NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

miRNA AND EPIGENETIC REGULATIONS OF THE IMMUNE RESPONSE

Bethesda Marriott Hotel, Bethesda, MD
December 11–12, 2008

Summary Report

THURSDAY, DECEMBER 11, 2008

INTRODUCTION

Olivier Blondel, Ph.D., Division of Diabetes, Endocrinology, and Metabolism, National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), National Institutes of Health (NIH), Bethesda, MD

The goal of the meeting is to learn about the state-of-the-art of microRNA (miRNA) regulation and the immune response. This is important because this field is exciting and needs further support in the coming years. As for epigenetic changes, there is a large investment at NIH for this research across diseases. There have been two initiatives in the past year, but these have been for basic research. Future initiatives will be focused on research that is more directly related to disease.

The field of miRNA regulation is moving quickly. The results to be presented at this meeting represent cutting-edge research in this area; at the current time, NIH has no initiatives in this area. A goal of this meeting is to inform NIH of potential initiatives to support research in this area.

The meeting is organized around four broad themes: (1) miRNAs and the immune response; (2) epigenetic regulation of the immune response; (3) miRNA and epigenetic regulations in autoimmune diseases; and (4) emerging therapeutic strategies in autoimmune diseases. In addition, two keynote addresses were planned to provide an overview of transcriptional control of T-cell activation and tolerance, and cytoplasmic determinants of T-cell fate.

KEYNOTE ADDRESS: A NEW EPIGENETIC MODIFICATION OF DNA

Anjana Rao, Ph.D., Professor, Department of Pathology, Immune Disease Institute, Harvard Medical School, Boston, MA

SESSION 1: miRNAS AND THE IMMUNE RESPONSE

Introduction to Session 1

Klaus Rajewsky, M.D., Senior Investigator, Immune Disease Institute, Harvard Medical School, Boston, MA

There are approximately 800 human miRNAs generated from RNA hairpins derived from transcripts in the nucleus. They are processed by a protein complex known as the Drosha-DGCR8, which is critical for exporting miRNA into the cytoplasm; a second enzyme, Dicer, processes these hairpins into a complex known as RISC, which is targeted to the three prime untranslated regions (3' UTR) of messenger RNA (mRNA). The two typical modes of miRNA control are through translational inhibition and destabilization of the message through de-adenylation leading to miRNA decay. These two modes afford researchers with two good targets for intervention regarding methylation.

An miRNA may have multiple binding sites within a 3′ UTR. Many 3′ UTRs carry several different miRNA binding sites, but others seem to be devoid of any. There is conservation of binding sites among related species, which allows the bioinformatic prediction of miRNA targets. Typically, several miRNAs are highly expressed in specific cell types, in different combinations. miRNA is involved in almost every process occurring in the immune system, from development processes to functions. It also appears to be involved in many diseases, including those related to autoimmunity to cancer. Human miRNA has one very distinguishing feature from transcription factors; they are able to evolve rapidly, which fits well with the needs of the immune system.

Gene Regulation by Transcription Factors and microRNAs

Dr. Rajewsky

The role of miRNA in the lymphocytes is critical, with no B-cell development occurring without miRNA. The mechanisms have partly been elucidated and involve a cluster of genes on chromosomes 13 and 14 between markers 17–92. Investigations using knock-outs of the miRNA-17–92 cluster (known as oncomirR-1) indicate that *Pten* and *Bim* are the targeted pathways within the cluster. *Bim* is at least partly responsible for apoptosis seen in B cells, and mouse studies have shown that there is partial rescue in B cells. When the *Bcl2* transgene is combined with a *BCR* transgene encoding a Bcl receptor, one gets an almost complete rescue.

The predominant mode of miRNA control is downregulation of genes, generally resulting in a 2- to 4-fold reduction in gene expression. This mode of regulation can have severe physiological consequences, as is known from the many cases of haploinsufficiency in many diseases. For example, an miRNA expressed exclusively in mature lymphocytes, miRNA-150, has been studied in transgenic mice for targeting of the transcription factor cMyb, which is essential for B-cell progenitor survival and promotes B1 cell development. miRNA provides a dose-dependent inhibition of cMyb and inhibition of B-cell development.

In general, the situation is much more complicated. The oncomiR-1 cluster is present in many cases of cancer. It is possible that the cluster is involved not just in apoptotic pathways, but also

in the control of cell cycle inhibitors. For example, in a mouse study, modest, chronic transgenic overexpression of the oncomiR-1 cluster in lymphocytes led to severe autoimmunity, lymphoproliferation, and premature death.

The targeting of multiple pathways seems to be typical for miRNA control. For example, miR-155 is involved in many features of the immune response. It controls T-cell differentiation (including Th1/2 differentiation and the maintenance of regulatory T cells [Tregs]), the germinal center B-cell response, AID-mediated chromosomal translocations, and cytokine production. In addition, overexpression of miR-155 can lead to myeloproliferation and B-cell lymphoma.

Laboratory experiments of a global analysis of miR-155 targets using stable isotope labeling with amino acids in cell culture (SILAC) in miR-155 knock-out cells, showed an upregulation of bins of genes containing 3′ UTR seeds. If a single mutation is entered in the seed, the correlation breaks down. This demonstrates that there are hundreds of genes through seed recognition and other types of regulation that we do not understand. This is true of many different concentrations.

The important question is how to use this information to find functional targets. A recent experiment looked at the targeted mutation of the miRNA binding site in the 3′ UTR, which is a direct way to test the importance of an individual miRNA target in miRNA control. miR-155 targets the gene insert AID, which has a single recognition site in its 3′ UTR. Introducing a targeted mutation into the binding site, Dorsett and colleagues (Immunity 2008) found that AID is upregulated 2-fold in the mutant compared to the wild type, and can produce oncogenic c-myc to IgH translocations in B cells. Results also showed that in the seed recognition mutant there is a 3- to 6-fold upregulation of translocations; in the miR-155 knock-out mouse, the number of translocations increases to 15-fold. This supports the hypothesis that miR-155 may have evolved to protect cells from this type of event.

In summary, miRNAs directly control large numbers of target proteins in a dose-dependent manner, mostly over modest ranges of concentrations; ectopic or modest over-expression of miRNAs can result in dramatic disease phenotypes; small changes in the expression levels and in key cellular proteins can have severe functional consequences; miRNAs may have evolved to exert their function in such contexts, often targeting several functionally connected proteins; and while hundreds of target genes are controlled by any given miRNA, only a few of them may represent functional targets in a given biological process.

MicroRNA-Mediated Regulation of T-Cell Sensitivity and Selection

Chang-Zheng Chen, Ph.D., Assistant Professor, Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA

There are three miR-181 genes in the mouse genome, each of which encodes two miRNAs of the same family. Chromosome 1 contains miR-181a-1 and miR-181b-1; chromosome 2 contains miR-181a-2 and miR-181b-2; and chromosome 8 contains miR-181c and miR-181d. The genetic makeup is conserved throughout many animal species, and they have identical seed nucleotides.

miR-181a controls T-cell receptor (TCR) signaling by targeting multiple phosphatases in the TCR signaling pathway, such as SHP-2, PTPN22, DUSP6, and DUSP5. If a target gene is overexpressed and is insensitive to miR-181a regulation (e.g., DUSP5), it can block T-cell signaling at various steps of the T-cell signaling pathway. These results argue that the TCR signaling threshold is controlled by miRNA genes, and miRNA may have evolved to carry out this function. For example, at high miR-181a expression, the phosphatase level is low and the basal kinase phosphorylation level is high; therefore, the threshold for T-cell activation is reduced. This implies that a binary switch applies both qualitatively and quantitatively. Regulation of T-cell sensitivity to antigens during normal development and maturation is another role played by miR-181a. *In vivo* experiments indicate that miR-181a expression in various T-cell populations seems to correlate with T-cell sensitivity to antigens. This suggests that miR-181a may be a T-cell sensitivity rheostat, and miR-181a expression seems to be higher in DP, naïve and memory T cells, the cells that have higher sensitivity.

miR-181a also is highly differentially expressed in thymic progenitors through coordinated regulation of Notch and pre-TCR signaling pathways by targeting signaling molecules in both pathways. *In vitro* and *in vivo* studies showed that one of the functional targets of miR-181a is Nrarp, a negative regulator of the Notch pathway. A summary of these investigations indicates that ectopic miR-181a expression potentiates early αβ T-cell development; miR-181a dampens expression of negative regulators of Notch and pre-TCR signaling pathways; multi-target suppression is required for miR-181a function; and silencing of miR-181a reduces DN to DP development.

