Instituto Juan March
de Estudios e Investigaciones

142 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Control of NF-κB Signal Transduction in Inflammation and Innate Immunity

Organized by

M. Karin, I. M. Verma and J. Moscat

Y. Ben-Neriah
B. Beutler
V. M. Dixit
M. Fresno
T. Ganz
G. Ghosh
G. Haegeman
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G. Natoli
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Introduction
Jorge Moscat

The mechanisms controlling NF-κB signaling constitute a paradigm of the membrane-nucleus cell communication. NF-κB is central to a number of cellular functions including cell proliferation, apoptosis and cellular differentiation. Also this transcription factor plays essential roles in innate and acquired immunity and its deregulation leads to important human diseases such as inflammation and cancer. NF-κB is formed by members of the Rel family of transcription factors, and are retained in the cytosol by the inhibitory molecule IκB. Upon activation by inflammatory cytokines, IκB is phosphorylated, ubiquitinated and degraded by the proteasome system which leads to the release and nuclear translocation of the classical RelA/p50 NF-κB. A complex formed by IKKβ and IKKγ is responsible for the phosphorylation of IκB in response to TNFα and IL-1. Genetic evidence in mice demonstrates the essentiality of these different components of the classic paradigmatic pathway. However, parallel to this cascade there is another pathway that starts with the activation of IKKα that phosphorylates NF-κB2 (p100) that leads to its cleavage and the release of the p52 subunit which, together with RelB, constitutes a second NF-κB transcriptional complex that presumably targets genes different from those responding to RelA/p50. Again, genetic evidence from knock out mice has demonstrated that both cascades are critical and independent. An important missing piece of this puzzle was the identification of the cytokines that trigger this novel non-canonical IKKα/NF-κB2 pathway. Data presented in this workshop demonstrate that BAFF, for B cells, and LT-β, for fibroblasts, activate this new pathway through an IKKγ-independent mechanism. These exciting evidences explain, at least in part, the immunological phenotype of the IKKα mutant mice and open new avenues for therapeutic intervention in diseases such as rheumatoid arthritis, asthma or some form of diabetes. In addition, the observation that the mutant IKKα mice display impairment in the proliferation of mammary gland epithelial cells, suggests that inhibitors of this kinase could be useful drugs in the treatment of breast cancer. In fact, results were presented in this meeting demonstrating that the incidence of tumors caused by the *neu* oncogene was severely inhibited in IKKα mutant mice. Mutations in the IKKγ gene have been associated with a human disease called Incontinentia Pigmenti. Strikingly, another human genetic disease has been linked to mutations in these pathways. Thus, mutations in IκBα that prevent its degradation are associated with a disease called Anhidrotic Ectoderma Dysplasia that show an impairment in T cell function as well as in the response to activation through the Toll system.

The genetic inactivation of the different NF-κB subunits gives rise to alterations in the function of B and T cells. Interestingly, knock out mice for the PKC isoforms ζPKC and θPKC display defects in B and T cells, respectively. In the case of ζPKC and B cells, the role of this kinase in the control of apoptosis may account for the phenotypic alterations detected in the ζPKC mutant mice. In the case of T cells, a novel pathway involving not only θPKC but also the adapters Bcl10 and MALT-1 was presented. The connection here with cancer is evident. Mutations in both Bcl-10 and MALT-1 genes are associated with lymphomas. On the other hand, the involvement of NF-κB in the acquired as well as the innate immune response is clear. In this regard, much work has been done in the model system of *Drosophila* where both the classical and the non-canonical pathways exist. However, although some components are conserved, others are not, indicating that sometimes findings in flies cannot be automatically translated to mammals and vice versa.

The last NF-κB workshop organized in the Juan March Foundation was in 1996. In the last six years our understanding of the pathway has increased spectacularly. New genes have been identified and knock out mice for virtually all the proteins of these cascades have been

generated, which allow us to know their function not only in cell cultures but also in a whole organism. In addition, the crystallographic study of the structures of at least some of the proteins of these pathways provides details of the intricate interactions that take place. In summary, much has been accomplished but more questions aroused in the intense and fruitful discussions that took place. Above all, it is important to understand the biology behind these fundamental cellular mechanisms but also important is to take advantage of its powerful therapeutic potential. Examples of this were presented. In the forthcoming years we will witness fabulous changes in the way patients are treated. This will only be possible with an exhaustive understanding of the signaling cascades operating under normal conditions, and subverted under the different pathologies.

Jorge Moscat

Session 1: Biochemical, functional and structural aspects of NF-κB
Chair: Michael Karin

Modes of NF-κB activation and downstream gene networks in oncogenesis and innate immunity

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Transiently activated NF-κB transcription factors are central regulators of the adaptive and innate immune response. However, under various pathological conditions, an aberrant persistent activation, refractory to the normal homeostatic control, represents a critical event for disease progression. NF-κB proteins provide a broad pathogenetic potential, based on the diverse processes controlled, such as inflammatory cytokine production, cell-cycle progression and apoptosis. Subgroups of lymphoma and other neoplastic diseases reveal constitutively activated NF-κB and constitutive activity of the NF-κB stimulating IκB kinase (IKK) complex. Constitutively activated NF-κB results in resistance to apoptosis, and enhanced proliferation and tumorigenicity. A genome-wide identification of NF-κB regulated genes in lymphoma cells with constitutive activation and in lipopolysaccharide stimulated pre B cells by high density microarrays has been undertaken to dissect pro-oncogenic and innate immune response gene networks. A the wide range of inducing agents activates the canonical IKK-IκBα and IKK-p105 pathway. A secondary, p100 dependent pathway will be described that is activated by a subset of cytokines and pathogens.

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Insights into function and regulation of NF-κB in B cell development

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NF-κB1 and NF-κB2 proteins are essential for full maturation of B cells. B cells lacking proteins encoded by these two loci are completely blocked from progressing past the early transitional (T1) stage of B cell development in spleen, the stage representing recent immigrants derived from bone marrow. In mice lacking the TNF family member BAFF (BLyS), B cells are blocked from progressing past the T1 stage as well. Transgene-mediated over-expression of BAFF in mice on the other hand leads to increased amounts of B cells and autoimmunity. Based on the similarities in phenotypes of NF-κB1/2 and BAFF knockout mice, we have investigated whether NF-κB may be a critical functional target of BAFF in B cells.

We have determined that BAFF can activate NF-κB activity in maturing and mature B cells, largely dependent on processing of the p100 form of NF-κB2 to p52. Processing not only generates p52 subunits but it also relieves the IκB-like inhibitory activity of p100, thereby inducing nuclear translocation of NF-κB complexes. Insights into the mechanisms underlying processing will be discussed. We demonstrate that this alternative activation pathway helps maturing B cells to progress, at least in part due to prolonged survival. Though important, NF-κB2 and its processing are not absolutely required in vivo, where a compensatory mechanism appears to partially overcome loss of NF-κB2. BAFF-induced processing contributes to progression and survival of maturing B cells at a stage when only a small fraction of such cells is positively selected into the long-lived, recirculating pool of mature B cells. Therefore, processing in response to BAFF may play an important role in regulating B cells homeostasis and specifically, in setting thresholds during enforcement of self-tolerance and in the shaping of the select repertoire of B cell specificities released into the periphery.

NF-κB regulation by IκB

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The inhibitor kappaB (IκB) proteins regulate transcriptional activities of NF-κB dimers by forming stable complexes with NF-κB. It was originally thought that these IκB/NF-κB complexes are inhibited simply because of their inability to enter the nucleus. It was proposed that the nuclear localization sequences (NLSs) of NF-κB dimers are masked by IκB thereby sequestering them in the cytoplasm. We have used structural, biochemical and cell-based experiments to investigate the molecular mechanism by which both classical IκB proteins such as IκBα and IκBβ, and non-classical IκB proteins such as p105 modulate sub-cellular localization of NF-κB. We find that in all binary IκB/NF-κB complexes, the IκB molecule masks only one NF-κB NLS whereas the second NLS remains free. This explains why IκBα/NF-κB complexes are dynamic and, shuttle between the cytoplasm and nucleus. Although binary IκBβ/NF and p105/NF-κB complexes also contain an exposed NLS, they are cytoplasmic. Cytoplasmic sequestration of these complexes requires accessory proteins. Association of these IκB complexes with accessory proteins ensures that NF-κB activation from various IκB/NF-κB complexes would require distinct signaling.

Regulation of NF-κB functions

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NF-κB activity is involved in regulation of many biological and pathological processes. Stimulation and activation of NF-κB depend on a complex regulatory network of associated inhibitors and coactivators under the control of kinases and proteases. IKK1 and IKK2 are key kinases in signal-induced phosphorylation of IκB proteins and subsequent NF-κB activation. We investigated the role of IKK1 and IKK2 in the NF-κB activation pathway by utilizing knockout mice lacking the IKK1 and IKK2. The global gene expressions induced by IKK1 and IKK2 were examined with microarray analysis and proteomics approach. We identified the genes specifically regulated by IKK1 in skin and their functions in keratinocyte differentiation are studied. We are also interested in NF-kB function in cell proliferation and death and its roles in tumor development. Chemotherapeutic agents are known to simultaneously induce the transcription factors p53 and NF-κB. To investigate a potential link between these opposing pathways, we are analyzing the p53 response in $IKK1^{-/-}IKK2^{-/-}$ MEFs. Our results uncover distinct functions of the highly homologous kinases IKK1 and IKK2 in regulating p53 stability and suggest a mechanism for the acquisition of resistance to chemotherapeutic agents, which activate both NF-κB and p53 in the absence of mutations in p53.

