Alliance for Lupus Research

Target Identification in Lupus Grant (with LOI)

Grant Application

Date Submitted: 8/25/2014 4:49:18 PM

Resubmission? No | Prior App:

TITLE OF PROJECT *(Titles exceeding 81 characters, including spaces and punctuation, will be truncated.)*

Characterization and Function of CD4 T Cell Subsets in Lupus

APPLICANT NAME

Craft, Joseph E

HIGHEST DEGREE(S)

M.D.

POSITION TITLE: Paul B. Beeson Professor of Medicine and Profe

ACADEMIC RANK: Full Professor

DIVISION: Rheumatology

DEPARTMENT: Internal Medicine and Immunobiology

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APPLICANT'S CURRENT INSTITUTION

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New Haven CT 06520-8031
United States

PROGRAM ELIGIBILITY INFORMATION: *(Responses to selected fields displayed below. For some grant programs this section may be blank.)*

U.S. Citizenship: U.S. Citizen

DATES OF PROPOSED PROJECT *(MM/DD/YYYY)*

From: 2/1/2015 Through: 1/31/2018

PROPOSED BUDGET

600000.00

Name Yale University

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47 College Street, Suite 203
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SIGNING OFFICIAL FOR Yale University

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Title Grant & Contract Manager

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E-MAIL ADDRESS

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HUMAN SUBJECTS ☐ No ☐ Yes

Human Subjects Assurance No. FWA00002571

IRB Status: Pending

IRB Date: 08/24/14

VERTEBRATE ANIMALS ☐ No ☐ Yes

Animal welfare assurance no. A3230-01

IACUC Status: Pending

IACUC Date: 08/24/14

RECOMBINANT DNA No

Status:

Date:

BIOHAZARDS No

APPLICANT ASSURANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

SIGNATURE OF APPLICANT *(In ink. "Per" signature not acceptable.)*

DATE

SIGNING OFFICIAL ASSURANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with the grantor's terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF SIGNING OFFICIAL *(In ink. "Per" signature not acceptable.)*

DATE

ADDITIONAL SIGNATURE (follow guidelines for required signatures): I certify that the statements herein are true, complete and accurate to the best of my knowledge.

DATE

ADDITIONAL SIGNATURE (follow guidelines for required signatures): I certify that the statements herein are true, complete and accurate to the best of my knowledge.

DATE

Applicant: Craft, Joseph E

Application Contacts

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Division				Division	Rheumatology		
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Division				Division			
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Tel:		Fax:		Tel:		Fax:	
E-mail				E-mail			
Role				Role			
Name				Name			
Institution				Institution			
Title				Title			
Division				Division			
Dept				Dept			
Address				Address			
Tel:		Fax:		Tel:		Fax:	
E-mail				E-mail			

GENERAL AUDIENCE SUMMARY

APPLICANT NAME	DATE SUBMITTED
Craft, Joseph E	8/25/2014 4:49:18 PM

TITLE OF PROJECT *(Titles exceeding 81 characters, including spaces and punctuation, will be truncated.)*

Characterization and Function of CD4 T Cell Subsets in Lupus

This General Audience Summary will become public information; therefore, do not include proprietary/confidential information.

A plethora of new agents have been used therapeutically in lupus, including in nephritis, with mixed results. Possible explanations for these outcomes, apart from possible flaws in clinical trial design, are initiation of therapy after tissue inflammation and subsequent organ injury have occurred, and/or heterogeneity in immunopathogenesis; regardless, there is a pressing need to improve our understanding of disease mechanisms and organ injury in lupus, as a path toward selection of appropriate agents for therapeutic intervention. We will attempt to do this in our proposal, characterizing the cells that promote inflammation in lupus with the longer-term goal to identify new therapeutic targets in disease.

SCIENTIFIC ABSTRACT

APPLICANT NAME	DATE SUBMITTED
Craft, Joseph E	8/25/2014 4:49:18 PM

TITLE OF PROJECT *(Titles exceeding 81 characters, including spaces and punctuation, will be truncated.)*

Characterization and Function of CD4 T Cell Subsets in Lupus

This Scientific Abstract will become public information; therefore, do not include proprietary/confidential information.

Pathogenic CD4 T helper (Th) cells are critical for disease promotion in systemic lupus erythematosus. These cells exert their effector function via autoreactive B cell help in secondary lymphoid organs (SLOs), including the spleen or lymph nodes, or by infiltration of tissues, such as the kidney. In both cases, autoantibody production and Th-cell infiltration, activation of innate immune cells ensues, mediated by autoantibody-autoantigen complex engagement and by tissue delivery of contact-dependent and soluble factors. The consequent inflammation leads to organ dysfunction and, sometimes, its loss. Thus, dissecting signals that promote CD4+ Th-cell differentiation and maintenance in lupus is critical for understanding pathways of disease initiation and progression, and for identification of new therapeutic targets, or confirmation of existing ones. CD4 Th-cell differentiation is initiated in SLOs after naïve T cells receive antigen (Ag) plus costimulatory signals delivered by dendritic cells (DCs), along with environmental cues including cytokines. The latter, acting via transcription factor induction and regulation, direct differentiation and maintenance of the classical Th-cell subsets, Th1, Th2, Th17, and follicular helper (Tfh) cells, among others. Th1, Th2, and Th17 cells drive inflammation in lupus via migration to and effector function in tissues, while Tfh cells remain in SLOs promoting autoreactive B cell maturation with generation of pathogenic memory B cells and long-lived autoantibody-producing plasma cells. Thus, tissue-effector Th subsets and SLO-resident Tfh cells contribute to tissue injury in SLE. The factors that promote Th1-cell, for example, and Tfh-cell differentiation in SLE, and as importantly, their maintenance and subsequent promotion of chronic tissue inflammation, are not clearly defined. We plan to do so in a lupus model, characterizing the steps required for pathogenic Th1- versus Tfh-cell differentiation and tissue maintenance and injury. While murine models of disease are excellent tools for studies of disease pathogenesis, their utility is magnified when their dissection is done in parallel with studies of SLE patients. We plan this combined approach. We will phenotypically and genetically characterize Th1 and Tfh cells in lupus, dissect the signals required for their development and maintenance, and determine their role in autoantibody formation and tissue injury, using novel cytokine reporter and gene-mutant mice. We will in parallel genetically characterize circulating Th1 and Tfh cells from patients with SLE, cells that in our preliminary studies have a phenotype similar to those we believe pathogenic in mice. Our aims are designed to meet a TIL goal to “ characterize key steps in the pathogenesis of lupus that will allow for the development of new therapeutic agents ” .

APPLICANT: Craft, Joseph E

PROPOSED BUDGET					
	Period 1	Period 2	Period 3	Period 4	Period 5
Start Date (mm/dd/yyyy)	Feb 01, 2015	Feb 01, 2016	Feb 01, 2017		
End Date (mm/dd/yyyy)	Jan 31, 2016	Jan 31, 2017	Jan 31, 2018		
Personnel Direct Costs					
Salaries & Wages	99547.00	101445.00	103399.00		
Fringe Benefits	23851.00	24548.00	24949.00		
SubTotal: Personnel Costs	123398.00	125993.00	128348.00		
Non - Personnel Direct Costs					
Consultant Costs	0.00	0.00	0.00		
Equipment	0.00	0.00	0.00		
Supplies (Medical or Laboratory)	50787.00	48192.00	45837.00		
Supplies (Office)	0.00	0.00	0.00		
Travel	1000.00	1000.00	1000.00		
Patient Care Out-Patient	0.00	0.00	0.00		
Alterations and Renovations	0.00	0.00	0.00		
Other Expenses	10000.00	10000.00	10000.00		
Consortium&Contractual Direct	0.00	0.00	0.00		
Sub Total: Non-Personnel costs:	61787.00	59192.00	56837.00		
TOTAL DIRECT COSTS	185185.00	185185.00	185185.00		
Indirect Costs (i.e. overhead costs, facilities and administrative costs)					
INDIRECT COSTS	14815.00	14815.00	14815.00		
TOTAL COSTS	200000.00	200000.00	200000.00		

BUDGET JUSTIFICATION

APPLICANT NAME	DATE SUBMITTED
Craft, Joseph E	8/25/2014 4:49:18 PM

TITLE OF PROJECT *(Titles exceeding 81 charecters, including spaces and punctuation, will be truncated)*

Characterization and Function of CD4 T Cell Subsets in Lupus

JUSTIFICATION: The first section should include the following line items: personnel, consultant costs; equipment and supplies (both office and medical of laboratory). (2000 character limit)

Joseph Craft, M.D., Principal Investigator (15% effort). Dr. Craft will be responsible for overall direction of the research plan, interpretation of data, writing all papers for publication,and provision of progress reports to the ALR. Insoo Kang, M.D., Investigator (5% effort), will work with Dr. Craft on collection and analysis of human samples. Jason Weinstein, Ph.D., Associate Research Scientist (25% effort), is a senior member of the Craft lab, with considerable experience in cellular and molecular immunology. He, John Ray, B.S., Graduate Student (100% effort; no salary requested -- National Science Foundation Fellowship Awardee), a fourth-year graduate student in the Craft lab, and Jessica Wang, B.S., Graduate Student (100% effort; tuition not requested), a third-year immunobiology graduate student in the lab, generated the preliminary data for this proposal and will be responsible for carrying out its aims. All meet with Dr. Craft on a weekly (one-to-one) basis and as a group to discuss work on this project. They also meet with Dr. Craft on an informal basis several times during the week. Ping Zhu, B.S., Research Associate (10% effort), will care for the animals needed for the project, as well as be responsible for ordering supplies, and handling general laboratory maintenance as specifically related to this project. Laura Kramer, R.N. (10% effort), will obtain human samples at the Yale Lupus Clinic. She has worked with Drs. Craft and Kang for several years, and is experienced in patient evaluation, sample collection, and handling clinical data and HIC protocols. Patrick Gallagher, M.D., Consultant, Professor of Pediatrics, Pathology, and Genetics at Yale, is an expert at functional genomics, including ChIP-seq and RNA-seq performance and characterization, is a longstanding collaborator of Dr. Craft ' s, with a joint paper submitted and currently under review. He will assist in generation and characterization of the genomic data.

BUDGET JUSTIFICATION

APPLICANT NAME	DATE SUBMITTED
Craft, Joseph E	8/25/2014 4:49:18 PM

TITLE OF PROJECT *(Titles exceeding 81 charecters, including spaces and punctuation, will be truncated)*

Characterization and Function of CD4 T Cell Subsets in Lupus

JUSTIFICATION: The second section should include all other line items including but not limited to: travel, Patient Care, other expenses, consortium and contractual costs. (2000 character limit)

Supplies: Costs for antibodies (cell purification and flow cytometry, including sorting) and consumable supplies (gloves, pipette tips, plastic ware, oligonucleotides, PCR reagents, general lab supplies) are based upon those generated in gathering the preliminary data and those projected going forward. We estimate that this will be approximately $35,000 for each year of the proposed award. RNA sequencing is the biggest expense here. Current charges are approximately $3000 per lane for the latter (HiSeq 2000 or 2500: 75 or 150 bp, paired-end sequencing (1 lane, 2 x 75 or 150), plus library construction and expenses related to isolation). On each lane, we can run up to 8 samples; hence, we anticipate being able to perform up to 40 total samples per year (experimental and controls, with appropriate replicates), for about $20,000 yearly. Animals: Animal housing and purchase costs are based upon those anticipated for completion of the aims. We have on hand in our breeding colony mice needed for these experiments, although we will supplement them as needed for B6 mice from Jackson Laboratory. We anticipate carrying approximately 60 cages of breeders and experimental strains at any one time, with housing costs for FY ’15 at Yale $0.89/day/cage, our anticipated costs are approximately $20,000 yearly. Flow Cytometry: Charges are based upon usage necessary to obtain the preliminary data and that anticipated going forward to analyze and sort cells for this project. Charges at Yale are $17-25/hr for analysis, based upon instrument used (we typically use the more expensive LSRIIs for analysis, given the multi-color parameters needed for our experiments), and from $37-78/hr (depending upon the instrument utilized) for sorting. We sort ourselves as much as possible, utilizing the lower rate. We anticipate charges of approximately $6,000 annually. Travel expenses are estimated at $1000 annually, to enable the PI to attend yearly ALR meetings to present and discuss data.

Proposal Narrative -- Supplement

This summary represents additional information for our ALR TIL proposal, “Characterization and Function of CD4 T Cell Subsets in Lupus”, submitted in August 2014.

As part of our proposal, we planned transcriptome (RNA-seq) analysis of CD4 T cells from mice and humans with lupus. We outlined our experience with this approach, including recently published work dissecting the CD4 T-cell transcriptome in follicular helper and Th1 cells following viral infection, a short-term tractable model of robust type I interferon production, analogous to that seen in SLE. This published study provides support for the feasibility of our work (Ray JP, Marshall HD, Laidlaw BJ, Staron MW, Kaech SM, **Craft J**. The transcription factor STAT3 and type I Interferons are mutually repressive insulators for differentiation of follicular helper and T helper 1 cells. 2014 *Immunity* 40:367-377).

Since submission of our proposal, we have published an additional manuscript substantiating our experience with this approach to analyze gene expression in human tonsillar follicular helper T cells (Weinstein JS, Lezon-Geyda K, Maksimova Y, Craft S, Zhang Y, Su M, Schultz VP, **Craft J*, Gallagher PG*.** 2014. Global Transcriptome Analysis and Enhancer Landscape of Human Primary T Follicular Helper and T Effector Lymphocytes. *Blood* [*in press*]. *co-senior and co-corresponding authors). This latter work was performed in conjunction with our consultant on the ALR grant, Dr. Patrick Gallagher, a geneticist at Yale, supporting the collaborative strength of our application. Dr. Jason Weinstein from my lab, and a key member of our ALR proposal, conducted the experiments in this paper.

Space constraints prohibit provision of the full text of this recently accepted paper; however, its abstract follows:

Abstract

T follicular helper (Tfh) cells are a subset of $CD4^+$ T helper (Th) cells that migrate into germinal centers and promote B cell maturation into memory B and plasma cells. Tfh cells are necessary for promotion of protective humoral immunity following pathogen challenge, but when aberrantly regulated, drive pathogenic antibody formation in autoimmunity and undergo neoplastic transformation in angioimmunoblastic T-cell lymphoma and other primary cutaneous T-cell lymphomas. Limited information is available on the expression and regulation of genes in human Tfh cells. Using a fluorescence activated cell sorting-based strategy, we obtained primary Tfh and non-Tfh T effector (Teff) cells from tonsils and prepared genome-wide maps of active, intermediate, and poised enhancers determined by ChIP-seq, with parallel transcriptome analyses determined by RNA-seq. Tfh cell enhancers were enriched near genes highly expressed in lymphoid cells or involved in lymphoid cell function, with many mapping to sites previously associated with autoimmune disease in genome-wide association studies. A group of active enhancers unique to Tfh cells associated with differentially expressed genes was identified. Fragments from these regions directed expression in reporter gene assays. These data provide a significant resource for studies of T lymphocyte development and differentiation and normal and perturbed Tfh cell function.

October 3, 2014

Dear Author:

I am pleased to inform you that your manuscript, "Global Transcriptome Analysis and Enhancer Landscape of Human Primary T Follicular Helper and T Effector Lymphocytes" has been accepted for publication in Blood pending completion of the copyright transfer agreement by each author of the manuscript.

If you are a first-time user of our online manuscript processing system, you

will need to register a new account using the e-mail address included with this submission (i.e., the exact e-mail address to which this message was sent). After you have registered, you will be able to change your e-mail address in your record, if you prefer.

If you have an existing account using an e-mail address different from the one included in this submission, or if the address included in the submission is now inactive, please reply to this message and provide the correct address. Go to http://submit.bloodjournal.org, click on “Create a new account” and follow the instructions provided. Be aware that it may take up to 15 minutes before your manuscript will appear in your "Pending Copyright Transfers" queue.

Please be sure to submit the electronic copyright form to Blood by clicking the "I Agree" button. The manuscript will not be prepublished or sent for production until all authors complete the transfer. If you do not find your manuscript in your "Pending Copyright Transfers" queue after one hour, please contact the editorial office at: prepublications@hematology.org.

Sincerely,
Blood Editorial Office

OTHER SUPPORT

CRAFT, JE

ACTIVE

5 R01 AR040072-25 (Craft) 8/31/1990 - 4/30/2015 2.28 calendar
NIH/NIAMS $211,680
Immune Responses in Lupus
The aims of this grant are to determine the roles of Bcl6 in PSGL1 regulation in the initial events that lead to T_{FH} cell migration and development, and to further dissect the requirements for T_{FH} cell differentiation and function. These studies are to be conducted in mice.

5 P30 AR053495-07 (Craft) 3/1/2006 - 8/31/2017 2.70 calendar
NIH/NIAMS $444,352
Yale Rheumatic Disease Research Core Center
The goal of this center application is to support novel cores in generation and preservation of genetically modified mice and in *in vivo* microscopy.

5 UL1 TR000142-08 (Sherwin) 9/30/2006 - 6/30/2016 1.20 calendar
NIH/NCATS $5,533,635
Yale University Clinical and Translational Science Award Program
The goal of this project is to develop educational and research programs in translational investigation at Yale. Dr. Craft receives support as director of the Investigative Medicine Program; he does not receive project support.

YA-002 Research Grant (Craft) 5/20/2013 - 5/19/2017 1.56 calendar
AbbVie Yale Collaboration in Immunobiology $249,800
Clinical Assessment and Therapeutic Blockade of T - B Cell Collaboration in Autoimmunity
The goals of this proposal are to track circulating T follicular helper-like (cTfh-like) cells in lupus patients in relationship to therapies that abrogate Tfh function and/or development, including abatacept.

Rheumatology Research Foundation (Craft) 07/01/14 – 06/30/16 2.4 calendar
Studying Monocytes and iPS cells in RA $185,185
The goals of this project are to derive iPSCs from peripheral blood mononuclear cells (PBMCs) of RA patients bearing the RA-risk *IRF5* SNP rs2004640; generate isogenic iPSCs by editing the RA-risk *IRF5* SNP rs2004640 to the non-risk SNP, using CAS9 (CRISPR-associated) nuclease and CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) gene editing; differentiate monocytes (MO) from iPSCs and isogenic iPSCs; define the function and transcriptomes of MO differentiated from iPSCs and isogenic iPSCs; transplant iPSCs into humanized mice, followed by assessment of their phenotype and function, and their role in induction and as therapeutic targets in inflammatory arthritis.

PENDING

Connecticut Innovations, Inc. (Craft) 11/01/14 – 06/30/18 .84 calendar
Therapeutic Role of IPSCs in Human Lupus $300,000
The goals of this project are to derive iPSCs from peripheral blood mononuclear cells (PBMCs) of lupus patients bearing the SLE-risk *IRF5* SNP rs2004640; generate isogenic iPSCs by editing the SLE-risk *IRF5* SNP rs2004640 to the non-risk SNP, using CAS9 (CRISPR-associated) nuclease and CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) gene editing; differentiate monocytes (MO) from iPSCs and isogenic iPSCs; define the function of MO differentiated from iPSCs and isogenic iPSCs; and transplant CD14+ myeloid progenitor cells (MPCs) differentiated from iPSCs and isogenic iPSCs into humanized mice, followed by assessment of their phenotype and function, and their role in induction of inflammatory skin disease by lupus serum.

Lupus Research Institute (Craft) 01/01/15 – 12/31/17 1.80 calendar
Pathogenic CD4+ T Cells in SLE: Differentiation and Effector Function $300,000

While much is known about the extrinsic, largely cytokine, signals that direct Th-subset differentiation and maintenance in vitro and following immunization of normal mice, the factors that promote Th1-cell, for example, and Tfh-cell differentiation in SLE, and their maintenance and promotion of chronic tissue inflammation are less clearly defined. Our goal in the current proposal is to use phenotypic and genetic approaches to characterize these cells from novel lupus-prone gene-mutant and cytokine reporter mice, and to dissect the events that lead to their development and maintenance in the inflamed kidney.

OVERLAP

There is no scientific or financial overlap of the above projects at this time. If the pending proposal is funded, Dr. Craft's effort on currently funded grants will be adjusted as needed so that his total effort does not exceed 11.5 calendar months.

OTHER SUPPORT

KANG, I

ACTIVE

YA-002 Research Grant (Craft) 5/20/2013 - 5/19/2017 2.40 calendar
AbbVie Yale Collaboration in Immunobiology $250,000
Clinical Assessment and Therapeutic Blockade of T - B Cell Collaboration in Autoimmunity
The goals of this proposal are to track circulating T follicular helper-like (cTfh-like) cells in lupus patients in relationship to therapies that abrogate Tfh function and/or development, including abatacept.

Rheumatology Research Foundation (Craft) 07/01/14 – 06/30/16 2.88 calendar
Studying Monocytes and iPS cells in RA $185,185
The goals of this project are to derive iPSCs from peripheral blood mononuclear cells (PBMCs) of RA patients bearing the RA-risk *IRF5* SNP rs2004640; generate isogenic iPSCs by editing the RA-risk *IRF5* SNP rs2004640 to the non-risk SNP, using CAS9 (CRISPR-associated) nuclease and CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) gene editing; differentiate monocytes (MO) from iPSCs and isogenic iPSCs; define the function and transcriptomes of MO differentiated from iPSCs and isogenic iPSCs; transplant iPSCs into humanized mice, followed by assessment of their phenotype and function, and their role in induction and as therapeutic targets in inflammatory arthritis.

Research Grant (Iwasaki) 4/15/13 – 4/14/15 .72 calendar
AbbVie Pilot Research Study Proposal $250,000
Role of Endogenous Retroviruses in Lupus Pathogenesis
The goal of the proposed project is to understand the disease pathogenesis of SLE and possibly develop a new strategy to diagnose, prevent and/or treat SLE in humans.

PENDING

R01 (Kang) 4/1/15 - 3/31/2020 2.16 calendar
NIH $499,999
Immune dysregulation at the Interface of Aging, Lung Function, and COPD
The goal of this project is to investigate the interface among aging, lung function and COPD

OVERLAP

None

OTHER SUPPORT

WEINSTEIN, J.

ACTIVE

5 T32 AR07107-39 (Craft) 7/1/76 - 8/31/16 12 calendar
NIH/NIAMS $60,772
Training Program in Investigative Rheumatology
The goal of this project is to train five M.D. and Ph.D. postdoctoral fellows yearly for careers in investigative rheumatology and immunology.

PENDING

K01 (Weinstein) 4/1/15 – 3/31/16 12 calendar
Transcriptional Regulation of T Follicular Helper Cells in Lupus $118,625
Molecules that are necessary for T lymphocyte effector function are critical for end-organ injury in systemic lupus erythematosus. Their identification and characterization will provide insight into therapeutic targets in disease.

OVERLAP

There is no scientific or financial overlap of the above projects at this time. If the pending proposal is funded, effort on currently funded grant will be adjusted accordingly.

1. SPECIFIC AIMS

Pathogenic $CD4^+$ T helper (Th) cells are critical for disease promotion in systemic lupus erythematosus (SLE, lupus). These cells exert their effector function via autoreactive B cell help in secondary lymphoid organs (SLOs), including the spleen or lymph nodes, or by infiltration of tissues, such as the kidney. In both cases, autoantibody production and Th-cell infiltration, activation of innate immune cells ensues, mediated by autoantibody-autoantigen complex engagement and by tissue delivery of contact-dependent and soluble factors. The consequent inflammation leads to organ dysfunction and, sometimes, its loss. Thus, dissecting signals that promote $CD4^+$ Th-cell differentiation and maintenance in lupus is critical for understanding pathways of disease initiation and progression, and for identification of new therapeutic targets, or confirmation of existing ones.

