004) Cooperation of Ras- and c-Myc-dependent pathways in regulating the growth and invasiveness of synovial fibroblasts in rheumatoid arthritis. *Arthritis and rheumatism* 50(9):2794-2802.
103. Morita Y, *et al.* (1998) Antisense oligonucleotides targeting c-fos mRNA inhibit rheumatoid synovial fibroblast proliferation. *Annals of the rheumatic diseases* 57(2):122-124.
104. Yamanishi Y, *et al.* (2005) p53 tumor suppressor gene mutations in fibroblast-like synoviocytes from erosion synovium and non-erosion synovium in rheumatoid arthritis. *Arthritis research & therapy* 7(1):R12-18.
105. Ospelt C, *et al.* (2008) Overexpression of toll-like receptors 3 and 4 in synovial tissue from patients with early rheumatoid arthritis: toll-like receptor expression in early and longstanding arthritis. *Arthritis and rheumatism* 58(12):3684-3692.
106. Brentano F, Schorr O, Gay RE, Gay S, & Kyburz D (2005) RNA released from necrotic synovial fluid cells activates rheumatoid arthritis synovial fibroblasts via Toll-like receptor 3. *Arthritis and rheumatism* 52(9):2656-2665.
107. Lee SY, *et al.* (2014) Interleukin-17 increases the expression of Toll-like receptor 3 via the STAT3 pathway in rheumatoid arthritis fibroblast-like synoviocytes. *Immunology* 141(3):353-361.
108. Seibl R, *et al.* (2003) Expression and regulation of Toll-like receptor 2 in rheumatoid arthritis synovium. *The American journal of pathology* 162(4):1221-1227.

109. Pierer M, *et al.* (2004) Chemokine secretion of rheumatoid arthritis synovial fibroblasts stimulated by Toll-like receptor 2 ligands. *Journal of immunology* 172(2):1256-1265.
110. Buckley CD (2011) Why does chronic inflammation persist: An unexpected role for fibroblasts. *Immunology letters* 138(1):12-14.
111. Filer A, *et al.* (2006) Differential survival of leukocyte subsets mediated by synovial, bone marrow, and skin fibroblasts: site-specific versus activation-dependent survival of T cells and neutrophils. *Arthritis and rheumatism* 54(7):2096-2108.
112. Burger JA, Zvaifler NJ, Tsukada N, Firestein GS, & Kipps TJ (2001) Fibroblast-like synoviocytes support B-cell pseudoemperipolesis via a stromal cell-derived factor-1- and CD106 (VCAM-1)-dependent mechanism. *The Journal of clinical investigation* 107(3):305-315.
113. Ohata J, *et al.* (2005) Fibroblast-like synoviocytes of mesenchymal origin express functional B cell-activating factor of the TNF family in response to proinflammatory cytokines. *Journal of immunology* 174(2):864-870.
114. Benito-Miguel M, *et al.* (2012) IL-15 expression on RA synovial fibroblasts promotes B cell survival. *PloS one* 7(7):e40620.
115. Shaw AT & Gravallese EM (2015) Mediators of inflammation and bone remodeling in rheumatic disease. *Seminars in cell & developmental biology*.
116. Bromley M & Woolley DE (1984) Chondroclasts and osteoclasts at subchondral sites of erosion in the rheumatoid joint. *Arthritis and rheumatism* 27(9):968-975.
117. Gravallese EM, *et al.* (1998) Identification of cell types responsible for bone resorption in rheumatoid arthritis and juvenile rheumatoid arthritis. *The American journal of pathology* 152(4):943-951.
118. Suzuki Y, Nishikaku F, Nakatuka M, & Koga Y (1998) Osteoclast-like cells in murine collagen induced arthritis. *The Journal of rheumatology* 25(6):1154-1160.
119. Romas E, *et al.* (2000) Expression of osteoclast differentiation factor at sites of bone erosion in collagen-induced arthritis. *Arthritis and rheumatism* 43(4):821-826.
120. Kuratani T, *et al.* (1998) Induction of abundant osteoclast-like multinucleated giant cells in adjuvant arthritic rats with accompanying disordered high bone turnover. *Histology and histopathology* 13(3):751-759.
121. Pettit AR, *et al.* (2001) TRANCE/RANKL knockout mice are protected from bone erosion in a serum transfer model of arthritis. *Am J Pathol* 159(5):1689-1699.
122. Redlich K, *et al.* (2002) Osteoclasts are essential for TNF-alpha-mediated joint destruction. *The Journal of clinical investigation* 110(10):1419-1427.