We have explored NF-κB signaling pathway in other species. Apparently, NF-κB cascade signaling is essential for vertebrate development. Deficiency in the expression of IKK and/or NF-κB proteins is involved with aggressive phenotypes like liver degeneration, skin and skeletal defects and hematopoiesis abnormalities. We have identified and partially characterized the majority of NF-κB homologs in zebrafish. Using appropriate techniques for the over-expression or "knock-down" these genes, we hope to identity accurately the role of these genes in the organogenesis and development in vertebrates. We will also discuss the identification of additional members of IKK complex involved in NF-κB activation.

In vivo analysis of NF-κB function by conditional mutagenesis

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Upon activation, members of the NF-κB transcription factor family induce the expression of many genes that are critically involved in cell survival and proliferation and in the regulation of immune and inflammatory responses. Activation of NF-κB by proinflammatory signals is mediated by the IκB kinase (IKK) complex, which contains two catalytic subunits termed IKK1(α) and IKK2(β) and a regulatory protein named NF-κB Essential Modulator (NEMO) or IKKγ. Targeted disruption of the gene encoding IKK1 revealed that this kinase is not essential for NF-κB activation by inflammatory cytokines but has a novel function in the induction of keratinocyte differentiation. Mice lacking IKK2 or NEMO die in utero displaying massive liver degeneration, a phenotype similar to that observed in p65/RelA knockout mice. Studies in mouse embryonic fibroblasts showed that NF-κB activation in response to proinflammatory signals is strongly reduced in the absence of IKK2 and is completely abolished by the lack of NEMO.

To investigate the function of IKK-induced NF-κB activation *in vivo*, we have used the Cre/loxP technology to generate mice carrying conditional mutations in the genes encoding IKK2 and NEMO. In order to study the function of NF-κB in the epidermis we have generated mice lacking IKK2 specifically in epidermal keratinocytes. Mice with epidermis-specific deletion of IKK2 develop a severe inflammatory skin disease, which is caused by a TNF mediated, αβ T cell-independent inflammatory response that develops in the skin a few days after birth. These results show that IKK2-mediated NF-κB activation in the epidermis control mechanisms that are critical for the maintenance of immune homeostasis in the skin. Ongoing experiments addressing the mechanisms of NF-κB function in different tissues of the adult mouse by using conditional targeting of IKK2 and NEMO will be discussed.

Session 2: NF-κB signaling - I
Chair: Jorge Moscat

Processing and function of NF-κB precursor proteins

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Mammalian cells express five NF-κB members, RelA, RelB, c-Rel, p50, and p52, which play both overlapping and specific roles in regulation of immune responses and many other biological processes. A unique feature of p50 and p52 is that they are produced through processing of precursor proteins, p105 and p100. The NF-κB factors are normally sequestered in the cytoplasm through physical association with IκBs as well as the p100 and p105 precursor proteins. The canonical pathway of NF-kB activation is triggered through IkB phosphorylation by the IKK signalsome, which is composed of IKKa, IKKb, and the adaptor protein IKKg or NEMO. This pathway typically leads to nuclear expression of p50/RelA and p50/c-Rel NF-kB heterodimers. However, the nuclear expression of p52 and RelB appears to rely on a noncanonical signaling pathway involving the processing of NF-kB2/p100.

We have previously shown that the processing of p100 is tightly suppressed by its C-terminal sequences. Active processing of p100 can be stimulated by the NF-kB inducing kinase (NIK), which acts by triggering phosphorylation and ubiquitination of p100 (Xiao et al., 2001). Subsequent studies suggest that IKKa, but not IKKb or IKKg, is required for NIK-induced p100 processing (Senftleben et al., 2001), thus suggesting the presence of a different IKK complex in mediating the noncanonical NF-kB signaling pathway. While the physiological processing of p100 occurs primarily in B cells, T cells transformed by the human T-cell leukemia virus (HTLV) exhibits high levels of p100 processing activity (Xiao et al., 2001). Interestingly, the HTLV-encoded Tax protein induces p100 processing by physically recruiting IKKa (not IKKb) to p100. Thus, a p100/Tax/IKKa complex can be readily detected in HTLV-infected T cells. Since overexpression of p52 is associated with lymphoid hyperplasia and lymphoma formation, the Tax-mediated abnormal processing of p100 may play a role in HTLV-induced T-cell malignancies.

Our recent studies focus on identification of additional factors regulating p100 processing. Using siRNA-mediated gene suppression technique, we demonstrated an essential role for β-TrCP in the ubiquitination and processing of p100 (Fong and Sun, 2002). By yeast two-hybrid screening, we have identified a novel factor physically interacting with NIK. Preliminary studies suggest that this factor is involved in both p100 processing and IkBa degradation. These new findings, as well as our recent studies on p105, will be discussed.

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Transcriptional activation of inflammatory genes requires a dual signalling pathway

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Many inflammatory gene promoters contain κB-responsive elements, which are absolutely required for their induction by inflammatory stimuli, such as TNF. Upon treatment of mouse fibroblast cells with TNF, the transcription factor NF-κB is released from its cytoplasmic complex, migrates to the nucleus and binds onto the κB sites of various cellular promoters. By mutation analysis of the IL6 promoter, we have shown before that NF-κB is the only transcription factor, which is responsive to TNF and, hence, absolutely necessary for its transcriptional activation by TNF.

Upon inhibition of the the ERK and p38 MAP kinases, which are also activated by TNF, induction of the IL6 gene is abrogated, but not the cytoplasmic activation of the transcription factor NF-κB, nor its DNA-binding capacity. From this we have concluded that phosphorylation of NF-κB by MAP kinases may represent an additional, but necessary signal to fine-tune inflammatory gene expression, and thus to codetermine the transactivation capacity of the induced transcription factor.

The NF-κB subunits, p50 and p65, are, however, not a direct substrate for TNF-induced phosphorylation by MAP kinases; in contrast, the p65 subunit can be specifically phosphorylated *in vitro* and *in vivo* by 'mitogen- and stress-stimulated kinase' 1 (MSK1), which is a nuclear kinase, activated by and located downstream of both the ERK and p38 MAP kinases. MSK1 phosphorylates NF-κB p65 at Ser position 276, which is a crucial position for NF-κB transcriptional activity in fibroblast cells. Upon TNF stimulation, MSK1 associates with NF-κB p65, as follows from co-immunoprecipitation experiments and ChIP analysis; however, this TNF-induced association is abrogated, when the cells were treated with inhibitors of the ERK and p38 MAP kinases together, or by inhibition of MSK1. Finally, in MEF cells from $MSK^{-/-}$ animals, in which the various MAP kinases pathways as well as the cytoplasmic activation of NF-κB have remained unaffected, the TNF-induced transactivation potential of NF-κB is completely gone and inflammatory gene expression is severely decreased.

Therefore, we conclude that in mouse fibroblast cells inflammatory gene expression is effected by a dual signalling pathway; i.e. cytoplasmic activation of the transcription factor complex NF-κB and concomitant phosphorylation of the transactivating subunit p65 by the nuclear kinase MSK1 at position Ser 276. Since MSK1 has already been described to also phosphorylate histon 3 tails, this is to our knowledge the first report indicating a nuclear kinase that phosphorylates the driving transcription factor as well as its neighbouring chromatin environment.

IKKi: a key integrator of transcriptional regulation at the crossroads of pro- and anti-inflammatory signaling

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IKKi is an IKK2-related kinase that is transcriptionally regulated in response to proinflammatory stimuli. Initial reports suggest that IKKi plays a role in the "core" signaling pathway that regulates NF-kB activation. In an effort to better elucidate the cellular function of IKKi we created stable cell lines that inducibly express either a wildtype or kinase inactive version of IKKi. Using this system we demonstrate the capacity of IKKi to modulate the expression of known NF-kB regulated genes in a stimulus and temporal-specific manner. These results were confirmed in IKKi -/- mouse embryo fibroblasts. Contrary to previous studies, IKKi appears to function independent of the "core" pathway leading to NF-kB activation. Moreover, we provide results that indicate IKKi functions in a novel capacity to integrate cross talk between pro- and anti-inflammatory signaling pathways.

