INNATE ANTI-VIRAL IMMUNITY AND VIRUS EVASION STRATEGIES

Opening Doors

Scientific workshop for young researchers.

Sigüenza (Guadalajara), 17 - 21 February 2008

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1. Introduction

The British Council in Spain, in collaboration with the Spanish Council for Scientific Research (CSIC) is organising a series of scientific workshops to provide opportunities for young researchers from the UK and Spain to meet face-to-face for the exchange of ideas, knowledge and information on priority topics and to explore future areas of research and collaboration.

This workshop on “Innate Anti-Viral Immunity and Virus Evasion Strategies” was the sixth in the series.

2. Presentation

Viral diseases represent a constant threat to man. New viruses are emerging that did not cause human diseases (SARS or hemorrhagic viral diseases), new variants of known viruses may overcome existing immunity and cause pandemia (influenza virus) and the control of viral infections established in the human population has proven more complex than predicted (HIV, herpesviruses). The outcome of infection depends on a delicate balance between the success of the host defence mechanisms to control the invading pathogen and the ability of the virus to overcome host defences. We are starting to understand at the molecular level the battle between viruses and the host defence systems, and it is becoming evident that the initial virus-cell interaction and the activation of innate immunity are critical to control viral diseases. The ‘Innate anti-viral immunity and virus evasion strategies’ Workshop discussed the initial molecular events occurring upon infection that may restrict virus replication, and included intracellular signalling, the interferon system, cytokine networks, natural killer cells and other innate immunity pathways. Viral evasion strategies to counteract the host anti-viral responses were also considered and will help to identify new strategies to fight infection. A variety of viral systems were discussed including emerging viruses, human pathogens and virus-host models. The workshop served as a forum for the exchange of ideas among participants and led to future collaborations between UK and Spanish scientists. The discussions provided important insights into viral pathogenesis and the control of viral diseases.

3. List of participants

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4. Programme

**INNATE ANTI-VIRAL IMMUNITY AND VIRUS EVASION STRATEGIES**

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<td>John Sinclair</td>
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5. Summary of discussions

Studies on the interaction of viruses with the immune system are complex due to the wide range of virus families that utilize divers replication strategies, infect different cells and tissues and cause a variety of diseases. Interestingly, it was evident through the workshop that, in spite of the high diversity of viruses, recent studies have shown that viruses target common immune mechanisms, underscoring the contribution of specific innate immune pathways to anti-viral defence. The discussions were organized around specific cellular and immune mechanisms, their contribution to anti-viral defence and the identification of virus-encoded evasion strategies.
Two Editors from leading journals, Christine Ferguson (PloS Biology, Cambridge) and Sheilagh Molloy (Nature Reviews Microbiology, London), participated in the workshop. This was particularly important and added value to the workshop since it offered a unique opportunity to the participants to discuss, both formally and informally, their research with Editors that have a broader view of current trends of scientific research and to establish contacts with leading journals that may be interested in their future research.

The interferon (IFN) response
IFNs were identified because of their anti-viral effect and it has been well established that they play a crucial role within the first hours and days after viral infection. However, our knowledge of the IFN-mediated mechanisms is still limited. Rick Randall (University of St. Andrews) reviewed our current understanding of the intracellular pathways triggered by IFNs and discussed some evasion strategies utilized by viruses. Influenza virus NS1 and parainfluenza virus type 5 V proteins intercept intracellular signalling at different points. It was suggested that the formation of intracellular inclusion bodies containing newly formed virus particles may prevent the activation of innate immune mechanisms. It became evident from the discussion that a better understanding of the pathways that lead to the production of IFN and the mechanisms employed by viruses to prevent this induction will be of relevance to design new therapeutic strategies against viruses. Kay Childs (University of London) expanded these concepts by discussing in detail the domains of the V protein from paramyxoviruses that interact with mda-5, a protein involved in the recognition of pathogens and that stimulates the IFN response. Complementary studies on the IFN-inducing pathway mediated by Toll-like receptor 3 (TLR3) leading to anti-viral responses were presented by Andrew Macdonald (University of Leeds). Adolfo García-Sastre (Mount Sinai School of Medicine, New York) presented new data supporting the contribution of the tumour suppressor protein p53 to enhance the IFN response and its anti-viral activity. The p53 protein was known to activate apoptosis that prevents viral replication, and the new data presented expands the anti-viral function of p53. Susana Guerra (Centro Nacional de Biotecnología, Madrid) showed that vaccine virus targets the IFN-stimulate gene ISG15, providing further evidence for the inhibition of IFN pathways by viruses.