The pre-miRNA loop nucleotides control the activity and specificity of miRNA genes. Twenty-two nucleotides are processed by Drosha or Dicer and target mRNAs for specific cleavage or translational repression. The question was asked whether miR-181a and miR-181c, which have only a one-nucleotide difference, have the same biological activity regarding T-cell development. Experiments indicated that miR-181a promotes early T-cell development, but miR-181c does not. This suggests that miRNA genes can encode identical mature miRNA and have different biological activity, which may be determined by the corresponding loop nucleotides. Regardless of the mechanism, the pre-miRNA loop is a functional motif of miRNA genes. A final assumption is that precursor miRNAs, including pri-miRNAs and pre-miRNAs, also may have functional roles in target gene repression.

Germinal Centers and Lymphomangenesis

Riccardo Dalla-Favera, M.D., Director, Herbert Irving Comprehensive Cancer Center, Institute for Cancer Genetics, Columbia University, New York, NY

The target structure for B-cell morphogenesis in humans is the germinal center. It has been hypothesized that miRNA is involved in germinal cell formation that may lead to lymphomagenesis. The laboratory has been investigating miRNAs, and possibly new miRNAs that are involved in this process, identification of alterations in miRNA expression associated with malignant transformation, and functional analysis of selected miRNAs. A cloning strategy was used, matches were made to human sequences, and candidate miRNA was computationally identified; 103 short-miRNA sequences were validated and 75 new miRNAs were identified. These were checked against existing B-cell libraries, and individually were found to be

expressed in different stages of B-cell development. Results of these investigations indicated that there is specific regulation of known and new miRNA during the germinal center reaction. In addition, changes in miRNA expression are associated with neoplastic transformation, as suggested by the assessment from the B-cell libraries.

An investigation of the role of miRNA in the pathogenesis of B-cell chronic lymphocytic leukemia (CLL) focused on a region of deletion of chromosome 13q14, which is the most common genomic aberration in CLL, and is present in 50 percent of CLL cases. The deletion can be homozygous in 20 percent of the cases, with the rest heterozygous. It is most prevalent in the subset of IgV-mutated CLL cases. There were no "classic" tumor suppressor genes identified in the deleted region, although miRNAs miR-15a/16-1 were found in the deleted region, are the target of the deletion, and are associated with CLL pathogenesis. The minimal deletion region (MDR) contains three essential regions, DLEU1, a non-conserved locus, long-coding RNA, and in DLEU2, a short distance from miR-15a/16-1. Every deletion associated with CLL deletes DLEU2 and miR-15a/16-1; therefore, it is difficult to find the target between these two. A laboratory investigation of the MDR found that the miRNAs had biological activity consistent with reversing (in part) the transformed phenotype. An investigation in MDR and miR-15a/16-1 knock-out mice further indicated that they acquire monoclonal B-cell lymphocytosis; a few mice develop typical CLL or diffused large B-cell lymphoma (DLBCL). Conclusions from these investigations are: (1) the 13q14 region controls the expansion of the B-cell compartment, (2) 13q14 deletions can cause CD5+ MBL, NHL, and CLL in mice, (3) mice with 13q14 deletions provide a faithful model of human lymphoproliferative diseases associated with this genetic lesion, and (4) miR15a/16-1 appear to be the critical target of 13q14 deletions.

Role of miRNAs in the Innate Immune Response

Mark Lindsay, Ph.D., Reader in Biopharmaceutics, Department of Translational Medicine, University of Manchester, Education and Research Centre, Wythenshawe Hospital, Manchester, United Kingdom

The innate immune (II) response is responsible for the acute defense against pathogens such as bacteria, fungi, and viruses and is also thought to contribute to the development of chronic lung diseases, such as asthma and chronic obstructive pulmonary disease (COPD). The II detects and destroys invading pathogens via the Toll-like and interleukin-1 receptor (TIR) superfamily. The role of miR-146a in II response has been extensively studied *in vitro* to determine its functional role and mechanisms-of-action(s). For example, a study in human alveolar cells found that IL1β induces miR-146a expression at 3 hours, and this induction precedes the release of IL8 and Rantes chemokines. It also was found that miR-146a was induced at relatively high concentration. To determine the pathways involved in miR-146a expression, multiple concentrations of p38 MAP, ERK-1/2, IKK2, and JNK-1/2 inhibitors were used *in vitro*. Results indicated that the IKK2 and JNK-1/2 inhibitors knocked out miR-146a expression. Therefore, it appears that IL1β stimulates miR-146a expression through the IKK2 and JNK-1/2 pathway. To determine the functional role of miR-146a, further investigations showed that at high IL1β, miR-146a regulates IL8 and RANTES release through a negative feedback loop. To better understand the mechanism, an *in vitro* investigation showed that increased miRNA-146a does not downregulate IRAK-1 and TRAF6 or proteins involved in secretion. Instead, it was shown that

the action of miR-146a was mediated through the regulation of protein(s) involved in the translational machinery.

Investigations have also been conducted in primary human airway smooth muscle cells and confirmed that IL1β stimulates miR-146a production. However, time course studies of the IL1β-induced response in primary human airway smooth muscle cells found that miR-146a expression was produced at a slower rate and at lower IL-1β concentration compared with those in alveolar epithelial cells. Release of IL8 and IL6 was found to correlate with miR-146a production. Investigations of intracellular pathways that regulate IL1β-induced miR-146a expression showed that this was reduced by IKK2, JNK-1/2, and MEK-1/2 inhibitors. Functional studies showed that miR-146a positively regulates IL6 but not IL8 release in airway smooth muscle cells.

To further investigate the role of miR-146a, a study on the pulmonary lipopolysaccharide (LPS)-induced response in mouse lung found that LPS induces TNFα release in mouse lung. Results indicate that miR-146a and miR-223 are upregulated by LPS.

Chromatin Control of the Antiviral Response

Alexander Tarakhovsky, Ph.D., The Rockefeller University, New York, NY

One of the methods for detecting viruses is RNA-sensing molecules, such as MDA-5 and RIG-1, which belong to the family of RNA helicases. These molecules signal through adaptor proteins such as IPS-1, which result in activation of transcription factors such as NFκB and IRFs to induce type-1 interferon genes. The role of chromatin is critical in the regulation of the processes that lead to an immediate, versus delayed, response of anti-viral gene immune response. After viral infection, H3K9me2 associated with the interferon promoter is rapidly demethylated. H3K9me2 levels decrease within the first 2 hours. This apparently helps the primary infected cells to amplify their response to produce a maximum amount of interferon and facilitate an anti-viral response by neighboring cells not infected by the virus. This creates a hypothesis that this is a potential model for epigenetic memory regarding chromatin signaling, something that has not been previously seen.

The mechanisms that elicit these changes involve a Jumonji (JMJ) family demethylase and the histone methyltransferase G9a. In this model, the virus activates JMJ or targets it to a particular locus. Laboratory studies showed that the demethylation is instigated by the JMJD2D demethylase. Further experiments confirmed that JMJD2D is induced by viral infection, is a novel histone demethylase, triggers IFN, and makes the cell more resistant to viruses.

To further understand the role of G9a on anti-viral expression, cells with G9a deficiency were studied, and it was found that there was a dramatic increase in IFNβ production and a reduction in cellular infection. This confirms that G9a-deficient cells support less viral replication.

SESSION 2: EPIGENETIC REGULATIONS OF THE IMMUNE RESPONSE

Chair: Matthias Merkenschlager, M.D., Ph.D., MRC Clinical Sciences Centre, Imperial College London, London, United Kingdom

The session focused on epigenetics to understand cell and gene regulation. During the differentiation of stem cells, epigenetic changes in gene expression occur that allow the cell to commit to one of several alternative fates. Epigenetic changes are carried through the cell cycle, similar to genetic changes, but do not lead to changes in the sequence of DNA. Methods of maintenance of gene expression patterns include DNA methylation of the CpG islands; nucleosomes and post-translational histone modifications; and higher-order organization, such as in non-histone chromatin proteins.