Mechanisms underlying specificity in NF-kB-regulated transcription

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A major issue in the regulation of NF-kB-dependent transcription is how specificity is achieved. We have proposed that one regulatory mechanism operates at the chromatin level by modulating the state of accessibility of NF-kB sites in target genes. In LPS-stimulated macrophages and dendritic cells this mechanism generates two distinct waves of NF-kB recruitment to target promoters: a fast recruitment to constitutively and immediately accessible (CIA) promoters and a late recruitment to promoters requiring stimulus-induced modifications in chromatin structure to make NF-kB sites accessible (promoters with regulated and late accessibility, RLA). NF-kB is rapidly recruited to every CIA promoter independently of the stimulus; moreover, transfected NF-kB subunits can be recruited to these promoters in the absence of any stimulation, thus suggesting a constitutive accessibility of the NF-kB sites. Conversely, NF-kB recruitment to RLA promoters occurs in a stimulus-specific manner, being individual stimuli intrinsically different in their ability to activate the signaling pathways that modulate NF-kB access to chromatin. Strong and sustained p38 MAP kinase activation is required to enhance the accessibility of a subset of NF-kB sites, thus indicating that a major function of p38 is to modulate NF-kB recruitment to selected chromatin targets in a promoter-specific fashion. Specificity also depends on the ability of individual NF-kB dimers to differentially activate transcription from distinct target genes. The mechanisms underlying transcriptional specificity and redundancy among various dimers will be discussed.

BR3, a receptor for BAFF, is a physiological activator for NF-kB2 in B lymphocytes

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BAFF, a member of the TNF-ligand family, activates NF-kB, is a potent survival factor for B cells, and has been implicated in systemic lupus erythematosus. It binds three members of the TNF-receptor family expressed on B cells: TACI, BCMA, and BR3/BAFF-R. We show that BR3/BAFF-R signalling promotes processing of NF-kB2/p100 to p52, a transcription factor critical for B cell maintenance. NF-kB2/p100 processing was abrogated in B cells from A/WySnJ mice that possess a mutant BR3 gene, but not in TACI- or BCMA-null mice. Furthermore, basal activation of NF-kB2/p100 in wild type animals was dramatically reduced by injecting a BAFF-neutralizing BR3-Fc chimeric protein. Selective activation of BR3 induced NF-kB2/p100 processing in B cells. This correlated with the ability of activated BR3 to rescue B cells from anti-IgM-induced apoptosis, a model for deletional self-tolerance. Remarkably, when NZB/WF1 mice that develop a fatal lupus-like syndrome were briefly treated with BR3-Fc, NF-kB2 processing was inhibited and the disease process attenuated. Since inhibiting the BR3-BAFF interaction has therapeutic ramifications, a structural analysis of the ligand-binding interface within BR3 was undertaken by NMR spectroscopy. The BAFF-binding site was contained within a discrete 26-residue core. When stabilized within a b- hairpin scaffold, six of the core residues were sufficient to confer binding to BAFF.

Session 3: NF-κB signaling - II
Chair: Inder M. Verma

IKK – A master regulator of innate and adaptive immune responses

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The IκB kinase (IKK) complex is composed of 3 subunits: IKKα, IKKβ, and IKKγ. IKKα and IKKβ, the catalytic subunits, display a high degree of biochemical and structural similarity, both functioning as IκB kinases *in vitro*. The physiological function of the different IKK subunits and the reason for duplication of the catalytic subunits was probed by gene disruption and knockin experiments. At their outset, these experiments demonstrated a critical function for IKKβ in activation of NF-κB in response to a large number of proinflammatory stimuli, including TNFα, IL-1, dsRNA, LPS and ISS-DNA. IKKβ is also essential for prevention of TNFα induced apoptosis and is indispensable for activation of innate immune responses. IKKβ is required for suppressing the apoptosis of TLR4-activated mouse macrophages and is an essential mediator of acute inflammatory response and tissue protection following exposure to certain physical stresses. All of these IKKβ- specific functions depend on IκB phosphorylation and degradation and are mediated through the canonical NF-κB activation pathway. By contrast, the biological functions of IKKα were found to be rather complex and perplexing. Although IKKα was found not to be required for activation of the canonical NF-κB pathway in response to proinflammatory stimuli, it is essential for skin and bone morphogenesis. The role of IKKα in epidermal differentiation, however, does not depend on its protein kinase activity nor on NF-κB. Recently, IKKα was found to have a second function – being required for activation of a second NF-κB pathway based on the processing of NF-κB2/p100 to p52. This function does depend on the kinase activity of IKKα and seems to be triggered only by select members of the TNF family. This function is required for adaptive immune responses and proper organization and development of lymphoid organs. It appears that the most critical function of IKKα in this context is exerted within the B lymphocyte compartment. A third function of IKKα that is also dependent on its kinase activity is in development of the mammary gland. This function is exerted via the canonical NF-κB pathway (i.e. IκB degradation) but is not triggered by standard proinflammatory stimuli. In summary, duplication of the IKK catalytic subunits has enabled the assumption of diverse biological functions that are differentially dependent on IKKα and IKKβ.

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Novel aspects of the function of the NF-κB-inducing kinase (NIK)

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The NF-κB inducing kinase (NIK) is a MAP3K kinase originally identified by virtue of its ability to bind to TRAFs, adapter protein family that participate in the triggering of several MAP3K cascades by receptors of the TNF/NGF and Toll/IL-receptor families as well in response ER stress[1]. NIK was shown to bind specifically to IKK1, a component of the core IκB phosphorylating complex, and to phosphorylate and activate IKK1[2]. This finding, as well as multiple reports over the recent 5 years of the ability of over-expressed NIK mutants to block NF-κB activation by a wide range of different inducers suggested some role of this kinase in signaling for NF-κB activation. However, clear notions of the exact nature of this role and the exact signaling pathways in which NIK participate just begin to emerge. So far, the only receptor conclusively shown to involve NIK in its signaling is the lymphotoxin β receptor (LTβ-R)[3 4 5]. However, lymphocytes, in which NIK is prevalent, do not express the LTβ-R. Findings of novel interactors and of the functional role of NIK in hematopoietic cells will be presented.

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Signaling via the SCF$^{\beta\text{-TrCP}}$ ubiquitin ligase: lessons from the NF-κB and the Wnt activation pathways

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Modulation of protein stability via the ubiquitin-proteasome system emerges as one of the most common regulatory processes in all eukaryotes[1]. Signal transduction is an example of such processes, in which ubiquitination events may contribute to either activation or inhibition/termination of a signaling cascade. β-TrCP, one of many different specificity determining components (E3) of the ubiquitin system, represents a multi-purpose signaling mediator: by regulating the ubiquitination of three related substrates, IκB, p105 and β-catenin, the same E3 contributes to the activation of NF-κB and to suppression of the Wnt/β-catenin signaling cascade, respectively[2]. β-TrCP recognizes and adheres to its substrates via a short, doubly phosphorylated peptide motif, DSGXX(X)S[1]. This recognition motif, which is shared between the three substrates, is generated through the phosphorylation activity of two distinct kinase complexes, IKK and the Axin complex. Using various proteomic tools, we identified several components of the IKK- and Axin-regulated ubiquitination machineries [3,4]. We will compare the degradation process of the different β-TrCP substrates and discuss its implications for the relevant signaling pathways. One of the interesting questions concerns the identification of the rate limiting step in each degradation process, whether it is the protein phosphorylation, β-TrCP binding and/or the rate of ubiquitination. Preliminary experiments indicate that the rate limiting step may vary for the different substrates. β-TrCP inhibitors are considered as possible means of modulating NF-κB activation in inflammatory and proliferative diseases. Therefore, an understanding the common and distinct features of IκB, p105 and β-catenin degradation may help in designing therapeutic modalities based on the targeting of ubiquitin system components.

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Regulation of NF-κB activation and function by ζPKC

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A number of studies using dominant negative mutants have implicated the atypical PKC isoforms in the control of NF-κB activation. More recently, the availability of mice in which ζPKC was inactivated by homologous recombination allows a more definitive study of the implication of this kinase in the NF-κB pathway. The lack of ζPKC, in embryonic fibroblasts (EFs), severely impairs κB-dependent transcriptional activity as well as cytokine-induced phosphorylation of p65. In addition, a cytokine-inducible interaction of ζPKC with p65 was detected which requires the previous degradation of IκB. Interestingly, the ζPKC-/- mice although grossly normal, show phenotypic alterations in secondary lymphoid organs reminiscent of those of the TNF receptor-1 and of the lymphotoxin-β receptor gene deficient mice. These alterations were detected in very young (2-week-old) mice and were much less apparent in older animals. Thus, although the overall structure of the spleen was preserved in ζPKC-/- mice, there was a defect in the marginal zone together with smaller B cell follicles in the white pulp as compared with age-matched wild type controls. In addition, defects were observed in peripheral and mesenteric LNs as well as in the PPs in which there was an impaired segregation between B and T cell zones and a decrease in FDCs. In older (4-8 week-old) KO mice, the defects in the LNs nearly disappear and those in the PP, although still detectable were much less dramatic, indicating that the loss of ζPKC causes a delay but not a complete blockade in the delivery of signals required for the proper development of these lymphoid organs.

The development of lymphoid organs is controlled by a dynamic interplay between hematopoietic and nonhematopoietic cells. In this regard, young ζPKC-/- mice show a reduced relative percentage of B cells in peripheral and mesenteric LNs which was significantly enriched in an immature B cell population. In PPs, although the relative percentage of T and B cells was normal in the ζPKC KO mice, there was a reproducible increase in immature B cells. In young and adult ζPKC-/- mice the microarchitecture of the spleen and other secondary lymphoid organs is little or no affected. However, these animals are unable to mount an optimal immune response in vivo. Therefore, we investigated whether these alterations could be accounted for by defects in B cell function.