123. Schett G, *et al.* (2003) Osteoprotegerin protects against generalized bone loss in tumor necrosis factor-transgenic mice. *Arthritis and rheumatism* 48(7):2042-2051.
124. Kong YY, *et al.* (1999) Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. *Nature* 402(6759):304-309.

125. Romas E, *et al.* (2002) Osteoprotegerin reduces osteoclast numbers and prevents bone erosion in collagen-induced arthritis. *The American journal of pathology* 161(4):1419-1427.
126. Herrak P, *et al.* (2004) Zoledronic acid protects against local and systemic bone loss in tumor necrosis factor-mediated arthritis. *Arthritis and rheumatism* 50(7):2327-2337.
127. Sims NA, *et al.* (2004) Targeting osteoclasts with zoledronic acid prevents bone destruction in collagen-induced arthritis. *Arthritis and rheumatism* 50(7):2338-2346.
128. Jarrett SJ, *et al.* (2006) Preliminary evidence for a structural benefit of the new bisphosphonate zoledronic acid in early rheumatoid arthritis. *Arthritis and rheumatism* 54(5):1410-1414.
129. Deodhar A, *et al.* (2010) Denosumab-mediated increase in hand bone mineral density associated with decreased progression of bone erosion in rheumatoid arthritis patients. *Arthritis care & research* 62(4):569-574.
130. Cohen SB, *et al.* (2008) Denosumab treatment effects on structural damage, bone mineral density, and bone turnover in rheumatoid arthritis: a twelve-month, multicenter, randomized, double-blind, placebo-controlled, phase II clinical trial. *Arthritis and rheumatism* 58(5):1299-1309.
131. Sugimoto T, *et al.* (2015) Three-year denosumab treatment in postmenopausal Japanese women and men with osteoporosis: results from a 1-year open-label extension of the Denosumab Fracture Intervention Randomized Placebo Controlled Trial (DIRECT). *Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA* 26(2):765-774.
132. Wei S, Kitaura H, Zhou P, Ross FP, & Teitelbaum SL (2005) IL-1 mediates TNF-induced osteoclastogenesis. *The Journal of clinical investigation* 115(2):282-290.
133. Jovanovic DV, *et al.* (1998) IL-17 stimulates the production and expression of proinflammatory cytokines, IL-beta and TNF-alpha, by human macrophages. *Journal of immunology* 160(7):3513-3521.
134. Kotake S, *et al.* (1999) IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis. *The Journal of clinical investigation* 103(9):1345-1352.
135. Hashizume M, Hayakawa N, & Mihara M (2008) IL-6 trans-signalling directly induces RANKL on fibroblast-like synovial cells and is involved in RANKL induction by TNF-alpha and IL-17. *Rheumatology* 47(11):1635-1640.
136. Yao Z, *et al.* (2006) Tumor necrosis factor-alpha increases circulating osteoclast precursor numbers by promoting their proliferation and differentiation in the bone marrow through up-regulation of c-Fms expression. *The Journal of biological chemistry* 281(17):11846-11855.
137. Fleischmann R, *et al.* (2012) Placebo-controlled trial of tofacitinib monotherapy in rheumatoid arthritis. *The New England journal of medicine* 367(6):495-507.
138. van Vollenhoven RF, *et al.* (2012) Tofacitinib or adalimumab versus placebo in rheumatoid arthritis. *The New England journal of medicine* 367(6):508-519.

139. Lee EB, *et al.* (2014) Tofacitinib versus methotrexate in rheumatoid arthritis. *The New England journal of medicine* 370(25):2377-2386.
140. Jimenez-Boj E, *et al.* (2007) Bone erosions and bone marrow edema as defined by magnetic resonance imaging reflect true bone marrow inflammation in rheumatoid arthritis. *Arthritis and rheumatism* 56(4):1118-1124.
141. Haavardsholm EA, Boyesen P, Ostergaard M, Schildvold A, & Kvien TK (2008) Magnetic resonance imaging findings in 84 patients with early rheumatoid arthritis: bone marrow oedema predicts erosive progression. *Annals of the rheumatic diseases* 67(6):794-800.
142. Hetland ML, *et al.* (2009) MRI bone oedema is the strongest predictor of subsequent radiographic progression in early rheumatoid arthritis. Results from a 2-year randomised controlled trial (CIMESTRA). *Annals of the rheumatic diseases* 68(3):384-390.