Epigenetic Regulation of Cytokine Expression in T-Helper Cells

Christopher Wilson, M.D., Professor and Chair, Department of Immunology, University of Washington School of Medicine, Seattle, WA

CD4 cells can adopt one of many fates tailored to provide protection against specific categories of infection or against self-injury. These fates reflect differences in overall gene expression established by transcription factor networks and epigenetic mechanisms that can be propagated from one generation to the next as cells divide. The focus of this presentation was on interferon gamma (IFN-γ), the signature cytokine of Th1 cells.

Recent research studies have identified conserved regulatory elements in the locus encoding IFN-γ (*IFNG* on human Chr12 and *Ifng* on mouse Chr10), which are sufficient for Th1-specific but not copy-number-dependent *IFN-γ* expression. These observations prompted a search for additional regulatory elements, using high-resolution, next generation sequencing-based detection of DNase hypersensitivity sites (DHSs), which mark regulatory elements of all types when active or poised for activity in specific cell types. This analysis revealed that most DHSs in the *IFNG* locus were Th1-specific. These studies also revealed previously unidentified regulatory elements located over an ~180-kb domain surrounding human *IFNG* and mouse *Ifng*, which are flanked by sites occupied by CTCF.

CTCF is a self-interacting, "insulator" protein, which mediates chromatin looping. In the context of the *Ifng* locus, CTCF binding helped to bring distal elements into proximity to *Ifng* and its promoter in three-dimensional space, whereas they are distant from each other in the linear DNA sequence. The Th1-specifying transcriptional regulator T-bet was required for Th1-specific CTCF binding. Both CTCF and T-bet were essential for formation of the Th1-specific three-dimensional architecture of this locus and for high-level IFN-γ expression in Th1 cells. These results demonstrate that the binding and actions of the ubiquitous regulator CTCF at the *Ifng* locus is conditioned on the presence of the Th1-specific master regulator T-bet, and that these two factors together orchestrate structure and function at this locus.

Chromatin Structure and Gene Regulation in T-cell Development and Function
Dr. Merkenschlager

The main purpose of cohesins is to define chromosome topology, and they have been reported to be involved in gene expression. In humans, there are developmental syndromes (Cornelia de Lange and Roberts syndrome) that are caused by mutations in cohesin subunits or cohesin-loading proteins. In these developmental disorders, chromosome segregation is working more-or-less normally, but developmental abnormalities arise for unknown reasons.

One approach to better understanding cohesins is to map various binding sites, and it was found that there was a strong preference for conserved, non-coding sequences, which often coincide with DNase hypersensitive regions. Very few of these sequences bind to cohesins; there seems to be selectivity. The sequence underlying many cohesin binding sites was found to be similar to the core binding motif of the DNA binding protein CTCF. Binding of CTCF often occurs between regions of different chromatin structure and serves an insulator function. It was found that CTCF binds first and recruits cohesin to the site and that once recruited, cohesin mediates CTCF's insulator functions.

CTCF prefers to bind to unmethylated DNA. This preference can determine site-specific cohesin recruitment, as has been confirmed for B- and T-cell-specific sites. Therefore, by changing the methylation status in development, cells can position CTCF complexes differentially in their genome. Because CpG methylation is heritable in the cell cycle, the positioning of CTCF and cohesin is likely to be an epigenetic trait.

In addition to universal functions that cohesins have in all types of organisms in holding the chromatids together, they may have co-opted additional functions, which differ between different animals. These studies establish a novel role for cohesins in linking epigenetic state and the functional organization of the genome.

Analysis of Epigenetic States During T-cell Differentiation and Activation
Keji Zhao, Ph.D., National Heart, Lung and Blood Institute, National Institutes of Health, Warren G. Magnuson Clinical Center, Bethesda, MD

The use of the chromatin IP (ChIP) assay allows for the study of the structure of chromatin and histone modifications in human CD4+ T cells. In the past year, using the ChIP assay, Dr. Zhao's group has mapped the genome-wide distribution of approximately 20 histone methylations, 18 histone acetylations, the histone variant H2A.Z, polymerase II and CTCF, and genome-wide nucleosome positions in human CD4+ T cells. Typical modification patterns are associated with active and inactive loci. Epigenetic differences have been identified in different lineages of naïve and CD4+ T helper cells such as in Th1, Th2, Th17, iTreg, and nTreg cells. Gene expression and repression are associated with H3K4me3 and H3K27me3 modifications, respectively, indicating that epigenetic changes have occurred.

Signature cytokine genes Ifng (Th1), Il4 (Th2), and Il17 (Th17) are associated with H3K4me3 in featured lineages, whereas most of the "master regulatory" genes are associated with bivalent modifications. This suggests that these regulators have the potential to be re-activated or

induced in various lineages. For example, T-bet can be induced in Treg cells under Th1 polarizing conditions, which is accompanied by expression of IFNγ. These data indicate that T-cell fates are flexible and an epigenetic mechanism underlies the T cell flexibility.

DNA Methylation Controls Foxp3 Gene Expression

Jochen Huehn, Ph.D., Department of Experimental Immunology, Helmholtz Centre for Infection Research, Braunschweig, Germany

Tregs play an active role in the regulation of various immune functions, such as autoimmunity, responses to allergens and infections, tumor rejection, and allo-tolerance. Tregs are characterized by the constitutive expression of CD25 and of the transcription factor Foxp3, the latter being considered to be the "master switch" for the development and function of these cells. The vast majority of $Foxp3^+$ Tregs is already formed during the maturation of T cells within the thymus; these Tregs show features of a stable T-cell line and are named natural Tregs. However, $Foxp3^+CD4^+$ Treg can also be generated *de novo* in a TGF-β-dependent process from naïve T cells by TCR triggering. Although it is known that stable Foxp3 expression is essential for the maintenance of suppressive properties of Tregs, only little knowledge exists on regulation of Foxp3 expression. The structure of the *Foxp3* locus contains a promoter region 6.5 kb upstream of the translational start site that is NFAT- and AP-1-dependent, a TGF-β enhancer region that is NFAT- and SMAD3-dependent, and an evolutionarily conserved element upstream of exon-1 that is enriched in CpG motifs and that contains several transcription factor binding sites. We have recently demonstrated that naturally occurring, but not *in vitro*, TGF-β-induced $Foxp3^+$ Treg display stable Foxp3 expression associated with a selective demethylation of the CpG motifs within the conserved element. Thus, this site was named TSDR, Treg-specific demethylated region. To determine whether the TSDR methylation status influences the stability of Foxp3 expression, experiments using the drug 5-aza-2′-deoxycytidin (Aza), which prevents *de novo* methylation of newly synthesized DNA, were performed. Inhibition of DNA methylation not only promoted induction of Foxp3 expression during priming even in the absence of exogenous TGF-β, but also conferred stability of Foxp3 expression upon restimulation. Most notably, such stable Foxp3 expression was found only for cells displaying enhanced TSDR demethylation. This observation also holds true for $Foxp3^+$ Tregs, which were *de novo* generated by DEC-205-mediated *in vivo* targeting of agonist ligands to dendritic cells. In contrast, *in vitro* TSDR methylation diminished its transcriptional activity in luciferase assays. Together, these data suggest that TSDR is an important methylation-sensitive enhancer element regulating Foxp3 expression and demonstrate that epigenetic imprinting in this region is critical for establishment of a stable Treg lineage. The knowledge about Foxp3-inducing signals and the stability of Foxp3 expression is of essential relevance for the *in vitro* generation or expansion of Tregs for therapeutic applications in autoimmune patients or allograft recipients.

A disadvantage of the use of Foxp3 protein expression as a marker for Tregs in the human system is that, upon activation, even conventional T cells transiently express Foxp3 and thus cannot be distinguished from Tregs permanently expressing Foxp3; this is a difference between mice and men. Because *Foxp3* TSDR demethylation was a unique feature of stable $Foxp3^+$ Tregs, a whole blood assay using real-time PCR (RT-PCR) to distinguish methylated from unmethylated CpG motifs within the TSDR has been developed. Using this method, the precise quantification of "true" and stable $Foxp3^+$ Tregs is possible, and the assay is highly selective and

has a high sensitivity. As a proof of concept, the method has been used to determine the number of Tregs in peripheral blood of melanoma patients under IL2 therapy and within solid tumor tissues of lung, colon, breast, and prostate carcinoma patients. These investigations may lead to a better understanding of the immune response in cancer patients.

