06
Cytokine interferon/mode of action

06-01/P

TUMOR NECROSIS FACTOR ALPHA INHIBITS THE Na^+-K^+ ATPASE IN RAT CARDIOMYOCYTES *VIA* PGE2

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There has been increasing evidence that tumor necrosis factor alpha (TNF-α) is synthesized by cardiomyocytes and contributes to their impaired function and to cardiac failure. Because the Na^+-K^+ ATPase is a key player in the contraction of cardiomyocytes, this work was undertaken to study the effect of TNF-α on the Na^+-K^+ATPase in rat heart. Animals were injected with 270ng/100 g body weight TNF-α and myocytes were isolated 4hrs later. A time response study showed an increase in the activity of the Na^+-K^+ ATPase in the left and right ventricles with no change in its protein expression. The effect of the cytokine on the activity of the pump disappeared in the presence of indomethacin suggesting an involvement of PGE2 in the action of TNF-α . Rats treated directly with PGE2 showed a dose dependent response. A decrease in the activity of the Na^+-K^+ ATPase was observed at a low dose and an increase at a high dose in both right and left ventricle without a change in its protein expression. PGE2 exerted the same effects on isolated cardiac myocytes. Since PGE2 is suspected to be the active mediator in TNF-α signaling, inhibiting its synthesis by inhibiting some suspected transcription factors was attempted. PDTC and curcumin, respective inhibitors of NF-KB and AP1 partially abrogated the effect of the cytokine whether used separately or in combination, suggesting that these two factors are involved in TNF-induced PGE2 synthesis but are not the only ones. It can be concluded that TNF affects the activity of the pump rather than its expression *via* PGE2.

06-02/P

ANTI-RETROVIRAL EFFECTS OF TYPE I INTERFERONS *IN VIVO*

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In mice type I Interferon belongs to a multigene family with 14 IFN-α subtype genes yet has only one IFN-β gene. IFN-α/β is produced very early post infection and induces an antiviral state in the cells. Interestingly, individual IFN- α subtypes differ extremely in their biological activity. However, nothing is known yet about the exact biological function of IFN-α subtypes in a retroviral infection. In the current study we have used the Friend retrovirus model to determine the role of type I interferons *in vivo*.
The important role of type I IFN in the early immune defence against Friend virus became evident when mice deficient in IFN type I receptor ($IFNAR^{-/-}$) and IFN-ß ($IFN\text{-}ß^{-/-}$) were infected with the virus. Lack of IFNAR and IFN-ß led to significantly higher viral loads compared to control mice during acute FV infection. This difference was induced by an antiviral effect of IFN-α and IFN-β that was most likely mediated by antiviral enzymes as well as by an effect on T-cell responses. To get more insight into the nature of IFN-α responses we determined the expression of various IFN-α subtypes during FV infection by quantitative PCR. Results show that IFN-α1, -α4, -α6 and -α9 were expressed during acute FV infection, but expression levels were different. All these IFN-α subtypes were biologically active against FV, but differed in their relative biological efficacies.
Therapeutical approaches show that exogenous IFN-α can be used to reduce FV replication during acute infection. Knowledge of the activity of the different IFN- α subtypes can improve the current usage in viral therapy.

06-03/P

POSTTRANSCRIPTIONAL REGULATION OF RNASE-L EXPRESSION BY ITS 3'UTR

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RNase-L mediates critical cellular functions including antiviral, antiproliferative, pro-apoptotic, and tumor suppressive activities; accordingly, its expression and activity must be tightly regulated. However, RNase-L activity is not associated with significant changes in RNase-L gene expression, and apart from the requirement of 2',5'-linked oligoadenylate moieties (2-5A) for RNase-L activation, little is known about the regulation of RNase-L activity. The mature RNase-L mRNA contains a conserved 1.9kb 3'UTR that comprises 40% of the full-length transcript, suggesting that this region may function to posttranscriptionally regulate RNase-L expression. Indeed, the 3'UTR mediated a potent inhibition of RNase-L and chimeric β-globin expression that reflected a four-fold decrease in mRNA stability. Sequence analysis of the 3'UTR revealed several AU-rich elements (AREs) that may mediate this regulation. Deletion analysis and RNA immunoprecipitation determined that the ARE binding protein, HuR, interacted with the penultimate ARE to increase RNase-L expression. Consistent with this regulation, HuR transfection, or the stress-induced increase in endogenous cytoplasmic HuR by UVC or heat shock, resulted in a 3'UTR-dependent increase in RNase-L expression. Further analysis revealed a distinct, upstream destabilizing element that may mediate its low expression in the absence of stress. This study identifies a novel mechanism of RNase-L regulation in which the 3'UTR functions to maintain its mRNA at low basal levels in resting cells, but acts to increase RNase-L expression in conditions of cell stress. Analysis of the extent to which this regulation is disrupted in human malignancies may reveal a broader role for RNase-L in tumor suppression.

06-04/O

DIFFERENTIAL SIGNALING PATHWAYS IN HEPATITIS C PATIENTS RESISTANT TO INTERFERON/RIBAVIRIN THERAPY AND THOSE SHOWING VIRAL CLEARANCE

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The Virahep-C study is a collaborative, multi-center trial of Peg-interferon/ribavirin therapy for patients with chronic hepatitis C virus, genotype 1. Virahep-C includes 401 participants enrolled at 8 clinical centers. One aim of the trial is to determine factors associated with resistance to therapy. Affymetrix DNA microarray analyses were performed on PBMCs collected from 69 participants who had samples taken at baseline and days 1, 2, 7,14 and 28 after initiation of treatment. Expression levels for approximately 300 genes were modified 2 fold or more at a p-value of < 0.001 within the first 1-2 days in patients who responded to treatment on day 28. Considerably fewer genes were induced in non-responding patients. Utilizing Gene Set Enrichment Analysis software, differences were found in the interferon signaling pathway genes before initiation of treatment between non-responding patients and responders. Non-responding patients had higher levels of interferon induced genes than non-responders ($p = 0.03$). Following initiation of treatment responders showed elevated levels of gene expression in the interferon, the G-signaling pathway (MAPK1), and pml pathway and in androgen and estrogen metabolism at day 1 and 2. By day 14 of treatment the major differences between the two groups of patients was in the interferon and caspase pathways. By day 28 the interferon pathway expression remained high, but a major difference was in genes associated with DNA damage and repair mechanisms. This DNA damage was associated with response to treatment. Based on these findings, we propose that the lack of response to peginterferon/ribavirin therapy may be due to prior induction of a sub class of interferon inducible genes, which may blunt subsequent interferon activity. Enzymes associated with DNA damage are induced in responding patients by one month into therapy, which may lead to leucopenia and other side effects. Funding provided by NIDDK, and Roche Laboratories Inc.

06-05/P

LINKAGE OF VIRAL TITER TO GENE EXPRESSION IN HEPATITIS C PATIENTS TREATED WITH INTERFERON/RIBAVIRIN.