$CD4^+$ Th-cell differentiation is initiated in SLOs after naïve T cells receive antigen (Ag) plus costimulatory signals delivered by dendritic cells (DCs), along with environmental cues including cytokines. The latter, acting via transcription factor induction and regulation, direct differentiation and maintenance of the classical Th-cell subsets, Th1, Th2, Th17, and follicular helper (Tfh) cells, among others[1]. The former three aid pathogen elimination via interaction with cells of the innate immune system, while the latter Tfh cells drive B cell maturation with pathogen-specific antibody (Ab) formation. Likewise, Th1, Th2, and Th17 cells promote inflammation in lupus via migration to and effector function in tissues[2-7], while Tfh cells remain in SLOs initiating autoreactive B cell maturation with generation of pathogenic memory B cells and long-lived autoantibody-producing plasma cells[8]. Thus, tissue-effector Th subsets and SLO-resident Tfh cells contribute to tissue injury in SLE.

While much is known about the extrinsic, largely cytokine, cues that direct Th-subset differentiation and maintenance *in vitro* in cell culture, and following infection or immunization of normal mice, the factors that promote Th1-cell, for example, and Tfh-cell differentiation in SLE, and as importantly, their maintenance and subsequent promotion of chronic tissue inflammation, are less clearly defined. Using a murine viral infection model -- so chosen because of its short-term flexibility and heightened type I interferon (type I IFN) signaling, the latter analogous to that which occurs in lupus – we have defined the signals that distinguish Th1- and Tfh-cell differentiation[9], a choice grounded in the observations that both subsets are pathogenic in murine and human lupus[2,3,7,10,11]. It is also based upon the findings that signals leading to Th2 vs. Tfh, or Th17 vs. Tfh, differentiation in conventional immune responses follow a similar paradigm; *i.e.*, Th1 vs. Tfh differentiation can be used to model differentiation of other peripherally acting Th-cell subsets compared to Tfh cells. We now plan to extend our studies to a lupus model, characterizing the steps required for pathogenic Th1- versus Tfh-cell differentiation and tissue maintenance and injury. While murine models of disease are excellent tools for studies of pathogenesis, their utility is magnified when their dissection is done in parallel with studies of SLE patients. We plan this combined approach, and will:

Phenotypically and genetically characterize Th1 and Tfh cells in lupus, dissect the signals required for their development and maintenance, and determine their role in autoantibody formation and tissue injury, using novel cytokine reporter and gene-mutant mice. We will in parallel genetically characterize circulating Th1 and Tfh cells from patients with SLE, cells that in our preliminary studies have a phenotype similar to those we believe pathogenic in mice.

BACKGROUND

$CD4^+$ Th cells are central to regulation of immunity. Tfh cells promote Ig affinity maturation and class switching upon migration to B-cell follicles and ultimately GCs, whereas other Th

subsets, such as Th1 and Th17, and Th2, migrate to tissues to mediate inflammatory or allergic responses, respectively. In a like manner, $CD4^+$ Th cells promote pathogenic Ab formation and tissue inflammation in lupus (reviewed in[1,8]). For example, Tfh cells, necessary for B cell maturation, drive autoreactive B cell responses via their canonical cytokine IL-21 in murine lupus[12], and interruption of the latter's signaling is therapeutically beneficial[13-16]. Production of IFN-γ, a product of Th1 cells, correlates with disease severity in multiple murine lupus strains, promoting renal disease[17-23] and activating myeloid and tissue cells that promote the inflammatory response[11,24]. Similarly, IL-4 and IL-17, produced by Th2 and Th17 cells, respectively, promote tissue injury in murine disease models[5,6,25-27]

$CD4^+$ Th cells promote pathogenic outcomes in patients with SLE. Th1 cells are found in nephritic kidneys, with their presence correlated with the severity of inflammation[2,3,10]. Tfh cells are also pathogenically important. GC B cell maturation, dependent upon Tfh-cell help and IL-21 secretion[28-30], is aberrantly regulated in SLE[31,32], with B cells bearing a GC or plasmablast phenotype detected in the circulation of patients with active disease[31,33-35]. $CD4^+$ T cells with a phenotype similar to tonsillar Tfh cells also have been isolated from the blood of patients, with such so-called circulating Tfh (cTfh) cells correlated with disease severity[36-38]. Despite their phenotypic similarities, however, the cTfh population lacks features of classical Tfh cells resident in human SLOs, including upregulation of the canonical Tfh cell transcription factor Bcl6[37]. These observations have called into question the precise relationship of the blood cells to the Tfh population, as we have reviewed[1]. Nonetheless, the expansion of blood borne cTfh-like cells in diseases in which autoantibodies are so prominent logically infers that the circulating population reflects the aberrant GC homeostasis of disease. Extrafollicular Tfh cells have also been found in nephritic kidneys of lupus patients, in aggregates with B cells, and are associated with the severity of tubulointerstitial inflammation[39]. These studies in aggregate imply that disruption of Th-cell effector function, including production of pathogenic cytokines or cytotoxicity by autoreactive Th1 cells, has therapeutic promise in SLE.

The signals that promote development and maintenance of $CD4^+$ Th cells in conventional immune responses have been defined *in vitro* or following infection of normal mice. Their differentiation is determined by interactions of naïve T cells with DCs and their cytokines, the latter signaling via the JAK (Janus kinase)-STAT (Signal Transducer and Activator of Transcription) pathway, with subsequent regulation of cell differentiation via expression of transcription factors, including the canonical T-bet, GATA3, RORγt, and Bcl6 necessary for development of Th1, Th2, Th17, and Tfh cells, respectively[1]. These transcription factors control expression of surface-bound and soluble proteins that dictate cell function, plus that of chemokine receptors and adhesion molecules that regulate tissue localization. Thus, effector $CD4^+$ Th subsets develop in response to environmental signals, including cytokines, and are defined by transcription factor expression, cytokine production, and function. Although $CD4^+$ Th cells are pathogenic in murine lupus, and appear to be in SLE, relatively less is known about the signals that promote their differentiation and effector function in disease.

3. PUBLISHED and PRELIMINARY DATA

Tfh- and Th1-cell development: A gradient model of differentiation. Cytokines are of particular importance in $CD4^+$ Th-cell differentiation. For example, IFN-γ and IL-12 promote Th1-cell development, signaling via STAT1 and STAT4, respectively, to initiate T-bet transcription, with IL-4 acting via STAT6 and subsequently GATA3 that of Th2 cells; IL-2 signaling via STAT5 is important for differentiation of both subsets. By contrast, STAT3 driving Bcl6 transcription following IL-6 activation is necessary for Tfh-cell development[40]. Yet, the nascent $CD4^+$ Th cell does not respond to singular signals; rather, it must integrate a variety of environmental cues as it differentiates. We have focused on this issue in our recent work,

determining how the differentiating cell balances cytokine signals during development of Th1 versus Tfh subsets, given their pathogenic roles in lupus. We began with the premise that both differentiate from the same naïve precursor following receipt of Ag stimulation by DCs in the T cell zone of SLOs. We used a murine viral infection model -- so chosen because of its short-term flexibility with ease of genetic manipulation, and because of its heightened type I interferon (type I IFN) production analogous to that which occurs in lupus – to demonstrate that these two Th-cell fates are determined by a gradient of inputs, promoting Th1-cell differentiation at the expense of that of Tfh cells and vice-versa[9] (Appendix) (**Fig. 1**).

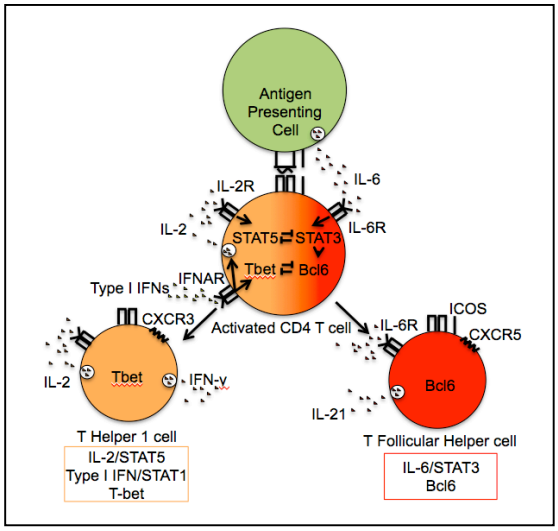

Fig. 1. Activated CD4$^+$ Tfh and Th1 cells differentiate from a naïve precursor based upon a gradient of inputs from the TCR stimulation, co-stimulation, and cytokines. IL-6 signaling through STAT3 promotes Tfh-cell differentiation via induction of Bcl6, whereas type I IFNs and IL-2 promote Th1-cell differentiation through T-bet and STAT5 upregulation, respectively. Once subset commitment is initiated, repression of the other subset occurs through STAT3 and STAT5 chromatin binding competition and T-bet and Bcl6 mutual repression (see text and Ray, *et al.*, I*mmunity*, 40:367-77, 2014).

Such cross-repression of Th1- and Tfh-cell development was surprising; however, upon reflection it made sense: the nascent T helper cell integrates environmental cues, as a rheostat would, rather than as an on-off switch, balancing the needs of the infected host for genesis of inflammatory Th1 cells, or Tfh cells that drive GC B-cell Ig class switching to produce isotypes appropriate to the invading pathogen. For Th1 immunogens such as viruses, the latter is inflammatory IgG2a. In a like manner, Th2 cells, and Tfh cells that drive Ig switch toward IgE, arise at the expense of one another, as we have shown in a recent collaboration with the Iwasaki lab at Yale[41]. In this latter case, nascent Th cells balance DC and environmental cues to generate Th2 and Tfh subsets responsive to challenge by helminths or allergens.

Tfh- and Th1-cell development in murine lupus. By contrast to our and other studies of CD4$^+$ Th-cell differentiation in normal immune responses, we know little of the signals driving, and their competing effects on, development of pathogenic Tfh and Th1 cells in SLE. To begin to investigate these signals, we used lupus-prone C57BL/6J (B6) *Sle1.Yaa* mice (from E. Wakeland, UTSW). B6.*Sle1.Yaa* mice have an introgressed *Sle1* locus from the highly penetrant NZM2410 strain, conferring loss of T and B cell tolerance to nuclear Ags[42-44], and carry a duplication of TLR7 via the Y-linked autoimmune accelerating (*Yaa*) locus[45-48]. The latter enhances disease susceptibility with robust type I interferon responsiveness[47,49], similar to many lupus patients[50-52]. B6.*Sle1.Yaa* CD4$^+$ T cells expand as early as age 6 weeks, with eventual association with aberrant GC responses and autoantibody-mediated glomerulonephritis[15,47,49], indicating onset of immunological abnormalities before end-organ disease. This sequence is analogous to that which occurs in SLE[53], underscoring the importance of dissecting initial signals that drive effector Th-cell differentiation in a tractable murine model. We initially examined Tfh and Th1 cells – identified as CD44^{hi}CXCR5^{hi}PD-1^{hi}PSGL1^{lo}Bcl6$^+$ and CD44^{hi}CXCR5^{lo}PD-1^{hi}PSGL1^{hi}T-bet$^+$, respectively[1] – from 7-month old B6.*Sle1.Yaa* mice, in parallel with lupus-prone MRL^{lpr} animals, finding that both populations from both strains, compared to cells from control B6 and B6.*Sle1* (non-*Yaa*) mice, were expanded in the setting of robust GC development (**Fig. 2A**), an expansion that began as early

as age 2 months (data not shown). Notably, both Tfh and Th1 cells co-secreted IL-21 and IFN-γ in a pattern analogous to that following lymphocytic choriomenigitis virus (LCMV) challenge of normal mice (**Fig. 2B**). Tfh cells also expressed both Bcl6 and T-bet, the canonical Tfh and Th1 transcription factors, including some that expressed both, whereas Th1 cells were only T-bet positive (**Fig. 2C**).

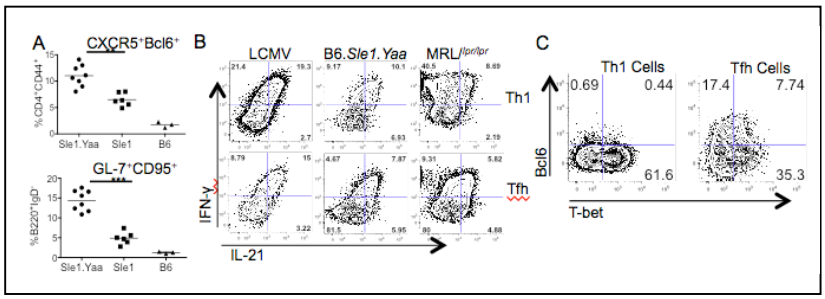

Fig. 2. Tfh and GC B cells are expanded in lupus-prone mice. B6.*Sle1* and B6.*Sle1.Yaa* mice were sacrificed at 6 months of age. **A**. Tfh ($Bcl6^{hi}CXCR5^{hi}$) and GC B cells ($B220^{+}IgD^{-}GL\text{-}7^{hi}CD95^{hi}$) cells from 6 months-old B6.*Sle1* and B6.*Sle1.Yaa* mice, compared to B6 controls. **B.** Intracellular IL-21 and IFN-γ protein expression by splenic $CD4^{+}CD44^{+}CXCR5^{lo}PD\text{-}1^{lo}$ Th1 and $CD4^{+}CD44^{+}CXCR5^{hi}PD\text{-}1^{hi}$ Tfh cells from B6 mice 7 days following infection with LCMV and from unimmunized B6.*Sle1.Yaa* and MRL^{lpr} mice. **C.** Intracellular Bcl6 and T-bet expression in Th1 and Tfh cells from B6.*Sle1.Yaa* mice at age 3 months.

As we considered these data, it was not surprising that Tfh cells expressed Bcl6 and secreted their canonical cytokine IL-21 needed for GC maintenance and plasma cell development[28,29,54], with T-bet driven IFN-γ presumably needed for B-cell class switching to the inflammatory IgG2a isotype[55]. Nonetheless, the expression of T-bet in lupus Tfh cells was puzzling, since this transcription factor is normally repressed by Bcl6 under homeostatic conditions[56]. Also puzzling was the Th1-cell production of IL-21, since the latter's transcription is dependent upon STAT3 (and ICOS, the inducible co-stimulator), as seen in Tfh (and in Th17) cells[57], with this STAT not known to have a role in Th1-cell development. These findings stimulated us to address the transcriptional regulation of Tfh and Th1 cells, using chromatin immunoprecipitation and RNA sequencing (ChIP-seq and RNA-seq) to examine their *TBX21* (T-bet) locus. We found this locus open and accessible in both (**Fig. 3**), consistent with their robust IFN-γ expression. These findings indicated that, in chronic inflammation, T-bet expression is not fully repressed by Bcl6 in Tfh cells and the regulation of cytokines by Th1 cells in chronic inflammatory states is not fully understood.

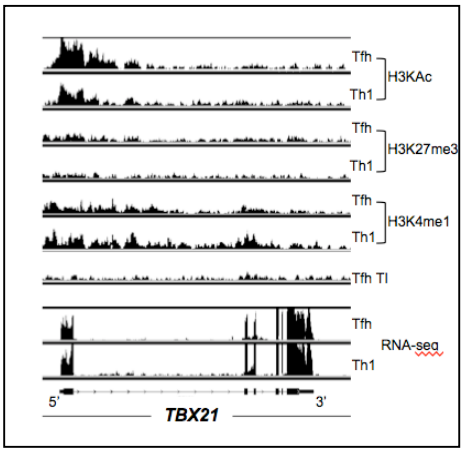

Fig. 3. T-bet expression in human Tfh cells promotes IFN-γ expression. A. Top Rows. ChIP-seq was performed using antibodies to H3K27Me3 (to identify repressive marks) and anti-H3KAc and -H3Kme1 (active marks) from sorted Tfh and Th1 cells. Peaks demonstrate activation (H3KAc and H3K4me1) before the promoter and within the exons of *TBX21* (T-bet). **Bottom Rows.** RNA-seq was performed on Tfh and Th1 cells sorted from human tonsils. Integrated genome browser view demonstrated increased peaks at the exons of *TBX21* transcripts in Tfh cells compared to Th1 cells, consistent with RNA expression.

As the field considers therapies to block the effector function of pathogenic $CD4^{+}$ Th cells in lupus, it seems important to better understand the regulation of their transcription factors and pathogenic cytokines. We next examined the kidneys of 4- and 7-month old B6.*Sle1.Yaa* animals, finding that they were infiltrated with both Tfh and Th1 cells (**Fig. 4**).

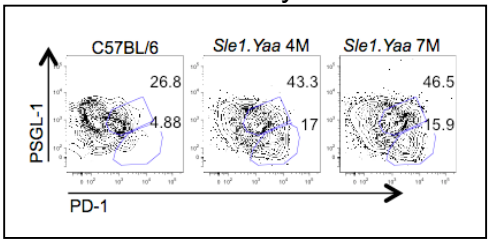

Fig. 4. Renal Tfh and Th1 cells in B6.*Sle1.Yaa* mice. Kidneys from B6.*Sle1.Yaa* mice aged 4 and 7 months, and from a 4-month old B6 control were digested with collagenase and lymphocytes were enriched. Representative flow cytometry plots of Tfh ($CD4^{hi}CD44^{hi}PSGL\text{-}1^{lo}PD1^{hi}$) and Th1 ($CD4^{hi}CD44^{hi}PSGL\text{-}1^{hi}PD\text{-}1^{mid}$) cells.

Renal Tfh cells may be promoting local B cell maturation with Ig production, as evidence suggests in SLE[39], and consistent with past work revealing B, and T cell infiltration in B6.*Sle1.Yaa* kidneys[49]. Th1 cells are presumably stimulating peripheral inflammatory responses via myeloid cell activation. Yet, the detailed phenotype, local regulation, and disease contributions of these populations are unclear.

Tfh and Th1 cells in SLE. To investigate the characteristics and function of Tfh and Th1 cells in SLE, we used flow cytometry to analyze blood samples of 49 patients, comparing them age- and gender-matched patients with Behçet's disease (BD), a chronic inflammatory illness, and healthy individuals. Cells were identified as we and others have described (reviewed in[1]), with cTfh-like cells CD45RA^{lo}CXCR5^{hi}PD-1^{hi} and Th1 cells CD45RA^{lo}CXCR5^{lo}ICOS^{hi}PD-1^{hi}, with the former secreting IL-21, and the latter expressing IFN-γ, their canonical transcription factor T-bet, and the peripheral homing chemokine receptor (and T-bet driven gene product) CXCR3. Both populations were expanded in the blood of SLE patients compared to diseased and healthy controls (**Fig. 5A**), suggesting a central role in pathogenesis. By comparison to T-bet expression in Th1 cells, the cTfh-like cells did not express the Tfh-cell transcription factor Bcl6 (data not shown), as previously demonstrated[56,58,59]. PD-1 was a particularly useful marker that enabled distinction of cTfh-like and Th1 cells in SLE patients compared to control groups, with its expression correlated with disease activity and circulating plasmablasts (**Fig. 5B,C**), and with anti-ds-DNA antibodies and hypocomplementemia (not shown).

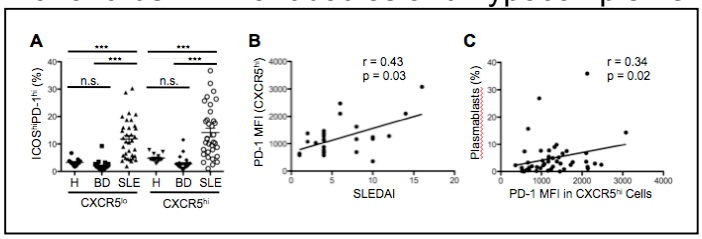

Fig. 5. Prevalence of circulating Tfh-like (cTfh-like; ICOS^{hi}PD-1^{hi}CXCR5^{hi}) cells in blood of healthy controls (Healthy; n = 16), patients with Behçet's disease (BD; n = 28), and patients with lupus (SLE; n = 49). A. Percentages of ICOS^{hi}PD-1^{hi} lymphocytes among CD4 CXCR5^{hi} cells in each population, with statistical analysis performed using one-way ANOVA (***p < 0.001). **B.** Correlation between the MFI of PD-1 on circulating CXCR5^{hi} cells and SLEDAI (Spearman r = 0.43, p = 0.03). **C.** Correlation between percentage of circulating plasmablasts among circulating B cells in SLE and PD-1 MFI on activated CXCR5^{hi} cells (Spearman r = 0.34, p = 0.02). Data points represent individual subjects.

PD-1 on Tfh cells engages its ligands PD-L1 and PD-L2 on GC B cells, and its signaling is critical for plasma cell maturation as well as Tfh-cell function[60], supporting a role for the cTfh-like cells in disease. By contrast, the association of PD-1 on Th1 cells with disease activity, plasmablasts, and anti-DNA antibodies was surprising, although this association may be secondary to the proinflammatory nature of these cells. Upon activation, they can also upregulate CXCR5 with B-cell helper function[61], inducing memory B cells to produce switched Igs[62].

As we analyzed the cytokine secretion by the cTfh-like and Th1 cells, we found that as in splenic *B6.Sle1.Yaa* Tfh cells (**Fig. 2B**), a portion of the former secreted IL-21^{+} as well IFN-γ^{+}, with these cells increased in SLE patients compared to healthy controls (**Fig. 6A, B**). Similarly, a portion of CXCR5^{lo}PD-1^{hi}IFN-γ^{+} Th1 cells were IL-21^{+}, and likewise expanded in SLE (**Fig. 6B**; flow plot not shown); *i.e.*, IL-21^{+}IFN-γ^{+} double positive (DP) cells existed among the expanded population of PD-1^{hi} cells in SLE, in both the CXCR5^{hi} Tfh and CXCR5^{lo} Th1 subsets. These were intriguing findings, considering that IL-21 and IFN-γ, the canonical Tfh and Th1-cell cytokines, respectively, appear critical for disease initiation and progression in murine lupus, as outlined above (Background). Similarly, in SLE patients, these cells may well promote pathogenic autoantibody secretion and amplify inflammation in target tissues such as the nephritic kidney. Yet, as in the mouse, they are not well defined, an important first step in determining their pathogenicity. The production of cytokines by both populations also argued that their PD-1 expression did not indicate exhaustion, as seen in chronic infections.

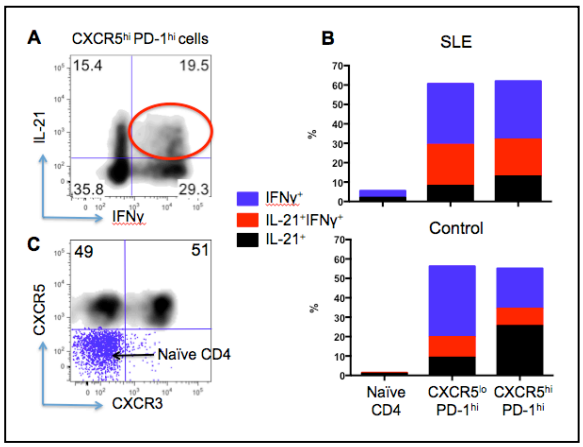

Fig. 6. Increased frequency of IL-21$^+$ IFN-γ$^+$ cells in cTfh-like and Th1 cells in the blood of SLE patients. Surface-stained PBMC were sorted by flow cytometry into naïve (CD45RA$^+$), Th1 (CD45RA$^-$CXCR5^{lo}PD-1^{hi}), and cTfh-like (CD45RA$^-$CXCR5^{hi}PD-1^{hi}) populations, followed by PMA/ionomycin stimulation and staining with anti-IL-21 and anti-IFN-γ. **A**. IL-21 and IFN-γ staining of cTfh-like cells from a representative SLE patient. Cells encircled in red are IL-21$^+$ IFN-γ$^+$ double positive (DP). **B**. Percentage of indicated cytokine$^+$ cells in each subset, with the DP cells increased in lupus Th1 and cTfh-like cells. **C**. Density plot of CXCR5 and CXCR3 expression of cTfh-like cells overlaid onto their naive counterparts (in blue).

4. EXPERIMENTAL DESIGN AND METHODS

Our goals are to phenotypically and genetically characterize Th1 and Tfh cells in lupus, dissect the signals required for their development and maintenance, and determine their role in autoantibody formation and tissue injury, using novel cytokine reporter and gene-mutant mice. We will also genetically characterize circulating Th1 and Tfh cells from SLE patients, cells that in our preliminary studies have a phenotype similar to those we believe pathogenic in mice.