Epigenetic Regulations in Dendritic Cells

Steven Kunkel, Ph.D., Endowed Professor of Pathology Research, Division of General Pathology and the Immunology Program, University of Michigan Med School, Ann Arbor, MI

Patients who survive sepsis suffer significant deficiencies in their immune responses caused by poorly understood mechanisms. We have explored this phenomenon by studying dendritic cells (DCs) recovered from animals surviving severe peritonitis-induced sepsis, using the well-established cecal ligation and puncture (CLP) model. Immediately following the initiation of sepsis, there is a depletion in DCs from the lung and spleen, which is followed by re-population of these cells back to the respective organs. DCs recovered from surviving animals exhibit a significant and chronic suppression of interleukin (IL)-12, a key host defense cytokine. The suppression of DC-derived IL-12 persisted for weeks after CLP and was not due to immunoregulatory cytokines, such as IL-10. Using chromatin immunoprecipitation (ChIP) techniques, we have demonstrated that the deficiency in DC-derived IL-12 was due to epigenetic alterations. Specifically, IL-12 expression was regulated by stable reciprocal changes in histone H3 lysine-4 trimethylation (H3K4me3) and histone H3 lysine-27 dimethylation (H3K27me2), as well as changes in cognate histone methyltransferase (HMT) complexes on the *Il12p35* and *Il12p40* promoters. These data implicate histone modification enzymes in suppressing DC-derived IL-12, which may provide one of the mechanisms of long-term immunosuppression subsequent to the septic response. Our data suggest that a significant number of bone marrow cells were depleted immediately after the immunologic event; with time, bone marrow-derived dendritic cells were re-seeded to distant organs and these cells were shown to have inappropriate chromatin remodeling. The replenished bone marrow cells were significantly altered in the expression of immune mediators; in addition, a different macrophage phenotype arises, typified by a classic set of M2 marker proteins (e.g., YM1, FIZZ1, mannose receptor, and arginase 1), which also appears to be regulated by chromatin remodeling.

The histone methyltransferases and demethylases play a role in fine-tuning the immune system by balancing methylation dynamics. Jumonji C domain-containing protein 3 (Jmjd3) is a newly found H3K27me3/me2 demethylase. When bone marrow macrophages are treated with IL4, Jmjd3 is upregulated. If IL4 is removed from the macrophage medium, Jmjd3 expression is reduced. This also was seen in an investigation using peritoneal macrophages. If siRNA is added to the medium of cells expressing jmjd3 in the presence of IL4, which reduces jmjd3 expression, there is a reduction in the expression of M2 marker genes (e.g., YM1 and FIZZ1). Because IL4 is dependent on STAT6 for the induction of certain proteins, knocking down STAT6 causes a reduction in the expression of jmjd3 and M2 marker genes.

In summary, these studies suggest that specific cytokines are important in influencing epigenetic-directed chromatin remodeling that alters the phenotype of bone marrow cells, which delivers epigenetically altered immune cells to distant tissue sites to participate in an inflammatory event. If something goes awry in this process, the cytokine profile may be either silenced or

permanently turned on, which may be what is happening in autoimmune diseases. These events may lead to chromatin remodeling in early progenitor cells susceptible to static and heritable (long-term) regulation of gene expression. Circulating cells, which are more differentiated, also are susceptible to chromatin remodeling, although these changes may be more dynamic.

Epigenetic Regulation of Haematopoietic Stem Cell Differentiation

Michele Goodhardt, Ph.D., Directeur de Recherche, CNRS, INSERM U662, Institut Universitaire d'Hématologie, Hopital Saint Louis, Paris, France

A central question in developmental biology is how the progressive restriction in development from primitive pluripotent stem cells to multipotent stem cells and finally to fully committed mature cells is encoded in the epigenetic memory of developing cells.

All blood cells are derived from multipotent hematopoietic stem cells (HSC) via a series of differentiation steps during which the multilineage potential of the stem cell is gradually lost as HSC differentiate into lymphoid or myeloid committed cells. To address the role of chromatin structure in the establishment of HSC multilineage potential and commitment to the lymphoid lineage, histone modifications were studied at a panel of lymphoid- and myeloid-affiliated genes in multipotent and lineage-committed hematopoietic cells isolated from human cord blood. Results show that transcriptionally poised lymphoid and myeloid genes are enriched for histone H4 acetylation and histone H3 lysine 4 dimethylation in HSC. Repressive H3 lysine 9 or 27 methylation marks are absent at these loci. During HSC differentiation, locus activation is associated with increased levels of histone H3 acetylation and H3 lysine 4 trimethylaton, while loss of histone acetylation and addition of repressive H3 lysine 9 or 27 methylation marks is observed at nonlineage-associated genes. These results suggest that high histone H4 acetylation and histone H3 lysine 4 dimethylation in the absence of active H3 lysine 4 trimethylation or repressive H3 lysine 9 or 27 methylation describes a transcriptional competent "ground state" for lymphoid and myeloid genes in primary HSC, keeping them silent but poised for expression at later stages of differentiation.

Another epigenetic factor that seems to be important in the regulation of gene activity is the location of the gene within the nucleus and, in particular, its position relative to repressive nuclear compartments (e.g., heterochromatin regions or the nuclear periphery). Studies on the β-globin locus showed that changes in histone modifications precede changes in subnuclear positioning during differentiation of HSC toward the erythroid lineage.

FRIDAY, DECEMBER 12, 2008

KEYNOTE ADDRESS: CYTOPLASMIC DETERMINANTS OF T-CELL FATE

Steven Reiner, M.D., Professor, Department of Medicine, Division of Infectious Diseases and Abramson Family Cancer Research Institute, Chair, Immunology Graduate Group, University of Pennsylvania, Philadelphia, PA

At the beginning of an immune response, naïve T cells patrol through the lymph nodes of the body and bump into antigen-presenting dendritic cells that convey information that something they are specific to has breached the initial body defenses and now poses a threat. In order for them to be useful, they must increase their numbers and acquire new or different functions (while at the same time restricting other functions). They also have the imposing problem of time and space, because the original contact with the presenting cell allows it to get the information, but because it divides within 30 hours, the daughter cells do not themselves physically encounter the information. The question is how the information gets imprinted in the original cell and is passed during replication. One hypothesis is that reshaping the chromatin in the original cell allows passage of the information to subsequent daughter cells to acquire new functions, possibly through remodeling of IFN-gamma, IL4, and IL17. It now is becoming appreciated that the fate of the initial cell may not just be simply reproduced into subsequent cells by division, but that there could be multiple fates possible in the subsequent cells based on conditions present at the time. This division of labor allows some immune cells to leave the lymph node to travel to tissue to exert their function, but others may need to stay in the lymph node and move over to a B-cell follicle to help there.

A central question is how the one cell can generate the cell diversity required to meet two opposing demands. In the short term there must be progeny to deliver acute function. In the long term, there must be progeny that replace the useful lymphocyte so that it can again produce daughter cells to deliver function and daughter cells that maintain the specific lineage. Asymmetric cell division may be an answer to the question of how a naïve cell produces differences in daughter cell traits. When a T cell comes into contact with a stimulus (e.g., an antigen-presenting cell or an area of antibodies ligating its stimulating receptors), it polymerizes actin and moves its microtubule cytoskeleton and signaling molecules toward the stimulus. The relevance of this during cell division is suggested by time-lapse multi-photon imaging studies that documented prolonged contact between a T cell and an antigen presenting cell before the T cell's first division. Dr. Reiner's group obtained evidence that this prolonged contact causes the T cell to remain polarized and divide in a stereotyped fashion, with one daughter close to the stimulus and the other daughter on the far side of the stimulatory cell. Those first two daughters also inherit unequal shares of the molecules that were coalesced at either side of the cell before division.

Further studies from Dr. Reiner's group have suggested that the proximal daughter cell deals with the acute response while the distal daughter retains the memory of this lineage. The distal daughter is then able to respond similarly upon reinfection—divide asymmetrically to produce one daughter dedicated to acute function and the other daughter to maintain the lineage. In this way the behavior of the lymphocyte is similar to the strategy used by other tissues involved in protection from the environment; to maintain lifelong function despite being composed of short-

lived cells, the stem cell behavior of a cell provides a way to meet the opposing demands of acute function and regeneration. For lymphcoytes being signaled during the response to self or the response to microbes, inequality in cytoplasmic inheritance could have substantial influence on the subsequent gene expression and epigenetic landscape of the incipient daughter cells.