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The treatment of hepatitis C with interferon/ribavirin results in a varied response in terms of outcome and decrease in viral titer. In some patients (responders) there is a sharp decrease in viral titer by day 28 of initiation of treatment whereas in other patients there is barely a detectable effect on the virus (null patients). Affymetrix microarrays were performed on RNA isolated from PBMC of patients undergoing interferon/ribavirin therapy. Samples were collected at day 0, and 1, 2, 7, 14, and 28 days after initiation of treatment. We applied a novel method to identify genes that are linked with the decrease in viral titer. This method was based on the relationship between interpatient proximities according to expression of a gene, viral titer, and total gene expression across all genes. Using this approach we identified 11 genes that provide an expression based clustering of patients that is similar to viral titer based clustering of patients. These genes include IRF7, MX1, OAS1 and OAS2, viperin and other ISG's of unknown function. These genes appear to play a major role in the clearance of hepatitis C virus during the early phase of treatment. Funding provided by NIDDK, and Roche Laboratories Inc.

06-06/P

STAT3 IS RESPONSIBLE FOR IL-27/WSX-1-MEDIATED SUPPRESSION OF CYTOKINE PRODUCTION BY ACTIVATED T CELLS

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Recent lines of evidence have demonstrated that IL-27, a newly identified IL -12-related cytokine, has two apparently conflicting roles in immune responses: one as an initiator of Th1 responses and the other as an attenuator of inflammatory cytokine production. Although the IL-27-mediated Th1 initiation mechanism has been elucidated, little is known about the molecular basis for the suppression of cytokine production. In the present study, we demonstrated that IL-27 suppressed the production of various pro-inflammatory cytokines by fully activated CD4+ T cells while it had no suppressive effect on the cytokine production by CD4+ T cells at early phases of activation except for IL-2 suppression. At these early phases of activation, IL-27 in association with IL-12 promoted IFN-gamma production. IL-27 also suppressed IL-17 production by activated CD4+ T cells thereby counter-acting IL-23, another IL-12-related cytokine with pro-inflammatory effects. In fully activated CD4+ T cells, STAT3 was preferentially activated by IL-27 stimulation while both STAT1 and 3 were activated by IL-27 in early activated CD4+ T cells. Lack of STAT3 in fully activated cells impaired the suppressive effects of IL-27. These data indicated that the preferential activation of STAT3 in fully activated CD4+ T cells plays an important role in the cytokine suppression by IL-27/WSX-1.

06-07/P

INHIBITION OF A DNA REPAIR PROTEIN INHIBITS IFN-γ-MEDIATED CLASS II HLA INDUCTION

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Studies using mutant cell lines and knock-out mice have clearly established that signal transduction in response to the Interferons (IFNs) requires activation of Janus kinases (JAKs) and Signal Transducers and Activators of Transcription (STATs). Although JAK/STAT signalling is essential, it is not necessarily sufficient for a full IFN-γ response, including the induction of Class II HLA. A FACS-based kinome-wide siRNA screen in human osteosarcoma cells (U2OS), which monitors, in parallel, quantitatively, the induction of Class I and II HLAs in response to IFN-γ has yieldied a number of candidate molecules. The Dharmacon siSMART kinome library comprises all known kinases and a number of related molecules. Out of the 779 genes analysed, 16 have shown a clear effect in response to IFN-γ. These include the previously identified PI3K and CamKII, as well as, reassuringly, JAK1 and JAK2. Interestingly, the screen has also implicated an enzyme traditionally involved in DNA repair in the regulation of IFN-γ-mediated Class II HLA induction.

* These authors have contributed equally to this work.

06-08/P

IL-27P28 GENE TRANSCRIPTION IN MACROPHAGES IS REGULATED BY IFNγ DIRECTLY THROUGH INTERFERON REGULATORY FACTOR-1

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IL-27 is a heterodimeric protein consisted of Epstein–Barr virus (EBV)-induced gene 3 (EBI3), an IL-12p40-related protein, and p28, an IL-12p35-related polypeptide. IL-27 is produced early by activated antigen-presenting cells in response to microbial infection. It is able to induce clonal proliferation of naïve but not memory $CD4^+$ T cells and synergizes with IL-12 in IFNγ production by naïve $CD4^+$ T cells. In contrast, how IL-27 gene expression is regulated during an immune response has not been established. Here we report the first study of the molecular regulatory mechanisms involved in the transcription of IL-27 p28 gene in IFNγ-activated macrophages. We identified five transcriptional initiation sites (TIS) within the p28 gene promoter and determined that the most adjacent one to the translation start site is the predominantly used TIS in response to LPS and IFNγ stimulation in macrophages. "Transcriptional pausing" is a major mechanism for initiating p28 transcription. Moreover, p28 mRNA expression in IRF-1-null macrophages stimulated by IFNγ was markedly decreased, indicating that IRF-1 is a critical factor for p28 transcription. Conversely, adenovirus-mediated gene transduction of IRF-1 in primary macrophages resulted in enhanced p28 gene expression. Furthermore, the IFNγ/IRF1-response element (IRF1-RE) was localized to a TTTTC motif at -56/-48 of the mouse *IL-27 p28* promoter, to which endogenous IRF-1 can physically bind *in vitro* and *in vivo*. This study uncovers a novel IFNγ-mediated activation pathway in IL-27p28 expression in macrophages through IRF-1, and contributes to the elucidation of an important host defense mechanism.

06-09/P

SITE AND SIZE OF INTERFERON PEGYLATION ALTERS AFFINITY TO THE IFNAR2-EXTRACELLULAR DOMAIN

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The ability to pegylate interferon alfa and extend the pharmacokinetics has contributed to substantial improvements in efficacy. The binding and downstream functions of interferon are directly mediated by the binding at the receptor. We have observed a difference in specific binding affinity at the receptor resulting in the loss of biological potency based on the site and size of the pegylation. There are expectations the binding event results in downstream activation of kinases and changes in gene expression and attenuation of the biological potency. Our results show, that the binding and downstream activity of pegylated interferon alfa-2b are altered due to observable differences in binding affinity to the IFNAR2-extracellular domain. The change in binding is determined by both the size of the polyethelyene glycol molecule and amino acid to which the pegylation is directed. In this study, the His34 and the Lys31, the most common sites for pegylated interferons, were prepared with a linear methoxy polyethylene glycol succinimidyl carbonate (SC-PEG) linker with an average PEG molecular mass of 5, 12, 20, and 30 kDa. The anti-viral specific activity and binding affinity to IFNAR2-extracellular domain were measured for the various Lys31 and His34 preparations and showed substantial differences in binding and activity. From the results, pegylation at position 34 does not influence the koff, but only kon, while at position 31 both kon and koff are affected. Moreover, the effect at position 31 on kon is significantly larger compared to the effect on position 34, making H34 an overall preferred position for pegylation. Findings show that the size of the pegylation has little effect on kon, showing that peg size should be driven by bioavailability requirements. The affinity and biological activity of pegylated interferons are directly linked to the alteration caused by pegylation site and the affinity to the IFNAR2-extracellular domain.