143. Nishimura K, *et al.* (2007) Meta-analysis: diagnostic accuracy of anti-cyclic citrullinated peptide antibody and rheumatoid factor for rheumatoid arthritis. *Annals of internal medicine* 146(11):797-808.
144. Saeki Y, *et al.* (2013) Baseline anti-citrullinated peptide antibody (ACPA) titers and serum interleukin-6 (IL-6) levels possibly predict progression of bone destruction in early stages of rheumatoid arthritis (ERA). *Rheumatology international* 33(2):451-456.
145. Hecht C, *et al.* (2014) Additive effect of anti-citrullinated protein antibodies and rheumatoid factor on bone erosions in patients with RA. *Annals of the rheumatic diseases*.
146. Berglin E, *et al.* (2004) A combination of autoantibodies to cyclic citrullinated peptide (CCP) and HLA-DRB1 locus antigens is strongly associated with future onset of rheumatoid arthritis. *Arthritis research & therapy* 6(4):R303-308.
147. Nielen MM, *et al.* (2004) Specific autoantibodies precede the symptoms of rheumatoid arthritis: a study of serial measurements in blood donors. *Arthritis and rheumatism* 50(2):380-386.
148. Harre U, Kittan NA, & Schett G (2014) Autoantibody-mediated bone loss. *Current osteoporosis reports* 12(1):17-21.
149. Mathsson L, Lampa J, Mullazehi M, & Ronnelid J (2006) Immune complexes from rheumatoid arthritis synovial fluid induce FcgammaRIIa dependent and rheumatoid factor correlated production of tumour necrosis factor-alpha by peripheral blood mononuclear cells. *Arthritis research & therapy* 8(3):R64.
150. Clavel C, *et al.* (2008) Induction of macrophage secretion of tumor necrosis factor alpha through Fcgamma receptor IIa engagement by rheumatoid arthritis-specific autoantibodies to citrullinated proteins complexed with fibrinogen. *Arthritis and rheumatism* 58(3):678-688.
151. Lu MC, *et al.* (2010) Anti-citrullinated protein antibodies bind surface-expressed citrullinated Grp78 on monocyte/macrophages and stimulate tumor necrosis factor alpha production. *Arthritis and rheumatism* 62(5):1213-1223.

152. Harre U, *et al.* (2012) Induction of osteoclastogenesis and bone loss by human autoantibodies against citrullinated vimentin. *The Journal of clinical investigation* 122(5):1791-1802.
153. Harre U, *et al.* (2015) Glycosylation of immunoglobulin G determines osteoclast differentiation and bone loss. *Nature communications* 6:6651.
154. Kaneko Y, Nimmerjahn F, & Ravetch JV (2006) Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. *Science* 313(5787):670-673.
155. Ideguchi H, Ohno S, Hattori H, Senuma A, & Ishigatsubo Y (2006) Bone erosions in rheumatoid arthritis can be repaired through reduction in disease activity with conventional disease-modifying antirheumatic drugs. *Arthritis research & therapy* 8(3):R76.
156. Finzel S, *et al.* (2011) Repair of bone erosions in rheumatoid arthritis treated with tumour necrosis factor inhibitors is based on bone apposition at the base of the erosion. *Annals of the rheumatic diseases* 70(9):1587-1593.
157. Finzel S, *et al.* (2013) Interleukin-6 receptor blockade induces limited repair of bone erosions in rheumatoid arthritis: a micro CT study. *Annals of the rheumatic diseases* 72(3):396-400.
158. Crane JL & Cao X (2014) Bone marrow mesenchymal stem cells and TGF-beta signaling in bone remodeling. *The Journal of clinical investigation* 124(2):466-472.
159. Biskobing DM, Fan X, & Rubin J (1995) Characterization of MCSF-induced proliferation and subsequent osteoclast formation in murine marrow culture. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 10(7):1025-1032.
160. Takahashi N, Udagawa N, & Suda T (1999) A new member of tumor necrosis factor ligand family, ODF/OPGL/TRANCE/RANKL, regulates osteoclast differentiation and function. *Biochemical and biophysical research communications* 256(3):449-455.
161. Monroe DG, McGee-Lawrence ME, Oursler MJ, & Westendorf JJ (2012) Update on Wnt signaling in bone cell biology and bone disease. *Gene* 492(1):1-18.
162. Sanchez-Duffhues G, Hiepen C, Knaus P, & Ten Dijke P (2015) Bone morphogenetic protein signaling in bone homeostasis. *Bone*.