SESSION 3: miRNA AND EPIGENETIC REGULATIONS IN AUTOIMMUME DISEASE

Chair: Kristin Abraham, Ph.D., Program Leader, Division of Strategic Coordination, Office of the Director, National Institutes of Health, Bethesda, MD

The Epigenetics of Autoimmunity

Bruce Richardson, M.D., Ph.D., Professor, Department of Medicine, University of Michigan, Ann Arbor, MI

Systemic Lupus Erythematosus (SLE) is an autoimmune disease that primarily affects women, and is believed to develop when genetically predisposed people encounter environmental triggering agents. Epigenetics—DNA methylation abnormalities—play an important role in SLE. For example, MECP2, which links methylcytosines in DNA to chromatin-inactivation complexes, is one of the identified genetic susceptibility loci. Further, various environmental factors such as procainamide (Dnmt1 inhibitor), hydralazine (PKC-δ inhibitor), ultraviolet light (ERK pathway inhibitor), aging, diet, and other environmental agents can prevent the replication of DNA methylation patterns during mitosis, resulting in DNA demethylation and lupus-like autoimmunity.

Past studies have shown that CD4+ T cells treated with the DNA methylation inhibitor 5-azacytidine become autoreactive by increasing LFA-1; the autoreactive T cells can be activated by self-class MHC class 2 molecules with the inappropriate antigen in the antigen-binding cleft. These experiments were conducted on cloned and polyclonal human T cells and in mice, and they represent a case where one can take a normal antigen-specific T cell, expose it to something that will inhibit DNA methylation, and make it autoreactive to self-class 2 MHC molecules. The disease that most closely resembles this is chronic graft versus host disease, which in mice can resemble a variety of conditions such as glomerulonephritis, vasculitis, rheumatoid arthritis, Sjogren's syndrome, and scleroderma. Serologically, all the mice develop anti-nuclear antibodies, anti-dsDNA, anti-thymocyte antibodies, a positive Lupus band test, and increased IgG, resembling SLE. The mechanism includes the sequelae of DNA hypomethylation, causing altered gene expression and altered T-cell function leading to autoimmunity. To determine if the same sequelae occur in human SLE, experiments have been conducted and confirmed that the same DNA sequences demethylate and are overexpressed *in vitro* and in SLE. Genes that are demethylated include CD11a, perforin, KIR, CD70, and CD40L. Interestingly, CD40L is on the X chromosome, and studies of demethylated CD40L in men and women indicate that treatment with 5-azacytidine increases CD40L on CD4+ T cells from women but not men through the demethylation of the CD40L promoter on the inactive X. Further studies suggest that the second X chromosome is being demethylated in women with SLE, and that women with SLE can overexpress CD40L, but men with lupus do not.

The JNK signaling pathway is normal but ERK phosphorylation is decreased in lupus T cells, and the ERK pathway regulates DNA methyltransferase expression. To determine if this is related to autoimmunity, a mouse model (Tet-on dnMEK/CD2-rtTA mice) has been developed. Doxycycline in this mouse model induces expression of the dnMEK in T cells, inhibiting ERK pathway signaling, decreasing DNA methyltransferase levels, and inducing anti-DNA antibodies. In the mouse model, demethylation and overexpression of methylation sensitive genes (i.e., CD70 and CD11a) also occur, and the interferon "signature" that is characteristic of SLE patients is seen. In experiments on the ERK pathway, it was found that in idiopathic SLE- and hydrazaline-treated T cells, a decrease in PKC-δ activation occurs, which accounts for the cascade of epigenetic sequelae characteristic of SLE. The conclusion is that the DNA demethylation model predicts epigenetic, cellular, and functional abnormalities in human SLE.

Targets of FoxP3 and Regulators of Treg Development and Function

Alexander Rudensky, Ph.D., Investigator, Howard Hughes Medical Institute and Professor, Department of Immunology, University of Washington, Seattle, WA

Foxp3 is the master regulator in the development and function of Tregs. Investigations in Foxp3^{null} mice show that after birth they have a cytokine storm and die in approximately 4 weeks. There also are human diseases caused by functional disorders in the Foxp3 pathway. Foxp3 takes part in various transcription pathways, and the genes that sense the environment, signal transduction, and a variety of functions including miRNAs, are influenced by levels of expression of Foxp3. Foxp3 is a transcriptional activator and repressor of gene expression, and Foxp3 binding regions of these genes are subjected to permissive or depressive histone modifications. To determine the stability of the Treg cell lineage, investigations were conducted using a mouse model, and it was reported that tamoxifen treatment to create a burst of CRE activity caused no cytokine production in approximately 35 percent of Foxp3+ cells; the rest remained stable. To elucidate how Foxp3-controlled functional programs ensure control of different classes of immune response, additional investigations were conducted. Transcription factor IRF4 was shown to be required for differentiation of Th2 lineages. When Foxp3 was knocked down using mature Tregs, within 2 days of transfection, IRF4 was significantly decreased. Additional experiments found that Foxp3^{cre} mice have delayed lymphoproliferative disease and tissue (pancreas) disease with suppression of Foxp3. It also was found that there was cytokine dysregulation in IFNγ, IL2, and others. Conclusions from the previous experiments are that IRF4 transcription factor is essential for Th2 differentiation.

Two classes of transcription factors—the IRF and STAT families—initiate differentiation of T cells and other cells of the immune system, and they act as environmental sensors. Studies in mice have shown that it is unknown whether IRF4 is modified in a transcriptional manner, either phosphorylated or methylated. At 5 weeks of age, mice with modified IRF4 have enlarged spleens and perturbations in some signaling pathways. Some STAT members are phophorylated. Investigations of STAT3 in Treg cells with a Th17 lineage were conducted. By deleting the conditional STAT3 allele in Tregs, mice with the deletion did not show allele proliferation and the mice died from colitis at 9 to 10 weeks of age. The cells were efficient at suppressing the proliferation of Tregs, yet cells isolated from Foxp3 knock-out mice failed to restrain the ability of Foxp3-deficient cells to produce IL17, and mice that received these cells suffered from colitis and skin inflammation. This is happening because Th17 responses are selectively dysregulated

in mice that harbor STAT3-deficient Tregs, whereas Th1 and Th2 responses were controlled. Therefore, it is surmised that STAT3 transcription is essential for Th17 receptor cell differentiation and endows Tregs with the ability to suppress Th17 responses. In addition, Tregs that lack STAT3 do not produce IL17, nor do they produce other effective cell cytokines, and IRF4 deficient Tregs do not produce IL4, IL5, IL10, or IL 13, and do not become impaired in their ability to suppress the corresponding type of immune response. It has been found that IRFs and STATs are involved in Foxp3 transcriptional complexes. The target gene for Foxp3 appears to be the Icos promoter. In conclusion, Treg cells employ effector-specific suppression modules that are controlled by transcription regulators of differentiation into the corresponding effector-cell lineages, and the modules are controlled by IRF and STAT families. The regulators are likely recruited into the Foxp3 transcriptional complexes in response to specific environmental queues as an adaptation of Treg cell suppressor programs to an inflammatory environment.

The Role of MicroRNAs in Regulatory T-cell Homeostasis
Dr. Rudensky

The population of Tregs is relatively stable and maintained as seen in the previous presentation. The role of miRNA-dependent gene regulation in non-Tregs is in the processes of division and survival, but the non-Tregs also provide stability by enforcing the Treg-suppressor program under inflammatory conditions. Investigations of the role of specific miRNA in Treg homeostasis is important to understand the diverse phenotypes resulting from ablation of gene regulation pathways through, for instance, the Dicer. For example, Foxp3 binds to the *MR155* encoding gene via the intron sequence, where it maintains a high level of expression in Treg cells. It has been reported that a subset of Tregs are reduced in the absence of *MR155*; this is not unlike the reduction observed in Foxp3-positive mice that have the Dicer-conditional allele in Tregs. Studies have shown that miRNA-deficient T cells produce smaller amounts of IL2 and are biased toward Th2 responses. Further studies in bone marrow chimeras found that the proportion of *MR155*-sufficient and miRNA-deficient Tregs was not different after 2 months, although Tregs were reduced at 4 months, indicating a delayed cell-intrinsic defect in homeostasis of *MR155*-deficient Treg cells. To address the question of whether this was senescence and whether it was reversible, an investigation was conducted by isolating *MR155*-deficient and *MR155*-sufficient cells; it was shown that reduced proliferation could be rescued in the *MR155*-deficient cells. This suggested that in a non-competitive environment that *MR155*-deficient Tregs are fine, but in a competitive environment they lose the ability to proliferate. It was found that IL2 signaling was related to this situation.