06-10/P

DISSECTING THE MOLECULAR MECHANISM OF TGF-BETA REGULATION OF IFN-GAMMA PRODUCTION

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Immunosuppressive cytokines, such as TGF-β are important to maintain a balanced immune response against pathogens. Despite a decent understanding of the cellular effects of these cytokines, there is a lack of insight into the molecular mechanisms underlying their inhibitory action. For TGF-β, there are conflicting reports as to how it interferes with the production of the pro-inflammatory cytokine: IFN-γ. In this study we make an attempt to widen our perspective on the proposed mechanism of inhibition of IFN-γ by TGF-β in T and NK cells. We have shown that TGF-β inhibits initially the mRNA and later the protein expression of IFN-γ in T and NK cells. Subsequently, we performed a sequential study of the effect of TGF-β on the pathway of induction of IFN-γ. The signalling pathway of the IFN-γ inducing cytokines IL-12 and IL-18 is not affected in presence of TGF-β. Both activation as well as the DNA binding capacity of transcription factors: NFκB and STAT4 (induced by IL-12 and IL-18, respectively) on the IFN-γ promoter are also not affected. Stability of IFN-γ mRNA in presence of TGF-β is unaffected as well. Hence, the immunosuppressive action of TGF-β is hypothesized to occur at the level of transcriptional repression. TGF-β has been shown to inhibit the expression of a Th1-specific transcription factor, T-bet. However, the initial suppression of IFN-γ precedes the inhibition of T-bet. Therefore, we presume that T-bet might be involved in sustaining the inhibitory effect of TGF-β only at the recall stimulus. Furthermore, we have performed a gene profiling analysis to identify genes that are regulated by TGF-β in T cells. Several genes were found to be upregulated by TGF-β, the predominant among them being the transcriptional regulators. We are currently exploring the role of these genes as the candidate gene responsible for the suppressive effect of TGF-β on IFN-γ production.

06-11/O

MOLECULAR BASIS FOR PKR ACTIVATION BY PACT OR DSRNA

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The mammalian protein kinase, PKR, is a critical component of the innate immune response against virus infection. Its cellular actions are mediated by modulating cell signaling and translational regulation. To be enzymatically active, latent PKR needs to be activated by binding to one of its activators, double-stranded (ds) RNA or PACT protein. Although the structures of the N-terminal dsRNA-binding domain (dsRBD) and the C-terminal kinase domain (KD) of PKR have been separately determined, the mode of activation of the enzyme remains unknown. In this study, we used a combined functional and structural approach to determine the mechanism of PACT-mediated PKR activation. To address this problem, we used biochemical, genetic and NMR analyses to identify within PKR, the PACT-binding motif (PBM) located in the KD and demonstrated an intramolecular interaction between PBM and dsRBD. This interaction is responsible for keeping PKR in an inactive conformation, because its disruption by point mutations of appropriate residues produced constitutively active PKR. Furthermore, a short decoy peptide, representing PBM, was able to activate PKR by interfering with the intramolecular interaction. While dsRNA activates PKR by directly binding to dsRBD present in the N-terminal region of PKR, PACT domain 3 activates PKR by directly binding to PBM present in the C-terminal PKR KD. These observations suggest a model for PKR activation upon binding of dsRNA or PACT, where PACT binds to PKR in a manner distinct from that of dsRNA. These two different bindings, to the two partners of the intramolecular interaction that keeps latent PKR in the inactive conformation, lead to the same allosteric changes in PKR and its consequent activation.

06-12/P

INSIGHTS INTO THE MOLECULAR MECHANISMS UNDERLYING THE PLEIOTROPIC ACTIVITIES OF TYPE I INTERFERONS

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Type I interferons (IFN) are multifunctional cytokines that can induce antiviral, antiproliferative and immunomodulatory effects. We have characterized an IFN-α2 mutant that matches IFN-β performances in both biochemical and biological assays, and demonstrated that the IFN-induced antigrowth and antiviral signals are not necessarily coupled (Jaitin *et al.* MCB 2006 26:1888-1897). We are currently studying the molecular basis that distinguishes these signals. We found that differential antiproliferative potencies of IFN-β and IFN-α2 correlate to differential downregulation of IFNAR receptors, a process that could actively participate in this IFN activity. In addition, we defined distinct aspects of IFN antiviral and antigrowth signals by comparing interferon-stimulated gene (ISG) expression profiles and expression kinetics, using microarray and real-time PCR experiments. One interesting aspect of interferon biology is that following four hours of cell incubation with interferon, full antiviral protection is achieved, and that this antiviral state is sustained *in vitro* for at least twelve hours after complete removal of interferon. We found that this antiviral state is sustained by a small number of genes, still upregulated twelve hours after IFN occlusion; analysis of the functional ontology of these genes allowed to define the essential functions that confer this antiviral state. Conspicuous "memory" genes were, in addition to known ISGs directly involved in antiviral response and MHC Class I, all the genes required for the proteasome-to-immunoproteasome switch. Their relative expression levels, together with expression profiles in cultures under continuous IFN treatment, provided information on IFN signal regulation. In addition, this group of genes was also interesting in terms of the genes not included in it. Indeed, other known ISGs, found to be involved in IFN-induced growth suppression, were induced only by continuous IFN incubations. The distinction between transient and permanent gene activation seems to be the key factor determining antiviral *versus* antiproliferative response of interferons.

06-13/O

ENGINEERING IFNα2 MUTANTS WITH ENHANCED RECEPTOR-BINDING AFFINITIES ELUCIDATE THE MECHANISM OF DIFFERENTIAL ACTIVATION BY INTERFERONS

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Type I Interferons are multifunctional cytokines that exhibit differential activities through a common receptor composed by the subunits IFNAR1 and IFNAR2. Here, we combined biophysical and functional studies to explore the mechanisms that allow the alpha and beta IFNs to act differentially through the same receptor. For this purpose, we have engineered IFNα2 mutants exhibiting 20 - to 100-fold increased affinities to IFNAR2 or IFNAR1. Optimization of the binding affinity of IFNα2 to IFNAR1 yielded an IFNβ-like IFNα2 protein, that forms a much more stable ternary complex *in vitro*. Strikingly, higher affinity towards IFNAR1 was accompanied by an increased antiproliferative (but not antiviral) activity i.e. very similar to the activity pattern of IFNβ. Moreover, microarray and real-time PCR experiments have shown that this mutant IFNα2 induces a similar gene expression profile as IFNβ. The antiproliferative activity of IFNα2 could be further increased beyond that of IFNβ by optimizing the binding affinity towards IFNAR1. The high degree of conservation of the mutated residues suggests that the IFNα subfamily have evolved to bind IFNAR1 weakly, apparently to sustain a differential level of biological activities with IFNβ. A second set of IFNα2 mutations was designed to display tighter binding towards IFNAR2. Here, we took advantage of the sequence and charge variability of the tail segment between interferons. We found that a positively charged IFNα8-like tail increases binding of IFNα2 to IFNAR2 by 20-fold, namely reaching the IFNAR2-binding affinity of IFNβ. This mutant displays increased antiviral and antiproliferative activity, albeit not to the extend of the increased affinity. Overall, the results here show a direct link between binding-affinity and some of the biological activities of interferons, but not others. The mechanism underlying this observation will be discussed.