163. Regard JB, Zhong Z, Williams BO, & Yang Y (2012) Wnt signaling in bone development and disease: making stronger bone with Wnts. *Cold Spring Harbor perspectives in biology* 4(12).
164. Morvan F, *et al.* (2006) Deletion of a single allele of the Dkk1 gene leads to an increase in bone formation and bone mass. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 21(6):934-945.
165. Li J, *et al.* (2006) Dkk1-mediated inhibition of Wnt signaling in bone results in osteopenia. *Bone* 39(4):754-766.
166. Bodine PV, *et al.* (2004) The Wnt antagonist secreted frizzled-related protein-1 is a negative regulator of trabecular bone formation in adult mice. *Molecular endocrinology* 18(5):1222-1237.

167. Yao W, *et al.* (2010) Overexpression of secreted frizzled-related protein 1 inhibits bone formation and attenuates parathyroid hormone bone anabolic effects. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 25(2):190-199.
168. Diarra D, *et al.* (2007) Dickkopf-1 is a master regulator of joint remodeling. *Nature medicine* 13(2):156-163.
169. Daoussis D & Andonopoulos AP (2011) The emerging role of Dickkopf-1 in bone biology: is it the main switch controlling bone and joint remodeling? *Seminars in arthritis and rheumatism* 41(2):170-177.
170. Matzelle MM, *et al.* (2012) Resolution of inflammation induces osteoblast function and regulates the Wnt signaling pathway. *Arthritis and rheumatism* 64(5):1540-1550.
171. Li X, *et al.* (2005) Sclerostin binds to LRP5/6 and antagonizes canonical Wnt signaling. *The Journal of biological chemistry* 280(20):19883-19887.
172. Staehling-Hampton K, *et al.* (2002) A 52-kb deletion in the SOST-MEOX1 intergenic region on 17q12-q21 is associated with van Buchem disease in the Dutch population. *American journal of medical genetics* 110(2):144-152.
173. Balemans W, *et al.* (2002) Identification of a 52 kb deletion downstream of the SOST gene in patients with van Buchem disease. *Journal of medical genetics* 39(2):91-97.
174. van Lierop AH, *et al.* (2013) Van Buchem disease: clinical, biochemical, and densitometric features of patients and disease carriers. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 28(4):848-854.
175. Balemans W, *et al.* (2001) Increased bone density in sclerosteosis is due to the deficiency of a novel secreted protein (SOST). *Human molecular genetics* 10(5):537-543.
176. Hamersma H, Gardner J, & Beighton P (2003) The natural history of sclerosteosis. *Clinical genetics* 63(3):192-197.
177. Chen G, Deng C, & Li YP (2012) TGF-beta and BMP signaling in osteoblast differentiation and bone formation. *International journal of biological sciences* 8(2):272-288.
178. Gilbert L, *et al.* (2000) Inhibition of osteoblast differentiation by tumor necrosis factor-alpha. *Endocrinology* 141(11):3956-3964.
179. Gilbert L, *et al.* (2002) Expression of the osteoblast differentiation factor RUNX2 (Cbfa1/AML3/Pebp2alpha A) is inhibited by tumor necrosis factor-alpha. *The Journal of biological chemistry* 277(4):2695-2701.
180. Jilka RL, Weinstein RS, Bellido T, Parfitt AM, & Manolagas SC (1998) Osteoblast programmed cell death (apoptosis): modulation by growth factors and cytokines. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 13(5):793-802.
181. Stashenko P, Dewhirst FE, Rooney ML, Desjardins LA, & Heeley JD (1987) Interleukin-1 beta is a potent inhibitor of bone formation in vitro. *Journal of bone*

and mineral research : the official journal of the American Society for Bone and Mineral Research 2(6):559-565.

182. Hengartner NE, Fiedler J, Ignatius A, & Brenner RE (2013) IL-1beta inhibits human osteoblast migration. *Molecular medicine* 19:36-42.
183. Liu XH, Kirschenbaum A, Yao S, & Levine AC (2005) Cross-talk between the interleukin-6 and prostaglandin E(2) signaling systems results in enhancement of osteoclastogenesis through effects on the osteoprotegerin/receptor activator of nuclear factor-{kappa}B (RANK) ligand/RANK system. *Endocrinology* 146(4):1991-1998.
184. Kaplan FS, Pignolo RJ, & Shore EM (2015) Granting immunity to FOP and catching heterotopic ossification in the Act. *Seminars in cell & developmental biology*.