To determine the mechanism for the loss of "competitive fitness" of *MR155* Tregs, the activation of STAT5 phosphorylation was investigated. It was found that reduced STAT5 phosphorylation occurred in those cells that were *MR155*-deficient Treg cells. A candidate gene was researched to identify a target for IL2 signaling. Resulting studies identified *SOCS-1*, a negative regulator of certain cytokine-receptor signaling specifically involved in attenuation of the IFNγ and IL2 receptors. *SOCS-1* is conserved across primates and humans and is upregulated in normal Tregs. Further studies indicated, however, that *MR155* deficiency does not affect the expression of *SOCS-1*, but the absence of *SOCS-1* results in a 5-fold increase in SOCS-1 protein in *MR155*-deficient Tregs. This indicates that *SOCS-1* is a target of *MR155* and can restore *MR155* expression in *MR155*-deficient Tregs. This raised the question of whether in the presence of

MR155, the amount of *SOCS-1* present in Tregs can affect Treg numbers. To address the question, mice that express the *SOCS-1* transgene and mice that generated conditional *SOCS-1* allele were investigated. It was found that overexpression of *SOCS-1* results in a decrease in Tregs and conditional of *SOCS-1* results in an increase in Treg numbers. Together, this suggests that Foxp3 integrates transcriptional regulation of structural genes and transcriptional regulation of miRNAs to enforce a regulatory network that imparts superior homeostatic properties to the cellular population, ensuring that Tregs survive and expand in conditions of limiting growth factors.

Foxp3 involvement in Treg homeostasis is as a suppressor of expression of the IL7 receptor, binds to the IL7 receptor alpha chain, and suppresses IL2, which makes the cells less competitive for IL7, the main cellular growth factor that maintains homeostasis. It also deprives Tregs from IL2 that would act as a cell-intrinsic cytokine. Foxp3 also binds to CD25 and the IL2 receptor alpha chain sends the level of expression IL2 receptors to higher levels. This illustrates the dependence of cells on the production of essential growth factors by other cells conditioned on their activation; the more activation present, the more growth factors and cytokines produced for Tregs. The signaling through cytokine receptors as a negative feedback induces expression of negative regulators of cytokine signaling such as SOCS family members. As *MR155* targets *SOCS-1*, the cells become sensitive to medium and low concentrations of IL2, ensuring the maintenance of the Treg subset in a competitive environment.

Genome-wide DNA Methylation Analysis in Monozygotic Twins Discordant for Type-1 Diabetes

Vardhman Rakyan, Ph.D., Barts and The London School of Medicine and Dentistry, London

It is well established that type 1 diabetes (T1D) has a strong genetic component, although the majority of identical monozygotic (MZ) twins remain discordant for the disease, and the concordance rate falls even further with increasing age at diagnosis of T1D. The question of how to get phenotypic diversity with genetic homozygosity has focused on epigenetic factors. These studies have shown that methylated genes may play a role and that transgenerational epigenetic inheritance is possible, as shown in studies on Axin-fused, an allele in a retrotransposon that has been shown responsible for mutant phenotypes, and is characterized by variable expression states. Three types of epialleles have been discussed: (1) obligatory, when gene type determines epigenetic type; (2) facilitated, when genotype includes the likelihood of epigenetic type; and (3) pure, when phonotype is induced by the environment.

Studies to identify epialleles in T1D have used the approaches of molecular genetics and functional genomics, focused primarily on DNA methylation. These studies have been conducted on discordant MZ twins; at present, no validated epigenetic factor has been identified to address discordance, although the investigation continues. Some results have shown that epialleles rise early in T1D, and methylated DNA immunoprecipitation approaches have been used for genome-wide DNA methylation profiling. Bayesian tools have been developed to conduct analyses of methylation patterns.

Epigenetic Manipulation of Foxp3 to Control Autoimmunity and Allograft Responses

Wayne Hancock, M.D., Ph.D., Children's Hospital of Philadelphia, University of Pennsylvania Medical School, Philadelphia, PA

There is much interest in understanding how the immune system fits together and how it controls overly exuberant responses and maintains homeostasis. Foxp3 does not exist in a vacuum but rather is closely involved with histone/protein deacetylases (HDAC), histone acetyltransferases (HAT), DNA methyltransferases (DNMT), and other molecules of interest. This presentation deals with aspects of HDAC biology in Tregs and the effects of HDAC targeting on Treg functions *in vitro* and *in vivo.*

Large numbers of HDAC inhibitors have been evaluated for their effects on Treg function. Some of these inhibitors, particularly the hydroxamic acid series of HDAC inhibitors, when used in the nanomolar range, enhance Treg function. Other drugs, such as benzamides, which include long-acting HDAC inhibitors, have little or no effect on Treg function. Zinc-dependent HDAC inhibitors primarily target either Class I or Class II HDAC. Biochemical effects of pan-HDAC inhibitors are usually due to effects on the Class I HDAC or the ability to recruit Class I HDACs by acting on Class II HDAC molecules. The exception would be a subfamily of Class II called Class IIB, the best example of which is HDAC6, which may be directly affected by selected inhibitors.

HDAC6, which has two deacetylase domains, may be a primordial precursor of the other HDAC. It exists in the cytoplasm and primarily controls cytoplasmic events, some of which relate to regulation of the acetylation of alpha-tubulin and a variety of other proteins, including HSP90. Blocking HDAC6 through the use of an HDAC6-specific inhibitor, such as Tubacin, increases Foxp3 protein expression, enhances Treg function *in vitro* and *in vivo*, and can prevent or treat murine colitis in a Treg-dependent manner. Targeting this HDAC has beneficial effects in transplantation and autoimmunity, and tools are now available to monitor and block HDAC6-dependent functions.

HDAC9—a Class IIA HDAC that probably works by recruiting other molecules—is of interest because its expression is increased by 30-fold in Treg versus regular T cells. HDAC9 decreases Foxp3 expression and function, and its neutralization promotes Treg survival by regulating expression of heat shock protein (HSP)70 and related HSPs. HSP70 induction as a result of HDAC9 neutralization or deletion is a new mechanism for enhancing Treg functions.

HDAC7, which is expressed in thymocytes, is important to thymic selection through regulation of *Nur77* expression. *Nur77* transgenics have marked increases in Tregs and accept fully MHC-mismatched cardiac transplants without immunosuppression. Targeting HDAC7, resulting in derepression of Nur77 or Nur77-independent pathways, may be of therapeutic significance in autoimmunity and transplantation.

In summary, methylation, acetylation, and other epigenetic mechanisms in Tregs are being probed using genetic and pharmacologic approaches. Various currently approved drugs influence Foxp3-dependent Treg functions by affecting epigenetic mechanisms, but additional HDAC-specific regulators are needed.

Regulation of Thymocyte Differentiation by HDAC7

Eric Verdin, Ph.D., Senior Investigator and Associate Director, Department of Virology and Immunology, The J. David Gladstone Institute, San Francisco, CA

HDAC7 is of interest because of its very high expression in the thymus. It is predominantly expressed in the cortex of the thymus, where it influences early thymocyte development. One of the hallmarks of Class II HDAC is their regulation via nuclear/cytoplasmic shuttling. The phosphorylation of HDAC7 by a kinase leads to its extrusion from the nucleus; a specific phosphatase exerts the opposite activity. The combined activity of the two enzymes regulates the shuttling. When in the nucleus, HDAC7 represses a specific set of cellular targets. The nuclear/cytoplasmic shuttling of HDAC7 acts as a switch that allows these genes to be transcriptionally activated. Experimental results suggest that HDAC7 may form part of the regulatory network that defines the transition between double positive and single positive T cells.