Rationale and Overview. We will use traditional phenotypes and transcriptome analysis to characterize Tfh and Th1 cells in the blood and SLOs of lupus-prone mice alongside those in inflamed kidneys, comparing cells in these locations and determining their changes during the course of disease, and to dissect the signals required for their development. Knowledge gained from these experiments will enable us in the longer term to investigate the roles of distinctive Tfh and Th1 effector molecules in tissue injury, and help us determine whether or not sampling the blood, or SLOs, accurately reflects the pathogenic potential of tissue-infiltrating cells in the kidney, and how these cells compare to those from the blood of patients with SLE (Aim 2). We also will assess the pathogenic roles of CD4$^+$ Th cells in driving autoantibody formation and end-organ disease, investigating the contribution of their canonical cytokines to tissue injury, as proof-of-principle they contribute to the ongoing inflammatory response. These experiments are facilitated by our development of novel cytokine reporter mice that enable tracking of cells in SLOs and in tissues. We also have genetic mutants to dissect the signaling pathways needed for development of pathogenic CD4$^+$ Th cells in lupus, with experience analyzing these genetically altered animals[9].

We will in parallel sequentially characterize cTfh-like and Th1 cells from patients with SLE, including during disease flares, with the goal to compare these cells genetically to those from mice with lupus. The best way to determine if cTfh-like cells truly reflect Tfh cell-driven abnormal GC biology, or if Th1 cells reflect tissue injury, would be direct comparison of the two populations, with their simultaneous isolation and analysis from the blood, SLOs and inflamed tissues of patients. Yet, it is challenging to access diseased secondary lymphoid tissues in SLE. Although tonsillar biopsies have been performed in such patients[32], these and lymph node biopsies are generally not indicated in clinical care, and therapeutic splenectomies in non-oncologic illnesses, for example in the autoimmune cytopenias of SLE, are rarely done. While kidney biopsies are performed in patients with clinical nephritis, the amount of tissue obtained is small, and perhaps more importantly in the context of our planned experiments, sequential samples are generally not available. Consequently, investigators have turned to blood cells to glean insights into the biology of diseased SLOs and end organs, as we will do here. In our analysis, though, we will directly compare circulating cells to those isolated from the blood, SLOs, and kidneys of pre- and post-diseased lupus mice, cells that in our preliminary studies have a phenotype similar to those from isolated form the blood of patients.

Characterization of Tfh and Th1 Cells Using Cytokine Reporter Mice. We will analyze Tfh and Th1 populations before and after onset of renal disease, at ages 2, 4, and 7 months. Cells will be characterized using traditional flow cytometric phenotypes in combination with RNA expression (RNA-seq). We will use novel B6.*Sle1.Yaa* IL-21 and IFN-γ reporters that, when used in combination with flow cytometric markers, will enable tracking of Tfh and Th cells in the blood, SLOs, and in kidneys, an approach not heretofore taken in lupus to our knowledge. We recognize that other Th effector cells, such as Th17 and Th2 cells, also contribute to tissue injury in lupus[5,6,63], although as argued above, events promoting Tfh- vs. Th1-cell differentiation are paradigmatic for those of other subsets, and in the interest of time and resources, we will focus on the latter herein. We have generated and characterized in normal mice a novel knock-in *Il21* reporter mouse TWIK (Twenty-one Inducible Katushka), in which the transcript of a fluorescent protein (Katushka, essentially red) was inserted into the 3' UTR of the *Il21* locus, and which faithfully marks IL-21 protein production[64]. We have crossed this animal to an IFN-γ reporter mouse (*Ifng/Thy1.1* BAC-In) in which IFN-γ^+ cells express a surface-bound Thy1.1 (CD90.1) reporter[65]. Thus, we can track IL-21 and IFN-γ-expressing Tfh- and Th1 cells, at least as determined by their cytokines, using flow cytometry and confocal microscopy. Our TWIK and *Ifng/Thy1.1* BAC-In reporter mice on the B6 background are being crossed to B6.*Sle1.Yaa* mice, and Tfh and Th1 cells will be analyzed using their cytokine expression in combination with classical flow cytometric markers that distinguish these cells, as we have described[1]: Th1 cells are $CD4^+CD44^+PSGL\text{-}1^{hi}CXCR5^{lo}TWIK^+Thy1.1^+$ and Tfh cells are $CD4^+CD44^+PSGL\text{-}1^{lo}CXCR5^{hi}TWIK^+Thy1.1$. Reporter negative cells will be analyzed in tandem. Samples will be taken from mice at ages 2, 4 and 7 months, the latter when animals are autoantibody$^+$ and proteinuric[49]. Assays for analysis of isolated cells – flow cytometry, serology, renal pathology -- are well established in our lab, as we have published[9,58,66-69]. All data will be presented as the mean ± SEM. The significance of the difference between groups will be analyzed with one-way ANOVA, with the significance of difference between two groups evaluated by the two-tailed Student's t test. Spearman correlation coefficient or Pearson correlation coefficient with two-tailed p value will be determined in the analysis of correlations. Numbers of mice for these, and experiments outlined below, are based upon our past studies[9,58,66-69]. Typically, we obtain significance using 5-10 animals per group per time point, with B6 controls analyzed in tandem.

Tfh and Th1 Cells – Transcriptome Analysis. RNA will be prepared from sorted murine and human populations, with cells identified as described in our Preliminary Data. RNA-seq has revolutionized the field of transcriptomics[70,71], as this method creates highly reproducible and quantitative measures of gene expression levels with five orders of magnitude dynamic range[72]. The data are also free of the cross-hybridization and noise often seen with older microarray technology. Furthermore, RNA-seq provides measures of allele-specific expression, post-transcriptional mutation detection, and presence of alternately or aberrantly spliced isoforms[73-75]. We have considerable experience with this approach, comparing RNA expression of murine Tfh to Th1 cells from virally infected mice[9], and of human tonsillar Tfh to Th1 cells (**Fig. 3**). We anticipate that a majority of the genes expressed by cells with Tfh and Th1 phenotypes in the blood, SLOs and the kidney will be similar, simplifying our search for differences. We will analyze existing databases to seek differences, focusing upon genes involved in Th1- and Tfh-cell development, and to search for transcription factor binding sites seeking those enriched in differentially expressed genes. We will group genes to compare pathways that may differ. We expect that our results will provide clarification of the pathways involved in cytokine expression in Tfh vs. Th1 cells in blood, SLOs, and kidneys.

Data Analysis. Tophat will be used to map reads and identify splice junctions[76]. Transcript

isoform or gene expression changes will be assessed with Cufflinks software using "reference" annotation[77]. Cufflinks will also be used to assemble novel transcript isoforms if long paired end reads are performed with high coverage. Some reads map to more than one transcript isoform, and Cufflinks uses a statistical model of the observed reads to derive a likelihood for the abundance of a set of transcripts. Other software (edgeR, DEGseq, DESeq and baySeq) packages will be used as appropriate (replicate data and multiple types of samples)[78-80]. edgeR will be used to create an overdispersed Poisson model to account for biological and technical variation and an empirical Bayes method is used to shrink genewise dispersions by borrowing information between genes. It can handle two or more groups and only requires one set of experimental replicates. The patterns of gene expression are characterized using a panel of software (GOstats, DAVID, Ingenuity, and gene set enrichment analysis) to identify pathways or gene categories that are significantly changed[81,82]. The expression patterns of gene sets of interest are visualized using heatmaps. The expression of different samples is analyzed using hierarchical clustering, signal density plots, signal boxplots, pairs plots and MVA plots using R scripts. This type of analysis is very useful in identifying sample problems, outliers, and to understand the overall relationships between different samples. The patterns of individual RNA sequencing samples are visualized using the Integrated Genome Browser (IGB) software or the UCSC genome browser[83,84]. IGB allows users to visually assess changes in signal levels and splicing patterns at different regions of the genome.

Expected Results, Pitfalls, Alternatives. Similar to our work in a viral system[9] and using tonsils (**Fig. 3**), we expect to identify genes differentially expressed between Tfh and Th1 cells, and perhaps between Tfh and Th1 cells in SLOs and the kidney, and likewise between cTfh and Th1 cells in SLE. Results will be meaningful with any outcome, as differences in the respective tissues will offer clues to pathogenic potential, with similarities suggesting common pathways and potential therapeutic options. We have experience performing RNA-seq on Tfh and Th1 cells from SLOs in a viral model[9], as well on Tfh and Th1 cells isolated from human tonsils (**Fig. 3**). Although the numbers of cells from inflamed kidneys of B6.*Sle1.Yaa* mice are more limited than from SLOs, we find that each 7-month old kidney provides approximately 4 x 10^3 of both Tfh and of Th1 cells. We can obtain 1 ug of transcriptome-grade RNA from 4 x10^6 cells, so each kidney provides around 1 ng RNA, or 20 ng from 10 mice. We also are developing experience with protocols that rely upon smaller RNA amounts (1 ng), doing RNA-seq using the Nugen Ovation system; thus, we should have adequate samples for analysis. Based on conservative calculations and experience handling patient samples, 50 ml blood (5 green top tubes) yields a minimum of 1.5 to 3 x 10^5 cTfh-like and Th1 cells in SLE, or 3-6 fold more than we found necessary (0.5 x 10^5 cells) in pilot experiments. For blood samples, we will use the Yale Lupus Cohort comprised of patients followed by the fulltime faculty at Yale (see Resources), as we have done in past clinical studies[85,86]. While identification of sufficient numbers of cTfh-like and Th1 cells in healthy controls, as a comparator to our SLE patients, may be an issue, our focus is on comparison of these cells in SLE, and to the mouse, which by contrast are expanded (**Fig. 5**), and which should yield sufficient numbers for proposed experiments. While we will not be able to study Tfh cells from SLOs of patients, we do routinely obtain them from non-autoimmune subjects at tonsillectomy (**Fig. 3**). While not matched to patients, this provides a ready source of control polyclonal cells from which to further dissect Tfh and Th1 cells in comparison to that of autoimmune cTfh cells.

Tfh and Th1 Cells – Inductive and Maintenance Signals. We next will determine the factors required for initial Tfh and Th1-cell differentiation in SLOs, studies that will be analogous to our work a short term viral model[9] (Appendix), in which we used a combination of STAT-deficient mutant mice and cytokine blockade to determine the balance of inflammatory signals

promoting Tfh- versus Th1-cell differentiation. We will compare Tfh and Th1 development in wild type and mutant B6.*Sle1.Yaa* mice, including floxed strains to enable T-cell intrinsic dissection of signaling pathways known to be operative in conventional immune responses, emphasizing our goal to determine the role of T cells in disease promotion. Dissection of these competing signals seems particularly important as therapies that interrupt cytokine signaling are in, or moving towards, clinical trials. Our gene mutant mice are on the B6 background, so crossing them to B6.*Sle1.Yaa* is straightforward as we need only to track 3 loci per cross, a relatively easy matter and one with which we have experience[18,66-68,87-94]. We will focus initially on roles of STAT1 and STAT3 in Tfh and Th1 development, based upon availability of floxed strains in our colony, and given their likely roles in murine. The latter is determined by our published data in the high-type I IFN viral model in which we demonstrated that STAT3 signaling is critical for Tfh-cell development, with type I IFN, presumably signaling via STAT1, promoting Th1-cell differentiation[9]. Floxed *Stat1* and *Stat3* (*Stat1*$^{fl/fl}$ and *Stat3*$^{fl/fl}$) mice are breeding to B6.*Sle1.Yaa* animals, as is our CD4-Cre strain. *Stat3* is on murine chromosome 11, so B6.*Sle1.Yaa* mutants should be produced at Mendelian rates. *Stat1* resides on ch. 1 at 26 cM, or a distance from *Sle1* at 76 cM to facilitate sufficient recombination events. Finally, while we plan to utilize B6.*Sle1.Yaa* mice in the proposed studies, we have experience with other lupus models, including MRL/*Fas*^{lpr} and (NZB x NZW)F_1[18,66-68,87-94], and will be cognizant of the need to investigate them, pending results in B6.*Sle1.Yaa* mice. As an adjunct to the experiments with mutant mice, we also will investigate the effects of cytokine blockade on Tfh and Th1 development in wild type B6.*Sle1.Yaa* animals, analyzing both pre-diseased and older mice. We will use blockade of cytokines signaling via STAT1 and STAT3 – anti-type I IFNs and IFN-g, and anti-IL-6, respectively -- to compare to gene mutant mice. Blockade will be done as we have published[9] (Appendix), over the course of 10-14 days.

Finally, we note that the signals that maintain these cells in chronic autoimmunity have not been tested. This is a difficult problem, as there is not a ready way to selectively delete signals for maintenance of these cells during disease, as opposed to developmentally. If, as we anticipate, dissect the effects of STAT mutations on Tfh compared to Th1 cell development, then these become an ideal system to do this, as long as they are tissue-specific and, preferably, inducible. We have tamoxifen inducible CD4-Cre mice, and will use these for tissue-specific deletion before and after disease onset. The efficiency of Cre deletion using these animals is around 70%, which based upon our analysis of ICOS-deficient B6.*Sle1.Yaa* mice is sufficient for disease amelioration (**Fig. 7**, below); thus, we anticipate we can determine the effects on individual *Stat* deletion on cellular and disease phenotypes.

Expected Results, Pitfalls, Alternatives. While one might attempt to predict the effects of extrinsic signals upon Tfh and Th1-cell differentiation in lupus based upon *in vitro* studies or examination of normal mice following immunization or infection, it is simply not known how these signals are balanced in the chronic inflammatory milieu typical of lupus. For example, IFN-γ is important in maintenance of Tfh cells in murine lupus[23], but this does not appear to be the case in our short term viral model; rather, type I IFNs drive Th1 development at the expense of Tfh cells, with type II IFN having a minimal effect[9]. We have extensive experience using *in vivo* antibody blockade to dissect the effects of cytokines on Tfh and Th1 development, and anticipate no problems. As needed, we can also use cytokine blockade to analyze the requirements of SLO and renal maintenance of these cells. We have contracted out production of the needed blocking antibodies. These experiments will be a nice parallel to those using inducible tissue-specific gene mutants. Finally, we recognize that signaling via other STATs may be important in development of Th1 cells, for example. In addition to STAT1, type I IFNs signal via STAT2 and STAT4; thus, we will investigate these molecules as time and

resources permit, and based upon the outcome of experiments using our STAT1 and STAT3 mutants. We have STAT2 and STAT4 mutants in our colony; these are not floxed, so any investigations using these animals would be through production of bone marrow chimeras.

Maintenance and Pathogenic Roles of Tfh and Th1 Cells. The developmental ontogeny of pathogenic Tfh and Th1 cells in lupus remains unclear. By 2 months of age B6.*Sle1.Yaa* mice have increased splenic Tfh and GC B cells. Despite the ongoing inflammatory response at this time point, these mice do not have phenotypic evidence of disease, such as autoantibodies or kidney injury (not shown). To determine the separable roles of Th1 and Tfh cells in disease pathogenesis, we will perform adoptive transfers of these Th-cell populations from wild type B6.*Sle1.Yaa* reporter^{+} mice into their congenic ICOS-deficient B6.*Sle1.Yaa*.ICOS$^{-/-}$ counterparts bred in our lab. Th cells require ICOS signaling for proper activation, differentiation, and effector cytokine expression[95], with wild type and lupus-prone mice deficient in this molecule having decreased Th1 and Tfh cells, with failure to develop GCs[67,96,97]. Our seven-month old B6.*Sle1.Yaa*.ICOS$^{-/-}$ mice fail to generate Bcl6^{+} Tfh cells and have a reduced percentage of GC B cells and anti-chromatin antibodies compared to ICOS-sufficient lupus mice (**Fig. 7**, data not shown). Thus, these animals are ideal recipients to determine the roles of Tfh and Th1 cells in disease initiation and maintenance. We will transfer cell-sorted splenic CD4^{+}CD44^{+}PSGL-1^{lo}CXCR5^{hi}PD-1^{hi}reporter^{+} Tfh or CD4^{+}CD44^{+}PSGL-1^{hi}CXCR5^{lo}PD-1^{lo} reporter^{+} Th1 cells from early (2 month) or aged (7 month) mice into their congenic ICOS$^{-/-}$ counterparts, with phenotypic assessment of splenic and renal cells as described above, in addition to monitoring the transferred population to insure stability of their phenotype. Our lab has considerable experience in the transfer of T cells to ICOS-deficient recipients with excellent results, with transferred populations temporally stable, and capable of driving a phenotype in recipients consistent with the function of the transferred cells[58].

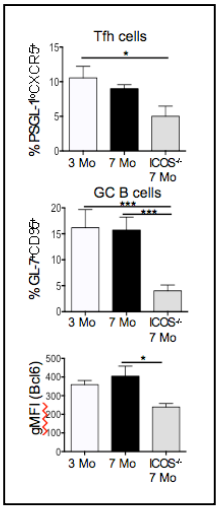

Fig. 7. Deficiency of ICOS in B6.*Sle1.Yaa* mice reduces Tfh and GC B cells in aged mice. Splenocytes from 3 and 7-month old B6.*Sle1.Yaa* ICOS$^{+/+}$ and 7-month old B6.*Sle1.Yaa* ICOS$^{-/-}$ mice were analyzed. **Top.** Percentages of splenic CD4^{hi}CD44^{hi}PSGL-1^{lo}CXCR5^{hi} Tfh cells in ICOS$^{+/+}$ and ICOS$^{-/-}$ B6.*Sle1.Yaa* mice. **Middle.** Percentages of splenic B220^{+}IgD^{-}GL-7^{hi}CD95^{hi} GC B cells. **Bottom.** Geometric mean fluorescence intensity (gMFI) of Bcl6 expression in splenic Tfh cells.

Expected Results, Pitfalls, Alternatives. These experiments will help determine if Th1 cells secreting IL-21 and IFN-γ, in the absence of Tfh cells, are capable of initiating and maintaining tissue inflammatory responses, or driving GC-like reactions in extrafollicular sites[98], or conversely, determine the degree to which Tfh cells producing the same cytokines promote organ injury in the absence of Th1 cells. As we assess the results of this experiment, we may also need to transfer cells on a more frequent basis, but this will be determined experimentally. We also plan to transfer both populations in tandem, to determine if disease phenotypes are exacerbated compared to single transfers. In parallel, we will use this transfer model to examine the disease contribution of Tfh-cell secreted-IFN-γ and -IL-21, using B6.*Sle1.Yaa* IFN-γ$^{-/-}$ and IL-21$^{-/-}$ mice currently breeding in our colony, again monitoring cells for stability of the transferred phenotype. This experiment will enable us to determine the separable contributions to autoantibody production and tissue injury of IFN-γ$^{-/-}$ or IL-21$^{-/-}$ produced by Tfh cells. We anticipate that cytokine production by Th1 cells will be unaltered in the absence of Tfh cells, and vice-versa, and that the phenotypes of CD4^{+} T cells transferred into ICOS$^{-/-}$ recipients will be stable, as we have shown[58]; however, if not, this result too will be informative, indicating the ability of these cells, in the inflammatory milieu of SLOs or the kidney, to modulate their phenotype, results that will be analyzed in the context of their transcriptomes.

5. MILESTONES

Year 1: Characterization of cytokine reporter mice, identification of signals involved in Tfh- and Th1-cell differentiation using antibody blockade, and initiation of RNA-seq experiments in mice and humans.

Year 2: Continuation of experiments from Year 1. Characterization of Stat mutant mice, and the pathogenic potential of Tfh and Th1 cells in adoptive transfer experiments. Mutant animals are currently breeding with anticipated completion by the winter of 2013-2014; however, we will need produce experimental animals.

6. SIGNIFICANCE

The events that initiate and sustain tissue injury in lupus are critical to understand, as their blockade is likely to be important therapeutically. Since there will undoubtedly be therapeutic toxicities or failures along the way, it seems reasonable to define the basis of this collaborative interaction in order to provide better understanding of disease and to maintain the pipeline of disease targets and therapeutic agents. Th1 cells promote tissue injury in lupus via migration to and effector function in peripheral tissues, whereas Tfh cells remain in SLOs promoting autoreactive B cell maturation with generation of pathogenic memory B cells and long-lived autoantibody producing plasma cells. Thus, both populations of cells, peripheral effector subsets and Tfh cells that reside in SLOs, contribute to tissue injury in SLE; however, the factors that promote differentiation of these cells in SLE, and as importantly, their maintenance and subsequent promotion of chronic tissue inflammation, are less clearly defined. Our goal herein is to use novel reporter and gene-mutant mice to address these uncertainties. We have demonstrated in preliminary studies that circulating Th cells from patients with SLE have similar phenotypes, including co-expression of IL-21 and IFN-γ. Our longer-term goal, then, is to compare these cells to those analyzed herein, with the anticipation that we can eventually glean clues about the pathogenic potential of these cells in SLE.

Expertise relevant to proposal. This proposal has arisen out of our studies of Tfh cells in which we have dissected their development and function in normal and in autoimmune responses[67,68](http://jimmunol.org/content/) (http://jem.rupress.org/content/), and along with others[56,59], identified Bcl6 as their canonical transcriptional regulator[58] (http://www.sciencemag.org/content/). We also have collaborated with Dr. Susan Kaech at Yale, our laboratory neighbor, to investigate their relationship to $CD4^+$ memory T cells that promote B cell maturation upon antigen rechallenge[99] (http://www.sciencedirect.com/science/article/) and to investigate STAT3-mediated T cell signaling[100](http://www.sciencedirect.com/science/article/). We have gone on to determine the relative roles of B-cell delivered Ag and costimulation in Tfh-cell differentiation[69] (http://www.jimmunol.org/content/) and in collaborative work with Ziv Shulman and Michel Nussenzweig at Rockefeller University demonstrated that Ag delivered by B cells drives Tfh-cell maturation within the GC[64]. More recently, as outlined in our proposal, we have determined the signaling gradient that promotes Tfh vs. Th1 differentiation in high type I IFN-states of the type found in SLE[9](http://www.sciencedirect.com/science/article/) and, in collaboration with the Iwasaki lab at Yale, investigated the DC signals that promote Th2 and its companion Tfh response[41](http://www.sciencedirect.com/science/article/). These observations have helped form the basis of this application. Given our expertise in the study of Tfh and Th1 cells in normal and autoimmune mice and humans with SLE, we believe we are well positioned to carry forward the proposed studies.

7. RELEVANCE TO THE MISSION OF THE ALR

A plethora of new agents have been used therapeutically in lupus, including nephritis, with mixed results. Explanations for these outcomes, apart from possible flaws in clinical trial design, are initiation of therapy after tissue inflammation and subsequent organ injury have occurred, and/or heterogeneity in immunopathogenesis; regardless, there is a pressing need to improve our understanding of disease mechanisms and organ injury in lupus, as a path toward selection of appropriate agents for therapeutic intervention.

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90 Peng, S. L. *et al.* Murine lupus in the absence of alpha beta T cells. *J Immunol* **156**, 4041-4049 (1996).

91 Peng, S. L. *et al.* alpha beta T cell regulation and CD40 ligand dependence in murine systemic autoimmunity. *J Immunol* **158**, 2464-2470 (1997).

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97 Bossaller, L. *et al.* ICOS deficiency is associated with a severe reduction of CXCR5+CD4 germinal center Th cells. *J Immunol* **177**, 4927-4932, doi:177/7/4927 [pii] (2006).

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99 Marshall, H. D. *et al.* Differential Expression of Ly6C and T-bet Distinguish Effector and Memory Th1 CD4+ Cell Properties during Viral Infection. *Immunity* **35**, 633-646 (2011).

100 Cui, W., Liu, Y., Weinstein, Jason S., Craft, J. & Kaech, Susan M. An Interleukin-21-Interleukin-10-STAT3 Pathway Is Critical for Functional Maturation of Memory CD8+ T Cells. *Immunity* **35**, 792-805 (2011).