Reference

Jaitin DA, Roisman LC, Jaks E, Gavutis M, Piehler J, van der Heyden J, Uze G, Schreiber G. *Mol Cell Biol* 2006; 26: 1888-97.

06-14/O

ABIN-3 IS AN IL-10-INDUCED GENE WITH ANTI-INFLAMMATORY PROPERTIES IN HUMANS BUT NOT IN MICE.

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Dysfunctional immune regulation can lead to an array of debilitating chronic inflammatory and autoimmune diseases. The anti-inflammatory cytokine interleukin-10 (IL-10) principally functions to limit inflammation as mice lacking IL-10 exhibit exacerbated inflammatory responses and spontaneous enterocolitis. IL-10 exerts its effects through receptor-mediated signaling and induction of target gene expression; however, our understanding of these effector genes and their mechanism of action remains incomplete. Screening for novel IL-10-induced genes in mouse macrophages, we identified the ortholog of human ABIN-3/LIND. ABIN-3 expression was induced specifically

by IL-10 in mouse and human mononuclear phagocytes in a manner that depended on co-stimulation with pro-inflammatory ligands. Consistent with other ABIN family proteins, human ABIN-3 could suppress NF-κB-dependent reporter gene expression in response to pro-inflammatory stimuli. Unexpectedly, however, mouse ABIN-3 did not share this activity and, furthermore, mice rendered deficient in ABIN-3 by gene-targeting displayed unaltered anti-inflammatory responses to IL-10. Comparative sequence analyses revealed that the mouse ortholog lacks a complete ABIN homology domain (AHD), which was required for the NF-κB inhibitory activity of human ABIN-3. Constitutive expression of the functional human ABIN-3 in monocytic cells suppressed pro-inflammatory gene responses, nuclear NF-κB DNA-binding activity, and cytoplasmic IκBα degradation. Human ABIN-3 acted downstream of RIP and TRAF proteins but upstream of IKK activation and interacted with the IKK regulatory subunit IKKγ/NEMO. Collectively, our results identify ABIN-3 as an IL-10-induced gene capable of attenuating NF-κB signaling in human mononuclear phagocytes and highlight a distinction in IL-10's mechanism of action between humans and mice.

06-15/O

SELECTIVE BLOCKADE OF IL-6 TRANS-SIGNALLING SUPPRESSES PROGRESSION OF COLLAGEN-INDUCED ARTHRITIS

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Interleukin (IL)-6 responses are transmitted *via* gp130, which serves as the universal signal-transducing receptor subunit for all IL-6-related cytokines. Although this classically occurs through IL-6 engagement of a membrane-bound receptor (IL-6R), it is evident that a soluble form of IL-6R affords IL-6 with a second mechanism of gp130 activation. This alternative mode of cell activation is termed IL-6 trans-signalling. Using a monoarthritic model of antigen-induced arthritis we have shown that IL-6 trans-signalling promotes synovial hyperplasia, STAT3 activation and mononuclear leukocyte recruitment. Documentation of these activities has been aided by the demonstration that two soluble form of gp130 (sgp130 and gp130-RAPS) selectively counteracts IL-6 trans-signaling *in vivo*. It was therefore reasoned that sgp130 might represent a promising therapeutic modality for the treatment of rheumatoid arthritis. To test the validity of this approach, we have now used collagen-induced arthritis (CIA) as a systemic model of inflammatory arthritis. CIA was induced in wild type mice and the efficacy and potency of a sgp130Fc fusion protein was examined in both prophylactic and therapeutic dosing regimes. On induction of CIA, sgp130Fc was administered (2-50 μg/mouse, i.p.) every second day over a 14-day period. This prophylactic approach resulted in a dose-dependent improvement in clinical score with 50 μg/mouse significantly reducing synovial hyperplasia, inflammatory infiltrate/exudate, joint erosion, and systemic serum amyloid-A levels. Similar benefit was also recorded when sgp130Fc was administered (i.p.) daily following the onset of established disease (>80% arthritis incidence). Collectively, these results suggest a central role for IL-6 trans-signaling in arthritis progression, and highlight the value of selectively targeting this mode of IL-6 activation as a therapeutic strategy.

06-16/P

INTERFERON-γ REGULATED TRANSCRIPTION THOUGH THE CAAAT/ENHANCER BINDING PROTEINS: A TALE OF KINASE DRIVEN PROTEIN: PROTEIN INTERACTIONS

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IFNs regulate transcriptional activation through a number of different elements, apart from the classical elements such as ISRE and GAS. Previous studies form our lab identified a novel IFN-responsive element, GATE. GATE binds to two transcription factors, GBF1 and GBF2. GBF2 was later identified as transcription factor CAAAT/enhancer binding protein-beta (C/EBP-β). Although GBF1 also induces gene expression through GATE, it is an extremely weak DNA binding protein on its own. Here we have determined the functional elements required for the biological activity of GBF1 and its interactions with C/EBP-β. In response to IFN-γ, C/EBP-β undergoes phosphorylation at a critical ERK phosphorylation motif. Mutational inactivation of the ERK phosphorylation site or an interference with the ERK1/2 prevented IFN-induced interactions between GBF1 and C/EBP-β; and inhibited transcription. These results identify a converging point for two transactivators that exert their effects through a single IFN-response element. We will also discuss the roles for mixed lineage kinases in regulating transcription through C/EBP-β.

06-17/P

PROTECTIVE ROLE OF INTERFERON-γ IN COLLAGEN-INDUCED ARTHRITIS CONFERRED BY INHIBITION OF GRANULOCYTE-CHEMOTACTIC PROTEIN-2

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In addition to its well-known proinflammatory action, IFN-γ exerts a protective role in experimental autoimmune diseases, such as collagen-induced arthritis (CIA), that rely on the use of complete Freund's adjuvant (CFA). Here we show that the increased severity of CIA in mice lacking a functional IFN-γ receptor (IFN-γR KO) as compared to wild-type mice is accompanied by increased levels of the CXC chemokine granulocyte chemotactic protein-2 (GCP-2) and increased influxes of neutrophils in the inflamed synovia. We demonstrated that the heat-killed mycobacteria present in CFA elicited production of GCP-2 in mouse embryo fibroblasts (MEF) cultures, and that this production was inhibited by IFN-γ. Inhibition of GCP-2 production by IFN-γ was signal transducer and activator of transcription-1 (STAT-1) -dependent, but interferon regulatory factor-1 (IRF-1) -independent. IFN-γR KO mice treated with neutralizing anti-GCP-2 antibodies were completely protected from CIA, indicating the *in vivo* importance of GCP-2 in the pathogenesis of CIA. Antibody-treated mice also had significantly decreased cellular immune responsiveness to collagen type II (CII) as evident from DTH reactivity against CII. Our data support the notion that one of the mechanisms whereby endogenous IFN-γ mitigates the manifestations of CIA consists in inhibiting production of GCP-2, thereby limiting mobilisation and infiltration of neutrophils, which are important actors in joint inflammation. These results may also be applicable to other experimental models of autoimmunity that rely on the use of CFA.