Experiments in transgenic and knockout mouse models have shown that mutation of three serine residues in the *N*-terminal domain of HDAC7 converts it into a super-repressor. Replacement of the HDAC catalytic domain with the VP16 activation domain converts HDAC7 from a corespressor to a coactivator. In HDAC7 knockout mice, development of CD4/CD8 subsets in the thymus is close to normal until the immature CD8 single-positive (ISP) stage, when the relative number of cells increases dramatically, followed by a dramatic decrease. Peripheral lymphocytes are lacking in these mice. In an HDAC7 phosphorylation mutant, the opposite results are observed, with a dramatic decrease in ISPs and constitutive repression of genes regulated by HDAC7. These data support the concept that HDAC7 plays a critical role in the transition between the ISP and double positive stages.

A microarray experiment in HDAC7-deficient thymocytes showed that 140 genes were upregulated and 132 were downregulated; most of these are probably secondary targets. Many intracellular signaling molecules are upregulated in HDAC7-deficient double positive thymocytes, but some negative regulators are downregulated. Developing thymocytes that lack HDAC7 have an enhanced propensity to apoptosis. One consequence could be a shortening of the time that the developing T cells could sample different T-cell receptors during development. HDAC7 regulates both positive and negative selection thresholds for T-cell response signal intensity. A lack of HDAC7 is associated with a restricted immune repertoire, leading to a relative immune deficiency. Blocking HDAC7 nuclear export in the thymus makes mice abnormally small and susceptible to autoimmune diseases. The results described above suggest that HDAC7 acts as an analog to a digital converter, inducing different effects depending on the strength of the signal. HDAC7, because of its nuclear to cytoplasmic shuttling, may integrate signals from the T-cell receptor and determine this output.

SESSION 4: EMERGING THERAPEUTIC STRATEGIES IN AUTOIMMUNE DISEASE

Chair: Judy Lieberman, M.D., Ph.D., Senior Investigator, Immune Disease Institute, Harvard Medical School, Boston, MA

Antibody Fragment-Mediated Delivery of siRNA Complexes in Specific Immune Compartments

Dr. Lieberman

Immune Targeting Nanoparticles Containing siRNAs to Curtail Inflammatory Bowel Diseases

Dan Peer, Ph.D., Head, Laboratory of Nanomedicine, Department of Cell Research and Immunology, Tel Aviv University, Tel Aviv, Israel

Cyclin D1 (CyD1) is the proverbial cell-regulating molecule that binds to the cyclin-dependent kinase governing the proliferation in normal and malignant cells. It also is a potent anti-inflammatory target. In inflammatory bowel diseases (IBD) such as Crohn's disease and ulcerative colitis, CyD1 is upregulated in epithelial cells and leukocytes. A strategy utilizing nanoparticles was developed to target gut mononuclear leukocytes and selectively silence CyD1 in an experimental colitis model, to determine if CyD1 contributes to the pathogenesis of inflammation and can serve as a therapeutic target. As shown in a previous presentation, systemic siRNA delivery is problematic because of poor uptake by cells, degradation, and rapid renal elimination. Leukocytes have additional challenges because they are resistant to transfection and have diverse distribution patterns often located deep in the tissues. There are few options for receptor targets for siRNA delivery. Integrins have been chosen for study because they are the largest family of cell adhesion molecules that mediate cell-to-cell and cell-to-matrix interactions, and because the biology of integrins is known. In addition, β2 and β7 integrins are exclusively expressed in leukocytes and are readily internalized, and some of them undergo conformational changes upon activation.

In gut leukocytes, β7 integrins are highly expressed. The vehicle used to study gut leukocytes was called integrin-targeted stabilized nanoparticles and used a β7 antibody, β7 I-tsNP, to deliver siRNA selectively to β7 integrin+ cells. To test the strategy to see if it could knock down a gene, it was tested against Ku-70, a DNA repair protein expressed in all cells. β7 I-tsNP was delivered by siRNA *in vitro*, and it was possible to knock down Ku-70 while being protected from degradation.

CyD1-siRNA knockdown by β7 I-tsNP in activated splenocytes leads to the selective suppression of Th1 cytokines TNF-α and IL12, but IL4 and IL10 from the Th2 family stay the same. This effect was not seen in CyD1-siRNA delivery with control IgG sNP and control Luci-siRNA delivery with β7 I-tsNP. To confirm these results, each of four sequences in the siRNA (designated CyD1-1, CyD1-2, CyD1-3, and CyD1-4) was tested, and the knockdown effects and Th1 suppression were found in each of the non-overlapping sequences. To determine if it is specific to CyD1 and if it is a cell-cycle dependent process, the cell cycle was blocked and each of the four sequences was knocked down sequentially. CyD1 knockdown and TH1 suppression were seen in four independent non-overlapping CyD1-siRNA sequences.

To target those sequences in the gut, an investigation ensued to determine uptake in gut cells. When labeled articles (β7 I-tsNP) were injected intravenously, there was essential silencing in the gut cells, with approximately 35 percent of the labeled anti-β7 I-tsNP getting into the targeted cells. A control isotype was tested and showed that only 1 percent was taken up in the gut. To see if it is possible to silence a gene, using I-tsNP and Ku-70 as markers in a β7 I-tsNP knockdown mouse, it was found that there was no knockdown of Ku-70. The safety of I-tsNP was confirmed in subsequent investigations.

To utilize CyD1 as a potential anti-inflammation target in a colitis model, β7 I-tsNP was injected into mice with colitis and into control mice. Results indicated that CyD1, TNF-α, and IL12 were reduced; IL10 remained high. It appears that this may be an avenue for further research to reduce inflammation associated with IBD, and that the nanoparticle platform could be utilized in other therapeutic settings.

Oligonucleotide-targeted Dendritic Cells in Autoimmunity

Nick Giannoukakis, Ph.D., Associate Professor, Department of Pathology and Immunology, University of Pittsburgh School of Medicine, Rangos Research Center, Pittsburgh, PA

Type 1 diabetes is a classic disease of autoimmunity, characterized, according to conventional thinking, by chronic inflammation that causes destruction of the beta cells in the islets of Langerhans. Hyperglycemia, however, does not indicate a complete eradication of all beta cell mass; in fact, 10 to 20 percent of beta cell mass is shown to remain in people with diabetes as long as 2 years after initiation of the disease. Islet inflammation alone is able to inhibit glucose sensing and insulin secretion. It is hypothesized that by reducing inflammation before, or just after, the onset of hyperglycemia, beta cell mass may be preserved and beta cell neogenesis possibly induced. An investigation was conducted using a co-stimulation blockade method to prevent and reverse new onset of disease. Pre-clinical data from the investigation indicate that it is possible to re-program the immune system to stop inflammation and the destruction of beta cells by increasing putative T- and B-regulatory cells in humans. The targets of the investigation were the expression of CD40, CD80, and CD86 in autologous dendritic cells. Knocking down these co-stimulatory proteins confers immunosuppressive properties to the dendritic cells, which are then administered into the recipients.

Pre-clinical data from experiments in NOD/LtJ, treated with bone marrow-derived dendritic cells treated with a mixture of CD40, CD80, and CD86 antisense oligodeoxyribonucleotides (AS-ODN), have shown that a single injection delayed the onset of T1D. Increased dosing frequency improved these results and delayed T1D for up to 40 weeks. In successfully treated mice, it was observed that they had larger lymph nodes with a high content of Foxp3 in close proximity to endogenously administered dendritic cells. Injections given systemically did not have an effect on the onset or progression of diabetes; those injected at an anatomic location that is subserved in part by the pancreatic lymph nodes had a delay in onset or progression. In addition, mechanistic studies showed an increase in successfully treated mice for Foxp3+ Tregs. The most important observation was that some mice injected with AS-ODN showed reversal of T1D, but not every mouse showed reversal. This was believed to be because of varying residual beta cell mass

among the mice. It was determined that reversal only occurred in those mice with no more than 400 mg/dL blood glucose at the time of injection.

In 2007, an NIH-funded, IRB- and FDA-approved safety study was begun to confirm that intradermal administration of autologous diabetes-suppressive dendritic cells is safe, non-toxic, and without side effects. Early results indicate that there are no statistically relevant differences in hematology, biochemistry, or immunocompetence compared to pre-treatment values, but increased prevalence of putative B regulatory cells, and perhaps slight increases in "adaptive" Foxp3+ CD25HIGH and CD4+ T cells, have been observed. Once safety is confirmed, efficacy will be investigated. Observations in studies to date have been: (1) AS-ODN treatment of dendritic cells results in greater than 90 percent suppression of steady state CD40, CD80, and CD86 mRNA levels by real time PCR, (2) AS-ODN treatment activates IL7 and IL7R alpha chain expression in these dendritic cells, and (3) changes in dendritic cell morphology are too rapid to be accounted for by gene expression changes alone. Of special note is the fact that IL2 and IL7 promote STAT5 binding to Foxp3 promoters. Further studies have shown that IL7 promotes the nuclear translocation of Foxp3 in GFP+ CD4+ CD25+ cells from Foxp3 promoter-GFP mice.