185. Ranganathan K, *et al.* (2015) Heterotopic Ossification: Basic-Science Principles and Clinical Correlates. *The Journal of bone and joint surgery. American volume* 97(13):1101-1111.
186. Nauth A, *et al.* (2012) Heterotopic ossification in orthopaedic trauma. *Journal of orthopaedic trauma* 26(12):684-688.
187. Sagi HC, Jordan CJ, Barei DP, Serrano-Riera R, & Steverson B (2014) Indomethacin prophylaxis for heterotopic ossification after acetabular fracture surgery increases the risk for nonunion of the posterior wall. *Journal of orthopaedic trauma* 28(7):377-383.
188. Rivera JC, Hsu JR, Noel SP, Wenke JC, & Rathbone CR (2015) Locally Delivered Nonsteroidal Antiinflammatory Drug: A Potential Option for Heterotopic Ossification Prevention. *Clinical and translational science* 8(5):591-593.
189. Kaplan FS, *et al.* (2008) Fibrodysplasia ossificans progressiva. *Best practice & research. Clinical rheumatology* 22(1):191-205.
190. Ramirez DM, Ramirez MR, Reginato AM, & Medici D (2014) Molecular and cellular mechanisms of heterotopic ossification. *Histology and histopathology* 29(10):1281-1285.
191. Kaplan FS, Chakkalakal SA, & Shore EM (2012) Fibrodysplasia ossificans progressiva: mechanisms and models of skeletal metamorphosis. *Disease models & mechanisms* 5(6):756-762.
192. Yu PB, *et al.* (2008) BMP type I receptor inhibition reduces heterotopic [corrected] ossification. *Nature medicine* 14(12):1363-1369.
193. Scarlett RF, *et al.* (2004) Influenza-like viral illnesses and flare-ups of fibrodysplasia ossificans progressiva. *Clinical orthopaedics and related research* (423):275-279.
194. Micha D, *et al.* (2016) Inhibition of TGFbeta signaling decreases osteogenic differentiation of fibrodysplasia ossificans progressiva fibroblasts in a novel in vitro model of the disease. *Bone* 84:169-180.
195. Shore EM & Kaplan FS (2011) Role of altered signal transduction in heterotopic ossification and fibrodysplasia ossificans progressiva. *Current osteoporosis reports* 9(2):83-88.

196. Gannon FH, *et al.* (2001) Mast cell involvement in fibrodysplasia ossificans progressiva. *Human pathology* 32(8):842-848.
197. Gannon FH, Valentine BA, Shore EM, Zasloff MA, & Kaplan FS (1998) Acute lymphocytic infiltration in an extremely early lesion of fibrodysplasia ossificans progressiva. *Clinical orthopaedics and related research* (346):19-25.
198. Kan L & Kessler JA (2014) Evaluation of the cellular origins of heterotopic ossification. *Orthopedics* 37(5):329-340.
199. Kaplan FS, *et al.* (2007) Hematopoietic stem-cell contribution to ectopic skeletogenesis. *The Journal of bone and joint surgery. American volume* 89(2):347-357.
200. Baccala R, Hoebe K, Kono DH, Beutler B, & Theofilopoulos AN (2007) TLR-dependent and TLR-independent pathways of type I interferon induction in systemic autoimmunity. *Nature medicine* 13(5):543-551.
201. Yoshida H, Okabe Y, Kawane K, Fukuyama H, & Nagata S (2005) Lethal anemia caused by interferon-beta produced in mouse embryos carrying undigested DNA. *Nature immunology* 6(1):49-56.
202. Kawane K, Tanaka H, Kitahara Y, Shimaoka S, & Nagata S (2010) Cytokine-dependent but acquired immunity-independent arthritis caused by DNA escaped from degradation. *Proceedings of the National Academy of Sciences of the United States of America* 107(45):19432-19437.
203. Ahn J, Gutman D, Saijo S, & Barber GN (2012) STING manifests self DNA-dependent inflammatory disease. *Proceedings of the National Academy of Sciences of the United States of America* 109(47):19386-19391.
204. Rathinam VA, Vanaja SK, & Fitzgerald KA (2012) Regulation of inflammasome signaling. *Nature immunology* 13(4):333-342.
205. Ishikawa H, Ma Z, & Barber GN (2009) STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity. *Nature* 461(7265):788-792.
206. Korganow AS, *et al.* (1999) From systemic T cell self-reactivity to organ-specific autoimmune disease via immunoglobulins. *Immunity* 10(4):451-461.