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The role of NF-κB in the regulation of innate and adaptive immunity to infection

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Although the NF-kB family of transcription factors are associated with the regulation of many functions of the innate and adaptive immune response relatively little is known about their actual role in resistance to infection. Infection with the opportunistic pathogen Toxoplasma gondii provides the opportunity to study the events that regulate innate responses (NK cell mediated) to infection and the subsequent development of protective adaptive responses (CD4 and CD8 cells). For this infection, the production of IL-12 by accessory cells is required for the production of IFN-g by NK and T cells which is necessary to control parasite replication. Using mice deficient in either NF-kB1, NF-kB2, C-Rel, RelB or which express the IkBΔN transgene in NK and T cells has revealed distinct roles for the individual family members in immunity to this pathogen. For example, mice deficient in RelB or which express the IkBΔN transgene have major defects in their ability to produce IFN-g and succumb at an early stage to this infection. In contrast, mice deficient in C-Rel have an early defect in their ability to produce IFN-g but eventually repair this defect but develop severe toxoplasmic encephalitis. Mice deficient in NF-kB1 or NF-kB2 develop strong protective immunity during the acute phase of this infection but appear to be unable to maintain this response and eventually succumb to the chronic phase of infection. Interestingly, one of the common features of the KOs of individual members is that rather than being involved in the decision to make Th1 or Th2 responses they appear to have a major block in their ability to proliferate and expand effector cell populations.

Session 4: NF-κB and immunity - I
Chair: Alain Israël

NF-κB signal transduction during bacterial and fungal infection

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A hallmark of the antimicrobial host defense in *Drosophila* is the challenge-induced massive synthesis of antimicrobial peptides by the fat body, a functional equivalent of the mammalian lines. The antimicrobial peptides are mostly small-sized, cationic, membrane-active molecules and exhibit various, sometimes overlapping, activity spectra at the micromolar range against bacteria and fungi. They contribute significantly to the resistance to infections, as illustrated by decreased survival rates of infected mutant flies deficient in antimicrobial peptides. In adult *Drosophila*, two NF-κB-like proteins control the transcription of the genes encoding these peptides : Dif (for dorsal-related immunity factor) and Relish. Dif is normally retained in the cytoplasm by binding to the Iκ-B-like inhibitor Cactus. Release of binding and nuclear translocation of the Rel homology domain occurs as a result of activation of the Toll transmembrane receptor. Relish, which has a C-terminal extension of ankyrin domain repeats (evocative of p105), requires a proteolytic cleavage for translocation of its N-terminal Rel-homology domain. This cleavage is dependent on activation of the Imd signaling pathway. Our information on the Toll and Imd pathways has significantly increased over the past two years and will be summarized in the presentation. Current efforts in our laboratory are centered around the link between sensing of microbial infections and activation of both pathways. We have now functionnally identified distinct proteins which activate Toll/ Dif during (i) infections by fungi and (ii) by Gram-positive bacteria and (iii) imd/Relish during Gram-negative bacterial infections. These results will be discussed in a phylogenetic perspective.

Signal transduction pathways activated by Toll-like receptors: a role for Mal in tailoring innate immunity

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Toll-like receptors belong to a superfamily of receptors, all of which possess a conserved domain in their cytosolic regions, termed the Toll/IL-1 receptor (TIR) domain. The TIR domain also occurs in the Type I IL-1 receptor and the IL-18 receptor. A key signal triggered by the TIR domain is NF-κB. There are 10 human TLRs and they are causing much excitement as they are one of the points of first contact between microbe and host during infection. TLR-4 is the long sought-for receptor for lipopolysaccharide from gram negative bacteria. TLR-2 recognises bacterial lipopeptides, peptidoglycan and zymosan from yeast. TLR-5 recognises flagellin, TLR-6 recognises mycobacterial lipopeptide, TLR-7 and TLR-8 recognise the anti-viral drug imiquimod and finally TLR-9 recognises CpG DNA. Common signalling pathways are activated by all of these, including NF-κB and the stress kinases p38 and JNK. These pathways are initiated by the recruitment of the TIR domain – containing adapter MyD88. A series of protein-protein interactions then ensue, involving the proteins Tollip, IRAK-4, IRAK, IRAK-2, TAB-2 and Traf-6. During this process, Traf-6 becomes ubiquitinated and interacts with the kinase TAK-1. TAK-1 associates with TAB-2, becomes ubiquitinated itself, and then activates MKK7, leading to JNK activation, or IKK-2 activation, leading to the activation of NF-κB.

A key question is whether receptors with TIR domains can generate specific signals and therefore tailor the innate immune response to the provoking pathogen. Evidence for this includes observations describing differences in the genes induced by TLR-2 and TLR-4 ligands in dendritic cells, and the observation that although IL-1RI, IL-18R, TLR-2 and TLR-9 signalling is completely impaired in cells from MyD88-null mice, that for TLR-4 is not. NF-κB and JNK are still activated in macrophages from these mice treated with Lipid A, although the responses are delayed. Importantly, LPS and Lipid A can still induce maturation of dendritic cells which are MyD88-deficient, and IRF-3 – dependent genes. It is these same genes that are specific for TLR-4. We have found another TIR domain adapter, named MyD88-adaptor-like (Mal), that may be the adapter responsible for the responses in MyD88-deficient cells. It acts to recruit IRAK-2 and associates only with TLR-4. Dominant negative forms of Mal only block TLR-4 signalling, having no effect on IL-1, IL-18, TLR-2 or TLR-9. Structural models of Mal and MyD88 in complexes with TLR TIR domains attest to the specificity of Mal for TLR-4. Mal may therefore allow TLR-4 to signal additional processes required for elimination of pathogens which are recognised by TLR-4. A possible role for Mal in the activation of IRF-3 has been proposed and we have preliminary evidence which suggests that the IRF-3 and NF-κB activation pathways may be closely linked, possibly at the level of the signalsome. These results will be discussed.

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Analysis of mutations in the study of the innate immune response

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Germline mutations have opened the door to understanding how innate immune sensing operates in Drosophila and in mammals. Both forward and reverse genetic methods have been applied in both species. Reverse genetic methods (in particular gene targeting) can prove informative if there is reason to suspect that a particular protein has a particular function. However, forward genetic methods can reveal function where none was suspected a priori. Toward this end, we have initiated a program of germline mutagenesis in mice, in which screens of innate immune function are used to detect both dominant and recessive mutations affecting key proteins in the innate immune response. The screens are of two types. First, the function of macrophages is examined ex vivo, testing their ability to respond to microbial inducers such as LPS, CpG-bearing DNA, peptidoglycan, flagellin, and other stimuli, their phagocytic potential, their production of reactive oxygen intermediates, and LPS tolerance. Second, the ability of animals to repel infection is studied. In this manner, mutations that affect critical immune functions are identified. Positional cloning can then identify these mutations. To date, more than 20,000 mutant mice have been produced, and more than 7,000 animals have been tested for innate immune competence by one of these types of screen. More than thirty phenodeviants have been identified, and two new transmissible mutations affecting LPS responses are currently being mapped meiotically. One of these, dubbed Lps2 because it presents a phenocopy of the classic Lps mutation, has been confined to a small chromosomal interval that is bereft of any genes that were previously known to be involved in LPS signaling.

Regulation of interleukin-1- and lipopolysaccharide-induced NF-κB activation by alternative splicing of MyD88

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MyD88 is an adaptor protein that is involved in interleukin-1 receptor (IL-1R) and Toll-like receptor (TLR)-induced activation of NF-κB. It is composed of a C-terminal Toll/IL-1R homology (TIR) domain and an N-terminal death domain (DD), which mediate the interaction of MyD88 with the IL-1R/TLR and the IL-1R associated kinase (IRAK), respectively. The interaction of MyD88 with IRAK triggers IRAK phosphorylation, which is essential for its activation and downstream signaling ability. Both domains of MyD88 are separated by a small intermediate domain (ID) of unknown function. We have identified a splice variant of MyD88, termed MyD88S, which encodes for a protein lacking the ID. MyD88S is mainly expressed in the spleen, and can be induced in monocytes upon LPS treatment. Although MyD88S still binds the IL-1R and IRAK, it is defective in its ability to induce IRAK phosphorylation and NF-κB activation. In contrast, MyD88S behaves as a dominant negative inhibitor of IL-1- and LPS-, but not TNF-induced NF-κB activation. These results implicate the ID of MyD88 in the phosphorylation of IRAK. Moreover, the regulated expression and antagonistic activity of MyD88S suggest an important role for alternative splicing of MyD88 in the regulation of the cellular response to IL-1 and LPS. Identification of the MyD88-ID binding kinase that is responsible for IRAK-phosphorylation is in progress, and will also be presented during the meeting.