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Patrick G. Gallagher	Professor Department of Pediatrics, Genetics, and Pathology
eRA COMMONS USER NAME (credential, e.g., agency login) PGG2PGG2	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY
Ohio State Univ., Columbus, Ohio	B.S.	1979	Zoology, Biochemistry
Northeastern Ohio Universities College of Medicine	M.D.	1985	Medicine

A. Personal Statement

A major focus of our laboratory is understanding the regulation of gene expression in normal and perturbed hematopoiesis. We have studied the role of specific transcription factors and epigenetic factors, including chromatin architecture and DNA methylation on the regulation of entire programs of genes, primarily those controlling expression of genes encoding erythrocyte membrane proteins. As detailed in the publications below, we have applied genomic strategies, including mRNA expression profiling, RNA seq, ChIP-chip and ChIP-seq technology, as well as novel phosphoproteomics and quantitative proteomics techniques, to our understanding of hematopoiesis. Our use of erythrocyte membrane genes as a model of regulation of complex genetic loci has had many benefits. The genes are large and are represented by many cell, tissue, and developmental stage-specific isoforms. Our studies have demonstrated that barrier insulators, elements critical for controlling tissue-specific gene expression, regulate erythroid-cell specific transcripts and that when mutated, lead to a disease phenotype. Another focus of the laboratory has been in the study of inherited disorders of the erythrocyte, including disorders of erythrocyte shape and metabolism. A special emphasis of this work has focused on the manifestation of these disorders in the fetus and newborn. Related studies include study of the genetic bases of volume regulation and ion transport in the erythrocyte in normal and disease states such as xerocytosis, hydrocytosis, and sickle cell disease. Our recent studies identified the hereditary xerocytosis gene as *FAM38A* encoding PIEZO1, the first report of mutations in a mammalian mechanosensory transduction channel. Together, these studies provide novel insights into the processing controlling erythropoiesis and will ultimately lead to a comprehensive understanding of the regulatory interactions that control specific gene expression programs during cell growth and development.

B. Positions, Honors, Activities

Positions

1985-88 Pediatric Intern & Resident, Children's Hosp Med Ctr, Univ of Cincinnati, Cincinnati, OH
1988-89 Chief Resident in Pediatrics, Children's Hosp Med Ctr, Univ. of Cincinnati, Cincinnati, OH
1989-92 Fellow in Neonatal - Perinatal Med, Dept. of Peds, Yale Univ Sch of Med, New Haven, CT
1990-94 Lecturer, Dept of Molecular Biochem & Biophysics, Yale Univ. Sch of Med, New Haven CT
1992-94 Assoc. Research Scientist, Depts. of Peds & Int Med, Yale Univ Sch of Med, New Haven, CT
1994-00 Assistant Professor, Department of Pediatrics, Yale Univ Sch of Med, New Haven, CT
1992-pres Attending Neonatologist, Yale-New Haven Hospital, New Haven, CT
1999-pres Asst Director, Neonatal-Perinatal Med Fellowship Training Program,Yale Univ SOM,New Haven,CT
2000-08 Associate Professor, Dept of Pediatrics, Yale Univ Sch of Med, New Haven, CT (without term)
2006-pres Director, Expression and Genomics Core, Yale Cntr of Excellence in Molecular Hematology
2008-pres Professor, Dept of Pediatrics, Yale Univ Sch of Med, New Haven, CT
2009-pres Professor, Dept of Genetics, Yale Univ Sch of Med, New Haven, CT
2011-pres Director, Yale Center for Blood Disorders
2011-pres Professor, Dept of Pathology, Yale Univ Sch of Med, New Haven, CT

Honors and Activities

1984: A.M.A. Scholarship in Clinical Nutrition, Combined Program - Harvard Medical School/MIT
1985: St. Elizabeth Award for Knowledgeable and Compassionate Care
1987: Resident Teaching Award, Dept. of Pediatrics, Univ. of Cincinnati
1988: Infectious Disease Society of America Kass Fellowship Award
1991: Trainee Travel Award, AAP/ASCI/AFCR 1992: Trainee Investigator Award, AAP/ASCI/AFCR
1993: Charles H. Hood Foundation Award 1993: NIH Clinical Investigator Development Award
1995, 99: Guest Ed, Seminars in Perinatology 1998: Distinguished Alumni Awd, NE Ohio Univ Col of Med
1996: Basil O'Connor Scholar Award, March of Dimes Birth Defects Foundation
1999-03 Ed Board, Gene Function Disease 2001:Mae Gaelani Faculty Teaching & Res Award
2001-pres: Ed Board, Ped Develop Path 2003-2008 : Editorial Board, Blood
2002: External reviewer, Telethon, Italy
2002-pres: Multiple Study Sections, NIH, ad hoc: Heme I, HP, ELB, VH, NHLBI PPG, NIDDK D, etc.
2003-: American Society of Clinical Investigation
2003-06: Am Soc of Hemat Scholar Awd Study Sect
2003: Ext Rev, S African Natl Res Fdn 2004-2008: Erythroid/Leukocyte Biol Stdy Sect,NIH
2007-09: Assoc Ed, Am J Hematol 2007-2009: Chair, ELB study Section, NIH
2008-13: Ed Board, J Biol Chem 2012: Doris Duke Res Foundation Study Section
2009-: Ed Board, Am J Hematol 2013-: Ed Board, PLoS One

C. Selected relevant publications.

1. An X, Schulz VP, Li J, Liu J, Wu K, Xue F, Hu J, Mohandas N, **Gallagher PG.** 2014. Global transcriptome analyses of human and murine terminal erythroid differentiation. *Blood.* 123:3466-77. PMID 24637361.

2. Bogardus H, Schulz VP, Maksimova Y, Miller BA, Li P, Forget BG, **Gallagher PG.** 2014. Severe hereditary spherocytosis due to uniparental isodisomy at the *SPTA1* locus. *Haematologica.* In press. PMID:24895341.

3. Su MY, Steiner LA, Bogardus H, Mishra T, Schulz VP, Hardison RC, **Gallagher PG.** 2013. Identification of biologically relevant enhancers in human erythroid cells. *J Biol Chem*. 288:8433444. PMCID: 3605659

4. Sriswasdi S, Harper SL, Tang HY, **Gallagher PG**, Speicher DW**.** 2014. Probing large conformational rearrangements in wild-type and mutant spectrin using structural mass spectrometry. *Proc Natl Acad Sci USA*. 111:1801-6. PMID:24453214

5. Guo S, Zi X, Schulz V, Cheng J, Zhong M, Koochaki SHJ, Megyola CM, Pan X, Heydari K, Weissman SM, **Gallagher PG**, Krause DS, Fan R, Lu J**.** 2014. Non-stochastic reprogramming from a privileged somatic cell state. *Cell.* 156:649-62. PMID:24486105

6. Zarychanski R, Schulz VP, Houston BL, Maksimova Y, Houston DS, Smith B, Rinehart J, **Gallagher PG.** 2012. Mutations in the mechanotransduction protein PIEZO1 are associated with hereditary xerocytosis. *Blood*. 120:1908-15. PMCID: PMC3448561.

7. Deng C, Li Y, Liang S, Cui K, Salz T, Yang H, Tang Z, **Gallagher PG**, Qiu Y, Roeder R, Zhao K, Bungert J, Huang S**.** 2013. USF1 and hSET1A mediated epigenetic modifications regulate lineage differentiation and HoxB4 transcription." *PLoS Genet*. 9:e1003524. PMCID: 3675019

8. Walker RH, Schulz VP, Tikhonova IR, Mahajan MC, Mane S, Arroyo Muniz M, **Gallagher PG.** 2012. Genetic diagnosis of neuroacanthocytosis disorders using exome sequencing. *Mov Disord*. 27:539-43. PMID: 22038564.

9. Steiner LA, Schulz VP, Maksimova Y, Wong C, **Gallagher PG.** 2011. Patterns of histone H3 lysine 27 mono-methylation and erythroid cell-type specific gene expression. *J Biol Chem*. 286:39453-65. PMCID: PMC3234769.

10. Stewart AK, Shmukler BE, Vandorpe DH, Rivera A, Li X, Hsu A, Karpatkin M, O'Neill A, Bauer DE, Heeney M, John K, Kuypers FA, **Gallagher PG**, Lux SE, Brugnara C, Westhoff CM, Alper SL**.** 2011. Loss-of-function and gain-of-function phenotypes of stomatocytosis mutant RhAG F65S. *Am J Physiol Cell Physiol*. 301:C1325-43. PMCID: PMC3233792.

11. **Gallagher PG**, Steiner LA, Liem RI, Owen AN, Cline AP, Seidel NE, Garrett LJ, Bodine DM**.** 2010. Hereditary spherocytosis due to mutation in a barrier insulator in the human ankyrin-1 gene. J Clin Invest. 120:4453-65. PMCID: PMC2993586.

12. Bouyer G, Cueff A, Egée VS, Kmiecik J, Maksimova Y, Glogowska E, **Gallagher PG**, Thomas SLY**.** 2011. Erythrocyte peripheral type benzodiazepine receptor /voltage-dependent anion channels are up regulated by *Plasmodium falciparum*. *Blood.* 118:2305-12. PMID: 21795748.

13. Steiner LA, Maksimova Y, Schulz V, Wong C, Mahajan MC, Weissman SM, **Gallagher PG.** 2009. Chromatin architecture and transcription factor binding regulate expression of erythrocyte membrane protein genes. *Mol Cell Bio*. 29:5399-412. PMCID: PMC2756878.

14. Rinehart J, Maksimova YD, Tanis JE, Stone KL, Hodson CA, Zhang J, Risinger M, Pan W, Wu D, Colangelo CM, Forbush B, Joiner CH, Gulcicek EE, **Gallagher PG**, Lifton RP**.** 2009. Sites of regulated phosphorylation that control K-Cl cotransporter activity. *Cell*. 138:525-36. PMCID: PMC2811214.

15. **Gallagher PG**, Nilson DG, Steiner LA, Maksimova YD, Lin Y, Bodine DM. 2009. An insulator with barrier element activity promotes alpha-spectrin gene expression in erythroid cells. *Blood.* 113:1547-54. PMCID: PMC2644083.

D. Research Support

Ongoing Research Support

5 R01HL106184-04 Gallagher (PI) 01/01/11-11/30/14
NIH NHLBI
Barrier Insulators in Erythropoiesis
This project studies the structure and function of barrier insulators in human erythroid cells.
Role: PI

5 T32 HD007094-38 Gallagher (PI) 07/04/77-04/30/16
NIH/NICHHD
Research in Perinatal Medicine
The major goal of this project is training in Perinatal medical research.
Role: PI

5 R01-HL65448-12 Gallagher (PI) 09/01/00-03/31/15
NIH NHLBI
Molecular Biology of Human Erythrocyte Alpha Spectrin
This project studies the expression of the human alpha-spectrin gene.
Role: PI

DDCF Gallagher (PI) 05/01/10-4/30/16
Doris Duke Innovations in Clinical Research
Erythrocyte Hydration Pathways as Modifiers in Sickle Cell Disease.
The goal of these studies is to identify mutations associated with erythrocyte hydration and assess their contribution to clinical phenotype in sickle cell disease.
Role: PI

Completed Research Support

RO1-DK62039 Gallagher (PI) 07/01/02-03/31/12
NIH NIDDK
Molecular Biology of Erythrocyte Ankyrin
The major goals of this project are to elucidate the molecular mechanisms involved in normal and abnormal expression of the human erythrocyte ankyrin (ankyrin 1) gene.
Role: PI

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors in the order listed on Form Page 2.
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NAME	POSITION TITLE
Joseph E. Craft	Paul B. Beeson Professor of Medicine Professor of Immunobiology Chief of Rheumatology; Director, Invest. Med. Prog.
eRA COMMONS USER NAME (credential, e.g., agency login) JOE_CRAFT	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY
University of North Carolina, Chapel Hill	A.B.	1969-1973	Chemistry
University of North Carolina School of Medicine	M.D.	1973-1977	Medicine
Yale University School of Medicine	Postdoctoral	1982-1985	Rheumatology/Immunol.

A. Personal Statement

Dr. Craft has a longstanding interest in dissecting the pathogenesis of systemic autoimmunity, focusing upon the activation and differentiation of CD4 T effector cell subsets in mice and in humans. His lab has characterized CD4 T cells that help B cells in murine models of lupus and in conventional immune responses, with the idea that information gleaned from the latter studies could be applied to our understanding of autoimmunity. In more recent studies, his lab has characterized separable CD4 T cell subsets that promote antibody and autoantibody help in secondary lymphoid organs and inflammation, including analysis of the transcriptional control and function of follicular helper and effector T cells, with additional studies on the role of CD4 T cell help for CD8 T cell development and function. Current studies involve further characterization of these cells in mice and in humans, and dissection of the mechanisms that lead to their activation, development, and effector function in inflammation and autoimmunity.

B. Positions and Honors

Positions and Employment

1977-80 Intern & Asst. Resident, Internal Medicine, Yale-New Haven Hospital (Chief, Samuel Their, MD)
1980-82 Instructor & Asst. Professor of Medicine (General Medicine), Yale University
1985-97 Assistant, Associate & Associate (tenure) Professor of Medicine (Rheumatology), Yale University
1991-pres. Chief, Section of Rheumatology, Yale School of Medicine
1997 Professor of Medicine, Yale University
1999 Professor of Immunobiology, Yale University
2004-pres. Director, Investigative Medicine Program (Ph.D. program for physician-scientists), Yale University

Other Experience and Professional Memberships

1980 ABIM certified, Internal Medicine; 1988 ABIM certified, Rheumatology; recertified 2008
1989-93 Arthritis Foundation: Applied Immunology Study Section; 1997-00, Cell. Immunol. Study Section
1993-pres. American Association of Immunologists
1995-00 NIAMS (NIH) Special Grants Review Committee, Chair 1997-00
2000-04 NIH, Immunological Sciences Study Section (now HAI Study Section), Chair 2002-04
1991-00 *The Journal of Immunology*, Associate & Section Editor
1997-09 *Arthritis & Rheumatism*, Advisory Editor
2003-04 Arthritis Foundation: Member, Executive Committee & Chair, Medical & Scientific Council
2000-10 ABIM Subspecialty Board in Rheumatology, Chair 2006-08, Chair Test Committee 2008-10
2000-08 Director of Medical Studies, Department of Immunobiology, Yale School of Medicine
2002-pres. Board of Lupus Clinical Trials Consortium
2003-07 Chair, Scientific Advisory Board, Alliance for Lupus Research
2006-pres. Immune Tolerance Network, Steering Committee
2007-10 Arthritis Foundation: Member, Research Committee
2010-14 Chair, Board of Scientific Counselors, NIAMS

Honors

1972 Phi Beta Kappa, University of North Carolina, Chapel Hill
1976 Alpha Omega Alpha, University of North Carolina School of Medicine

1984 Postdoctoral Fellow, Arthritis Foundation
1985 Fellow, American College of Rheumatology
1985-89 Pew Scholar in the Biomedical Sciences
1994 Elected, American Society for Clinical Investigation; 1996 Elected, Interurban Clinical Club
1998 M.A. (Honorary) Yale University
1998 Elected Fellow, American Association for the Advancement of Science
2000 NIH (NIAMS) MERIT Award
2001 Elected, Kunkel Society; 2002-05 Kirkland Scholar
2004 Bohmfalk Teaching Prize (for outstanding teaching in the basic sciences), Yale School of Medicine
2004-pres. Society of Distinguished Teachers, Yale School of Medicine
2010 Paul B. Beeson Professor of Medicine

C. Selected Publications since 2008 (from 172 total – 118 original articles; 54 reviews/chapters).
Most relevant to the current application (in chronological order)

1. Odegard J, Marks B, DiPlacido L, Poholek A, Dong C, Favell R, **Craft J**. ICOS-dependent extrafollicular helper T cells elicit IgG production via IL-21 in systemic autoimmunity. *J Exp Med.* 2008. 205:2873-2886. PMCID: PMC2585848.
2. Johnson RJ*, Poholek AC*, Yusuf I, DiToro D, Eto D, Barnett B, Dent AL, **Craft J**, Crotty S. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of follicular helper (T_{FH}) CD4 T cell differentiation. *Science.* 2009. 325:1006-1010 (*co-first authors). PMCID: PMC2766560 (Accompanied by a *Perspectives* article entitled "The Yin and Yang of follicular helper T cells" published in the same issue.)
3. Marks BR, Nowyhed H, Choi J-Y, Poholek AC, Odegard J, Flavell RA, **Craft J**. Thymic self-reactivity selects natural interleukin 17–producing T cells that can regulate peripheral inflammation. *Nat Immunol.* 2009. 10:1125-1132. PMCID: PMC2751862. (Accompanied by News and Views article entitled "The importance of being earnestly selfish" published in the same issue.)
4. Odegard J*, DiPlacido L*, Greenwald L, Kashgarian M, Dong C, Flavell R, **Craft J**. ICOS controls effector function, but not trafficking receptor expression, of kidney-infiltrating effector T cells in murine lupus. *J Immunol*. 2009. 182:4076-4084. (*co-first authors) PMCID: PMC2746004.
5. Poholek AC, Hansen K, Hernandez S, Eto D, Chandele A, Weinstein J, Dong X, Odegard JM, Kaech SM, Dent AL, Crotty S, **Craft J**. *In vivo* regulation of Bcl6 and T follicular helper cell development. *J Immunol.* 2010. 185:313-326. PMCID: PMC2891136.
6. **Craft J**. Dissecting the immune cell mayhem that drives lupus pathogenesis. *Sci Transl Med.* 2011. 3:73ps9. PMCID: PMC3694130
7. Marshall HD, Chandele A, Jung YW, Meng H, Poholek AC, Parish IA, Rutishauser R, Cui W, Kleinstein SH, **Craft J**, Kaech SM. Differential expression of Ly6C and T-bet distinguish effector and memory Th1 CD4(+) cell properties during viral infection. *Immunity.* 2011. 35:633-646. PMCID: PMC3444169
8. Cui W, Yiu L, Weinstein J, **Craft J**, Kaech SM. An IL-21/IL-10/STAT3 pathway is critical for functional maturation of memory CD8+ T cells. *Immunity.* 2011. 35:792-805. PMCID: PMC3431922
9. **Craft J**. Follicular helper T cells in immunity and in systemic autoimmunity. *Nature Rev Rheum. 2012.* 8:337-347. PMCID: PMC3604997
10. Look M, Stern E, Wan Q, DiPlacido LD, Kashgarian M, **Craft J**, and Fahmy TM. Nanogel-based delivery of mycophenolic acid ameliorates systemic lupus erythematosus in mice. *J Clin Invest.* 2013. 123:1741-1749. PMCID: PMC3613921
11. Bertino SA and **Craft J.** Roquin Paralogs Add a New Dimension to ICOS Regulation. *Immunity* 2013 38:624-6. PMCID: PMC3786165
12. Kumamoto Y, Lineham M, Weinstein J, Laidlaw B, **Craft J**, Iwasaki A. $CD301b^+$ dermal dendritic cells drive T_H2 immunity. *Immunity*. 2013. 39:733-743. PMCID: PMC3819035
13. Weinstein J, Bertino SA, Hernandez SG, Poholek AC, Teplitzky TB, Nowyhed HN, **Craft J**. B cells in T follicular helper cell development and function: Separable roles in delivery of ICOS ligand and antigen. *J Immunol*. 2014 192:3166-3179. PMCID: PMC3991608 [Available on 2015/4/1]
14. Ray JP, Marshall HD, Laidlaw BJ, Staron MW, Kaech SM, **Craft J**. The transcription factor STAT3 and type I Interferons are mutually repressive insulators for differentiation of follicular helper and T helper 1 cells. 2014 *Immunity.* 40:367-377. PMCID: PMC3992517 [Available on 2015/3/20] (Accompanied by a *Perspectives* article entitled "Tfh cell differentiation: Missing Stat3 uncovers interferons' interference" published in the same issue.)

15. Shulman Z, Gitlin AD, Weinstein JS, **Craft J**, Nuzzenzweig M. T follicular helper cell Ca^{2+} signaling during B cell selection in germinal centers. *Science.* (in press).

D. Research Support

Ongoing Research Support

5 R01 AR40072-24 Craft (PI) 8/31/90 - 6/30/15
NIH/NIAMS
Immune Responses in Lupus
The aims of this grant are to determine the roles of Bcl6 in PSGL1 regulation in the initial events that lead to Tfh cell migration and development, and to further dissect the requirements for Tfh cell differentiation and function.
Role: PI

5 P30 AR053495-07 Craft (PI) 8/17/07 - 7/31/17
NIH/NIAMS
Yale Rheumatic Disease Research Core Center
The goal of this center application is to support novel cores in generation and preservation of genetically modified mice and in *in vivo* microscopy. The PI does not receive project support.
Role: PI

5 T32 AR07107-39 Craft (PI) 7/1/76 - 8/31/16
NIH/NIAMS
Training Program in Investigative Rheumatology
The goal of this project is to train five M.D. and Ph.D. postdoctoral fellows yearly for careers in investigative rheumatology and immunology. The PI does not receive salary or project support.
Role: PI

5 UL1 TR000142-08 Sherwin (PI) 9/30/06 - 6/30/16
NIH/NCATS
Clinical and Translational Science Award
This project will develop educational and research programs in translational investigation at Yale. Dr. Craft is included on this award as Director of the Investigative Medicine Program. He does not receive project support.
Role: Director, Investigative Medicine PhD Program

Research Grant Craft (PI) 5/20/13 - 5/19/17
AbbVie Yale Collaboration in Immunobiology
Clinical Assessment and Therapeutic Blockade of T - B Cell Collaboration in Autoimmunity
The goals of this proposal are to track circulating T follicular helper-like (cTfh-like) cells in lupus patients in relationship to therapies that abrogate Tfh function and/or development, including abatacept.
Role: PI

Rheumatology Research Foundation Craft (PI) 7/1/14 - 6/30/16
Studying Monocytes and iPS cells in RA
The goals of this project are to derive iPSCs from peripheral blood mononuclear cells (PBMCs) of RA patients bearing the RA-risk *IRF5* SNP rs2004640; generate isogenic iPSCs by editing the RA-risk *IRF5* SNP rs2004640 to the non-risk SNP, using CAS9 (CRISPR-associated) nuclease and CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) gene editing; differentiate monocytes (MO) from iPSCs and isogenic iPSCs; define the function and transcriptomes of MO differentiated from iPSCs and isogenic iPSCs; transplant iPSCs into humanized mice, followed by assessment of their phenotype and function, and their role in induction and as therapeutic targets in inflammatory arthritis.
Role: PI

Completed Research Support

5 R01 AR44076-16 Craft (PI) 7/1/96 - 2/29/13
NIH/NIAMS
Genetic Analysis of T Cells in Lupus
The aims of this project are to dissect the nature of the defect(s) in B cell maturation that occurs in the absence of inducible costimulator (ICOS), to characterize $CD4^+$ T cells that promote B cell maturation and peripheral inflammation in lupus-prone mice, and to determine the role of B7RP-1 in these events.
Role: PI

5 R01 AI075157-05 Craft (PI) 2/1/08 - 1/31/13
NIH/NIAID
Dissecting the Role of IL-15 in $CD8^+$ T Cell Homeostasis in Human Lupus
The goal of this project is to determine the mechanisms for memory $CD8^+$ T cell expansion in patients with SLE investigate the pathological implications of memory $CD8^+$ T cell expansion in patients with SLE.
Role: PI

Disease Targeted Research Grant Craft (PI) 7/1/12 - 6/30/13
American College of Rheumatology Research and Education Foundation
A Novel B Cell Marker and Therapeutic Target in Rheumatoid Arthritis
The goals of this proposal are to dissect the role of PSGL1 expression in inflammatory arthritis.
Role: PI

Target Identification in Lupus (TIL) Craft (PI) 3/1/11 - 2/28/14
Alliance for Lupus Research (ALR)
Follicular Helper T Cells: Characterization and Function
The goal of this proposal is to characterize follicular helper cells and their ability to help B cells.
Role: PI