06-18/P

LISTERIA MONOCYTOGENES-INDUCED IFN-β PRODUCTION IS DETRIMENTAL FOR THE HOST AND OCCURS THROUGH AN UNKNOWN CYTOSOLIC PATHWAY.

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Type I interferons (IFNs) are potent antiviral cytokines. Contrastingly, in response to bacterial challenge, their activity can be either beneficial or detrimental for the host. Infection of macrophages with

the Gram-positive facultative intracellular bacterium *Listeria monocytogenes* leads to the synthesis of large amounts of IFN-β, which sensitises macrophages to *Listeria*-induced cell death. Induction of IFN-β is dependent on TBK-1 and IRF-3, but independent of Toll-like receptors and their adapters MyD88, TRIF and TRAM. Recently, it was proposed that the ligand for IFN-β induction after infection with intracellular bacteria is in fact the bacterial DNA. Here we show that induction of IFN-β as well as MAPK and NFκB activation still occur in the absence of the nucleotide oligomerization domain (NOD) proteins 1 and 2, candidate receptors for intracellular bacteria. In addition, the recently identified mitochondria-associated adapter protein MAVS is dispensable for IFN-β synthesis after infection with *L. monocytogenes* or transfection of bacterial DNA, suggesting that *L. monocytogenes* stimulates an unknown cytosolic pathway in macrophages. The importance of IFN-β production and signalling is emphasised by our observation that IFN-β deficient mice are resistant to a normally lethal dose of *L. monocytogenes*. This suggests that the majority of sensitising type I IFNs is not produced by IFN-producing cells (pDCs). Furthermore, when comparing two different wild-type strains of *L. monocytogenes*, induction of higher amounts of IFN-β in macrophages correlates with increased virulence *in vivo*. Taken together, our data stress the impact of the signalling pathway induced by *L. monocytogenes* leading to the production of IFN-β.

06-19/P

SYNERGISTIC ANTIVIRAL ACTIVITY OF INTERFERON ALPHA AND MEMBERS OF THE IL-6 FAMILY

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Interferon-alpha (IFN-alpha) mediates the main defense against virus infection and is currently used as treatment in patients infected with Hepatitis C Virus (HCV). In spite of this only 55% of HCV patients respond to the treatment, so it would be very interesting to potentiate the antiviral activity of the IFN-alpha. One way to increase this antiviral activity is to potentiate the signaling pathway activated after the IFN-alpha binds to its receptor. This can be done using the IFN-alpha in combination with other cytokines that share elements of their signaling pathways with the IFN-alpha. We have analyzed the effect produced by using the IFN-alpha in combination with cytokines that strongly activate STAT-1 and STAT-3, like IL-6, Cardiotrophin-1 (CT-1) and Oncostatin M (OSM). When we use this combination of cytokines and we measure their antiviral activity in the HCV replicon system and in cells infected with ECMV we have observed that there is a potentiated antiviral activity when the IFN-alpha is used in combination with the other cytokines comparing to the use of the IFN-alpha alone. This effect correlates with a strengthened and more prolonged activation of STAT-1 and STAT-3 that is reflected in a stronger induction of diverse Interferon Stimulated Genes. When we have studied in detail the interaction of the IFN-alpha with these cytokines we have observed that in the three cases the interaction is synergistic, being stronger the IFN-alpha/CT-1 and IFN-alpha/OSM than the IFN-alpha/IL-6 interaction. This results suggest that the combination of the IFN-alpha with IL-6, CT-1 and OSM can improve the HCV treatment or at least to decrease the amount of IFN-alpha used, that could be reflected in a reduction of the secondary effects induced by the IFN-alpha treatment.

06-20/P

EX VIVO ANALYSIS OF BLOOD CELLS FROM IFN-β-INJECTED MS PATIENTS REVEALS DIFFERENTIAL STAT AND KINASE ACTIVATION PATTERNS

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Treatment of cells with interferon (IFN)-β activates IFN-stimulated gene factor 3 (ISGF3: IRF9 and phosphorylated STAT1 and STAT2), leading to induction of virtually all IFN responsive genes. However, other crucial pathways must function in addition. To study the response to IFN-β by human blood cells *in vivo,* we used a flow cytometry-based assay that allows the measurement of activated kinases and STATs in single cells. Activation of STAT1, STAT3, STAT5 and p38 mitogen-activated protein kinase (P38MAPK) was determined in monocytes, B cells, $CD4^+$ and $CD8^+$ T cells of 6 relapsing-remitting multiple sclerosis (MS) patients at 8 different time-points within 2.5 hours after intramuscular IFN-β1a (Avonex) injection. Four of six patients showed a kinetically-variable pattern for both STAT and P38MAPK activation, with initial peak responses for both STATs and kinases 30-70 minutes after injection. In contrast, the other 2 patients showed only P38MAPK activation during this time period. Monocytes of all 6 patients showed P38MAPK activation. In contrast, only 83%, 33% or 0% of the MS patients studied activated P38MAPK in their $CD8^+$ T cells, B cells, or $CD4^+$ T cells, respectively. We also found that STAT1, STAT3 and STAT5 were activated differentially in blood cell subsets. Notably, cell subsets showed different combined STAT and P38MAPK activation patterns, perhaps accounting for differential cell-type-specific gene activation by IFN-β. We also speculate that differences between individuals might underlie different therapeutic responses to IFN-β1a. We now plan to establish whether differential activation of STAT and P38MAPK correlate with gene induction and with responsiveness to IFN-β treatment in MS. In a separate study, we will investigate STAT and P38MAPK activation in blood cell subsets stimulated with IFN-β1a *in vitro*, in order to establish the response to IFN-β (currently unknown) in healthy controls, and to examine how differential activation patterns account for cell-type-specific responses to IFN-β.

06-21/P

IFN-α-MEDIATED STAT4 ACTIVATION IN MURINE IMMUNE CELLS *IN VITRO* AND PATHOGENIC CELL-MEDIATED IMMUNITY IN THE BRAIN

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Whether IFN-α-mediates STAT4 activation in the mouse and its biological significance remain controversial issues. We examined STAT4 activation in murine splenocyte and in purified T- and B-cell populations. In splenocytes, IFN-α treatment was associated with significantly increased STAT4 phosphorylation that was not dependent on STAT1, STAT2 or IL-12p40. Induction of IFN-γ gene expression was observed following IFN-α treatment of splenocytes from wild type as well as STAT1 and STAT2 deficient mice. In $CD4^+$ or $CD8^+$ T-cells or $CD19^+$ B-cells purified from the spleen, exposure to IFN-α induced STAT4 phosphorylation which was strongest in $CD8^+$ T-cells. These findings indicated that IFN-α is capable of the direct activation of STAT4 in primary murine splenocytes and purified lymphocyte populations and this correlates with the induction of IFN-γ gene expression. The functional relevance of these *in vitro* observations was further established in transgenic mice (termed GIFN) with CNSrestricted production of IFN-α that lack STAT2. GIFN STAT2 KO mice developed severe neurological disease with extensive T-cell infiltration of the brain. Concomitant with this immune pathology there was marked activation of STAT4 in the brain. Indicative of functional STAT4 signalling, IFN-γ gene expression was also detectable in the brain of GIFN STAT2 KO mice and localized to the infiltrating T-cells. We conclude that not only does IFN-α directly activate STAT4 in murine lymphocytes but that this process is capable of driving a pathogenic cell-mediated immune response *in vivo*.