Induction of human defensins by microbial stimuli

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Epithelia react to microbial pathogens by mounting a defensive response that includes the local production of antimicrobial peptides as well as the recruitment of neutrophil phagocytes that deliver additional antimicrobial peptides into the tissues. Evidence from several animal models makes a strong case for the role of antimicrobial peptides in host defense, since the ablation of the antimicrobial peptide response impairs local host defense against bacteria. We investigated the cellular pathways that induce the production of human β-defensin 2 (HBD-2) in the epidermis. In earlier studies we had shown that keratinocytes must be differentiated to acquire responsiveness to microbial or inflammatory stimuli, and that IL-1 was the most potent stimulus to HBD-2 production. In recent studies with organotypic epidermal cultures, E. coli lipopolysaccharide (LPS) was a weak direct inducer of human β-defensin-2 (HBD-2) mRNA and peptide but the induction was greatly amplified when monocyte-derived cells (MDC) acted as intermediaries between LPS and the epidermis. IL-1 receptor antagonist (IL-1 Ra) completely reversed the effect of MDC on epidermal HBD-2, indicating that, from among the many products of MDC, IL-1 was the dominant inducer of HBD-2 synthesis. In DNA microarray expression studies, HBD-2 was one the most abundant mRNAs induced in epidermis by LPS-treated MDC but the expression of most other genes was suppressed, and both these effects were also reversed by IL-1Ra. Thus epidermal response to LPS is potently amplified by MDC through IL-1 mediated signaling, leading to a selective increase in the synthesis of the antimicrobial peptide HBD-2. The inhibitor profile of IL-1-mediated HBD-2 induction indicates that multiple pathways of IL-1 signaling are involved. In the aggregate, these data support a key role for both IL-1 and HBD-2 in the host defense reaction of the epidermis.

Session 5: NF-κB and immunity - II
Chair: Jules A. Hoffmann

Genetic and biochemical analysis of the NF-κB signaling cascade

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The NF-κB family of transcription factors is critically involved in the immune, inflammatory as well as anti-apoptotic responses. Homo- or heterodimeric complexes of members of the NF-κB family are usually retained in the cytoplasm of non-stimulated cells by inhibitory molecules collectively referred to as IκB's. Activation of the NF-κB signaling cascade (through TNF, LPS, IL1 or multiple other stimuli) results in phosphorylation of the IκB's, followed by their degradation through the ubiquitin-proteasome pathway. Recently a major progress has been made as the kinases responsible for IκB phosphorylation have been identified. These kinases are part of a multisubunit high molecular weight complex, which also contains a structural/regulatory subunit, NEMO/IKKγ. We have recently demonstrated that mutations in the NEMO gene that abolish NF-κB activation lead to the human disease Incontinentia Pigmenti, which is characterized by prenatal male lethality and defects in the skin, nails, teeth and hair in affected females (2). More recently we have shown that milder NEMO mutations allow males to survive, allowing us to demonstrate that impaired but not abolished NF-κB signaling in humans results in two related syndromes which associate specific developmental and immunological defects (1). We have now identified a new mutation that affects another component of the NF-κB cascade.

Another pathway we have more recently begun to analyze is activation of NF-κB by the TCR. We have demonstrated that the IKK kinase complex is recruited to the T cell-APC contact region and to the immunological synapse after T-cell activation. This observation led us to evaluate the effect of targeting NEMO directly either to the TCR, or to the plasma membrane. I will present the results obtained in these 2 reconstituted systems. These studies offer a powerful mean of analysing the NF-κB cascade independently of the other TCR-induced activation pathways, and of separating the IKK-dependent from the IKK-independent events in this cascade.

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Regulation of Nuclear Factor κB transactivation in T cells

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Activation of the transcription factor NF-κB is controlled at two levels in resting T cells: an initial activation induced by the triggering of the TcR-CD3 complex and a second phase controlled by paracrine- or autocrine-secreted TNFα. The initial phase is regulated by p65 (RelA), whereas the second one is mainly dependent on c-Rel (1). A mutant clone, D6, derived from Jurkat T cells fails to activate NF-κB upon TNFα stimulation. This mutant clone showed a defect in the intermediate-late translocation of c-Rel to the nucleus promoted by TNFα stimulation, whereas early translocation is not affected. Activation or translocation of p65-containing complexes was not altered in this mutant clone. Sequencing of the c-Rel gene from this clone revealed a mutation of Ser-471 to Asn in the transactivation domain. The mutant S471N transactivation domain fused to the Gal4 DNA binding domain could not be activated by TNFα, unlike the wild type (2).

Furthermore, transactivation by c-Rel was dependent on phosphorylation of several serines in the transactivation domain, indicating that it is a phosphorylation-dependent Ser-rich domain. By Ser→Ala mutational and deletion analysis, we have identified two regions in this domain: 1) a C-terminal region (amino acids 540-588), which is required for basal activity; and 2) the 422-540 region, which responds to external stimuli as tumor necrosis factor (TNF) α or phorbol myristate acetate plus ionomycin. Ser from 454 to 473 were shown to be required for TNFα-induced activation, whereas Ser between 492 and 519 were required for phorbol myristate acetate plus ionomycin activation (3). Phosphatidylinositol 3-kinase (PI3K), protein kinase C (PKC) ζ and NIK were identified as downstream signaling molecules of TNFα-activation of c-Rel transactivating activity. We have identified the critical role of different Ser for NIK, PKCζ- and PI3K-mediated responses (3). Interestingly, c-Rel mutants in some of those Ser not only did not respond to TNFα but also acted as dominant negative forms of nuclear factor κB activation.

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Carma1 (Bimp3/CARD11) is a critical lipid raft-associated regulator of T-cell receptor-induced NF-κB activation

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Carma1 is a lymphocyte specific member of the membrane-associated guanylate kinase (MAGUK) family of scaffolding proteins which coordinate signaling pathways emanating from the plasma membrane. Via its caspase recruitment domain (CARD), Carma1 was recently shown to interact with Bcl10, an essential regulator of NF-κB activation and lymphocyte proliferation. Here we investigate the role of Carma1 in T-cell activation, and find that T-cell receptor (TCR) stimulation induces a physical association of Carma1 with the TCR and with Bcl10, which is paralleled by co-capping1of Bcl10 and Carma1 with the TCR complex. Carma1 is constitutively associated with lipid rafts, while cytoplasmic Bcl10 translocates into lipid rafts upon TCR engagement. A Carma1 mutant, defective for Bcl10 binding, has a dominant negative effect on TCR-induced NF-κB activation and IL-2 production, but does not affect Ca^{2+} mobilization nor interfere with the activation of the ERK, p38 and JNK pathways. Together, our data identify Carma1 as a critical lipid raft-associated regulator of TCR-induced NF-κB activation.

Bcl10 is a positive regulator of antigen receptor-induced activation of NF-kappaB

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Bcl10, a CARD-containing protein identified from the t(1;14)(p22;q32) breakpoint in MALT lymphomas, has been shown to induce apoptosis and activate NF-kappaB *in vitro*. We show that one-third of bcl10-/- embryos developed exencephaly, leading to embryonic lethality. Surprisingly, bcl10-/- cells retained susceptibility to various apoptotic stimuli *in vivo* and *in vitro*. However, surviving bcl10-/- mice were severely immunodeficient and bcl10-/- lymphocytes are defective in antigen receptor or PMA/Ionomycin-induced activation. Early tyrosine phosphorylation, MAPK and AP-1 activation, and Ca2+ signaling were normal in mutant lymphocytes, but antigen receptor-induced NF-kappaB activation was absent. Thus, Bcl10 functions as a positive regulator of lymphocyte proliferation that specifically connects antigen receptor signaling in B and T cells to NF-kappaB activation.

Recognition and signaling in *Drosophila* immunity

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The immune response in *Drosophila* and other insects has received much interest as a model for innate immune reactions (Hultmark 1993; Engström 1999; Imler and Hoffmann 2000; Khush et al. 2001). Several different signaling pathways are involved in the induction of the immune response in *Drosophila*. Best described are two pathways, both of which involve members of the Rel/NF-κB family. One of them, Relish, mediates the induction of a broad range of antibacterial and antifungal peptides such as the cecropins, attacins, diptericins etc (Dushay et al. 1996; Hedengren et al. 1999). Relish is rapidly activated in response to Gram-negative and Gram-positive bacteria as well as by most fungi. Thus, the Relish pathway is the major mediator of the humoral antimicrobial response in *Drosophila*. A second pathway acts via the Rel factor Dif. Activation of this pathway has a minor effect on the expression of most of the antimicrobial peptides and a major effect on one antifungal peptide, drosomycin (Rutschmann et al. 2000). In addition, Dif plays a role which is not yet well understood in the differentiation of blood cells.

The Dif activating pathway shares many components, such as the membrane receptor Toll, with that of Dorsal activation in the embryo. In contrast, the mechanism for Relish activation has only recently become accessible for investigation. As an important step in its activation, Relish is very rapidly cleaved into two fragments, within 15 seconds after a bacterial challenge. This cleavage is mediated by a novel signal-dependent endoproteolytic pathway which depends on the caspase Dredd and on a protein kinase related to human IKK (Stöven et al 2000; Silverman et al. 2000). We have now studied this reaction more in detail. We have also studied the upstream events in Relish activation. They are are so far unrelated to those of the Dif pathway and do not depend on Toll.

Thus, the *Drosophila* system includes three Rel/NF-κB factors, Relish, Dif and Dorsal, at least two of which are mediators of immune responses. In addition, there is a single NF-AT homolog for which the function is less clear. The application of the power of *Drosophila* genetics on this relatively simple system is a fruitful way to address questions of general relevance for our understanding of innate immunity.