Subsequent experiments were conducted to test the hypothesis that there is a connection between dendritic cells and Foxp3 Tregs. Three steps to test the hypothesis were designed to: (1) determine that IL7 production by and IL7R alpha expression on AS-ODN-treated dendritic cells are TLR-dependent, (2) show that TLR signals could lead to IL7 and IL7R alpha gene expression, and (3) illustrate that TLR activation, therefore, may partly modulate dendritic cell "tolerogenic" activity, partly via IL7 paracrine effects on Foxp3 Treg; this increases survival. In addition, investigations of imprinting through epigenetics as a mechanism of AS-ODN action have been conducted in mice and humans on the *IGF2* gene. Results indicate that AS-ODN, acting through TLRs, can affect methylation of *IGF2-H19* DMR, allelic usage of *IGF2,* and IGF-II production, and that these changes are heritable in daughter cells.

Folate-Mediated Targeting of Drugs and siRNAs to Activated Macrophages *In Vivo*

Philip Low, Ph.D., Corley Distinguished Professor, Department of Chemistry, Purdue University, West Lafayette, IN

The focus of this research is on the use of targeting ligands to deliver drugs to pathologic cells without promoting uptake in non-pathologic cells. Folate (folic acid) enters cells either via a folate transporter or a folate receptor (FR) present on cell surfaces, the latter accounting for only approximately 1 percent of cells in the body. Cancer tissues, activated macrophages, activated monocytes, and the proximal tubules of the kidney express FR. By attaching drugs or other molecules to folate, movement into a cell with the FR becomes very quick. This method has been used to deliver drugs for cancer therapy and various inflammatory diseases such as arthritis. Basic investigations of delivery have shown large percentages of drugs find their way into the target cells.

Flow cytometry studies on multiple samples from different mice show a linear correlation between reactive oxygen species (ROS) production and the expression of the FR-β on macrophages; therefore, FR-β is a marker of activated macrophages. It also has been confirmed

that the uptake of the drugs is mediated by the FR-β. Targeting activated (but not resting) macrophages is a valid strategy for the delivery of anti-inflammatory drugs. In humans with rheumatoid arthritis, studies have shown that inflamed joints may be targeted by tagging anti-inflammatory drugs to folate. It is possible to use the FR-β on macrophages to target chemotherapeutic drugs, siRNAs, nanoparticles, radioimaging agents, immunotherapeutic agents, and various optical imaging compounds to sites of inflammation. Current clinical trials of five distinct folate-linked drugs are being conducted, specifically for cancer drugs that selectively target tumor tissues. So far, toxicity of these drugs in clinical trials has been low. There is as yet no adequate siRNA delivery system, but it is expected that one will be developed in the near future.

Issues related to the delivery of folate-linked drugs include the penetration of tissue needed to allow binding to folate receptor-expressing pathologic cells. Size, shape, and water-solubility influence the targeting ability of folate conjugates. Studies of these factors in live mice, monitored by multiphoton itravital microscopy, have shown the impact that particle size and shape can have on the rate and magnitude of solid tissue penetration. The use of folate and other targeting ligands to deliver siRNAs has been investigated, but in general, no adequate system has been developed. Cholesterol-tagged drugs have been shown to enter the target cells, and tend to stay in the cells longer, although their specificity to diseased cells is not high enough. One new targeting method is the use of prostate-specific membrane antigen, the protein on the surface of prostate cancer cells. The targeting ligand provides very good targeting to some prostate cancer cell lines and will be investigated further.

Delivery of Oligonucleotides in Inflammation

Steffen Panzner, Ph.D., Founder and Chief Scientific Officer, novosom AG, Halle, Germany

The challenges of delivering oligonucleotides (OGNs) to target cells include getting them to the place you want, having them escape TLR detection, crossing the membrane, and escape from the endosome. The method used in these investigations for delivery involved novel amphoteric liposomes, which can change charge according to surrounding pH level (negative charge at neutral pH, e.g., in the blood). When applied *in vivo*, cationic liposomes or polymers face tolerability issues in serum due to aggregate formation and related toxicity. In contrast, amphoteric liposomes retain their particle size in serum. Amphoteric liposomes accumulate mainly in the liver, spleen, and sites of inflammation, whereas *in vivo* distribution of cationic particles is limited by their rapid interaction with endothelial surfaces, resulting in substantial lung accumulation. A novel liposome (NL) was developed by Novosom for the delivery of OGNs and tested in a variety of models.

Cationic liposomes are strong inducers for TLR responses when combined with OGNs. In contrast, NL-OGN engendered no release of IFNα and IL12p40 when tested over a range of concentrations in contact with PBMC cells from human donors.

Amphoteric liposomes were able to resist fusion at pH levels in the blood stream, but became extremely fusogenic if the pH dropped down in the endosome, leading to an endosomal escape and the delivery of the encapsulated OGN into the cytosol of the cell. This NL-OGN produces an ideal vector for the delivery of oligonucleotide-based therapeutics.

Targeting CD40 antisense was investigated in inflammation to test delivery of NL-OGN. CD40 also has good access for delivery into dendritic cells and macrophages. NL-OGN was shown to accumulate in inflamed cells and in macrophages in the spleen. In peripheral blood, macrophages (CD11b+) are almost completely transfected with CD40/OGN, and no uptake was observed in B cells (CD19+) or T cells (CD3+). In the spleen, macrophages (CD11b+, CD11c-) and myeloid dendritic cells are transfected, whereas other cells, such as splenocytes, remain unaffected. NL-OGN is very selectively taken up in phagocytic active cells. Analyses of spleen and macrophages indicated that NL-OGN-CD40 significantly reduces CD40 on macrophages in the spleen and inflamed sites.

NL-OGN-CD40 therapy has been tested for delivery in various diseases and conditions. For example, in a collagen induced arthritis (CIA) model in DBA/1 mice, NL-OGN was tested against murine anti-TNFα antibodies and dexamethasone and was found to have equal clinical benefit as glucocorticoids and achieved reductions close to baseline; in comparison, the TNFα antibody only stabilizes disease at a much higher dose. Benefits of NL-OGN were seen in pannus formation, inflammatory infiltrates, cartilage damage, reduction of joint space, and bone erosion in the arthritis model. In addition, the levels of cytokines TNFα, IL6, and IL17 were dramatically reduced, and the safety profile was excellent. In a colitis mouse model, NL-OGN was tested against prednisone and was found to achieve disease reversion on a macroscopic and microscopic level. The higher number of CD40+ macrophages found in mesenteric lymph nodes at advanced disease stages was significantly reduced following NL-OGN-CD40 treatment. Prednisolone treatment did not lead to such a significant effect.

Novel vectors also have been developed for delivery of siRNA. In an *in vivo* study, naïve mice were intravenously injected with different NL-OGN-CD45, and uptake was seen for monocytes and splenic macrophages and dendritic cells. In a test against a saline control, animals injected with the NL-OGN-CD45 had reduced expression of CD45 in macrophages by an order of 1 magnitude. Optimized NL-OGN-CD45 achieved a 90 percent reduction of CD45 in spleen macrophages.

In summary, delivery of siRNA to cells of the immune system can be achieved with novel vectors, and cellular transfection is the dominant requirement for knockdown activity with siRNA (even low amounts yielded strong effects). Subsequent studies will determine how to achieve the best delivery and the strongest effects.

For miRNA, an investigation on delivery of let-7e, which is linked to the regulation of TLR4, was conducted. When animals are challenged with LPS-TLR4, let-7e gets over-expressed to bring TLR4 down again. Mice treated with NL-OGN-let7e show a more substantial reduction of TLR4 after LPS challenge. While modulation of TLR4 levels was modest, strong effects were observed on IL6, which is the effector gene. In contrast, injection of NL-OGN-let7e antimir can lift the blockade on TLR4 production, resulting in restored or somewhat higher TLR4 levels after LPS challenge. NL-OGN-let7e antimir had no impact on IL6 release.