Cellular factors controlling viral infection
IFNs are induced in response to infection and prevent viral replication. However, there are many other factors within the cell that can limit viral replication and this is an area of very active research these days that may also lead to new strategies to control infection. This should be considered innate anti-viral immunity at the cellular level. Greg Towers (University College London) discussed a cellular protein, TRIM5, that is up regulated in response to IFN, is recruited to the incoming retroviral cores and directs them to the proteasome for degradation, restricting the replication of retroviruses including human immunodeficiency virus (HIV). A new form of TRIM5 fused to cyclophilin A was described which may improve the anti-viral activity and he suggested that other viral infections may be susceptible to this mechanism. José Alcami (Instituto de Salud Carlos III, Madrid) discussed the role of the transcription factor NF-kB in immunity, T cell activation and HIV infection. NF-kB is required for adequate expression of HIV in human CD4+ T cells. New insights into the molecular mechanisms that control NF-kB activity were discussed, including the transport of IkB between the nucleus and cytoplasm, and the cleavage of NF-kB by caspases. Marla Rosa López-Puertas (Instituto de Salud Carlos III, Madrid) showed the activation of NF-kB through TLR2 and its implication in HIV infection. John Sinclair (University of Cambridge) presented evidence for the control of human cytomegalovirus gene expression by cellular sub nuclear structures known as ND10. These structures associate with viral DNA and repress viral gene expression but human cytomegalovirus has evolved mechanisms to control these cellular factors. Two presentations discussed the
interaction of influenza virus, the virus that causes flu, with the cellular machinery. Amelia Nieto (Centro Nacional de Biotecnología, Madrid) provided evidence that hCLE, a cellular protein that modulates RNA polymerase II activity, interacts with the PA subunit of the influenza virus polymerase complex and accompany mRNAs through their transport from the site of synthesis to the site of translation. Susana de Lucas (Centro Nacional de Biotecnología, Madrid) discussed the role of the cellular protein Staufen, which interacts with the influenza virus protein NS1, in the morphogenesis of new virus particles.

**Virus-encoded cytokine receptors**
The cytokine network plays a role in the coordination of the immune response, and it has been found that large DNA viruses (poxviruses and herpesviruses) encode proteins that mimic host cytokines or their specific receptors in order to intercept with this complex network. The sequence similarity between viral proteins and host receptors suggests that viruses may have 'stolen' these immune receptors from the host during evolution. Antonio Alcamí (Centro de Biología Molecular Severo Ochoa, Madrid) reviewed our current knowledge of the mimicry of cytokine receptors by viruses and presented new data on a chemokine binding protein from herpes simplex virus, a human pathogen of clinical relevance. Ali Alejo (Centro de Investigación en Sanidad Animal, Madrid) illustrated the critical role of the virus-encoded cytokine receptors using ectromelia virus, a poxvirus that causes a generalized disease in mice (mouse pox) similar to smallpox. Some poxvirus-encoded receptors for tumour necrosis factor have an additional chemokine binding domain that potentiates the anti-inflammatory activity of the tumour necrosis factor inhibitor and is a critical virulence factor. These results underscored the need of animal models of viral pathogenesis to evaluate the role of viral immune modulatory proteins during infection. Viral proteins have been optimized during evolution as potent inhibitors of the immune response, and Nicola Abrescia (University of Oxford) highlighted the need to determine the structure of the viral proteins to understand their function. Crystallographic studies have shown that some viral proteins have similar folding to the cellular counterpart but retained low amino acid sequence similarity. Another example of mimicry of immune receptors by viruses was presented by David Blackbourn (University of Birmingham) who showed that Kaposi’s sarcoma herpes virus encodes a homologue of the cellular receptor OX2 that inhibits antigen-specific T cell activity to evade the host anti-viral response.

**Natural killer (NK) cell response to viral infection**
The important role of NK cells to control viral infection is underscored by the many mechanisms identified in viruses to evade NK cell killing. Gavin Wilkinson (University of Cardiff) reviewed and discussed NK cell evasion strategies encoded by human cytomegalovirus, a virus particularly focused on the inhibition of cellular responses. Further mechanisms employed by human cytomegalovirus to down regulate the expression of NK cell receptors in the infected cell were presented by Rebecca Aicheler (University of Cardiff) and Neil Bennett (University of Cambridge). It became evident from these studies that NK cell responses are important in the control of human cytomegalovirus.

**Viral pathogenesis**
The balance between the host mechanisms of defence and the evasion strategies encoded by viruses is determinant to decide who wins the battle between host and virus, and influences viral pathogenesis. Luis Enjuanes (Centro Nacional de Biotecnología, Madrid) showed the effect that deletion of different genes from SARS corona virus had on the infection of hamsters, and identified the structural protein E as a virulence factor. Marta De Diego (Centro Nacional de Biotecnología, Madrid) extended these observations in a mouse model of SARS corona virus that has been developed, identifying proinflammatory genes expressed in infections that may
contribute to a higher inflammation. Richard Elliot (University of St. Andrews) discussed the pathogenesis of a different family of viruses, bunya viruses that cause encephalitis and hemorrhagic fever, and include new viruses emerging in the human population. The NSs protein controls the expression of cellular genes and contributes to the ability of these viruses to overcome the host innate response. It was discussed that the NSs protein is variable among bunya viruses and this affects zoonosis.