06-22/P

HOW INTERFERON BETA SENSITISES MACROPHAGES TO DIE UPON INFECTION BY *LISTERIA MONOCYTOGENES*

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The invasion of the cytoplasm of murine macrophages by *Listeria monocytogenes* results in the macrophage producing interferon beta (IFN β). This sensitises the cells to die later in the infection: if IFN β signalling is prevented, cell survival is greatly increased. We are investigating how the combination of this extracellular signal and the presence of intracellular bacteria result in cell death. None of many single genes that are induced by IFN β upon infection, e.g. TRAIL, PKR, iNOS, is required for cell death to occur. Further work has therefore focused on defining the process of cell death that occurs and identifying the contribution of IFN β to this process. The macrophage death can be characterized as necrotic, but with accompanying caspase activation. Caspase activation requires the mitochondrial pathway, rather than extracellular ligands and occurs alongside necrosis: blocking caspase activation does not inhibit cell death. IFN β signalling increases both of these processes, and we are now investigating its exact contribution.

06-23/P

LACK OF IFN-GAMMA EXAGGERATED SODIUM ARSENITE-INDUCED RENAL INJURY BY SUPPRESSION OF INTRARENAL MRP1 EXPRESSION

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Subcutaneous injection of sodium arsenite (NaAs; 12.5 mg/kg) into BALB/c (WT) mice, caused acute renal dysfunction characterized by severe hemorrhages, acute tubular necrosis, and cast formation, with increases in serum blood urea nitrogen (BUN) and creatinine levels. Concomitant enhancement IFN-γ expression in the renal tubular epithelial cells prompted us to examine its roles in this pathology. IFN-γ deficient (IFN-$\gamma^{-/-}$) mice exhibited higher serum BUN and creatinine levels, and exaggerated histopathological changes, compared with WT mice. Eventually, IFN-$\gamma^{-/-}$ mice exhibited a high mortality (87.5%) within 24 hr after NaAs challenge, whereas most of WT mice survived. The intrarenal arsenic concentration was significantly higher in IFN-$\gamma^{-/-}$ mice later than 10 hr after NaAs treatment, with attenuated intrarenal expression of multidrug resistance-associated protein (MRP)1, a main transporter for NaAs efflux, compared with WT mice. NF-E2-related factor (Nrf)2 protein, a transcription factor crucial for MRP1 gene, was similarly increased in the kidneys of both strain mice after NaAs treatment. In contrast, the absence of IFN-γ augmented transforming growth factor (TGF)-β -Smad3 signal pathway, and eventually enhanced the expression of activating transcription factor (ATF)3, which is presumed to repress Nrf2-mediated MRP1 gene expression. Prior treatment of the mice with recombinat IFN-γ attenuated NaAs induced-renal injury in both strain mice. These findings show that IFN-γ can protect renal tissue against NaAs-induced acute injury, probably by maintaining Nrf2-mediated intrarenal MRP1 gene expression.

06-24/P

IL-17A ACTIVATES PI-3K/AKT AND STAT3 BUT NOT NF-κB DURING CYTOKINE INDUCTION AND PROLIFERATION IN LEUKEMIA CELLS

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Human IL-17A is produced mainly by memory activated CD4+ T cells though the receptor for IL-17A is ubiquitously expressed. Hence, IL-17A acts a large number of target tissues. The biologic functions of IL-17A include induction of cytokine, PGE_2 and I-CAM production. In non-hematopoietic cells cytokine induction by IL-17A is mediated by NF-kB mechanism. IL-17A has been implicated in many human diseases including arthritis, inflammation, lung diseases and cancer. However, its mechanisms of action are not completely understood. Using U937 leukemia cells, we investigated whether the NF-kB pathway mediates the effects of IL-17A in leukemia cells. Our results showed that though IL-17A strongly stimulates PK-3K/Akt activation, it is unable to activate NF-kB in these cells. Using DNA/protein transcriptional array and DNA binding ELISA technology, we confirmed that even though IL-17A stimulates activation of several transcriptional factors (c-Myb>EGR-1>STAT1>STAT3>SRE>GRE>CDP>smad3/4) between 2 to 9-fold, it does not regulate the function of NF-kB. These results point to lack of NF-kB participation in IL-17A mechanisms. Using cytokine/antibody array and cell viability assays, we showed that IL-17A stimulates marked induction (2 to 8-fold) of cytokines in order of IL-2>IL-3>IL-10>RANTES>IL-15>IL-1>IL-8. The cytokine induction and proliferative effects of IL-17A were partially dependent on PI-3K activation. Also, IL-17A stimulates STAT3 DNA binding activity by six fold. Luciferase activity assays in STAT3 reporter stable HeLa cell lines revealed that IL-17A stimulates STAT3 transcription by 4-fold within 8 hr. Furthermore, stimulation of proliferation and STAT3 activation by IL-17A were partially inhibited by the Jak2 inhibitor, AG490. Taken together, these results suggest that in leukemia cells, specific biologic effects (cytokine induction and proliferation) of IL-17 are mediated by the PI-3K/Akt and Jak2/STAT3-dependent mechanisms independent of NF-kB. Supported by NCI/NIGMS grants to Dr. S.E. Adunyah.

06-25/O

INCREASED STAT1 AND STAT1 PHOSPHORLYATION IN PBMC OF PATIENTS WITH SYSTEMIC LUPUS ERYTHEMATOSUS (SLE).

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Both type I and type II interferons can induce SLE flares. Moreover, serum IFNα is increased and associated with SLE disease activity, while murine models suggest a role of IFNγ. Both interferons signal *via* phosphorylating Stat1, an IFN-inducible protein. We prepared PBMC of 50 SLE patients and 30 healthy individuals (HC). Stat1 immunofluorescence staining was performed immediately after preparation and, in 8 healthy individuals, after 24 hours of incubation in medium with or without IFNα, IFNγ, IL-10, or combinations of these cytokines. Staining for phosphorylated Stat1 (pStat1) was performed immediately or after 15 minutes of incubation in medium with or without IFNα or IFNγ. Cells were analyzed by fluorocytometry. Stat1 mean fluorescence intensity was increased in SLE as compared to HC PBMC (lymphocytes: 16.2±13.2 *versus* 5.3±1.9 (mean±SD), $p < 0.0001$; monocytes: 17.8±12.6 *versus* 7.4±3.3, $p < 0.0001$), and correlated with disease activity (lymphocytes: $r = 0.65$, $p < 0.0001$). Within 24h, IFNα increased Stat1 to levels comparable to those of SLE patients. In contrast, the effect of IFNγ was limited to monocytes, while no significant change in lymphocytic Stat1 was seen. IL-10 significantly reduced Stat1 in both lymphocytes and monocytes, and diminished IFNα-induced Stat1 increase. Incubation with IFNα for 15 minutes resulted in Stat1 phosphorylation in SLE and HC PBMC. In contrast, IFNγ significantly induced Stat1 phosphorylation in SLE lymphocytes only (SLE: from 1.56±0.25 to 1.72±0.37, $p < 0.002$ *versus* HC: from 1.39±0.16 to 1.39±0.17, p = n.s.). Monocytes of both SLE patients and healthy individuals increased pStat1 upon stimulation with IFNγ, but this effect was much more pronounced in SLE (SLE: from 4.1±1.2 to 6.9±3.3, $p < 0.0001$; HC: from 3.5±0.9 to 4.4±1.5, $p < 0.005$). Thus, Stat1, the major signaling molecule of both types of IFNs, is upregulated in SLE and associated with disease activity. PBMC of SLE patients are strongly IFNγ-reactive, but healthy lymphocytes are unresponsive and healthy monocytes are hyporesponsive to IFNγ.