5 R21 AR062842-02 Craft (PI) 4/1/12 - 3/31/14
NIH/NIAMS
A Novel B Cell Marker and Therapeutic Target in Lupus
The goal of this project is to test the notion that PSGL-1 expression on antibody-secreting cells plays an important role in their trafficking to the bone marrow and splenic red pulp, niches for their survival, and that PSGL-1 expressing plasmablasts are expanded in the blood of patients with SLE.
Role: PI

5 U19 AI082713-05 Herold (PI) 5/1/09 - 4/30/14
NIH/NIAID
Autoimmunity Center of Excellence (U19); Administrative Core A
The goal of the Autoimmunity Center is to develop new therapeutic approaches for the treatment of autoimmune diseases. Dr. Craft does not receive project support.
Role: Member of Executive Committee

PR093356 Kang (PI) 7/1/10 - 6/30/14
Department of Defense
Studying the role for CD4+ T cell subsets in human lupus
The major goal of the project is to determine the role of IL-27 in induction of Th1, Th2, Th17, and Treg cells in human SLE.
Role: Investigator

5 R21 AR063942-02 Craft (PI) 9/7/12 - 8/31/14
NIH/NIAMS
Manipulation of Follicular Helper T Cells in Immunity and Autoimmunity
The goal of this proposal is genetically manipulate Tfh cells, and genes of interest within them, to evaluate their respective roles in normal and pathogenic immune responses.
Role: PI

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Insoo Kang	Associate Professor of Medicine
eRA COMMONS USER NAME (credential, e.g., agency login) insookang	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Hallym University, Korea		1986	Pre-Med
Hallym University College of Medicine, Korea	M.D.	1990	Medicine
Yale University Affiliated Program at Hospital of St. Raphael, New Haven, CT	Med Res	1991-1994	Internal Medicine
Yale University School of Medicine, New Haven, CT	Postdoc Fellow	1994-1997	Rheumatology

A. Personal Statement

Dr. Insoo Kang is Associate Professor of Medicine (Rheumatology) at Yale. He is a physician scientist with a research interest in understanding the pathogenesis of human autoimmune diseases using biological samples and clinical data. The primary focus of his research has been on T cell immunity, especially defining novel T cell subsets in humans based on cytokine and cytokine receptor expression. His lab identified two novel subsets of effector memory (EM) $CD8^+$ T cells with high and low levels of IL-7Rα expression (IL-7Rα^{high} and ^{low}) in human peripheral blood. This work was published in *Blood* with an editorial comment. He further demonstrated the unique characteristics of such cell subsets as well as the expansion of IL-7Rα^{low} EM CD8+ T cells in lupus patients, which is likely driven by repetitive immune stimulation. Also, his lab identified a subset of $CD4^+$ T cells with the expression of IL-1 receptor 1 (IL-1R1) that potently produced IL-17 in human naïve and memory $CD4^+$ T cells. This work, which first showed a potential role for IL-1R1 as a molecule identifying Th17 cells in humans, was published in *Blood* and cited as a "Must Read" paper by *Faculty of 1000 Biology*. Recently, his lab reported an increased frequency of Th17 cells that correlated with disease activity in lupus patients as well as the potential role of lupus immune complexes (e.g. dsDNA and U1-snRNP) in promoting Th17 cell responses via inducing IL-1β from human monocytes. The latter studies were published in *the Journal of Immunology*. Dr. Kang has received a total of 12 grants on studying human immunity from the NIH, the Department of Defense (DOD) and private foundations which include an R01, a K08 (from NIAMS), two R21s and an investigator-initiated DOD research grant. He has substantial experience in mentoring postdoctoral fellows and junior investigators (associate research scientists) (total 12). Five of them currently hold faculty or staff scientist positions at academic or governmental research institutions.

B. Positions and Honors

Positions and Employment

1991-94	Medical Intern and Resident, Yale University Affiliated Program at Hospital of St. Raphael, New Haven, CT
1994-97	Postdoctoral Fellowship in Rheumatology, Yale University School of Medicine, New Haven, CT
1997-98	Full-time Instructor, Section of Rheumatology, Department of Internal Medicine, Hallym University, Kang Dong Sacred Heart Hospital, Seoul, Korea
1998-99	Assistant Professor of Medicine, Section of Rheumatology, Department of Internal Medicine, Hallym University, Kang Dong Sacred Heart Hospital, Seoul, Korea
1999-2004	Assistant Professor of Medicine, Section of Rheumatology, Department of Internal Medicine, Yale University School of Medicine, New Haven, CT
2004-2008	Program Director, Yale University Investigative Rheumatology Fellowship Training Program, New Haven, CT
2005-present	Associate Professor of Medicine, Section of Rheumatology, Department of Internal Medicine, Yale University School of Medicine

Other Experience and Professional Memberships

1994	Member, American College of Physicians
1994	Diplomate of American Board of Internal Medicine
1996	Diplomate of American Board of Internal Medicine, Rheumatology
1997	Fellow, American College of Rheumatology
1998	Diplomate of Korean Board of Internal Medicine
2004	Member, American Association of Immunologists
2011	Member, Scientific Advisory Board, American Federation of Aging Research
2013	Associate Editor, *the Journal of Immunology*

Honors

1988-90	Gochon Scholarship for Academic Excellence
1990	President's Award of Hallym University for Valedictorian
1995-97	Physician-Scientist Development Award of Arthritis Foundation
1997	Senior Rheumatology Fellow Award of American College of Rheumatology
2002-2005	Arthritis Investigator Award of American College of Rheumatology
2002-2004	Research Scholarship of the Hartford Foundation Center for Excellence in Aging
2004-2009	Career Development Award (K08, NIAMS)

C. Selected peer-reviewed publications (selected from 43 peer reviewed publications).

1. **I Kang,** T Quan, H Nolasco, S Park, M Hong, G Howe, J Craft. 2004. Defective control of latent EBV infection in patients with systemic lupus erythematosus. *J Immunol* 172:1287-1294.
2. **I Kang ***, M Hong, H Nolasco, S Park, J Dan, J Choi, J Craft. 2004. Age-associated change in the frequency of memory CD4+ T cells impairs long-term CD4+ T cell responses to influenza vaccine. *J Immunol* 173:673-681. (*Corresponding Author)
3. H Kim, M Hong, J Dan, **I Kang**. 2006. Altered IL-7Rα expression with aging and the potential implications of IL-7 therapy on CD8+ T cell immune responses. *Blood*. 107:2855-2862 (accompanied by commentary entitled "IL-7 comes of age" published in the same issue).
4. H Kim, K Hwang and **I Kang**. 2007. The role of DNA methylation in differentially regulating IL-7Rα expression in human T cells. *J Immunol.* 178:5473-5479
5. H Kim*, K Hwang *, **I Kang.** 2007. Dual roles of IL-15 in maintaining IL-7R alpha low CCR7- memory CD8+ T cells in humans via recovering the PI3K/AKT pathway. *J Immunol*. 179:6734-6740 (cited in "IN THIS ISSUE" in the same issue). *Equally contributed to this work.
6. K Hwang, H Kim and **I Kang.** Aging and human CD4+ regulatory T cells. 2009. *Mech Age Dev.* 130:509-517 PMCID: PMC2753872
7. W Lee, S Kang, J Choi, S Lee, K Shah, E Eynon, R Flavell, **I Kang**. Regulating Human Th17 Cells Via Differential Expression of IL-1 Receptor. 2010. *Blood.* 115:530-540 (cited as a Must Read paper in Faculty of 1000 Biology). PMCID: PMC2810985
8. J Lee, W Lee, S Kim, N Lee, M Shin, S Kang, **I Kang**. 2011. Age-associated alteration in naïve and memory Th17 cell response in humans. *Clin Immunol.* 140:84-91. PMCID: PMC3115516
9. W Lee, M Shin, Y Kang, N Lee, S Jeon, **I Kang**. The relationship of cytomegalovirus (CMV) infection with circulatory IFN-α levels and IL-7 receptor α expression on CD8+ T cells in human aging. 2012. *Cytokine.* 58(3):332-5. Published online before print. PMCID: PMC3340433
10. M Shin, Y Kang, N Lee, S Kim, K Kang, R Lazova, **I Kang**. U1-snRNP activates the NLRP3 inflammasome in human monocytes. 2012. *J Immunol.* 188:4769-75. PMCID: PMC3347773
11. S Kang, S Kim, N Lee , W Lee, K Hwang, M Shin, S Lee, W Kim, **I Kang**. 2012. 1,25(OH)2 vitamin D3 promotes FOXP3 expression via binding to vitamin D response elements in its conserved non-coding sequence region. *J Immunol.* 188:5276-82. PMCID: PMC3358577
12. M Shin, J Lee, N Lee, W Lee, S Kim, **I Kang**. 2013. Maintenance of CMV-specific CD8+ T cell responses and the relationship of IL-27 to IFN-γ levels with aging. *Cytokine*. 61:485-90. PMCID: PMC3563774
13. M Shin, Y Kang, N Lee, E Wahl, S Kim K Kang, R Lazova, **I Kang**. 2013. Self dsDNA induces IL-1β production from human monocytes by activating NLRP3 inflammasome in the presence of anti-dsDNA antibodies. *J Immunol.* 190:1407-15. PMCID: PMC3563755
14. K Kang, N Lee, M Shin, S Kim, Y Yua, S Mohantya, R Belshed, R Montgomery, A Shaw, **I Kang.** 2013. An altered relationship of influenza vaccine-specific IgG responses with T cell immunity occurs with aging in humans. *Clin Immunol.* 147:79-88. PMCID: PMC3634098

15. N Lee, M Shin, K Kang, S Yoo, S Mohanty, R Montgomery, A Shaw, **I Kang**. 2014. Human monocytes have increased IFN-γ-mediated IL-15 production with age alongside altered IFN-γ receptor signaling. *Clinical Immunol.* 152:101-110. PMCID: PMC4018768

D. Research Support

Ongoing Research Support

Research Grant Craft (PI) 5/20/13 – 5/19/17
AbbVie Yale Collaboration in Immunobiology
Clinical Assessment and Therapeutic Blockade of T - B Cell Collaboration in Autoimmunity
The goals of this proposal are to track circulating T follicular helper-like (cTfh-like) cells in lupus patients in relationship to therapies that abrogate Tfh function and/or development, including abatacept.
Role: Co-Investigator

Rheumatology Research Foundation Craft (PI) 7/1/14 - 6/30/16
Studying Monocytes and iPS cells in RA
The goals of this project are to derive iPSCs from peripheral blood mononuclear cells (PBMCs) of RA patients bearing the RA-risk IRF5 SNP rs2004640; generate isogenic iPSCs by editing the RA-risk IRF5 SNP rs2004640 to the non-risk SNP, using CAS9 (CRISPR-associated) nuclease and CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) gene editing; differentiate monocytes (MO) from iPSCs and isogenic iPSCs; define the function and transcriptomes of MO differentiated from iPSCs and isogenic iPSCs; transplant iPSCs into humanized mice, followed by assessment of their phenotype and function, and their role in induction and as therapeutic targets in inflammatory arthritis.
Role: PI

Research Grant Iwasaki (PI) 4/15/13 – 4/14/15
AbbVie Pilot Research Study Proposal
Role of Endogenous Retroviruses in Lupus Pathogenesis
The goal of the proposed project is to understand the disease pathogenesis of SLE and possibly develop a new strategy to diagnose, prevent and/or treat SLE in humans.
Role: Investigator

Completed Research Support

1 R01 AG028069-05 Kang (PI) 9/1/07 - 6/30/12
NIH/NIA
Aging and IL-7-mediated CD8+ T cell survival
The major goal of the project was investigating the effect of aging on CD8+ T cell survival in elderly humans.
Role: PI

PR093356 Kang (PI) 7/1/10 - 7/31/13
Department of Defense
Studying the role for CD4+ T cell subsets in human lupus
The major goal of the project is to determine the role for CD4+ T cell subsets in developing human lupus.
Role: PI

1 R21 AT 005241-02 Kang (PI) 9/30/09 – 8/31/12
NIH/NCACM
Studying the effects of vitamin D on FOXP3 and IL-17 expression in human CD4+ T cells.
The major goal of the project was investigating how vitamin D affects the regulation of FOXP3 and IL-17 in human CD4+ T cells.
Role: PI

5 R01 AI 075157-04 Craft (PI) 2/1/08 - 1/31/13
NIH/NIAID
Dissecting the role of IL-15 in CD8+ T cell homeostasis in human lupus.
The major goal of the project was investigating the role of memory CD8+ T cells in the development of lupus
Role: Co-Investigator

5 U19-AI082713-03 Herold (PI) 4/1/09 - 3/31/14
NIH/NIAID
Yale Autoimmunity Center of Excellence (program director: K. Herold)
Project #1: lymphoid neogenesis and CD4+ T cell differentiation in primary Sjögren's syndrome. The major goal of the project was to define lymphoid neogenesis and CD4+ T cell differentiation in primary Sjögren's syndrome.
Role: Leader for Project #1

5 P30 AR053495-07 Craft (PI) 8/17/07 – 7/31/14
NIH/NIAMS
Yale Rheumatic Disease Research Core Center
Studying altered function of monocytes in lupus
The major goal of this project is to study monocytes in lupus
Role: PI (pilot grant)

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Jason Scott Weinstein	Postdoctoral Fellow
eRA COMMONS USER NAME (credential, e.g., agency login) JWEINSTEIN	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY
Rutgers University		1997-1998	Biology
Northeastern University	B.S.	1998-2003	Biology
University of Florida	Ph.D.	2003-2009	Immunology
Yale University		2009-Present	Immunology

A. Personal Statement

My interest is in examining the cellular relationship between the innate and adaptive immune system. As an undergraduate I worked in a lab humanizing antibodies and developing small molecules for treatment of sepsis. In graduate school I focused on the characterization of autoreactive B cells in pristane-induced lupus. After primarily working with B cells, I broadened his training in immunology to focus more on T cells and their effects on autoimmunity. Current studies involve analysis of the transcriptional regulation and function of follicular helper T cells and how they contribute to the pathogenesis of autoimmunity.

B. Research and/or Professional Experience

Employment

2009-2009 Student Tutor - University Athletic Association (biology, microbiology, genetics)
2009-pres. Postdoctoral Fellow, Section of Rheumatology, Yale University

Honors

2005 American College of Rheumatology Medical Student Achievement Award (MSAA)
2006 American College of Rheumatology Medical Student Achievement Award (MSAA)
2008 UF Medical Guild Research Competition 1st place Immunology/Microbiology
2008 Bronze Medal, 2008 Medical Guild Graduate Student Research Competition
2008 Graduate Student for Outstanding Research Award

Professional Societies and Public Advisory Committees *(selected):*

2002 Northeastern Biology Club, President
2004 Graduate Student Organization, elected member of Advisory Board

C. Selected peer-reviewed publications *(in chronological order)*

1. **Weinstein JS**, Nacionales DC, Lee PY, Kelly-Scumpia KM, Yan X, Scumpia PO, Vale-Cruz DS, Sobel E, Satoh M, Chiorazzi N, Reeves WH. 2008. Co-localization of Antigen-specific B and T Cells within Ectopic Lymphoid Tissue following Immunization with Exogenous Antigen. *J Immunol.* 181:3259-3267. PMCID: PMC2769209

2. ***Weinstein JS**, Nacionales DC ,Yan X, Albesiano E, Lee PY, Kelly-Scumpia KM, Lyons R, Satoh M, Chiorazzi N, Reeves WH. 2009. B Cell Proliferation, Somatic Hypermutation, Class Switch Recombination, and Autoantibody Production in Ectopic Lymphoid Tissue in Murine Lupus. *J Immunol.* 182:4226-4236. *co-first author. PMCID: PMC3395367

3. Reeves WH, Lee PY, **Weinstein JS**, Satoh M, Lu L. 2009. Induction of autoimmunity by pristane and other naturally occurring hydrocarbons. *Trends Immunol*. 30:455-464. PMCID: PMC2746238

4. Lee PY, Li Y, Kumagai Y, Xu Y, **Weinstein JS**, Kellner ES, Nacionales DC, Butfiloski EJ, van Rooijen N, Akira S, Sobel ES, Satoh M, Reeves WH. 2009. Type I interferon modulates monocyte recruitment and maturation in chronic inflammation. *Am J Pathol*. 175:2023-2033. PMCID: PMC2774066

5. Kelly-Scumpia KM, Scumpia PO, Delano MJ, **Weinstein JS**, Cuenca AG, Wynn JL, Moldawer LL Type I interferon signaling in hematopoietic cells is required for survival in mouse polymicrobial sepsis by regulating CXCL10. *J Exp Med*. 207:319-326. PMCID: PMC2822595

6. Scumpia PO, Kelly-Scumpia KM, Delano MJ, **Weinstein JS**, Cuenca AG, Al-Quran S, Bovio I, Akira S, Kumagai Y, Moldawer LL. 2010. Cutting edge: bacterial infection induces hematopoietic stem and progenitor cell expansion in the absence of TLR signaling. *J Immunol*. 184:2247-2251 PMCID: 20130216

7. Poholek AC, Hansen K, Hernandez SG, Eto D, Chandele A, **Weinstein JS**, Dong X, Odegard JM, Kaech SM, Dent AL, Crotty S, Craft J. 2010. In vivo regulation of Bcl6 and T follicular helper cell development. *J Immunol*. 2010. 185:313-326. PMCID: PMC 2891136

8. Kelly-Scumpia KM, Scumpia PO, **Weinstein JS**, Delano MJ, Cuenca AG, Nacionales DC, Wynn JL, Lee PY, Kumagai Y, Efron PA, Akira S, Wasserfall C, Atkinson MA, Moldawer LL.2011. B cells enhance early innate immune responses during bacterial sepsis. *J Exp Med*. 208:1673-1682. PMCID: PMC2149216

9. Cui W, Liu Y, **Weinstein JS**, Craft J, Kaech SM. 2011. An interleukin-21-interleukin-10-STAT3 pathway is critical for functional maturation of memory CD8+ T cells. *Immunity*. 35:792-805. PMCID: PMC3431922

10. Xu Y, Lee PY, Li Y, Liu C, Zhuang H, Han S, Nacionales DC, **Weinstein J**, Mathews CE, Moldawer LL, Li SW, Satoh M, Yang LJ, Reeves WH. 2012. Pleiotropic IFN-dependent and -independent effects of IRF5 on the pathogenesis of experimental lupus. *J Immunol.* 188:4113-4121. PMCID: PMC3580234

11. **Weinstein JS**, Hernandez SG, Craft J. 2012. T cells that promote B-Cell maturation in systemic autoimmunity. *Immunol Rev*. 247:160-171. PMCID: PMC3334351

12. Kumamoto Y, Linehan M, **Weinstein JS**, Laidlaw BJ, Craft JE, Iwasaki A. 2013. CD301b^{+} dermal dendritic cells drive T helper 2 cell-mediated immunity. *Immunity*. 39:733-743. PMCID: PMC3819035

13. Gao Y, Nish SA, Jiang R, Hou L, Licona-Limón P, **Weinstein JS**, Zhao H, Medzhitov R. 2013. Control of T helper 2 responses by transcription factor IRF4-dependent dendritic cells. *Immunity*. 39:722-732. PMCID: PMC4110745 [Available on 2014/10/17]

14. **Weinstein JS**, Delano MJ, Kelly-Scumpia KM, Nacionales DC, Lee PY, Scumpia PO, Switanek JL, Moldawer LL, Reeves WH. 2013. Long term Anti-Sm-RNP antibodies are generated from plasma cell residing within TMPD induced ectopic lymphoid. *J Immunol*. 190:3916-27. PMCID: PMC3622197

15. **Weinstein JS**, Bertino SA, Hernandez SG, Poholek AC, Teplitzky TB, Nowyhed HN, Craft J. 2014. B cell provision of ICOSL is not required for T follicular helper cell differentiation under conditions of excess B cell activation. *J Immunol*. 192:3166-179. PMCID: PMC3991608

D. Research Support

Ongoing Research Support

5 T32 AR07107-39 Craft (PI) 7/1/76 - 8/31/16
NIH/NIAMS
Training Program in Investigative Rheumatology
The goal of this project is to train five M.D. and Ph.D. postdoctoral fellows yearly for careers in investigative rheumatology and immunology.
Role: Trainee

Completed Research Support

Postdoctoral Fellowship Weinstein (PI) 2010 - 2013
Arthritis Foundation
Follicular Helper T Cells In Immunity and Autoimmunity
The goal of this project was to establish the role of Bcl6 as the transcriptional regulator of follicular helper T cells.
Role: PI

FACILITIES AND OTHER RESOURCES

Laboratory

The Section of Rheumatology occupies part of the 4th and 5th floors of the newly built The Anlyan Center (TAC) Building at Yale. The building was opened in February 2003. Space devoted to this project is 1,800 square feet of workspace, tissue culture room, general equipment room, flow cytometry room, and walk-in cold room. The office of Dr. Craft is on the 5th floor of TAC building and adjacent to his laboratory.

Clinical

Over 100 patients who meet the ACR criteria for SLE have been identified and used in our. We anticipate that this cohort plus new patients (we see 15-25 new lupus patients yearly) will provide adequate numbers. We obtain demographic and clinical data, with disease activity assessed using SELENA-SLEDAI. Healthy controls matched for age (± 2 years) and gender of patients with SLE are recruited as we have published (Kang, *et al*., *J Immunol*. 173: 673-681, 2004; Shah, *et al.*, *Arthritis Res Ther*. 12:R53, 2010). For healthy controls, those taking immunosuppressive drugs or with a disease affecting the immune system (infection, cancer, immunodeficiency, autoimmunity, and diabetes) are excluded. Subjects are recruited according to HIPAA guidelines, with informed consent.

Animal

All research involving animals is conducted via the Yale Animal Resources Center (YARC). The group maintains and oversees a variety of species. Mice will be kept SPF, with veterinary services available 24 hours per day. TAC has a new vivarium, with a capacity of 70,000 mouse cages, all housed in ventilated racks with microbiologic isolation at the cage level.

Computer

The Craft laboratory has 5 up-to-date Macintosh computers, linked together on the Internet; all postdocs and graduate students in the lab also have up-to-date Macintosh laptop computers, likewise linked. His office also has an up to date Macintosh computer.

Office

Dr. Craft's office is 200 square feet, and is directly adjacent to his laboratory.

Other

In the Yale Section of Rheumatology and Department of Immunobiology are other investigators with considerable experience in cellular and molecular immunology techniques. These highly interactive faculty include Richard Flavell, Susan Kaech, Linda Bockenstedt, Erol Fikrig, Richard Bucala, Al Bothwell, Ann Haberman, João Pereira, Carla Rothlin, Peter Cresswell, Jordan Pober, Bing Su, Kevan Herold, Nancy Ruddle, Ruslan Medzhitov, Warren Shlomchik, Paula Kavathas, Akiko Iwasaki, Tian Chi, and David Schatz. The Craft lab is on one of the two floors shared with the Department of Immunobiology in TAC, with the laboratory directly adjacent to those of João Pereira and A. Haberman and R. Flavell, and down one flight of stairs from that of Dr. Kaech. Other faculty on the same floor include W. Shlomchik, Mamula, Chi, Kavathas, Fikrig, and Bucala, with these labs downstairs from those of Cresswell, Kaech, Iwasaki, Schatz, Medzhitov, Rothlin, and Bothwell.

Flow cytometry and cell sorting will be carried out through the YALE Flow Cytometry Core (http://medicine.yale.edu/labmed/cellsorter/), which consists of 12 user-operated analyzers (four LSRIIs, LSRII Green, three FACSCaliburs, and four Stratedigm). The Caliburs can analyze up to 4 colors with 488 nm and 633 nm excitation lasers. The Stratedigm STD-8 and STD-13 are capable of analyzing 8 and 13 colors, respectively. The LSRIIs and LSRII Green have a capability of 12 colors. For cell sorting, we have the following instruments: four FACS Arias, a Beckman Coulter MoFlo, and a SONY SY3200, all operated by full time staff members, with two of the FACS Arias available for user operation with appropriate training. Cells can be sorted into 5 or 15 ml tubes or into various plates as single or multiple cells per well or onto microscopic slides for analysis. In addition, all sorters are capable of simultaneous 4–way sorting. All sorters except MoFlo are also equipped for sorting live human, primate or other potentially biohazardous cells. Users of the Sony Reflection can take advantage of using two sort heads and a lower charge for the second head to be running. Machines are checked daily for optimal performance, with staff members available during usual working hours for troubleshooting. Internet sign-up for analysis time is available from any computer via the internet for users after

completing the training.