Virus modulation of cell signalling and cellular responses
It has become evident in recent years that viral replication within the cell triggers a variety of signalling pathways that may restrict virus replication, and viruses have learned how to inhibit these pathways or utilize them for their own benefit. A number of good examples were discussed in different models. Emma Poole (University of Cambridge) described how the UL144 protein from human cytomegalovirus triggers NF-κB activation, Benjamin Hale (University of St. Andrews) discussed the activation of the cellular kinase PI3K by the NS1 protein from influenza virus and María del Prado Sabina (Centro de Biología Molecular Severo Ochoa, Madrid) presented data on the regulation of the cell cycle by African swine fever virus. Another level of control of viral infections is more evident at the organism level and involves immune cells that fight against invading pathogens. Indeed, viruses have learned how to control these cellular responses and examples from different viral systems were discussed. Rafael Aldabe (Universidad de Navarra) described the recruitment of regulatory T cells as a mechanism to dampen T cell responses in chronic hepatitis C infection. Two presentations described the modulation of dendritic cell activity by viruses infecting pigs. María Montoya (Universidad Autónoma de Barcelona) discussed the distinct regulation of cytokine production by porcine circovirus type 2 and porcine reproductive and respiratory syndrome virus. A mechanism to cause immunosuppression by foot-and-mouth virus based on the production of interleukin 10 was described by Noemi Sevilla (Centro de Investigación en Sanidad Animal, Madrid).

Interaction of viruses with ‘primitive’ immune systems
Studies on the evolution of immunity are relevant to understand the function of the immune system since all multicellular organisms had to develop defence mechanisms to avoid infection. It has become evident in recent years that the defence mechanisms employed by ‘primitive’ or less-sophisticated organisms are conserved in more-complex animals and these pathways highlight innate immune responses that are rapidly activated. Some interesting examples were discussed. Beatriz Novoa (Instituto de Investigaciones Marinas, Vigo) presented studies on the interaction of several viruses infecting fish species of commercial interest with the innate immune system and pointed out that specific fish reagents are becoming available and will help the research in this area. Carolina Tafalla (Centro de Investigación en Sanidad Animal, Madrid) showed that the induction of the IFN response in rainbow trout monocytes infected with viral hemorrhagic septicaemia virus may limit viral replication, as described with animal viruses. Some animal viruses are transmitted by mosquitoes or ticks, where they cause no disease. Alain Kohl (University of Edinburgh) presented studies on the interaction of these viruses with immune pathways in mosquito cells, illustrating common signalling pathways in arthropods and mammals. Adrian Valli (Centro Nacional de Biotecnología, Madrid) discussed RNA silencing as an important anti-viral response that was discovered in plants, and presented data on the suppression of the RNA silencing activity in plants by potyviruses. This innate mechanism of anti-viral immunity is likely to work in mammals and understanding the molecular events and the tricks that viruses use to evade RNA silencing is an area that will expand in the near future.
6. Collaborations

Gavin Wilkinson (Cardiff University) and Antonio Alcamí (Centro de Biología Molecular “Severo Ochoa”) are going to collaborate on the identification of new immune modulatory proteins encoded by human cytomegalovirus.

David Blackbourn (University of Birmingham) and Antonio Alcamí (Centro de Biología Molecular “Severo Ochoa”) are going to collaborate on the identification of proteins encoded by kaposi’s sarcoma herpesvirus and related gammaherpesviruses that bind cytokines or chemokines.

John Sinclair and Emma Poole (Cambridge) and Antonio Alcamí (Centro de Biología Molecular “Severo Ochoa”) are going to continue collaborating on Modulation of the host immune response by human cytomegalovirus proteins.

Andrew Macdonald (University of Leeds) has started collaborating with Richard Elliott (University of St Andrews), and even spent some time in his laboratory, working on novel regulatory proteins of the antiviral innate immune response. Furthermore, he has strengthened an existing collaboration with Alain Kohl (University of Edinburgh) on the same area.