06-26/P

IL-27 PROMOTES TH1 DEVELOPMENT AND INFLAMMATION PARTLY BY AFFECTING REGULATORY T CELLS

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The heterodimeric cytokine IL-27 is a member of the IL-12 family of cytokines. It mediates its effects by signaling through a heterodimeric receptor consisting of IL-27RA and gp130. IL-27RA gene-knockout mice mount accelerated Th2 responses and transiently delayed Th1 responses to immune challenges, suggesting that IL-27 has a role in the polarization of CD4 T cell responses. Paradoxically, although IL-27RA knockout mice can mount Th1 responses capable of clearing certain intracellular pathogens, they ultimately succumb to a lethal inflammatory immune response. This suggests that IL-27 is also involved in immune regulation; however, it remains unclear whether some aspects of the IL-27RA knockout immune phenotype may be attributed to developmental defects. We have investigated the mechanisms whereby IL-27 mediates its effects both *in vitro* and *in vivo*. We have found that IL-27 activates Stat1 and Stat3, induces expression of T-bet and the IL-12 receptor and inhibits expression of GATA-3 by CD4 T cells *in vitro*, suggesting that this may be the mechanism whereby IL-27 promotes the development of T helper type 1 (Th1)-polarized CD4 T cell responses. However, we have also observed *in vitro* that IL-27 enhances the development of CD4 T cells capable of producing IL-10, an immunoregulatory cytokine, *via* an as-yet undefined mechanism. We have additionally examined the effects of IL-27 over-expression *in vivo* in both *IL27*-transgenic mice and mice injected with IL-27 expression-vectors. IL-27 over-expression correlated with significant alterations in immunoglobulin isotypes and immune cell populations and, in the transgenics, multi-organ inflammatory infiltrates and increases in inflammatory cytokines. We also observed significant alterations in regulatory ($CD4^+FoxP3^+$) T cells, which were most dramatic in *IL27*-transgenic mice that had the highest levels of transgene expression and severity of immune pathology. Taken together, our data demonstrate that IL-27 has pleiotropic activities that together promote Th1-development and inflammation.

06-27/O

G1P3 (ISG 6-16), AN INTERFERON STIMULATED SURVIVAL FACTOR ANTAGONIZES TRAIL/APO2L INDUCED APOPTOSIS IN MYELOMA CELLS

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Although interferons (IFNs) have therapeutic activity against multiple myeloma (MM) in clinical settings, their clinical usefulness is still a matter of debate. TRAIL/Apo2L is one of the potent and robust IFN-α2b induced proapoptotic genes in myeloma cells (>100 fold compared to control); however, < 20% of cells undergone apoptosis. We hypothesized that induction of an interferon-stimulated gene (ISG) with pro-survival activity might antagonize antitumor activity of TRAIL. Consistent with this, IFN-α2b antagonized TRAIL induced apoptosis and increased myeloma cell viability at early time points (24 hr-36 hr). IFN-α2b stabilized mitochondria antagonizing the intrinsic apoptotic pathway induced by TRAIL through mitochondrial perturbation resulting in caspase-3 activation and apoptosis. During expression array studies, G1P3 (ISG 6-16) was identified as one of the highly expressed ISGs; therefore, we evaluated its role in myeloma cell survival. Like TRAIL, G1P3 mRNA (encodes a ~13 kD protein) was highly up-regulated by IFN-α1, IFN-α2 and IFN-β in all myeloma cell lines tested. Both endogenous and ectopically expressed G1P3 localize in mitochondria. G1P3 specific siRNA sensitized myeloma cells to IFN-α2b induced apoptosis suggesting a pro-survival role for G1P3. Consistent with this, ectopic expression of G1P3 antagonized TRAIL mediated mitochondrial perturbation, caspase-3 activation and apoptosis in myeloma cells. Overall, our data demonstrate a direct role for an ISG with survival function antagonizing the effect of TRAIL, providing a basis for improving the therapeutic efficacy of IFNs. Furthermore, the functional significance of G1P3 (ISG 6-16), one of the first ever identified ISGs, has finally begun to be clarified.

06-28/P

IL-31 AND OSM SUPPRESS PROLIFERATION OF LUNG EPITHELIAL CELLS: IDENTIFICATION OF RECEPTOR SPECIFIC SIGNALING MECHANISMS

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Interleukin-31 (IL-31), a T-cell derived IL-6-type cytokine, is the most recent member of the hematopoietin family. It has been shown to induce pruritis and dermatitis in transgenic mice and enhances airway hypersensitivity. IL-31 signals through the heteromeric receptor complex of IL-31Rα and oncostatin M receptor-β (OSMRβ), and activates STAT-1, -3 and -5. It is hypothesized that the cytoplasmic domains of the receptor subunits, through phosphorylation of their tyrosine residues, define the signaling specificity of IL-31Rα. This hypothesis is tested in this study by determining the effects of tyrosine-to-phenylalanine mutations in the IL-31Rα subunit, on signal transduction, gene regulation, proliferation, and cellular morphology. We selected lung epithelial cells as a model system, as they are positive for IL-31Rα and OSMRβ. The lung epithelial A549 cell line responds to IL-31 by phosphorylation of STAT3 to ~5% of OSM level. These cells have been converted to high responsiveness by stable transduction with an expression vector for the wild type or mutated forms of the IL-31Rα. In clonal lines with defined expression of receptor proteins, we show that signaling specificity of IL-31 involves activation of STATs, ERK, JNK, transcriptional induction of fibrinogen genes, and a particularly strong recruitment of Akt and Hsp27 pathways. IL-31 proved to be exceptionally effective in suppressing proliferation, as compared to OSM. It caused a partial arrest of the cells in the G1 phase of the cell cycle. In addition, IL-31 also brought about significant changes in cellular morphology by reducing cell-cell adhesion. Interestingly, mutation of the single STAT3-binding site on IL-31Rα significantly reduces both growth inhibition and altered morphology. The cell lines generated will be used to define the still unknown mechanisms by which IL-6 type cytokines, including IL-31, suppress proliferation and affect morphology of epithelial cells.