RNA-seq experiments require ultra-high throughput sequencing. This will be performed at the Yale Center for Genome Analysis (YCGA). Resources and sequencing protocols at the YCGA, available in detail at http://medicine.yale.edu/keck/ycga/index.aspx. High throughput sequencing will be carried out using the Illumina platform at the Yale Center for Genome Analysis, which also houses one of the four centers of the NIH Neuroscience Microarray Consortium. Yale University has recently invested significant amount of funding to establish YCGA that brings cutting edge high throughput genomic technologies under one roof to provide a centralized resource to carry out large scale genomic studies. YCGA is a full service facility and is currently equipped with multiple microarray platforms including Affymetrix, Illumina, NimbleGen, Exiqon, in-house spotted arrays, Sequenom and ABI real time PCR (http://www.yale.edu/westcampus/science_ycga.html). In FY 2008, using three genome analyzers, the Center completed >100 full runs of sequencing for 20 investigators from 4 other institutions, involving multiple applications such as transcriptome analysis (mRNA-Seq), DNA-protein interactions (ChIP-Seq), DNA methylation analysis (methyl-Seq), and targeted and whole genome resequencing. The Center currently operates multiple next generation sequencing platforms: 11 Illumina Genome Analyzers, 7 HiSeqs and one 454/Roche system. The YCGA is closely associated with Yale's W.M. Keck Foundation Biotechnology Resource Laboratory that is one of the largest of its kind in academia, is a world leader in providing genomics and proteomics services. A dedicated building, with over 5000 sq. ft. of laboratory and office space with all modern amenities, has been made available for YCGA. The Center has 20 full time staff including three Ph.D. and three MS level staff appointments. Dr. Shrikant Mane is the director of this resource and he has over 20 years experience in molecular and cell biology. He received his doctoral degree in cancer biology and previously established and directed the Affymetrix core facility at the Moffitt Cancer Center in Florida. The day to day operation of the Illmina high-throughput sequencing system is overseen by Dr. Mahajan, Ms. Sheila Umlauf, M.S., and Ms. Irina Tikhonova, M.S., while under the supervision of Dr. Shrikant Mane. Ms. Tikhonova and Westman together have over 25 years of experience in molecular biology and have been the Associate Directors of the Keck Microarray Resource for the past three years and have received extensive training from Illumina. All necessary infrastructures such as high performance computation and bioinformatics support are already in place. DNA sequence data generated at the YCGA is then transferred for further analysis using the Yale High Performance Computing (HPC) Cluster, also called the Yale Biomedical Supercomputer, which is a collection of 2,966 CPUs distributed on eight different servers with shared access to a large Lustre filesystem holding more than 100TB of space. In order to accommodate the massive data that is being generated by recently purchased 10 Illumina Genome Analyzers, the YCGA has purchased additional dedicated 768 cores/CPUs cluster and 1.2 PB of storage. Servers are UNIX- or LINUX-based, with installation of all standard programming languages and environments, including Perl, Python,R, SQL, Matlab, Mathematica, BioPerl, BioRuby. We have also installed on this system own software for parallel processing of familial data in linkage analysis (Allegro, MERLIN, FASTLINK) and copy-number analysis (QuantiSNP, PennCNV, and GNOSIS – an algorithm developed in our laboratory). Two Ph.D. computer scientists and one M.S. level staff support the IT and High Performance Computational needs of the Center. The bioinformatics support is provided by three Ph.D. and two MS level staff. The Center also has established a data analysis pipeline as per Illumina recommendations, and has developed a Yale Sequencing Database which enables users to track the samples and view raw data as well as archive final output files. All necessary infrastructures such as high performance computation and bioinformatics support are already in place. The Sequencing Resource also has established a data analysis pipeline as per Illumina recommendations, and has developed a Yale Sequencing Database which enables users to view raw data as well as archive final output files. The Resource is currently equipped with twelved Illumina Genome Analyzer-*II* (GA) sequencers. In order to generate exceptionally good quality sequence data, the Resource has developed standard operating procedures (SOPs) and enforces strict quality control (QC) parameters as follows:

Yale SCHOOL OF MEDICINE

Department of Internal Medicine

INSOO KANG, MD
Associate Professor of Medicine
Section of Rheumatology

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August 18, 2014

Joseph Craft, M.D.
Professor, Departments of Medicine and Immunobiology
Yale University School of Medicine
333 Cedar Street, 541D TAC
New Haven, CT 06520

Dear Joe:

I am pleased to serve as co-investigator on your ALR grant to study the characteristics of T helper cells in lupus. I have substantial experience in analyzing T cell phenotype and function in humans including lupus patients and healthy controls, which will help your study.

Having our labs and offices directly adjacent on the same floor of TAC building and research expertise complementing each other, I believe our proposal will advance our understanding on the role of T cells in lupus.

I am looking forward to working with you.

Sincerely,

Insoo Kang

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July 26, 2014

Joseph Craft, M.D.
Professor, Departments of Medicine and Immunobiology
Yale University School of Medicine
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New Haven, CT 06520
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Dear Joe:

This letter is to confirm my enthusiastic willingness to serve as a consultant on your grant analyzing follicular helper T cells in SLE. This work will extend our current, ongoing collaboration investigating the global transcriptome and the enhancer landscape of human primary Tfh cells. Our joint paper describing our results is in revision at *Blood.*

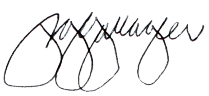
As you know, my laboratory has been studying the regulation of erythroid gene expression for many years. We have studied the role of specific transcription factors and epigenetic factors, including chromatin architecture and DNA methylation, on the regulation of individual genes and on entire programs of genes, primarily those controlling expression of genes encoding erythrocyte membrane proteins. As detailed in our publications, we have applied genomic strategies, including mRNA expression profiling, RNA-seq, microRNA profiling, ChIP-chip and ChIP-seq technology, as well as novel phosphoproteomics and quantitative proteomics techniques, to our understanding of erythropoiesis. Our use of erythrocyte membrane genes as a model of regulation of complex genetic loci has had many benefits. The genes are large and are represented by many cell, tissue, and developmental stage-specific isoforms. Our studies have demonstrated that barrier insulators, elements critical for controlling tissue-specific gene expression, regulate erythroid-cell specific transcripts and that when mutated, lead to a disease phenotype.

In addition, our laboratory has significant experience and expertise in analyzing genomic data using a variety of platforms and algorithms and is already actively engaged in analyses of data sets from hematopoietic cell data sets including yours, is prepared to similarly analyze your new data as it comes on line. Our laboratory has its own Yale network node, allowing direct access to the Yale Bulldog supercomputing resource.

I look forward to continuing to work with you on the studies as we analyze potentially pathogenic $CD4^+$ T helper cells during their differentiation and in ongoing disease states. I and my laboratory are prepared to assist you in any way we can, including making all of our clinical, scientific, and bioinformatics protocols, assays, and pipelines available to you for your proposed project. We will continue our biweekly joint laboratory meetings, our frequent telephone and email discussions, as well as those very frequent interactions of our postdoctoral fellows and technical staffs, to exchange information, updates, etc. and discuss results have been very productive and I look forward to continuing them.

Good luck with your proposed studies.

Sincerely,



Patrick G. Gallagher, M.D.
Professor of Pediatrics, Genetics, and Pathology

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Transcription Factor STAT3 and Type I Interferons Are Corepressive Insulators for Differentiation of Follicular Helper and T Helper 1 Cells

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SUMMARY

Follicular helper T (Tfh) cells are required for the establishment of T-dependent B cell memory and high affinity antibody-secreting cells. We have revealed herein opposing roles for signal transducer and activator of transcription 3 (STAT3) and type I interferon (IFN) signaling in the differentiation of Tfh cells following viral infection. STAT3-deficient CD4$^+$ T cells had a profound defect in Tfh cell differentiation, accompanied by decreased germinal center (GC) B cells and antigen-specific antibody production during acute infection with lymphocytic choriomeningitis virus. STAT3-deficient Tfh cells had strikingly increased expression of a number of IFN-inducible genes, in addition to enhanced T-bet synthesis, thus adopting a T helper 1 (Th1) cell-like effector phenotype. Conversely, IFN-αβ receptor blockade restored Tfh and GC B cell phenotypes in mice containing STAT3-deficient CD4$^+$ T cells. These data suggest mutually repressive roles for STAT3 and type I IFN signaling pathways in the differentiation of Tfh cells following viral infection.

INTRODUCTION

Follicular T helper (Tfh) cells are a subset of CD4$^+$ T cells required for the T-dependent germinal center (GC) response leading to the production of antigen-specific memory B and plasma cells (Crotty, 2011; McHeyzer-Williams et al., 2012). Proper regulation of Tfh cell differentiation in secondary lymphoid organs (SLOs) is critical for controlled immune function. Poor response of these cells is associated with a defective GC reaction (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009), whereas their overabundance can lead to pathogenic autoantibody production and autoimmune disease (Linterman et al., 2009; Vinuesa et al., 2005). Upregulation of B cell lymphoma 6 (Bcl6), the canonical Tfh cell transcription factor, and downregulation of its transcriptional repressor B-lymphocyte-induced maturation protein 1 (Blimp-1), are required for initiation of the Tfh cell development program (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009). Expression of Bcl6, concomitant with downregulation of the chemokine receptor CCR7 and P-selectin glycoprotein ligand-1 (PGSL-1) in concert with CXCR5 upregulation, enables Tfh cells to emigrate from the T cell zone of SLOs to the B cell follicle where they can promote GC reactions (Haynes et al., 2007; Marshall et al., 2011; Poholek et al., 2010). Bcl6 upregulation in nascent Tfh cells occurs in a two-step process dependent upon inducible T cell costimulator (ICOS) signaling via ICOS-ligand (ICOS-L), delivered first by dendritic cells in the T cell zone of SLOs, and second by interactions with B cells at the T-B border in the spleen and interfollicular regions of lymph nodes (Choi et al., 2013; Coffey et al., 2009; Kerfoot et al., 2011).

Previous work has suggested a role for the inflammatory milieu in promoting the Tfh cell phenotype, particularly those cytokines that are known to signal through signal transducer and activator of transcription 3 (STAT3). For example, the cytokines interleukin-6 (IL-6), IL-21, and IL-27 have been implicated in Tfh cell development, albeit with differing roles. IL-6 is required for development of Tfh cells early following viral challenge (Choi et al., 2013), while also promoting their maintenance later in chronic viral infections (Harker et al., 2011), with IL-27 needed for their maintenance upon protein immunization (Batten et al., 2010). IL-21 has also been reported to be important for Tfh cell differentiation (Nurieva et al., 2008; Vogelzang et al., 2008), although such a role has not been universally found, a difference perhaps reflecting mode of immunization (Linterman et al., 2010; Zotos et al., 2010). In the absence of IL-6, IL-21 is more important in later stages following protein immunization or viral challenge (Eto et al., 2011; Karnowski et al., 2012), yet it is not required early in Tfh cell differentiation (Choi et al., 2013). As would be expected from these results, STAT3 has been reported to be required for the development of CXCR5$^+$ CD4$^+$ T cells, following challenge with the antigen KLH in complete Freund's adjuvant and their subsequent function in promoting the development of peanut agglutinin$^+$ (PNA$^+$) GC B cells (Nurieva et al., 2008). Human subjects with dominant-negative mutations in STAT3 also display reduced numbers of CXCR5$^+$ circulating CD4$^+$ T cells, related to Tfh cells in SLOs further suggesting the potential importance of this signaling pathway in Tfh cell differentiation (Ma et al., 2012). Yet, work using adoptive transfers of viral-specific T cell receptor (TCR) transgenic CD4$^+$ T cells reported a requirement for STAT3 in Tfh cell development only within the first 48 hr following viral infection, with normal Tfh cell differentiation ensuing by 3 days after infection (Choi et al., 2013). This

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finding is inconsistent with the broader roles of STAT3 cytokines in Tfh cell development and maintenance.

Here, we have demonstrated a critical role for STAT3 in Tfh cell development and function following acute viral infection. STAT3 expression in $CD4^+$ T cells is required for their differentiation into Tfh cells and promotion of GC B cell development and virus-specific antibody responses. We also identify a role for STAT3 in downmodulating type I interferon (IFN) signaling, as STAT3-deficient Tfh cells display a marked increase in Th1 cell-associated and IFN-inducible transcripts. Accordingly, suppression of type I IFN signaling by antibody blockade of the IFN-$\alpha\beta$ receptor promoted Tfh cell differentiation in wild-type (WT) mice and mice containing STAT3-deficient $CD4^+$ T cells. The treatment also rescued the GC and pathogen-specific antibody defect found in the STAT3 mutant mice. This effect was specific to type I IFNs, as blockade of IFN-γ did not substantially alter Tfh cell percentages, nor affected GC B cell percentages after infection. These findings demonstrate the importance of STAT3 and the STAT3-activating cytokines in promoting Tfh cell differentiation and function during viral infection, and furthermore, reveal contrasting roles for STAT3 and type I IFNs in determining the balance between Tfh and Th1 cell differentiation.

RESULTS

Mice Containing STAT3-Deficient $CD4^+$ T Cells Have a Reduction in Tfh Cell Differentiation and Impaired GC Formation

Previous work using an antigen-adjuvant system demonstrated that STAT3 has a T cell-intrinsic effect on the development of $CD4^+$ $CXCR5^{hi}$ cells and the promotion of GC B cell differentiation (Nurieva et al., 2008). However, its role in Tfh cell development and function following viral infection appears limited to the first 2 days following challenge (Choi et al., 2013), an unexpected finding given that multiple STAT3-dependent cytokines have been implicated in Tfh cell differentiation and continue to be produced after day 3 of infection (Batten et al., 2010; Eto et al., 2011). To address this issue, we ablated STAT3 from all $CD4^+$ T cells by breeding $Stat3^{fl/fl}$ mice to $Cd4^{cre}$ mice ($Stat3^{fl/fl}Cd4^{cre}$ mice; abbreviated $Stat3^{-/-}$ in the Figures) (Schmidt-Supprian and Rajewsky, 2007; Welte et al., 2003; Wolfer et al., 2001), and infected them with the Armstrong strain of lymphocytic choriomeningitis virus (LCMV). $Stat3^{fl/fl}$ *cre*-negative littermates were used as controls and are designated as WT for simplicity. At 8 days postinfection (dpi) we observed similar frequencies of LCMV GP_{66}-specific $CD4^+$ $CD44^+$ T cells in both $Stat3^{fl/fl}Cd4^{cre}$ and WT littermate control mice despite differences in splenocyte numbers (see Figures S1A and S1B available online). Next, we compared the phenotypes of $CD4^+$ Th1 and Tfh cells that formed in $Stat3^{fl/fl}Cd4^{cre}$ and WT $CD4^+$ T cells that have been previously characterized during LCMV infection (Hale et al., 2013; Marshall et al., 2011). Increased expression of PSGL-1 and Ly6C and decreased expression of the chemokine receptor CXCR5 helped to distinguish Th1 cells that expressed high amounts of T-bet, IFN-γ, and granzyme B and were localized in the red pulp (Figure 1A, red gates). Another subset of PSGL-1^{hi} $Ly6C^{lo}$ cells were mostly localized in the T cell zone and expressed lower amounts of T-bet and IFN-γ; compared to the PSGL-1^{hi} $Ly6C^{hi}$ cells, some of these cells also expressed CXCR5 (Figure 1A, blue gates) (Hale et al., 2013; Marshall et al., 2011). Conversely, decreased expression of Ly6C and PSGL-1 and increased expression of CXCR5 distinguished Tfh cells (Figures 1A; see Figure S1A available online, green gates) (Hale et al., 2013; Marshall et al., 2011). Notably, all PSGL-1^{lo} cells expressed CXCR5, supporting previous evidence that these cells are follicular T cells (Figure S1A) (Poholek et al., 2010). Compared to the WT $CD44^+$ or GP_{66}-specific $CD4^+$ T cells, the $Stat3^{fl/fl}Cd4^{cre}$ cells displayed a reduction in the proportion and numbers of GP_{66}^+ and polyclonal $Ly6c^{lo}$ PSGL-1^{lo} cells and $Ly6c^{lo}$ PSGL-1^{lo} $CXCR5^{hi}$ Tfh cells at 8 dpi (Figures 1A–1E; Figures S1A and S1C–S1E). The mean fluorescence intensities (MFI) of CXCR5 in the polyclonal and GP_{66}^+ $Ly6c^{lo}$ PSGL-1^{lo} populations were also reduced in cells from the $Stat3^{fl/fl}Cd4^{cre}$ mice compared to WT littermates (Figures S1F and S1G). In association with a reduction in $Ly6c^{lo}$ PSGL-1^{lo} $CXCR5^{hi}$ Tfh cells in $Stat3^{fl/fl}Cd4^{cre}$ mice, GC B cells observed by flow cytometry were considerably reduced at 8 dpi compared to the WT mice (Figures 1F–1H). These findings were further corroborated by confocal microscopy, which revealed that mice with STAT3-deficient $CD4^+$ T cells have fewer GCs per B cell follicle (Figures 1I and 1J). The large reduction in GC B cells seemed disproportionate to the diminution of Tfh cells, so we analyzed the deletion efficiency of *Stat3* from $CD4^+$ T cells versus B cells, finding 97% deletion in $CD4^+$ T cells, with none in B cells (Figure S1H). A similar impairment in Tfh cell differentiation and GC B cell responses was observed at 14 dpi (Figures S2A–S2H). Thus, STAT3 activity in $CD4^+$ T cells is critical for formation of Tfh cells and activation of GC responses during viral infection.

$Stat3^{fl/fl}Cd4^{cre}$ Mice Fail to Promote Robust Isotype-Switched LCMV-Specific Antibody Production

With the defect in GC formation in the $Stat3^{fl/fl}Cd4^{cre}$ mice, we wondered whether early plasmablast responses were also affected. These occur in extrafollicular regions of SLOs, before highly affinity matured and isotype switched B cells exit the GC (Zotos et al., 2010). The plasmablast response to LCMV infection was analyzed with ELISAs to measure the generation of antigen-specific antibodies in the serum at 8 dpi. We found that there was a significant decrease in LCMV-specific immunoglobulin M (IgM) and IgG in the $Stat3^{fl/fl}Cd4^{cre}$ mice, compared to WT animals (Figures 2A and 2B). By day 14 dpi, IgM responses were rescued, whereas IgG responses remained hindered, suggesting that LCMV-specific plasmablasts and plasma cells were either delayed or reduced in class-switch recombination to IgG (Figures 2C and 2D). We also found that IgG2a antibodies were decreased at 14 dpi, although this decrease was not significant (Figure 2E). These data show there is a significant defect in the IgG antibody response following LCMV infection of $Stat3^{fl/fl}Cd4^{cre}$ mice.

STAT3-Deficient Tfh Cells Phenotypically Resemble Th1 Cells

We next examined the effects of STAT3-deficiency on Tfh cell properties such as Bcl6 expression and IL-21 production, a Tfh cell-producing cytokine that promotes GC longevity, B cell survival (Linterman et al., 2010; Zotos et al., 2010), and class switch recombination to IgG1 (Ozaki et al., 2002). Bcl6 protein was significantly decreased at 5 dpi (Figures 3A and 3B); however, we found no differences at 8 dpi (data not shown). We also

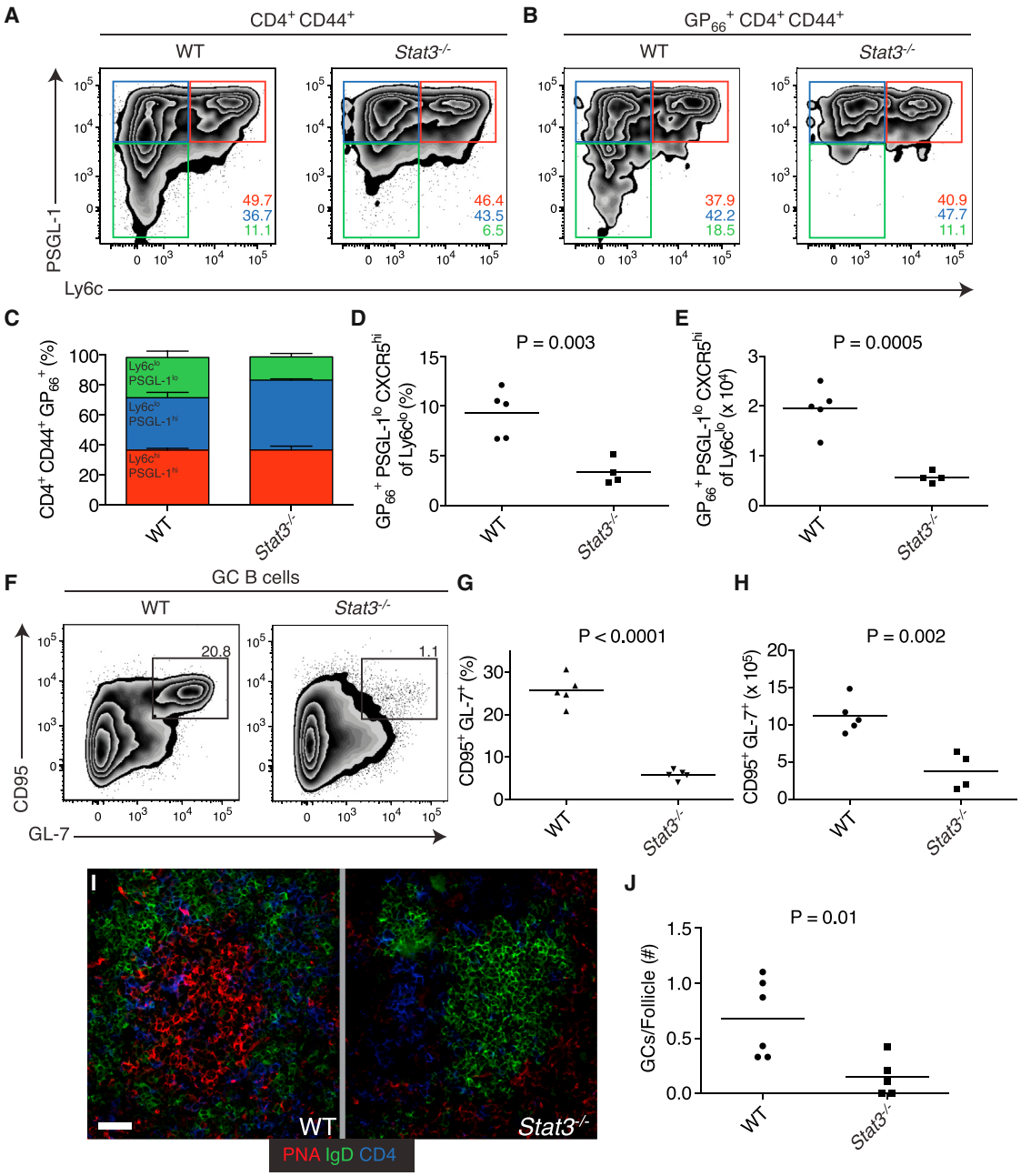

Figure 1. *Stat3*$^{fl/fl}$*Cd4*^{cre} Mice Have an Impaired Tfh Cell Response following Acute Viral Infection

(A and B) Flow cytometry plots of CD44$^+$ (A) and GP$_{66}^+$ LCMV epitope-specific (B) CD4$^+$CD44$^+$ T cells gated on Ly6c and PSGL-1. Th1 terminal effector precursors are Ly6c^{hi} PSGL-1^{hi} cells (red), Th1 central memory precursors are Ly6c^{lo} PSGL-1^{hi} (blue), and Tfh cells are Ly6c^{lo} PSGL-1^{lo} (green).