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POSTERS

ROCK and NFκB-dependent activation of cyclooxygenase-2 by Rho GTPases: effects on tumor growth and therapeutic consequences

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Rho GTPases are overexpressed in a variety of human tumors contributing to the oncogenic and metastatic phenotype of the tumoral cells. These proteins integrate different pathways that lead to regulation of cell adhesion, cell growth and apoptosis, cell cytoarchitecture and transcriptional regulation, all of which relate to neoplastic transformation (1-4). Several studies have described an essential role of transcriptional regulation in Rho GTPases-induced oncogenesis (5-8). We have shown that oncogenic RhoA, Rac1 and Cdc42 promote the expression of inducible cyclooxygenase-2 in several cell lines at the transcriptional level. Furthermore, the endogenous level of COX-2 in human colorectal cell line HT29 is completely abolished by expression of dominant negative mutant Cdc42. We further demonstrate that expression of COX-2 by Rho GTPases is dependent on transcription factor NFkB (9). With respect to RhoA, this effect is dependent at least on the ROCK, but not the PKN, effector pathway. Treatment of RhoA-transformed epithelial cells with Sulindac and NS-398, two well characterised Non Steroidal Anti-inflammatory Drugs (NSAIDs), leads to growth inhibition and apoptosis of RhoA-transformed cells as determined by proliferation studies and cell cytometry. Accordingly, tumor growth in syngeneic mice of oncogenic RhoA-expressing epithelial cells is strongly inhibited by NS-398 treatment. The inhibitory effect of NSAIDs over RhoA-induced tumor growth is not exclusively dependent on COX-2, since DNA-binding of NFκB is also abolished upon NSAIDs treatment, resulting in complete loss of COX-2 expression. Thus, altogether these results describe a ROCK/NFκB-target gene in the context of Rho GTPases that contributes to tumor growth. At last, we provide evidence that inhibition of Cdc42 in HT29 cells significantly delays tumor growth in vivo. Thus, we suggest that treatment of human tumors that overexpress Rho GTPases with NSAIDs and/or drugs that target NFκB could constitute a valid antitumoral strategy.

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IκBα and p65 regulate the cytoplasmic shuttling of nuclear corepressors: cross-talk between Notch and NFκB pathways

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Notch and NFκB pathways are key regulators of numerous cellular events such as proliferation, differentiation, or apoptosis. In both pathways, association of effector proteins with nuclear corepressors is responsible for their negative regulation. We have previously described that expression of a p65-NFkB mutant that lacks the transactivation domain (p65DTA) induces cytoplasmic translocation of N-CoR leading to a positive regulation of different promoters. We will present evidence that cytoplasmic sequestration of p65 by IκBα is sufficient to both translocate nuclear corepressors SMRT/N-CoR to the cytoplasm and upregulate transcription of Notch-dependent genes. Moreover, p65 and IκBα are able to directly bind SMRT and this interaction can be inhibited in a dose dependent manner by the CREB binding protein (CBP) coactivator, suggesting that corepressors and coactivators compete for p65 binding. In agreement with this, TNFα treatment results in downregulation of the Hes1 gene.

Finally, we present evidence on how this mechanism may influence cell differentiation in the 32D myeloid progenitor system.

Inherited disorders of the NF-κB signalling pathway and immunodeficiency in humans: the example of the anhydrotic ectodermal dysplasia with immunodeficiency (EDA-ID) and other related syndromes

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NF-κB is a family of transcription factor playing an important role in the immunity, by regulating the expression of genes encoding cytokines, adhesion and effector molecules. NF-κB pathways are activated by a wide range of stimuli, including cytokines (TNFα, IL-1β, IL-18) or microbial or viral products (LPS, PG, dsRNA, bacterial DNA). Those stimuli lead to the activation of a high molecular weight complex that phosphorylates IκB, NF-κB cytoplasmic inhibitors, which contains two catalytic subunits, IKKα and IKKβ, and NEMO (IKKγ), a structural and regulatory subunit with no catalytic activity.

Anhydrotic ectodermal dysplasia with immunodeficiency (EDA-ID) is a rare complex syndrome in which the patient has no sweat glands, sparse scalp hair, and rare conical teeth, associated with severe primary immunodeficiency, exposing the patients to severe life-threatening infections. We and others described mutations in *IKBKG*, the gene encoding NEMO located on the X-chromosome, in male EDA-ID patients. Those patients present poor responses to LPS, IL-1β, IL-18, and TNFα with reduced IκBα degradation and NF-κB nuclear translocation. There is substantial allelic heterogeneity, which seems to correlate with clinical heterogeneity.

However, *IKBKG* mutations have been excluded in several patients with EDA/ID. In addition, other patients without any developmental signs of EDA/ID are highly susceptible to a broad range of microbial infections, were shown to respond poorly to Toll-like receptors (TLR) ligands as well as IL-1 and IL-18, suggesting that they may also be mutated in the NF-κB signalling pathway. The molecular basis of EDA-ID in patients carrying a wild-type copy of NEMO and of severe bacterial infections in patients without EDA-ID are currently under investigation.

The NF-κB pathway in endoglin promoter

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Endoglin is an endothelial membrane glycoprotein involved in cardiovascular morphogenesis and vascular remodeling. It associates with transforming growth factor-β (TGF-β) signaling receptors to bind TGF-β family members, forming a functional receptor complex. The gene encoding endoglin is the target for the autosomal dominant disorder known as Hereditary Hemorrhagic Telangiectasia (HHT) type 1. Since the underlying mechanism for HHT1 is endoglin haploinsufficiency, the study of the regulation of endoglin gene expression appears to be critical to correct the disease. Endoglin has been reported to be increased after inflammatory processes but the reason for this remains unknown, hence the putative involvement of the NF-κB pathway in this regulation was assessed. When searching for consensus motifs of transcription factors within the endoglin promoter, we found two putative NF-κB consensus sites at -218 and +27. Reporter endoglin promoter constructs containing both putative NFκB sites, or just the most proximal, were activated by TNF-α, but inhibited by N-acetyl cysteine (NAC), when transfected in macrophages (cell line Raw 264.7) or in endothelial cells (HMEC-1). Mobility shift assays, using two different oligonucleotides encoding both NF-κB sites, revealed an atypical pattern containing just one band, with the same electrophoretic mobility as the p50/p50 homodimers. Furthermore, cotransfection experiments with expression vectors encoding different components of NF-κB family showed transactivation of the endoglin promoter by Bcl3; this latter being able to rescue the p50-dependent inhibition of the promoter activity in Raw 264.7 cells. These results suggest that NF-κB may regulate the transcriptional activity of endoglin, but in a manner different from the typical way, the mechanism of which remains to be elucidated.

Impaired lymph node organogenesis in NF-κB2-deficient mice

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The development of the highly specialised structures of secondary lymphoid organs such as lymph nodes involves a series of cell-cell interactions between hemopoietic and non-hemopoietic stromal cells. The signals that are essentials in this cross talk are mediated by members of the Tumor Necrosis Factor family of ligands Lymphotoxin $\alpha 1\beta 2$ ($Lt\alpha 1\beta 2$), TRANCE/RANKL, Tumor Necrosis Factor a (TNF-α) and their receptors Lymphotoxin β R, TRANCE-R and TNF-RI respectively and the chemokine CXCL13/ BLC and its receptor CXCR5. Interestingly, signalling through the LtβR, TRANCE-R, and the TNF-RI converge in the activation of the NF-κB family of transcription factors.

The Rel/NF-κB factors regulate the expression of a large number of genes involved in immune and inflammatory reactions. Recent reports have shown that signals through the LtβR activate the kinase NIK and result in proteolytic cleavage of the precursor of NF-κB2 p100 to its DNA binding subunit p52, thus increasing the formation of p52 containing dimers. We and others have generated *nfkb2*$^{-/-}$ mice and shown that NF-κB2 is required for proper spleen architecture and Follicular Dendritic Cell maturation.

Here we present evidence that NF-κB2 is also required for lymph node organogenesis and cell recruitment to the lymph node anlage. Lymph nodes are markedly reduced in size in the *nfkb2*$^{-/-}$ adult mice and their architecture is altered. Inguinal lymph nodes from *nfkb2*$^{-/-}$ neonates contained very few lymphoid cells. Using an *in vitro* assay for lymph node colonization we showed that *nfkb2*$^{-/-}$ lymphocytes are capable of colonizing *rag1*$^{-/-}$ lymph nodes similar to wild type cells. In contrast, lymphocytes from wild type mice are not able to repopulate *nfkb2*$^{-/-}$ lymph nodes indicating that at least part of the defect in lymph node organogenesis in *nfkb2*$^{-/-}$ mice resides in the stroma.

Further studies on the identification of *nfkb2*-target genes in stromal cells of the lymph nodes will be discussed.

Characterization of Rel/NF-κB transcriptional factors and IKK complex in zebrafish

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Rel/NF-κB family members are cytoplasmic transcriptional factors that, after their respective activation (phosphorylation and degradation of associated IκB proteins), are translocated to the nucleus and then, regulate the expression of a large number of target genes involved with physiological processes like programmed cell death (apoptosis), embryogenesis, inflammatory response, cell growth and differentiation.