207. Li QZ, *et al.* (2007) Protein array autoantibody profiles for insights into systemic lupus erythematosus and incomplete lupus syndromes. *Clinical and experimental immunology* 147(1):60-70.
208. Sedimbi SK, Hagglof T, & Karlsson MC (2013) IL-18 in inflammatory and autoimmune disease. *Cellular and molecular life sciences : CMLS* 70(24):4795-4808.
209. Wu C, *et al.* (2009) BioGPS: an extensible and customizable portal for querying and organizing gene annotation resources. *Genome biology* 10(11):R130.
210. Mangialaio S, *et al.* (1999) The arthritogenic T cell receptor and its ligand in a model of spontaneous arthritis. *Arthritis Rheum* 42(12):2517-2523.
211. Xiao TS & Fitzgerald KA (2013) The cGAS-STING pathway for DNA sensing. *Molecular cell* 51(2):135-139.

212. Jeremiah N, *et al.* (2014) Inherited STING-activating mutation underlies a familial inflammatory syndrome with lupus-like manifestations. *The Journal of clinical investigation* 124(12):5516-5520.
213. Rice GI, *et al.* (2009) Mutations involved in Aicardi-Goutieres syndrome implicate SAMHD1 as regulator of the innate immune response. *Nature genetics* 41(7):829-832.
214. Rice GI, *et al.* (2012) Mutations in ADAR1 cause Aicardi-Goutieres syndrome associated with a type I interferon signature. *Nature genetics* 44(11):1243-1248.
215. Rice GI, *et al.* (2013) Synonymous mutations in RNASEH2A create cryptic splice sites impairing RNase H2 enzyme function in Aicardi-Goutieres syndrome. *Human mutation* 34(8):1066-1070.
216. Stetson DB (2012) Endogenous retroelements and autoimmune disease. *Current opinion in immunology* 24(6):692-697.
217. Baum R, *et al.* (2015) Cutting edge: AIM2 and endosomal TLRs differentially regulate arthritis and autoantibody production in DNase II-deficient mice. *Journal of immunology* 194(3):873-877.
218. Gall A, *et al.* (2012) Autoimmunity initiates in nonhematopoietic cells and progresses via lymphocytes in an interferon-dependent autoimmune disease. *Immunity* 36(1):120-131.
219. Tabeta K, *et al.* (2006) The Unc93b1 mutation 3d disrupts exogenous antigen presentation and signaling via Toll-like receptors 3, 7 and 9. *Nat Immunol* 7(2):156-164.
220. Ishikawa H & Barber GN (2008) STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. *Nature* 455(7213):674-678.
221. Chan MP, *et al.* (2015) DNase II-dependent DNA digestion is required for DNA sensing by TLR9. *Nature communications* 6:5853.
222. Green NM, *et al.* (2009) Murine B cell response to TLR7 ligands depends on an IFN-beta feedback loop. *Journal of immunology* 183(3):1569-1576.
223. Raza K, *et al.* (2005) Early rheumatoid arthritis is characterized by a distinct and transient synovial fluid cytokine profile of T cell and stromal cell origin. *Arthritis research & therapy* 7(4):R784-795.
224. Lichanska AM, *et al.* (1999) Differentiation of the mononuclear phagocyte system during mouse embryogenesis: the role of transcription factor PU.1. *Blood* 94(1):127-138.
225. Zhuang H, *et al.* (2014) Toll-like receptor 7-stimulated tumor necrosis factor alpha causes bone marrow damage in systemic lupus erythematosus. *Arthritis & rheumatology* 66(1):140-151.
226. Rongvaux A, *et al.* (2014) Apoptotic caspases prevent the induction of type I interferons by mitochondrial DNA. *Cell* 159(7):1563-1577.
227. West AP, *et al.* (2015) Mitochondrial DNA stress primes the antiviral innate immune response. *Nature*.
228. Jakobs C, Perner S, & Hornung V (2015) AIM2 Drives Joint Inflammation in a Self-DNA Triggered Model of Chronic Polyarthritis. *PloS one* 10(6):e0131702.

229. Schett G & Gravallese E (2012) Bone erosion in rheumatoid arthritis: mechanisms, diagnosis and treatment. *Nature reviews. Rheumatology* 8(11):656-664.
230. Baum R & Gravallese EM (2014) Impact of inflammation on the osteoblast in rheumatic diseases. *Current osteoporosis reports* 12(1):9-16.