(C) Population distribution of terminal effector precursors, central memory precursors, and Tfh cells based on Ly6c and PSGL-1, gating as in (A).

(D and E) GP$_{66}$-specific Tfh cell percentages (D) and numbers (E), gated as Ly6c^{lo} PSGL-1^{lo} CXCR5^{hi}.

(F–H) Flow cytometry plots (F), percentages (G), and numbers (H) of GC B cells, gated as B220$^+$ IgD^{lo} CD95^{hi} GL-7$^+$.

(I) Immunohistochemistry of spleen sections, identifying GCs with anti-PNA (red), anti-IgD (green), and anti-CD4 (blue). Scale bar represents 25 μm.

(J) Quantification of GCs per B cell follicle.

(A–H) Representative of at least four experiments with at least four animals per genotype per experiment. + SEM (C).

(I and J) Representative of sections from at least five animals of each genotype. Statistics conducted with Student's unpaired t test. WT, wild-type (*Stat3*$^{fl/fl}$ *cre*-negative) mice. See also Figure S1.

observed a decrease in the percentages of CD4$^+$ CD44$^+$ CXCR5^{hi} IL-21 and IFN-γ double producers (IL-21 IFN-γ DP) at 8 dpi (Figures 3C and 3D). This trend was shared with IL-21-single producers (IL-21 SP), but there was no difference in IFN-γ SP between *Stat3*$^{fl/fl}$ *cre*-negative WT and mutant mice (Figures 3E and 3F).

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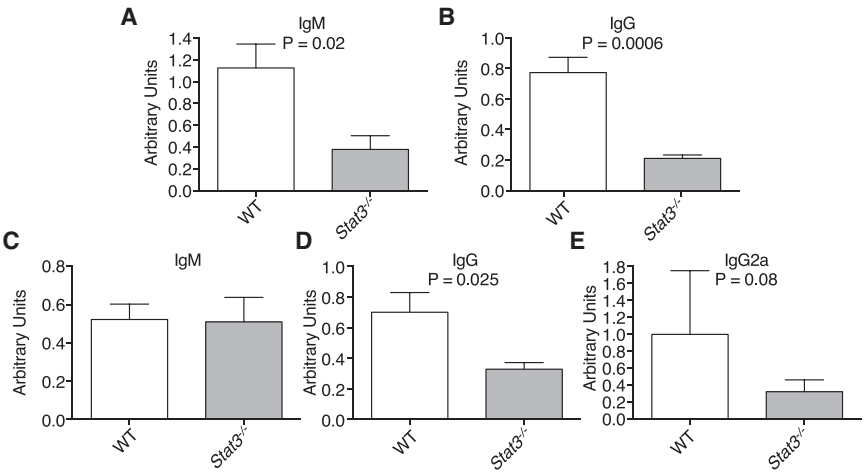

Figure 2. *Stat3*$^{fl/fl}$*Cd4*^{cre} (*Stat3*$^{-/-}$) Mice Are Hindered in their Ability to Create LCMV-Specific Antibodies

(A and B) IgM and IgG anti-LCMV at 8 dpi.

(C–E) IgM, IgG, and IgG2a anti-LCMV at 14 dpi.

(A–E) Representative of at least two experiments with at least four animals per genotype per experiment. Statistics conducted using Student's unpaired t test. + SEM (A–D). See also Figure S2.

Repression of Tfh cell differentiation following viral infection has been shown to occur through IL-2-mediated STAT5 signaling via the high-affinity IL-2Rα chain (CD25), with promotion of Blimp-1 expression and suppression of Bcl6 (Choi et al., 2011; Johnston et al., 2012; Nurieva et al., 2012; Pepper et al., 2011). Accompanying the decrease in Bcl6 in the STAT3-deficient CD4$^+$ T cells, we found increased CD25 expression at 5 dpi, a time at which WT Tfh cells normally turn down expression of this receptor (Figures 3G and 3H). We also found an increase in T-bet expression at this time point and at 14 dpi (Figures 3I and 3J; Figures S2I and S2J), with no difference at 8 dpi (data not shown), in line with the increase in Th1 cell development. These data show that STAT3 promotes Tfh cell differentiation in part by inducing Bcl6 and suppressing T-bet and IL-2 signaling.

Gene-Expression Analysis Reveals a STAT1-Driven Transcriptome in STAT3-Deficient Tfh Cells

Because STAT3-deficient Tfh cells had characteristics of Th1 cells, we next asked whether their global gene expression was also consistent with that phenotype. Therefore, we performed RNA-sequencing (RNA-seq) on sorted polyclonal CD4$^+$CD44$^+$ T cells from WT and STAT3-deficient CD4$^+$ T cells, again by using Ly6c and PSGL-1 expression to distinguish the three effector populations, with further gating on the CXCR5^{hi} group within the Ly6c^{lo} PSGL-1^{lo} subset to distinguish Tfh cells (Figure 1A; Figure S1A). We used a multidimensional scaling plot to relate the similarities and differences among the populations relative to one another (Figure S3A), finding that *Stat3*$^{fl/fl}$ *cre*-negative WT Tfh cells (Ly6c^{lo} PSGL-1^{lo} CXCR5^{hi}) had vastly different gene expression than either their Th1 Ly6c^{lo} PSGL-1^{hi} or Th1 Ly6c^{hi} PSGL-1^{hi} counterparts, validating their separation by surface marker expression (Marshall et al., 2011). In addition, we used unbiased K-means clustering to determine how global STAT3-deficient Tfh cell gene expression compares to the three WT populations (Figure 4A; Figure S3B). We found that STAT3-deficient Tfh cells had a transcriptome more similar to that of WT Th1 Ly6c^{lo} PSGL-1^{hi} cells than the WT Tfh cells, as determined by dendrogram (Figure 4A) and expression patterns (Figure 4A; Figure S3B). Integrated Pathway Analysis (IPA, Ingenuity® Systems, www.ingenuity.com) revealed that helper T cell differentiation was the most deregulated pathway in STAT3-deficient Tfh cells with upregulation of genes associated with T helper cell lineages such as regulatory T cells (i.e., *Foxp3* and *Il10*) (Figure 4B; Figure S3C). To corroborate our findings that STAT3-deficient Tfh cells were phenotypically similar to Th1 cells, we also found upregulated expression of genes associated with this lineage (i.e., *Tbx21*, *Ifngr1*, *Il12r*, and *Il2ra*) (Figure 4B). In juxtaposition, six out of seven genes with decreased expression in this canonical pathway were Tfh-cell associated (*Bcl6*, *Il21*, *Il4*, *Icos*, *Cxcr5*, and *Il6st*) (Figure 4B). In accordance with the downregulation of *Bcl6* and other Tfh-cell associated transcripts, we found an increase in *Prdm1*, which encodes Blimp-1 (Figure 4C). We validated *Bcl6*, *Il21*, *Il4*, and *Prdm1* expression by qPCR (Figures S4A–S4D). Also in line with our RNA-seq data, we observed an increase in Foxp3$^+$ CD44$^+$ Ly6c^{lo} CXCR5^{hi} follicular regulatory T (Tfr) cells, with no differences in GP$_{66}^+$ Tfr cells at 8 dpi, although these cells seem to be rather uncommon at this time point in LCMV infection (Figures S4E and S4F).

STAT3-deficient Tfh cells also had a marked increase in RNA expression of IFN-inducible genes over their WT littermates (Figure 4C), including transcripts downstream of both type I and type II IFN signal transduction (Figure 4C; Figure S4G). We then determined potential upstream regulators by using IPA analysis, finding that IFN regulatory factor-7 (IRF7) and STAT1 were the top two transcription factors affiliated with the changes in gene expression between STAT3-deficient versus WT Tfh cells (Table S1). We also found suppressor of cytokine signaling-1 (SOCS1) and SOCS3 phosphatase activity to be potentially decreased in the STAT3-deficient Tfh cells based on the IPA analysis (Table S1). As both SOCS1 and SOCS3 can act as repressors of STAT1 (Song and Shuai, 1998), an abundance of STAT1-mediated transcription might occur due to this mechanism in the absence of STAT3. However, SOCS1 and SOCS3 were not substantially reduced in the absence of STAT3 according to our RNA-seq results (Figure 4C), and upon further analysis with qPCR, we found that these mRNAs were actually upregulated in cells from *Stat3*$^{fl/fl}$*Cd4*^{cre} animals (Figures S4H and S4I), a phenomenon that might be due to an increase in type I IFN signaling. Similar results were found upon repeating the RNA-seq analysis with sorted cells with CD4$^+$ CD44$^+$ CXCR5^{hi} PD-1^{hi} gating strategy (data not shown).

Type I IFN Signaling Aids in Th1 Cell Differentiation In Vivo, at the Expense of that of Tfh Cells

Our RNA-seq data indicate that there is an increase in STAT1-mediated transcription in the absence of STAT3. To determine

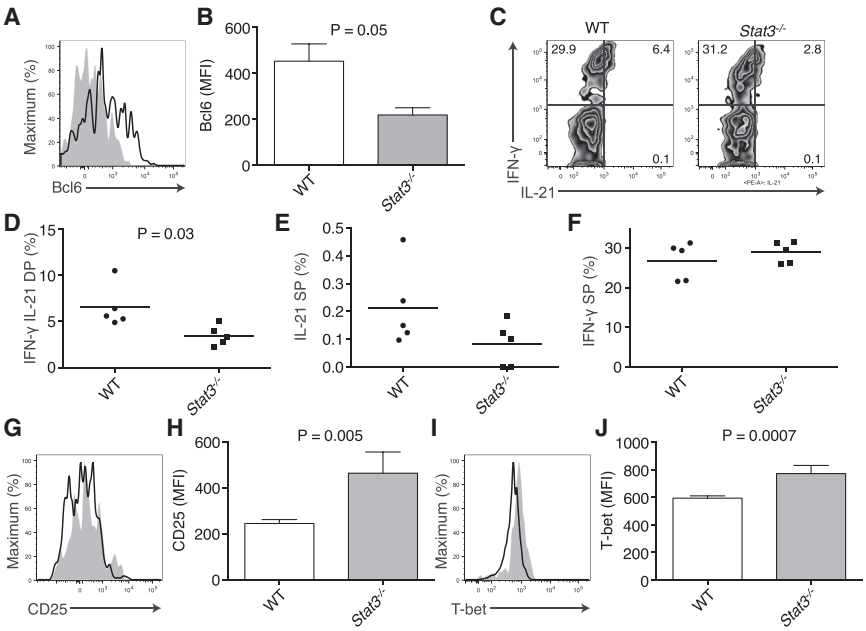

Figure 3. Tfh Cells Deficient in STAT3 Are Less Tfh-like Than Controls, with Characteristics of Th1 Cells

(A and B) Flow cytometry plot (A) and quantification (B) of Bcl6 protein expression in Ly6c^{lo}PSGL-1^{lo}CXCR5^{hi} cells at 5 dpi (WT black line, *Stat3*$^{fl/fl}$*Cd4*^{cre} [*Stat3*$^{-/-}$] shaded gray).

(C–F) Flow cytometry plots (C) and population quantification (D–F) of IL-21 and IFN-γ-expressing cells of the CD4^{+} CD44^{+} CXCR5^{hi} population at 8 dpi (SP, single positive; DP, double positive).

(G and H) Flow cytometry plot (G) and quantification (H) of CD25 protein expression in Ly6c^{lo} PSGL-1^{lo} CXCR5^{hi} cells at 5 dpi.

(I and J) Flow cytometry plot (I) and quantification (J) of T-bet expression in Ly6c^{lo} PSGL-1^{lo} CXCR5^{hi} cells at 5 dpi.

(A–J) Representative of at least two experiments, with at least three animals per genotype per experiment. Statistics conducted with Student's unpaired t test. + SEM (A–D). See also Figure S3.

the role of STAT1 in determining Tfh cell differentiation, we obtained a validated STAT1 small hairpin RNA vector and retrovirally transduced viral epitope GP$_{66}$-specific SMARTA transgenic (Stg) CD4^{+} T cells with it (Choi et al., 2013; Oxenius et al., 1998), and in parallel, with a control vector. Upon transduction, there was substantial silencing of STAT1 (79%, Figures S5A and S5B), and a large reduction in clonal expansion of transduced cells (Figure S5C). We found a slight but reproducible reduction in T-bet expression after transfer into WT congenic hosts (Figure S5D), which was not surprising given that STAT4 has also been shown to drive T-bet expression (Nakayamada et al., 2011). Despite differences in clonal expansion, there were no differences in Tfh cells or Bcl6 expression at 8 dpi (Figures S5E and S5F). It is possible that the use of TCR transgenic T cells nullifies the Tfh cell phenotypes that would otherwise be seen in a polyclonal repertoire (Tubo et al., 2013); however, in line with our data analyzing the polyclonal population (Figures 1A and 1B), we found that STAT3-deficient Stg^{+} cells also had a deficiency in the Tfh cell subset at 8 dpi (Figure S5G). Similar to polyclonal STAT3-deficient T cells, they had no significant differences in Bcl6 protein at 8 dpi (Figure S5H), but there was a marked increase in T-bet expression (Figure S5I). Thus, despite the increase in STAT1-mediated gene transcription in STAT3-deficient T cells, Tfh cell phenotypes were modulated by specific STAT1 signaling cytokines in addition to general STAT1 signaling, because this transcription factor is also known to operate downstream of Tfh-cell inducing cytokines (i.e., IL-6).

Our RNA-seq data suggested a role for both type I and type II IFN signaling in promoting the changes in gene transcription we observed (Figure S4G). Previous work has found that IFN-γ induces Tfh cell formation, and that in the absence of its receptor, *Roquin*$^{san/san}$ lupus-prone mice are rescued from their disease phenotype with reductions in Tfh cell numbers, GCs, and formation of autoantibodies (Lee et al., 2012). To ascertain the effect of IFN-γ on Tfh cell differentiation in our model, we blocked this cytokine by using the XMG1.2 neutralizing antibody (Abrams et al., 1992). Despite a large reduction in splenocyte numbers and T-bet expression upon IFN-γ blockade (Figures S5J and S5K), we found that there was only a marginal, and insignificant, increase in Tfh cell percentages and Bcl6 expression at 8 dpi (Figures S5L and S5M), without an effect on GC B cell percentages (Figure S5N). Thus, despite its role in driving Tfh cells in the *Roquin*$^{san/san}$ autoimmunity model, IFN-γ does not appear to have a large effect on the differentiation of Tfh cells following acute LCMV infection.

We then examined the effects of type I IFNs on Tfh cell differentiation. Type I IFNs are induced following LCMV infection and they regulate STAT1- and IRF7-mediated gene expression (Malmgaard et al., 2002). To determine whether T cell-intrinsic type I IFN signaling is responsible for suppressing Tfh cell formation in favor of Th1 cell differentiation, we crossed *Ifnar1*$^{-/-}$ mice to Stg^{+} mice, and transferred *Ifnar1*$^{-/-}$ Stg^{+} CD4^{+} T cells into infected WT C57BL/6 animals. As previously described (Havenar-Daughton et al., 2006; Way et al., 2007), the expansion of *Ifnar1*$^{-/-}$ Stg^{+} CD4^{+} T cells was severely compromised at 8 dpi (data not shown), indicating an effect on CD4^{+} T cell proliferation and differentiation. In addition, there was an increase in the percentages of Tfh cells along with increased Bcl6 protein, compared to those mice given WT Stg^{+} cells, but with no effects on T-bet expression (Figures 5A–5C; Figure S5O).

Given these findings, we wondered whether type I IFN signaling could actively suppress Tfh cell differentiation, which could potentially explain the propensity of *Stat3*$^{fl/fl}$*Cd4*^{cre} CD4^{+} T cells to develop a Th1-like phenotype. Indeed, upon type I IFN treatment of LCMV epitope-stimulated Stg^{+} WT CD4^{+} T cells, there was an increase in expression of CD25 and phospho-STAT5 (p-STAT5) downstream of IL-2 stimulation (Figure 5D). We found this effect to be dependent on IL-2 and reversed upon treatment with an IL-2 blocking antibody (Figure 5E), suggesting that direct type I IFN signaling might promote IL-2 responsiveness and Th1 differentiation during viral infection in vivo (Johnston et al., 2012; Nurieva et al., 2012; Oestreich

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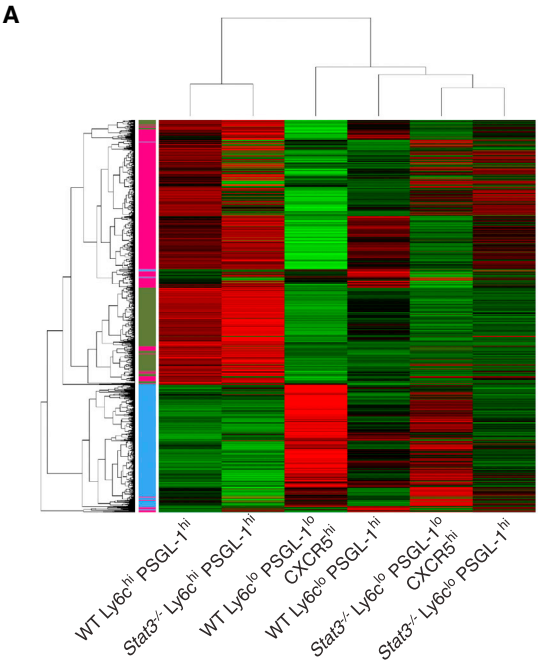


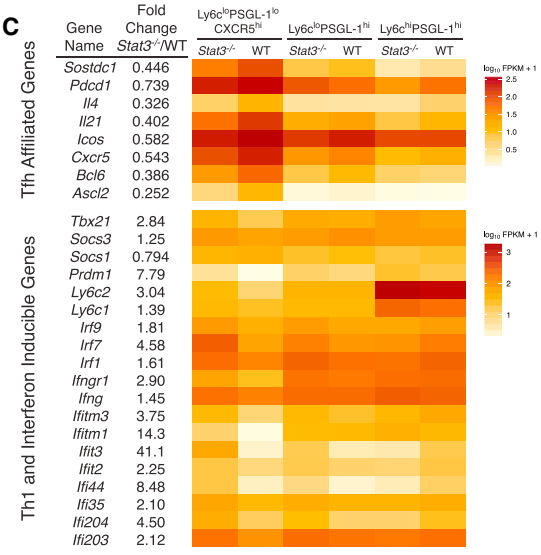
Figure 4. STAT3-Deficient Tfh Cells Have a Th1-like Transcriptome

(A and B) Expression analysis of Ly6c^{lo} PSGL-1^{lo} CXCR5^{hi}, Ly6c^{lo} PSGL-1^{hi}, and Ly6c^{hi} PSGL-1^{hi} cells from *Stat3*$^{fl/fl}$*Cd4*^{cre} (*Stat3*$^{-/-}$) and *Stat3*$^{fl/fl}$ *cre*-negative (WT) mice. (A) Unbiased K-means clustering and dendrogram based on differential gene expression of the three WT populations (see Figure S4B). The bar on the left denotes from which cluster genes come based on Figure S4B. The blue cluster is most associated with WT Tfh cells, green with both WT Th1 cell (PSGL-1^{hi}) populations, and magenta with WT terminal effector cells. Reduced expression is in green with induced expression in red. (B) List of differentially expressed genes from the IPA T helper cell differentiation pathway, with those in red associated with Th1 cells and those in green with Tfh cells. (C) Tfh-cell associated and Th1 and IFN-inducible genes upregulated in STAT3-deficient Tfh cells. Fold changes listed as the ratio of *Stat3*$^{fl/fl}$*Cd4*^{cre} (*Stat3*$^{-/-}$) to WT Tfh cell expression. See also Figure S4 and Table S1.

B

	Gene	Fold Change
Upregulated	*Cd86*	8.51
	Il10ra	8.47
	Il10	6.77
	Cd80	3.86
	Ifngr1	2.90
	Tbx21	2.84
	Il2ra	2.68
	Foxp3	2.45
	Il12rb2	2.39
	Tnfrsf1b	2.20
	Il4r	1.83
Downregulated	*Icos*	1.72
	Il6st	1.82
	Cxcr5	1.84
	Il21	2.48
	Hla-dob	2.49
	Bcl6	2.89
	Il4	3.06

C



et al., 2012). It is possible that T cells deficient in STAT3 are more sensitive to type I IFN and IL-2 signaling, because this transcription factor has been shown to suppress STAT5 signaling (Oestreich et al., 2012). In accordance with this hypothesis, STAT3-deficient cells had IL-2-dependent enhanced expression of CD25 with peptide stimulation and IFN-β treatment compared to WT cells (Figure 5E). STAT3 binding in the *Bcl6* locus occurs in conditions with low IL-2, whereas high amounts of IL-2 allow STAT5 to outcompete STAT3 for binding sites, hampering *Bcl6* expression (Oestreich et al., 2012). To determine how type I IFNs might affect STAT binding to the *Bcl6* locus, we used chromatin immunoprecipitation-qPCR (ChIP-qPCR) to determine binding of STAT1, STAT3, and STAT5 in the presence of IL-6 or type I IFNs. We used previously confirmed (*Bcl6 4*) and newly generated qPCR primer sets surrounding STAT-binding sites (Figure 5F) (Oestreich et al., 2012). While STAT3 bound the *Bcl6* locus in the presence of IL-6, STAT5 bound robustly in the presence of type I IFNs in all primer sets tested (Figure 5G), with little binding of STAT1 in either context. This suggests that type I IFN-induced IL-2 signaling leads to STAT5 binding at the expense of STAT3. Thus, in the absence of STAT3 following acute viral infection, IL-2 and STAT5 signaling is likely dominant, leading to a reduction in Tfh cell differentiation.