Alterations of Rel/NF-κB expression have been correlated with hepatic degeneration by apoptosis and abnormalities from immune and hemopoietic systems in mammals. More recently, blocking NF-κB activity has been shown to cause neural tube defects and affect limb development, indicating a possible role of Rel/NF-κB transduction signaling on maturation and differentiation of germ layers like endoderm, responsible by organogenesis during embryonic development.

Nowadays, genetic studies related to the experimental model zebrafish have been realized to get a better comprehension of the diverse events associated with vertebrate embryogenesis. Related to the conventional murine model (knock-out mice generation), zebrafish has advantages like (i) small size, (ii) high reproduction capacity, (iii) low generation time (iv) easiness of observation and embryo manipulation in all developmental stages and (v) low cost for breeding.

Due to the few number of studies focusing specifically the relationship of Rel/NF-κB factors and IκB kinase complex (IKK) with the gradative evolution of the germ layers during vertebrate development, here we describe some preliminary data that show the functional importance of these factors in organogenesis process, using zebrafish as a vertebrate model.

Pseudosubstrate regulation of the SCFbTrCP ubiquitin ligase by hnRNPU

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NF-κB is a master transcription factor, which induces many genes under stress and inflammatory conditions and in recent years has also been shown to have anti-apoptotic function. Under normal conditions NF-κB is associated with its inhibitor IκB, which sequesters it to the cytoplasm.

In order to activate NF-κB, IκB must first be degraded. This is achieved by a recently elucidated signal transduction cascade involving phosphorylation by the IKK kinase and ubiquitination by the E3RS/b-TrCP SCF ubiquitin ligase. Ubiquitin-mediated degradation is a highly selective regulatory process with many different ligases conveying substrate specificity. Not much is known about how this specificity is achieved or how ligases are regulated. In the context of E3RS we are interested in its possible regulation by novel associated proteins and its subcellular compartmentalization. Understanding the regulation of E3RS might enable future development of therapeutic agents, which can block its action and compromise NF-κB activation. Such inhibition might be useful in cases of inflammatory disease and in augmenting chemotherapy by sensitizing cancer cells to apoptosis. Preliminary results have shown E3RS to be nuclear localized by association with the abundant nuclear protein hnRNP-U.

hnRNP-U binds E3RS in a substrate-like manner and this binding can be abolished by pIκB (phosphorylated IκB). Also, hnRNP-U can provide very efficient ubiquitin ligase activity through its association with E3RS. Our future plans are to try and explain the different localization of the ligase and its substrate and determine how when and where they meet and where the ubiquitination actually takes place. We also would like to determine how important is the binding of hnRNP-U for E3RS function *in vivo*.

Glutathionylation of the p50 subunit of NF-κB: a mechanism for redox-induced inhibition of DNA-binding

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The response of the transcription factor NF-κB to perturbations in the redox homeostasis of cells may play an important role in modulating functions in which NF-κB is involved. p50 (structural component of NF-κB) contains a cysteine residue (Cys 62) in its DNA binding domain whose reduced state is essential to allow the binding to DNA. In diverse oxidative stress models, different oxidants were shown to induce an inhibition in the p50 DNA binding activity by oxidation of reactive protein thiols, including the conserved Cys 62. However the nature of these postranscriptional modifications remains unknown. The aim of this work was to identify oxidative modifications in Cys 62 that were involved in the redox regulation of p50. We observed that one of these modifications was the formation of a mixed disulfide with glutathione (S-glutathionylation). The S-glutathionylation of p50 occurs in response to oxidative changes in the redox pair GSH/GSSG and specifically targets to Cys 62. This modification is associated with an inhibition in its DNA-binding activity and is reversible. On the other hand, mass spectrometry studies (MALDI-TOF and nano ES QIT MS) show that other type of oxidative modifications (sulfenic, sulfinic and sulfonic acids) of one or more cysteine residues may co-exist. Structural simulations based on molecular modeling suggest the existence of specific electrostatic interactions between glutathione and p50 that could facilitate this S-glutathionylation. Moreover, by using cellular extracts from cells that have been exposed to oxidants it is possible to analyze the molecular modifications of a protein of interest. Accordingly, the use of bidimensional electrophoresis have allowed an optimal detection of different p50 species after an oxidant treatment of HeLa cells. The analysis of these species may permit the identification of oxidative modifications, including S-glutathionylation, involved in the redox regulation of p50. This modification could represent a mechanism by which oxidative stress signals could be transduced into gene expression changes.

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p38 MAPK promotes myogenesis by activating NF-κB in myoblast cells: analysis of the crosstalk between both pathways

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Skeletal muscle differentiation is regulated by extracellular growth factors, such as insulin, what transmit largely unknown signals into the cells. Some of these growth factors induce mitogen-activated protein kinase (MAPK) cascades within muscle cells. It has been shown that insulin stimulates muscle differentiation with the concomitant activation of PI3K and p38 MAPK. We have observed that constitutive expression of active MKK6, MKK6(E) (which selectives activates p38) into C2C12 myoblasts, was able to promote myogenesis in the absence of insulin, while addition of specific p38 inhibitor, SB203580, to C2C12-MKK6(E) cells blocked this process. On the other hand, it has been reported that NF-κB is activated during insulin-induced differentiation. In this work, we show that concomitant to the promotion of myogenic differentiation, overexpression of MKK6(E) induced NF-κB nuclear translocation, DNA binding and a NF-κB-driven luciferase activity, mimicking the insulin-induced effects on NF-κB activation. Furthermore, inhibition of p38 activity by SB203580 blocked MKK6(E)-induced NF-κB activation. However, overexpresion of MKK6(E) had no effect on the transactivation potential of p65 as assayed by transfection of the Gal4-65/pGal4-luciferase plasmids followed by measurement of luciferase activity. The MKK6(E)-induced differentiation was also reversed by inhibition of NF-κB activation (using a dominant negative form of IκBalpha), demonstrating the crosstalk between p38 MAPK and NF-κB signaling pathways during skeletal muscle differentiation.

Finally, using DNA fragmentation assays, we have observed that these signaling pathways play also a role in myocyte survival during differentiation. Altogether, we show that activation of the p38 MAPK pathway activates NF-κB, mainly by increasing NF-κB-DNA binding, resulting in the promotion of myoblast differentiation and survival.

Age-related NF-κB activation in human microvascular endothelial cells A role of NF-κB in endothelial vascular dysfunction

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Experimental evidence indicates that almost the totality of cardiovascular risk factors, such as aging and hypertension, are characterised by the presence of endothelial dysfunction, which is mainly induced by the production and release of oxygen-derived free radicals (ROS), which cause nitric oxide-breakdown (2,3). At molecular level, ROS can modulate the expression of certain number of redox-regulated genes in vascular cells.

Among them, AP-1 and NF-κB that are possibly the best characterised. NF-κB appears to be over-expressed in several human diseases, particularly those characterised by a chronic inflammatory state. Current evidences suggesting a strong correlation between aging and over-expression of several inflammatory cytokines have been made (1). Our interest have been focused to evaluate this hypothesis, isolating and characterising several human micro-vascular endothelial culture cell lines obtained from the Omental tissue of surgical patients with different ages (from 23 to 81 years old). Our results clearly shown that NF-κB basal activity was significant increased with patient's age. Analogously, several genes closely regulated by NF-κB, such as iNOS, COX-2, and several pro and inflammatory factors-mRNAs are also significantly increased with aging.

Taken together, all these effects on vascular gene expression can initiate vascular remodel and inflammation processes in human endothelial cells that could explain at least partially, the age-related endothelial dysfunction observed experimentally.

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The transcription factor NF-κB at the cross-roads between nitric oxide and prostaglandin signaling pathways

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The transcription factor NF-κB is key to the induction of numerous pro-inflammatory genes. Nitric oxide (NO) and prostaglandins (PG) are important inflammatory mediators. The increased generation of NO and PG that occurs during inflammation is due to the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), two NF-κB target genes, which is triggered by pro-inflammatory stimuli.

There is significant cross-talk between NO and PG-mediated pathways. This is exemplified by the feed-back regulation of both iNOS and COX-2 by their products. We have observed that both NO and PG regulate the expression of iNOS and COX-2. NF-κB plays a central role in this regulation. NO and PG may modulate the signaling pathways leading to NF-κB activation by acting at multiple levels. In addition, several reactive nitrogen species (RNS) and prostaglandins with cyclopentenone structure (cyPG) may regulate NF-κB activity through the direct posttranslational modification of NF-κB subunits. The covalent interaction between the p50 subunit and RNS or cyPG maps to a conserved cysteine residue located in the DNA binding domain (Cys 62 in human p50), and results in the inhibition of DNA binding. The modification of p50 by cyPG appears to be irreversible. However, the extent of this modification, and thus, of the cyPG-elicited inhibition of NF-κB-DNA binding, may depend on the cellular content of GSH and/or the occurrence of other oxidative modifications in the targeted cysteine residue. The covalent modification of p50 by cyPG occurs in intact cells and contributes to the anti-inflammatory effects that these prostanoids, in particular 15-deoxy-$\Delta^{12,14}$-PGJ_2, display in several experimental systems, including HeLa and renal mesangial cells.