231. Walsh NC, Crotti TN, Goldring SR, & Gravallese EM (2005) Rheumatic diseases: the effects of inflammation on bone. *Immunological reviews* 208:228-251.
232. Takayanagi H, *et al.* (2002) RANKL maintains bone homeostasis through c-Fos-dependent induction of interferon-beta. *Nature* 416(6882):744-749.
233. Parfitt AM, *et al.* (1987) Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 2(6):595-610.
234. Baum R, *et al.* (2015) Synergy between Hematopoetic and Radioresistant Stromal Cells is Required for Autoimmune Manifestations of DNase II-/- IFNaR-/- Mice.
235. Macias MP, *et al.* (2001) Expression of IL-5 alters bone metabolism and induces ossification of the spleen in transgenic mice. *The Journal of clinical investigation* 107(8):949-959.
236. Macki M, Bydon M, Papademetriou K, Gokaslan Z, & Bydon A (2013) Presacral extramedullary hematopoiesis: an alternative hypothesis. *Journal of clinical neuroscience : official journal of the Neurosurgical Society of Australasia* 20(12):1664-1668.
237. Liu CJ, *et al.* (2005) The interferon-inducible p204 protein acts as a transcriptional coactivator of Cbfa1 and enhances osteoblast differentiation. *The Journal of biological chemistry* 280(4):2788-2796.
238. Luan Y, *et al.* (2007) The retinoblastoma protein is an essential mediator of osteogenesis that links the p204 protein to the Cbfa1 transcription factor thereby increasing its activity. *The Journal of biological chemistry* 282(23):16860-16870.
239. Luan Y, *et al.* (2008) p204 protein overcomes the inhibition of core binding factor alpha-1-mediated osteogenic differentiation by Id helix-loop-helix proteins. *Molecular biology of the cell* 19(5):2113-2126.
240. Triliana R, *et al.* (2015) Skeletal characterization of an osteoblast-specific vitamin D receptor transgenic (ObVDR-B6) mouse model. *The Journal of steroid biochemistry and molecular biology*.
241. Gattu AK, *et al.* (2013) Determination of mesenchymal stem cell fate by pigment epithelium-derived factor (PEDF) results in increased adiposity and reduced bone mineral content. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 27(11):4384-4394.
242. Nagata K (2003) HSP47 as a collagen-specific molecular chaperone: function and expression in normal mouse development. *Seminars in cell & developmental biology* 14(5):275-282.
243. Yu B, *et al.* (2012) Parathyroid hormone induces differentiation of mesenchymal stromal/stem cells by enhancing bone morphogenetic protein signaling. *Journal of*

bone and mineral research : the official journal of the American Society for Bone and Mineral Research 27(9):2001-2014.

244. Miyanishi M, *et al.* (2007) Identification of Tim4 as a phosphatidylserine receptor. *Nature* 450(7168):435-439.
245. Matsunobu T, *et al.* (2009) Critical roles of the TGF-beta type I receptor ALK5 in perichondrial formation and function, cartilage integrity, and osteoblast differentiation during growth plate development. *Developmental biology* 332(2):325-338.
246. Doherty MJ, *et al.* (1998) Vascular pericytes express osteogenic potential in vitro and in vivo. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 13(5):828-838.
247. Modder UI & Khosla S (2008) Skeletal stem/osteoprogenitor cells: current concepts, alternate hypotheses, and relationship to the bone remodeling compartment. *Journal of cellular biochemistry* 103(2):393-400.
248. Pignolo RJ, Shore EM, & Kaplan FS (2011) Fibrodysplasia ossificans progressiva: clinical and genetic aspects. *Orphanet journal of rare diseases* 6:80.
249. Lanchoney TF, Cohen RB, Rocke DM, Zasloff MA, & Kaplan FS (1995) Permanent heterotopic ossification at the injection site after diphtheria-tetanus-pertussis immunizations in children who have fibrodysplasia ossificans progressiva. *The Journal of pediatrics* 126(5 Pt 1):762-764.
250. Paludan SR & Bowie AG (2013) Immune sensing of DNA. *Immunity* 38(5):870-880.
251. Smith S & Jefferies C (2014) Role of DNA/RNA sensors and contribution to autoimmunity. *Cytokine & growth factor reviews* 25(6):745-757.
252. Davignon JL, *et al.* (2013) Targeting monocytes/macrophages in the treatment of rheumatoid arthritis. *Rheumatology* 52(4):590-598.