Type I IFN Blockade Partially Rescues the *Stat3*$^{fl/fl}$*Cd4*^{cre} Tfh Cell, GC B Cell, and Pathogen-Specific Antibody Phenotypes

Although type I IFNs appear to insulate against Tfh cell in favor of Th1 cell differentiation during viral infection, we hypothesized that this effect might be exacerbated in the absence of STAT3 signaling. To determine whether blockade of type I IFNs could rescue the Tfh cell defect in LCMV-infected mice bearing STAT3-deficient CD4$^+$ T cells, we used the MAR1-5A3 (MAR1) IFN-αβR blocking antibody (Sheehan et al., 2006). Unlike the *Ifnar1*$^{-/-}$ CD4$^+$ T cells, pretreating WT and *Stat3*$^{fl/fl}$*Cd4*^{cre} mice with the MAR1 antibody at a dosage of 600 μg per mouse did not compromise expansion of CD44$^+$ or GP66$^+$ CD4$^+$ T cells following viral challenge (data not shown). Upon pretreatment with MAR1, *Stat3*$^{fl/fl}$*Cd4*^{cre} mice and their WT littermates had substantial increases in Ly6c^{lo} PSGL-1^{lo} Tfh cells, as well as Ly6c^{lo} PSGL-1^{hi} Th1 cells, while there was a decrease in Ly6c^{hi} PSGL-1^{hi} Th1 cells among both GP66$^+$ and total CD44$^+$ cells (Figures 6A–6E; Figures S6A–S6E). Notably, the *Stat3*$^{fl/fl}$*Cd4*^{cre} mice treated with MAR1 had similar Ly6c^{lo} PSGL-1^{lo} cell percentages to WT IgG treated mice, suggesting that STAT3 and type I IFNs act as mutually

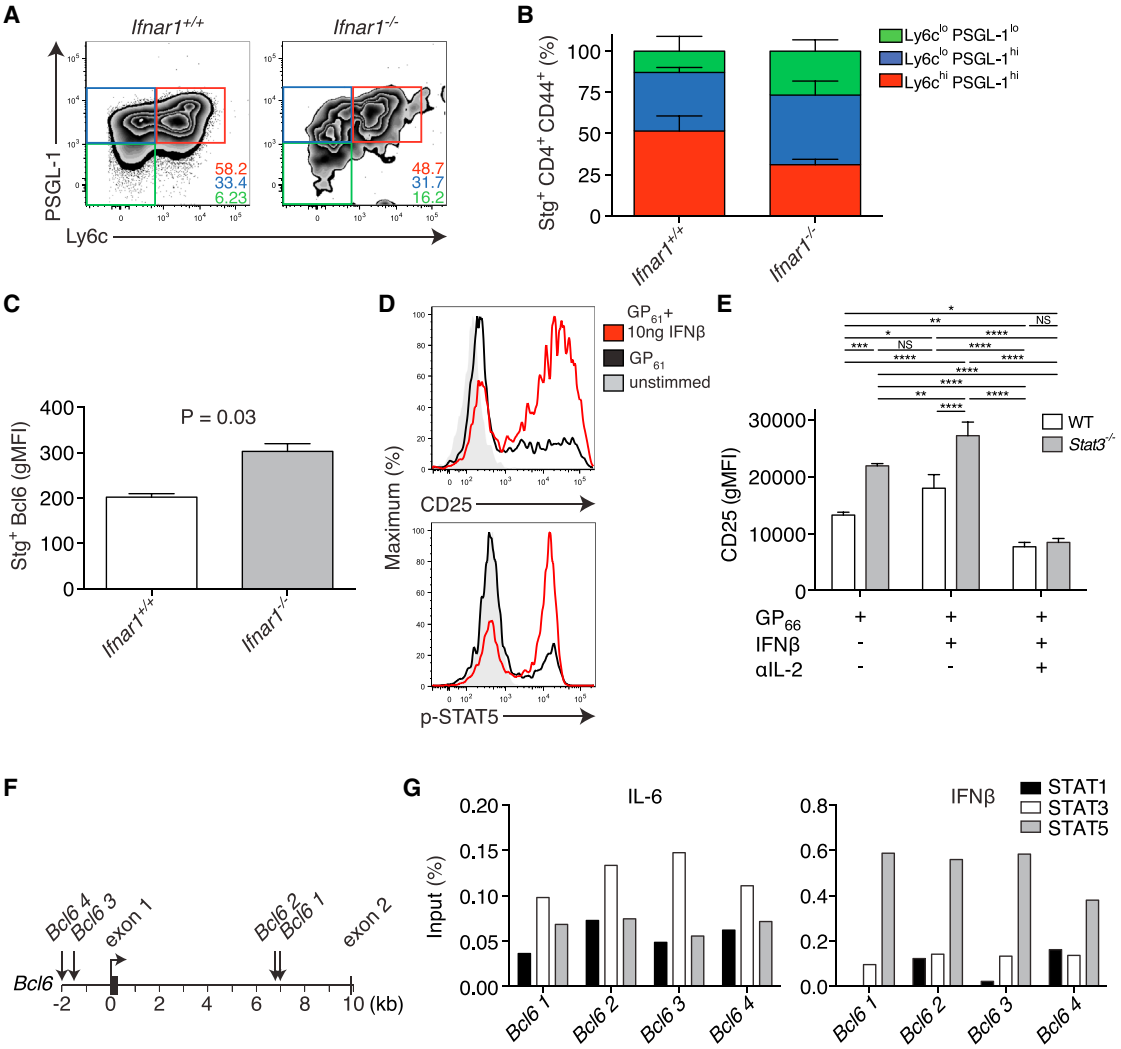


Figure 5. Type I IFNs Inhibit Tfh Cell Differentiation

(A) Flow cytometry plots of *Ifnar1*$^{+/+}$ and *Ifnar1*$^{-/-}$ CD4$^+$ CD44$^+$ Stg$^+$ cells gated on Ly6c and PSGL-1 at 8 dpi.

(B) Ly6c and PSGL-1 populations, gated as in (A).

(C) Bcl6 protein expression in *Ifnar1*$^{+/+}$ and *Ifnar1*$^{-/-}$ Stg$^+$ cells.

(D) Flow cytometry plots of CD25 and p-STAT5 expression of WT Stg$^+$ cells, either unstimulated (unstimmed) or stimulated with the cognate LCMV peptide GP_{61} for 4 days, or with this peptide + 10 ng IFN-β for 4 days. p-STAT5 samples were stimulated with IL-2 for 25 min prior to fixation.

(E) CD25 protein expression for WT and *Stat3*$^{fl/fl}$*Cd4*^{cre} (*Stat3*$^{-/-}$) Stg$^+$ T cells stimulated with the cognate LCMV peptide GP_{66} for 3 days, with this peptide + 10 ng IFNβ for 3 days, or with peptide, 10 ng IFN-β, and αIL-2.

(F) Schematic showing the location of primer sets (arrows) used to determine STAT binding in the *Bcl6* locus. Tic marks are 1 kb and indicate the distance from the transcription start site.

(G) ChIP-qPCR of STAT1, STAT3, or STAT5 for STAT binding within the *Bcl6* locus on WT Stg$^+$ T cells treated with 10 ng/mL IL-6 or IFN-β.

(A–D) Representative of at least three experiments, with at least three animals per genotype per experiment.

(E and G) Representative of at least two experiments. Statistics conducted with Student's unpaired t test or one-way ANOVA with Tukey posttest; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 (E). + SD (C and E). See also Figure S5.

repressive insulators for the differentiation of Tfh and Th1 cells, respectively (Figures 6A–6C; Figures S6A–S6C). The WT MAR1-treated mice also had a remarkable increase in Tfh cells beyond that of treated *Stat3*$^{fl/fl}$*Cd4*^{cre} mice, suggesting that STAT3 might have direct effects on Tfh cell differentiation other than insulating against type I IFNs. To corroborate these data, we found at 8 dpi a significant increase in Bcl6 protein expression in both *Stat3*$^{fl/fl}$*Cd4*^{cre} and WT GP_{66}^+ and total CD4$^+$ CD44$^+$ T cells upon MAR1 treatment, compared to a lack of differential expression between untreated *Stat3*$^{fl/fl}$*Cd4*^{cre} and WT mice (Figure 6F; Figure S6F). CXCR5 expression was unaffected upon treatment in GP_{66}-specific T cells, although increased among total WT CD44$^+$ cells (Figure 6G; Figure S6G). We found a repeatable, albeit insignificant, reduction in IFN-γ production upon MAR1 treatment (Figure 6H). The effects on IL-21, however, were more variable, with a possible increase in IL-21 SP

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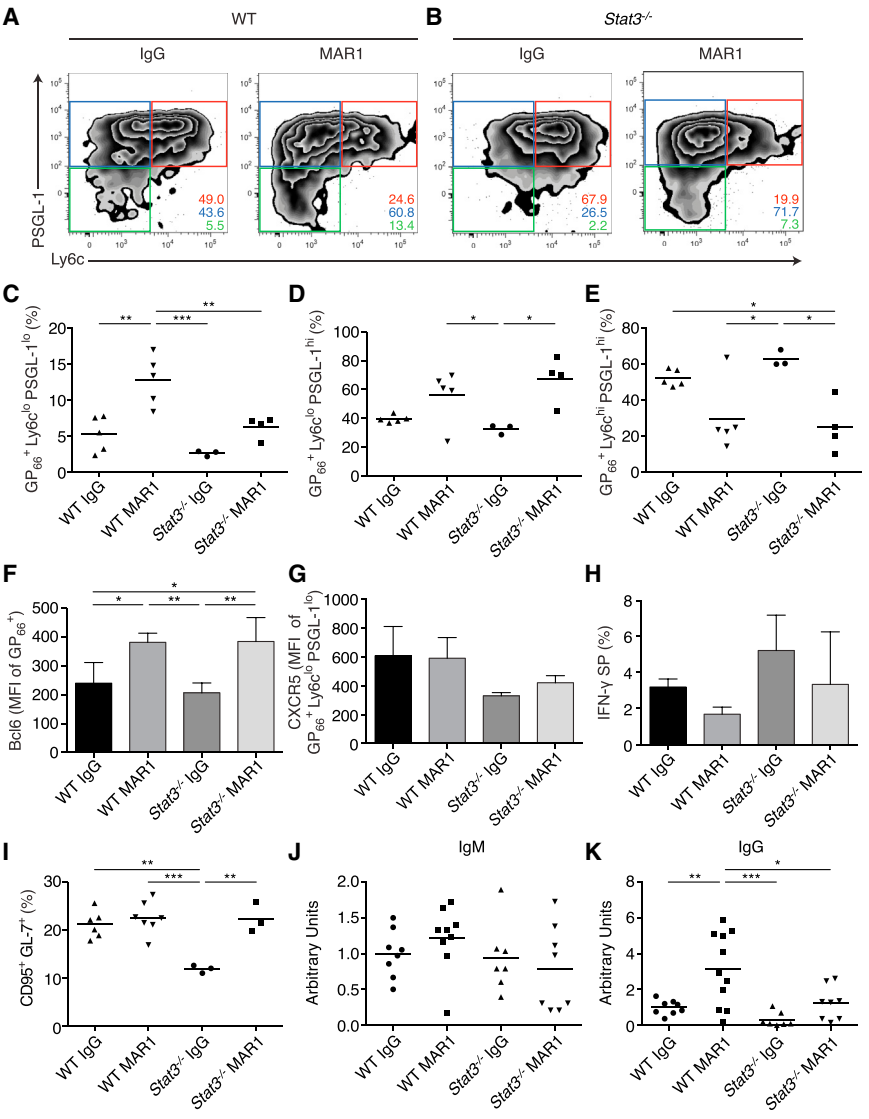

Figure 6. Inhibition of Type I IFN Signaling Partially Rescues STAT3-Deficient Tfh Cell Phenotypes

(A and B) Flow cytometry plots of WT (A) or *Stat3*$^{fl/fl}$*Cd4*^{cre} (*Stat3*$^{-/-}$) (B) CD4$^+$ CD44$^+$ GP$_{66}^+$ Ly6c and PSGL-1 expressing cells, with treatment of the MAR1 blocking antibody or isotype control (IgG).

(C–E) Comparisons of percentages of GP$_{66}^+$ Ly6c and PSGL-1 populations between isotype and MAR1-treated WT and *Stat3*$^{fl/fl}$*Cd4*^{cre} (*Stat3*$^{-/-}$) mice, using the gating in (A).

(F) Bcl6 MFI of GP$_{66}^+$ cells.

(G) CXCR5 MFI of GP$_{66}^+$ Ly6c^{lo} PSGL-1^{lo} cells.

(H) IFN-γ SP cells gated on CD4$^+$ CD44$^+$ CXCR5$^+$.

(I) GC B cell percentages gated on B220$^+$ IgD^{lo} CD95$^+$ GL-7$^+$ cells.

(J and K) Serum concentrations of LCMV-specific IgM (J) and IgG (K).

(A–K) Representative of two to three experiments, with at least three animals per condition per genotype. (J) and (K) are pooled from two separate experiments, where serum concentrations were normalized to WT IgG treated. Statistics conducted with one-way ANOVA with Tukey posttest. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. + SEM (F–H). See also Figure S6.

and IL-21 IFN-γ DP cells in both WT and *Stat3*$^{fl/fl}$*Cd4*^{cre} mice (Figures S6H and S6I).

To analyze the downstream effect of increased Tfh cells in the MAR1-treated mice, we studied GC B cell percentages and pathogen-specific antibody responses. We found that, unlike the effect on Tfh cells, MAR1 had a minimal effect on GC B cell percentages in WT mice. However, in the animals containing STAT3-deficient T cells, GC B cells were rescued to WT percentages (Figure 6I). In line with this finding, whereas effects on IgM were no different, pathogen-specific IgG antibody responses were rescued to the concentrations of WT control animals (Figures 6J and K). This suggests that type I IFNs play an inhibitory role in plasmablast and plasma cell responses in addition to deterring the GC response. We also found a moderate increase in SOCS3 protein expression in the STAT3-deficient Tfh cells (Figure S6J), in agreement with our qPCR data (Figure S4I). This was reduced by MAR1 treatment (Figure S6J), suggesting that type I IFNs elicit the increase in SOCS3 expression found in the *Stat3*$^{fl/fl}$*Cd4*^{cre} mutant.

These data reveal a fine balance in signaling pathways following acute viral infection with a requirement for STAT3 for Tfh cell differentiation and type I IFNs to promote that of the Th1 response.

DISCUSSION

We found that STAT3 is required for Tfh cell differentiation and function during acute viral infection. Mice with Tfh cells that lack STAT3 display impaired GC B cell and plasmablast formation leading to the absence of antigen-specific antibody responses, with promotion of Th1 cell differentiation mediated by type I IFN signaling. Thus, there exists in vivo tight cytokine regulation of Tfh versus Th1 cell differentiation.

STAT3 has been previously noted to aid in Tfh cell differentiation following immunization with a nominal antigen (Nurieva et al., 2008). In a like manner, it has been shown to make a transient contribution to Tfh cell development following LCMV infection (Choi et al., 2013), with the downstream effects of this process in terms of GC responses consequently not analyzed (Choi et al., 2013). However, we found a defect in the development of STAT3-deficient polyclonal and Stg$^+$ Tfh cells at day 8 following viral challenge, indicating that STAT3 affects Tfh cell differentiation later than day 3. This earlier published work also demonstrated that STAT1 aids STAT3 in transient promotion of Tfh cell differentiation (Choi et al., 2013). Our finding that type I IFNs perturb Tfh cell development in the absence of STAT3 suggests that these cytokines utilize STAT1 in a cytokine-specific manner in this process. Indeed, we did not see promotion of

Tfh cells upon silencing of STAT1; so, it is possible that while STAT1 can deter Tfh cell differentiation through signal transduction mediated by type I IFNs, it might also promote Tfh cell differentiation through other cytokines (i.e., IL-6).

There are several possible mechanisms for type I IFN and STAT3 cross-repression that can explain our findings, given the complex regulatory network that involves competition for binding partners, binding sites, and upregulation of repressive SOCS proteins. For example, STAT3 has the capacity to bind to the same consensus region as STAT1, potentially blocking its transcription and vice versa (Wang et al., 2011). The N-terminal domain of STAT3 also has been shown to inhibit type I IFN signaling following viral infection of mouse embryonic fibroblasts through sequestration of STAT1. However, we did not find promotion of Tfh cell differentiation in STAT1-silenced Stg^+ T cells following viral infection. STAT3 and type I IFN cross regulation also could occur via their respective downstream signaling attenuators, SOCS1 and SOCS3. For example, in the absence of STAT3, a lack of either could lead to an increase in STAT1 or STAT5 phosphorylation, reminiscent of that seen in $CD8^+$ T cells in the absence of STAT3 (Cui et al., 2011). However, this mechanism also seems unlikely, as we observe an increase in the expression of SOCS1 and SOCS3 transcripts and SOCS3 protein expression in the $Stat3^{fl/fl}Cd4^{cre}$ in a type I IFN-dependent manner.

Another possible mechanism for type I IFN and STAT3 cross-repression, and one apparent from our results, is through type I IFN promotion of CD25 expression and subsequent STAT5 phosphorylation. In the absence of STAT3, CD25 expression is increased, and upon stimulation of WT STg^+ cells with their cognate peptide and type I IFNs, its expression is also increased, accompanied by enhanced p-STAT5, in an IL-2-dependent manner. STAT5 competes with STAT3 for consensus DNA binding sites, including at the *Bcl6* locus (Oestreich et al., 2012). We found that STAT5 outcompeted STAT3 for binding at the *Bcl6* locus in the presence of type I IFNs. STAT3-deficient Tfh cells appear to have an increase in *Blimp-1* expression, and STAT5 has also been shown to upregulate *Blimp-1*, potentially inhibiting Tfh cell differentiation through cross-repression of Bcl6 and with promotion of Th1 cell differentiation (Johnston et al., 2009, 2012; Nurieva et al., 2012; Oestreich et al., 2012). Thus, increased IL-2 signaling via type I IFNs likely upregulates Blimp-1 and deters Bcl6-mediated Tfh cell differentiation (Johnston et al., 2012; Nurieva et al., 2012; Oestreich et al., 2012). In the presence of STAT3, Tfh cell differentiation was promoted upon type I IFN blockade during acute viral infection. Therefore, it is likely that STAT3 plays multiple roles, one as an insulator against type I IFNs and another as an enhancer of Tfh cell differentiation. Likewise, type I IFNs might also play dual roles, by both promoting Th1 cell differentiation through STAT1 induction of *Tbx21* and Tfh cell differentiation through upstream (or indirect) induction of autocrine IL-2, subsequently leading to STAT5 activation and direct competition with STAT3 for binding sites in the *Bcl6* locus.

Patients with dominant-negative STAT3 mutations have characteristic decreases in circulating "Tfh-like" cells in their blood, as well as lower serum antigen-specific IgG concomitant with increased IgE (Ma et al., 2012). Many of these patients suffer recurrent infections from *Candida albicans* and *Staphylococcus aureus* due to a scarcity of Th17 cells, with STAT3 signaling necessary for the development of this subset (de Beaucoudrey et al., 2008; Ma et al., 2008; Milner et al., 2008; Renner et al., 2008). Given these findings and that such patients have deficiencies in memory T cells, it is prudent to further characterize the T and B cell intrinsic components of STAT3 signaling in developing long term immunity. Patients with the autoimmune syndrome systemic lupus erythematosus (SLE) have high titers of autoantibodies and an abundance of Tfh cells (Craft, 2012); thus, blockade of STAT3 signaling might be therapeutically beneficial, with a decrease in T cell-mediated autoantibody production. Although we show here that STAT3 plays a role in Tfh cell differentiation in acute viral infection, this transcription factor also is required for regulating Th2 type immunity (Stritesky et al., 2011). We and others have shown that in the absence of STAT3, there is a drastic reduction in IL-4 production, with STAT3 important for opening the IL-4 locus to enable STAT6 binding (Stritesky et al., 2011). In Th2 cell-associated infections, IL-4 production by T cells is important for inducing antigen-specific IgE production by plasma cells. Given the importance of STAT3 in promoting Th2 cell type immunity, it will be critical to explore the effects of $CD4^+$ T cell STAT3 signaling in exacerbating allergic disease such as asthma. STAT3 inhibitors are in development, and this could be a useful therapeutic for those who suffer from Tfh-cell mediated autoimmune diseases such as SLE, or those with allergic illnesses, given the effect of STAT3 on IL-4 production.

EXPERIMENTAL PROCEDURES

Mice and LCVM Infection

Mice were housed in specific pathogen-free conditions at the Yale School of Medicine (New Haven, CT). C57BL/6 (B6) mice were purchased from the National Cancer Institute (Bethesda, MD), with other strains provided as noted: $Stat3^{fl/fl}$ (B6.129S1-*Stat3tm1Xyfu*/J) mice from Xin-Yuan Fu at the Indiana University School of Medicine, $Cd4^{cre}$ (B6.Cg-*Tg(Cd4-cre)1Cwi/BfluJ*) from Christopher Wilson at the University of Washington in Seattle, SMARTA Tg (*Tg(TcrLCMV)1Aox*) from Hans Hengartner at the University of Zürich in Switzerland, and $Ifnar1^{-/-}$ ($Ifnar1^{tm1Agt}$) from Michel Aguet at the University of Zürich in Switzerland. All were used at 6–8 weeks of age. The Institutional Animal Care and Use Committee of Yale University approved all procedures involving mice. They were infected with LCMV Armstrong by intraperitoneal (i.p.) injection of 2×10^5 PFU per mouse.

Antibodies for Flow Cytometry and Cell Sorting

These protocols are described in detail in the Supplemental Experimental Procedures.

Microscopy

These protocols are described in detail in the Supplemental Experimental Procedures.

ELISA for Antibodies to LCMV

Anti-LCMV Ab was measured by ELISA with sonicated cell lysate from LCMV-infected BHK-21 cells as capture Ag. Ninety-six-well Polysorp microtiter plates (Nunc) were coated overnight with lysate in PBS. AP-conjugated goat anti-mouse IgM, IgG, and IgG2a secondary Abs were used for detection (Southern Biotech). ODs were converted to units based on standard curves with sera from WT mice infected with LCMV (Softmax Pro 3.1 software; Molecular Devices).

RNA-Seq

Sorted Tfh cells were lysed with their RNAs isolated with the miRNeasy kit (QIAGEN). Samples were sequenced on an Illumina HiSeq 2000 with 75-bp paired-end reads. FASTQ format sequencing reads were aligned to the MM9 mouse genome using TopHat version 2.0.6 and Bowtie version 1.3.0.

Reads were compared between samples with Cufflinks version 2.0.2. Heatmaps were made with CummeRbund version 2.0.0. Quantitative real-time PCR was used to confirm expression of RNA transcripts. IPA Ingenuity® Systems was used to analyze deregulated pathways and potential upstream regulators.

Quantitative PCR

These protocols are described in detail in the Supplemental Experimental Procedures.

Retroviral Transduction and Cell Transfer

These protocols are described in detail in the Supplemental Experimental Procedures.

Immunoblot

These protocols are described in detail in the Supplemental Experimental Procedures.

Chromatin Immunoprecipitation

These protocols are described in detail in the Supplemental Experimental Procedures.

Type I IFN, IFN-γ, and IL-2 Blockade

For type I IFN blockade, WT or *Stat3*$^{fl/fl}$*Cd4*^{cre} mice were treated at day −1 with 600 μg of MAR1-5A3 IFN-αβR blocking antibody or IgG1 isotype control antibody (both from Leinco Technologies) in PBS via i.p. injection (Sheehan et al., 2006), before infection with LCMV Armstrong at day 0. For IFN-γ blockade, WT mice were treated at day 0 and day 3 after LCMV Armstrong infection with 250 μg of XMG1.2 IFN-γ blocking antibody or PBS vehicle control (Abrams et al., 1992). IL-2 blocking antibody (JES6-1A12) was used in vitro at a concentration of 10 μg/mL (Abrams et al., 1992).

Statistics

Data were analyzed with the Student's unpaired t test or with one-way ANOVA with Tukey posttest for multiple comparisons with Prism 6®. The number of asterisks represents the degree of significance with respect to p values, and the p value is presented within each figure or figure legend.

ACCESSION NUMBERS

The GEO accession number for the RNA-Seq data reported in this paper is GSE55596.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2014.02.005.

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Alliance for Lupus Research

Target Identification in Lupus Grant (with LOI)

Grant Application

Date Submitted: 7/14/2014 4:24:43 PM

Resubmission? No | Prior App:

TITLE OF PROJECT *(Titles exceeding 81 characters, including spaces and punctuation, will be truncated.)*

Defining unique traits of lupus Th17 cells and their modulations by IL-27

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HIGHEST DEGREE(S)

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PROGRAM ELIGIBILITY INFORMATION: *(Responses to selected fields displayed below. For some grant programs this section may be blank.)*

U.S. Citizenship: U.S. Citizen

DATES OF PROPOSED PROJECT *(MM/DD/YYYY)*

From: 2/1/2015 Through: 1/31/2018

PROPOSED BUDGET

$600,000.00

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HUMAN SUBJECTS ☐ No ☒ Yes

Human Subjects Assurance No. FWA00002571

IRB Status: Pending

IRB Date: 08/25/2014

VERTEBRATE ANIMALS ☐ No ☒ Yes

Animal welfare assurance no. A3230-01

IACUC Status: Pending

IACUC Date: 08/25/2014

RECOMBINANT DNA No

Status:

Date:

BIOHAZARDS Yes

APPLICANT ASSURANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

SIGNATURE OF APPLICANT *(In ink. "Per" signature not acceptable.)*

DATE: 8/18/2014

SIGNING OFFICIAL ASSURANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with the grantor's terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF SIGNING OFFICIAL *(In ink. "Per" signature not acceptable.)*

Marybeth Brandi

DATE: 8/20/14

ADDITIONAL SIGNATURE (follow guidelines for required signatures): I certify that the statements herein are true, complete and accurate to the best of my knowledge.

DATE

ADDITIONAL SIGNATURE (follow guidelines for required signatures): I certify that the statements herein are true, complete and accurate to the best of my knowledge.

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