06-29/O

THE COMBINATION OF ITMN-191, AN ORALLY ACTIVE INHIBITOR OF THE HCV NS3/4A PROTEASE INHIBITOR, AND PEG-INTERFERON ALFA-2A RESULTS IN SYNERGISTIC ANTIVIRAL EFFECTS *IN VITRO*

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Background: In recent years, the discovery and early clinical development of HCV protease inhibitors has advanced to demonstrate potent antiviral effects of this class of moelcule both in preclinical systems (HCV replicons) as well as in clinical trials in chronic Hepatitis C patients. Current standard of care for chronic Hepatitis C patients involves the use of Pegylated IFN-alpha in combination with the nucleoside analog ribavirin. Addition of HCV protease inhibitors to the current standard of care is the most likely evolution of the family of protease inhibitors as montherapy would result in rapid evolution of drgu resistance. We have developed a macrocylic inhibitor of the HCV NS3 protease call IATMN-191. ITMN-191 is a highly potent, orally absorbed inhibitor of the NS3/4A protease that concentrates in the liver of rats and cynomolgus monkeys at levels suggestive of human efficacy. This compound is currently undergoing preclinical development. The objective of this study was to evaluate the *in vitro* antiviral activity of ITMN-191 in combination with peginterferon alfa-2a (Pegasys®). *Methods:* Two replicon systems were used for anti-HCV drug-drug interaction study. A HCV genotype-1b replicon (K2040) and a derivative of K2040 with reduced sensitivity to

ITMN-191 (R191M320). Drug-drug interaction data were analyzed by the Loewe additivity model and the Bliss independence drug interaction model[1,2]. *Results/Conclusions:* The combination of ITMN-191 and PEG-IFN alfa-2a synergistically inhibited HCV RNA replication in Huh7 cells. Analysis of fixed dose ratios by the Loewe additivity model showed that combination of the two drugs yielded combination index (CI) values indicative of synergy at EC_{50}, EC_{75} and EC_{90} (0.3, 0.4 and 0.4, respectively) and isobologram analysis supported synergistic interaction. The drug reduction index (DRI) values were >1, reflecting a potential clinical benefit when ITMN-191 when administered in combination with PEG-IFN alfa-2a. Analysis of variable ratio drug combinations by the Bliss independence model indicated that the synergy volumes observed in ITMN-191 combinations were significant (>50uM^2). Peak synergy volumes occurred at low concentrations, all of which may be therapeutically relevant. The extent of synergy observed by either method is quantitatively larger than that observed for the experimental HCV protease inhibitors VX-950 and SCH 503034. Importantly, the human minimum plasma concentration (C_{min}) of PEG-IFN alfa-2a greatly improved ITMN-191 potency in a replicon fully sensitive ITMN-191 and in replicon with reduced sensitivity to ITMN-191. Additionally, the replicon with reduced sensitivity to ITMN-191 was hypersensitive to PEG IFN alfa-2a relative to the parental replicon (>10 fold). In conclusion, these data suggest the combination of ITMN-191 and PEG-IFN alfa-2a may yeild virologic response in HCV patients supperior to that of PEG-IFN alfa-2a alone or ITMN-191 alone. Future clinical study is warrented to adress this hypothesis.

References

1. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enz Regul* 1984; 22: 27-55.
2. Prichard MN, Prichard LE, Shipman CJ. Strategic design and three-dimensional analysis of antiviral drug combinations. *Antimicrob Agents Chemother* 1993; 37: 540-5.

06-30/P

THE EFFECT OF SCHEDULING ON THE PHARMACODYNAMIC MARKERS OF MURINE INTERFERON-GAMMA IN MICE

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To investigate whether sustained interferon-gamma serum concentrations might increase therapeutic benefit, or might lead to adverse effects (e.g., down-regulating interferon-gamma-regulated genes due to feedback inhibition), we studied the effect of two dosing regimens using murine recombinant interferon-gamma (rMuIFN-gamma) in CD-1 mice. Groups were treated for two weeks with either 100 μg/kg subcutaneous injections of rMuIFN-gamma given three times a week (TIW), or were implanted with subcutaneous continuous infusion pumps that delivered rMuIFN-gamma at ~1.78 μg/kg/hr. In both arms, the total dose administered was 600 μg/kg. Sera were analyzed to determine rMuIFN-gamma concentrations and define its pharmacokinetics. Pharmacodynamics were established through monitoring various CXC chemokines: IP-10, ITAC, and MIG, as well as IL-6. We found all biomarker concentrations depended on rMuIFN-gamma levels, after a short delay. The TIW dosing regimen gave large peak-to-trough fluctuations, attaining maximal concentrations shortly after dosing, and then declining to undetectable levels before the next dose; continuous infusion resulted in sustained biomarker levels. For the first time, we show that continuous rMuIFN-gamma infusion does not down-regulate crucial CXC chemokines; rather the sustained serum rMuIFN-gamma concentration results in sustained biological responses in the mouse, suggesting that a long-acting form of interferon-gamma administered over two weeks may not cause tachyphylaxis, and may have clinical benefit.

06-31/P

INTERFERONγ CAN ACTIVATE AP-1 FOR STAT1-INDEPENDENT GENE TRANSCRIPTION

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The biological responses of cells to interferon(IFN)-γ are mediated by the several hundreds of genes whose transcription it modulates. Many of these genes are directly regulated by the activation of Janus kinases (JAKs) that trigger DNA binding of Signal transducer and activator of transcription (STAT)-1. However, IFNγ retains some functionality in STAT1-/- animals and about 1/3 of interferon stimulated genes (ISGs) are still induced in STAT1 cells. The molecular mechanisms of STAT1-independent biology and gene transcription in response to IFNγ are currently unknown. We have found that IFNγ rapidly and transiently activates AP-1 binding transcription factors. IFNγ-induced AP-1 DNA binding activity could be detected in cells lacking expression of JAK1, JAK2 or STAT1 suggesting that this pathway contributes to STAT1-independent transcription of ISGs. AP-1 DNA binding was critically dependent on expression of c-jun and therefore we studied expression of ISGs in c-jun-/- cells. The induction of several ISGs, including IFI-205, RANTES and iNOS, was impaired in IFNγ-treated c-jun-/- cells, but others, such as IP-10 and SOCS3, were unaffected. Consistent with these observations, chromatin immunoprecipitation demonstrated that c-jun was enriched on the iNOS promoter, but not the c-jun independent SOCS3 promoter following treatment with IFNγ. We also investigated the role of mitogen activated protein (MAP) kinase pathways in IFNγ-induced AP-1 activation. Our data excludes the involvement of c-Jun N-terminal kinase and p38 MAP kinase, but inhibitors that target the extracellular regulated kine (ERK) pathway inhibited phosphorylation of c-Jun and AP-1 DNA binding. These data suggest that IFNγ activates a pathway involving ERK MAP kinases leading to activation of AP-1 DNA binding. This pathway acts in parallel with the JAK/STAT pathway, affects the transcription of a subset of ISGs and may partially explain how IFNγ can act absence of STAT1.