Is there a receptor for macrophage activation by eukaryotic antibiotic peptides?

Guerrero Esther1; Vila del Sol, Virginia2; Andreu, David3; Fresno, Manuel2; Rivas, Luis1

We previously demonstrated that the cecropin A-melittin hybrid peptide CA(1-8)M(1-18) was capable to induce NOS2 expression in Raw 264.7 cells involving activation of the NF-κB pathway. We have extended this observation into a wide variety of natural and synthetic eukaryotic antibiotic peptides. Although the maximal induction for NOS2 was very similar for all the peptides tested, the peptide concentration needed to reach this maximum was highly dependent on the respective peptide.

Nevertheless there was a clear correlation between NOS induction with peptide damage to the plasma membrane. This NOS2 induction was abrogated by inhibitors of the purinergic P2X7 receptor, confirmed by EMSA experiments. Furthermore the peptide synergized with extracellular ATP.

NOS2 induction was capable to kill intracellular *Leishmania* amastigotes, as it was inhibited by L-NMMA. Altogether, an universal mechanism among eukaryotic antibiotic peptide and macrophage activation was described; this mechanism is cytokine independent, and may represent an early event of the innate immune response against macrophage invasion by intracellular pathogens.

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PKCβ controls NF-κB activation in B cells through selective regulation of the IκB kinase α

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Activation of the NF-κB transcription complex by signals derived from the surface expressed B cell antigen receptor controls B cell development, survival and antigenic responses. Activation of NF-κB is critically dependent on serine phosphorylation of the IκB protein by the multi-component IκB kinase (IKK) containing two catalytic subunits (IKKα and IKKβ) and one regulatory subunit (IKKγ). Using mice deficient for protein kkinase C (PKC)β we show an essential role of PKCβ in the phosphorylation of IKKα and the subsequent activation of NF-κB in B cells. Defective IKKα phosphorylation correlates with impaired B cell antigen receptor-mediated induction of the pro-survival protein Bcl-xL. Lack of IKKα phosphorylation and defective NF-κB induction in the absence of PKCβ explains the similarity in immunodefeciencies caused by PKCβ or IKKα ablation in B cells. Furthermore, the well established functional cooperation between the protein tyrosine kinase Bruton's tyrosine kinase (Btk), which regulates the activity of NF-κB and PKCβ suggests PKCβ as a likely serine/threonine kinase component of the Btk-dependent NF-κB activating signal transduction chain.

Cell stress and MEKK1-mediated c-Jun activation modulate NFκB activity and cell viability

Isabel Sánchez-Pérez, Salvador Aznar Benitah, Montserrat Martínez-Gomariz, Juan Carlos Lacal and Rosario Perona

Chemotherapeutic agents such as cisplatin induce persistent activation of N-terminal c-Jun Kinase, who in turn mediates induction of apoptosis. By using a common MAPK Kinase, MEKK1, cisplatin also activates the survival transcription factor NFκB. We have found a cross talk between c-Jun expression and NFκB transcriptional activation in response to cisplatin.

Fibroblast derived from c-jun knock out mice are more resistant to cisplatin induced cell death, and this survival advantage is mediated by upregulation of NFκB dependent transcription and expression o1 MIAP3. This process can be reverted by ectopic expression of c-Jun in c-jun-/- fibroblasts, which decreases p65 transcriptional activity back to normal levels. Negative regulation of NFκB dependent transcription by c-jun contributes to cisplatin induced cell death, which suggests that inhibition of NFκB may potentiate the antineoplastic effect of conventional chemotherapeutic agents.

NF-kappaB activation: Not only the usual suspects !

Svenja Stöven

Signaling pathways leading to Rel/NF-kappaB factor activation are evolutionary conserved. Many of their components have homologues in several biological model organisms. Striking similarities have emerged especially between NF-κB activation during innate immune responses in man and flies. At first glance the *Drosophila* imd-pathway (immune deficiency) appears to be a mirror-image of mammalian TNF receptor signaling. However, there are interesting differences:
In a collaboration with Kathryn Andersons group/ New York we were able to identify a peptidoglycan recognition protein (PGRP) as the putative membrane receptor in the imd-pathway (1). PGRPs form a protein family with members in several insect and mammalian species, but they do not show homology to TNF receptors.

The target NF-kappaB protein in the imd-pathway is Relish, a compound Rel protein like p105. As its mammalian counterpart, Relish is phosphorylated by an IKK complex (2). But, unlike other NF-kappaB proteins the activation of Relish does not require the proteasome. Instead, the protein is split into the active NF-kappaB part and a stable IkappaB part by endoproteolysis (3). Moreover, this process requires the caspase-8 homologue Dredd, and we can now present evidence that Relish is indead cleaved by a caspase. These results demonstrate the variation in the imd-pathway compared to TNF receptor signaling, where caspase activity induces apoptosis.

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Physiologic and pathologic functions of the MAP3K Tpl2/Cot

Michael Waterfield, Gutian Xiao, Edward Harhaj, and Shao-Con Sun

Our studies involve the role of the protoncoprotein Cot in Human T-cell Leukemia Virus (HTLV) induced T-cell transformation. Cot is a serine/threonine kinase that has been shown to activate multiple signalling pathways such as the mitogen activated protein kinase cascade (MAPK) and the c-jun N-terminal kinase (JNK) pathway. In addition, Cot stimulates NFAT and NF-κB dependent transcription. Transgenic expression of a carboxy-terminal truncated form of Cot has been shown to induce T-cell lymphomas in mice. Due to Cot's ability to induce T-cell lymphomas, its expression was examined in a panel of tumor cell lines derived from patients with HTLV. All but one of the cell lines was shown to overexpress Cot at the protein and RNA level. To examine Cot's possible role in cell transformation, transient transfection experiments were performed in Jurkat T-cells. Cot was expressed alone and in conjunction with the Tax oncoprotein encoded by HTLV. Interestingly, Cot and Tax synergize to enhance the transcription of multiple promoters and enhancers (IL-2, NF-κB, NFAT) involved in the regulation of T-cell activation and proliferation. By luciferase assay, NF-κB dependent transcription was increased 10 fold in jurkat cells that express both Cot and Tax compared to those that express Tax alone. In addition, Cot was shown to bind to Tax by coimmunoprecipitation. Our hypothesis is that Tax binding to Cot causes it to enter a state of aberrant activation; similar to Tax binding NEMO. Currently, my project is aimed at further elucidating the *in vivo* function of Cot, finding the mechanism of Cot/Tax synergy, and determining if Cot is required for HTLV induced T-cell transformation.

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51 **Workshop on Mechanisms of Expression and Function of MHC Class II Molecules.**
Organizers: B. Mach and A. Celada.

52 **Workshop on Enzymology of DNA-Strand Transfer Mechanisms.**
Organizers: E. Lanka and F. de la Cruz.

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Organizers: A. Sher, M. Fresno and L. Rivas.

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56 **Workshop on Programmed Cell Death in the Developing Nervous System.**
Organizers: R. W. Oppenheim, E. M. Johnson and J. X. Comella.

57 **Workshop on NF-κB/IκB Proteins. Their Role in Cell Growth, Differentiation and Development.**
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Organizers: E. K. Weir and J. López-Barneo.

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63 **Workshop on TGF-β Signalling in Development and Cell Cycle Control.**
Organizers: J. Massagué and C. Bernabéu.

64 **Workshop on Novel Biocatalysts.**
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65 **Workshop on Signal Transduction in Neuronal Development and Recognition.**
Organizers: M. Barbacid and D. Pulido.

66 **Workshop on 100th Meeting: Biology at the Edge of the Next Century.**
Organizer: Centre for International Meetings on Biology, Madrid.

67 **Workshop on Membrane Fusion.**
Organizers: V. Malhotra and A. Velasco.

68 **Workshop on DNA Repair and Genome Instability.**
Organizers: T. Lindahl and C. Pueyo.

69 **Advanced course on Biochemistry and Molecular Biology of Non-Conventional Yeasts.**
Organizers: C. Gancedo, J. M. Siverio and J. M. Cregg.

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Organizers: J. C. Alonso and N. D. F. Grindley.

72 **Workshop on Plant Morphogenesis.**
Organizers: M. Van Montagu and J. L. Micol.

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Organizers: G. Morata and W. J. Gehring.

*74 **Workshop on Plant Viroids and Viroid-Like Satellite RNAs from Plants, Animals and Fungi.**
Organizers: R. Flores and H. L. Sänger.

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76 **Workshop on Initiation of Replication in Prokaryotic Extrachromosomal Elements.**
Organizers: M. Espinosa, R. Díaz-Orejas, D. K. Chattoraj and E. G. H. Wagner.

77 **Workshop on Mechanisms Involved in Visual Perception.**
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78 **Workshop on Notch/Lin-12 Signalling.**
Organizers: A. Martínez Arias, J. Modolell and S. Campuzano.

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Organizers: J. L. R. Arrondo, F. M. Goñi, B. De Kruijff and B. A. Wallace.

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