253. Bauernfeind F & Hornung V (2013) Of inflammasomes and pathogens--sensing of microbes by the inflammasome. *EMBO molecular medicine* 5(6):814-826.
254. Goldbach-Mansky R (2009) Blocking interleukin-1 in rheumatic diseases. *Annals of the New York Academy of Sciences* 1182:111-123.
255. Bresnihan B, *et al.* (1998) Treatment of rheumatoid arthritis with recombinant human interleukin-1 receptor antagonist. *Arthritis and rheumatism* 41(12):2196-2204.
256. Jiang Y, *et al.* (2000) A multicenter, double-blind, dose-ranging, randomized, placebo-controlled study of recombinant human interleukin-1 receptor antagonist in patients with rheumatoid arthritis: radiologic progression and correlation of Genant and Larsen scores. *Arthritis and rheumatism* 43(5):1001-1009.
257. Roldan R, Ruiz AM, Miranda MD, & Collantes E (2008) Anakinra: new therapeutic approach in children with Familial Mediterranean Fever resistant to colchicine. *Joint, bone, spine : revue du rhumatisme* 75(4):504-505.
258. Fitzgerald AA, Leclercq SA, Yan A, Homik JE, & Dinarello CA (2005) Rapid responses to anakinra in patients with refractory adult-onset Still's disease. *Arthritis and rheumatism* 52(6):1794-1803.

259. Vasques Godinho FM, Parreira Santos MJ, & Canas da Silva J (2005) Refractory adult onset Still's disease successfully treated with anakinra. *Annals of the rheumatic diseases* 64(4):647-648.
260. Gao D, *et al.* (2015) Activation of cyclic GMP-AMP synthase by self-DNA causes autoimmune diseases. *Proceedings of the National Academy of Sciences of the United States of America* 112(42):E5699-5705.
261. Lee BL & Barton GM (2014) Trafficking of endosomal Toll-like receptors. *Trends in cell biology* 24(6):360-369.
262. Bayik D, Gursel I, & Klinman DM (2016) Structure, mechanism and therapeutic utility of immunosuppressive oligonucleotides. *Pharmacological research*.
263. Kaminski JJ, *et al.* (2013) Synthetic oligodeoxynucleotides containing suppressive TTAGGG motifs inhibit AIM2 inflammasome activation. *Journal of immunology* 191(7):3876-3883.
264. Woo SR, Corrales L, & Gajewski TF (2015) The STING pathway and the T cell-inflamed tumor microenvironment. *Trends in immunology* 36(4):250-256.
265. Woo SR, *et al.* (2014) STING-dependent cytosolic DNA sensing mediates innate immune recognition of immunogenic tumors. *Immunity* 41(5):830-842.
266. Ohkuri T, *et al.* (2014) STING contributes to antiglioma immunity via triggering type I IFN signals in the tumor microenvironment. *Cancer immunology research* 2(12):1199-1208.
267. Corrales L, *et al.* (2015) Direct Activation of STING in the Tumor Microenvironment Leads to Potent and Systemic Tumor Regression and Immunity. *Cell reports* 11(7):1018-1030.
268. Lefevre S, Meier FM, Neumann E, & Muller-Ladner U (2015) Role of synovial fibroblasts in rheumatoid arthritis. *Current pharmaceutical design* 21(2):130-141.
269. Gray EE, Treuting PM, Woodward JJ, & Stetson DB (2015) Cutting Edge: cGAS Is Required for Lethal Autoimmune Disease in the Trex1-Deficient Mouse Model of Aicardi-Goutieres Syndrome. *Journal of immunology* 195(5):1939-1943.
270. Ivashkiv LB & Donlin LT (2014) Regulation of type I interferon responses. *Nature reviews. Immunology* 14(1):36-49.
271. Unterholzner L (2013) The interferon response to intracellular DNA: why so many receptors? *Immunobiology* 218(11):1312-1321.
272. Paludan SR (2015) Activation and regulation of DNA-driven immune responses. *Microbiology and molecular biology reviews : MMBR* 79(2):225-241.
273. Nagata S, Hanayama R, & Kawane K (2010) Autoimmunity and the clearance of dead cells. *Cell* 140(5):619-630.
274. Crisan M, *et al.* (2008) A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell stem cell* 3(3):301-313.
275. Sharma S, Fitzgerald KA, Cancro MP, & Marshak-Rothstein A (2015) Nucleic Acid-Sensing Receptors: Rheostats of Autoimmunity and Autoinflammation. *Journal of immunology* 195(8):3507-3512.