Ben Hale (University of St Andrews) has applied for a postdoc position in Adolfo García-Sastre’s lab (Mount Sinai School of Medicine)

7. Abstracts

Rick Randall - The interferon antagonists of influenza A viruses and parainfluenza virus type 5; what they tell us about the biology of the viruses

Adolfo García-Sastre – The tumor suppressor gene p53 is a positive regulator of the type I interferon response

Kay Childs - Inhibition of mda-5 by the V proteins of paramyxoviruses

Greg Towers - TRIM5, Cyclophilin A, Retroviruses and the Red Queen

Jose Alcamí - Factors controlling HIV latency and replication in immune cells

Antonio Alcamí – Modulation of immunity by soluble cytokine receptors encoded by poxviruses and herpesviruses

Ali Alejo – A critical role of the poxvirus CrmD protein, a TNF and chemokine binding protein, in immune evasion and pathogenesis

Nicola Abrescia – Structural studies of Vaccinia Virus immunomodulatory proteins

Gavin Wilkinson - Cytomegalovirus modulation of the NK cell activation

Rebecca Aicheler - Identification of a novel human cytomegalovirus gene that suppresses expression of the NKG2D Ligands MICA and MICB

Neil Bennet - Down-regulation of NKG2D ligands by the UL142 gene of human cytomegalovirus

John Sinclair - Intrinsic cellular repression of cytomegalovirus gene expression is mediated through chromatin structure

Amelia Nieto - Characterization of proteins associated with hCLE, a factor that interacts with cellular and influenza virus RNA polymerases. Implications in viral infection

Susana de Lucas - Role of human Staufen protein in influenza virus infection

Luis Enjuanes - Recombinant SARS-CoV with attenuated phenotype as vaccine candidates

Marta L. DeDiego - Pathogenicity of severe acute respiratory coronavirus deletion mutants in hACE-2 mice
Influenza viruses and parainfluenza virus type 5 (PIV5) are both enveloped viruses with negative strand RNA genomes that superficially appear quite similar. They also have relatively small genomes with limited coding capacity; influenza A viruses specifies 11 proteins whilst PIV5 encodes only 8 proteins. Most of these viral proteins are involved with virus replication and/or structural proteins. However, like all other viruses, influenza A viruses and PIV5 have to deal with the cells innate antiviral defences, including the interferon (IFN) response. The IFN response is an extremely powerful antiviral response that if allowed to work correctly could probably control most virus infections in the absence of an adaptive immune response. However, all viruses must have some means by which they (at least partially) circumvent the IFN response. Influenza A viruses and PIV5 do this by encoding small IFN antagonists, termed the NS1 and V proteins respectively. In keeping with its rapid and virulent means of spread (and hence disease causing potential) the NS1 protein of influenza virus intercedes at multiple points to inhibit the IFN response, including interfering with the correct processing and export of cellular mRNA from the nucleus to the cytoplasm. This results in an inhibition of cellular protein synthesis and consequently infected cells cannot produce or respond to IFN. However, it also means that infected cells die fairly rapidly after virus infection. In contrast PIV5 has a completely different mechanism for circumventing the IFN response. PIV5 does not inhibit cellular protein synthesis, but rather its V protein interacts with and inhibits the activity of mda-5, a molecule involved in the detection of viruses which, when activated, stimulates the cell to produce IFN. The V protein also targets STAT1 for proteasome-mediated degradation, and thus prevents infected cells from responding the IFN. Nevertheless, the ability of PIV5 to
circumvent the IFN response is not absolute, as cells still produce a small amount of IFN in response to virus infection. Thus, uninfected cells that surround a virus-infected cell may enter an IFN induced antiviral state that can limit virus replication and slowdown the spread of infection. However, PIV5 can dismantle the IFN-induced antiviral state. Upon infection of cells in an antiviral state, the V protein targets STAT1 for degradation and, in the absence of continuous IFN signaling the cell cannot maintain its antiviral state indefinitely, normal virus replication is established between 24 – 48h post infection. During the period that it takes for the cell to go out of its IFN-induced antiviral state time, PIV5 must maintain its genome within the hostile environment of the cell. Evidence was presented that PIV5 does so by forming intracellular inclusion bodies, and the suggestion was made that the formation of inclusion bodies may be a virus defence mechanism that helps protect the virus from both innate and adaptive immune responses. Furthermore, by having a "less aggressive" means for circumventing the IFN response, PIV5 infected cells do not necessarily die, and that thus the virus may have evolved a mechanism by which to establish more prolonged, or even persistent infections in vivo. Clearly, as is the case for influenza viruses and PIV5, the way in which viruses interact with the IFN, and other cellular responses, must be critical factors that dictate the type of life cycle and types of disease that viruses cause.

The tumour suppressor gene p53 is a positive regulator of the type I interferon response

Adolfo García-Sastre1,2,3 César Muñoz-Fontela4, Salvador Macip4, Luis Martinez-Sobrido1, Lauren Brown2, Joseph Ashour1, Sam W. Lee5 and Stuart A. Aaronson4

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Tumour suppressor p53 is activated by a number of stimuli including DNA damage and oncogenic stress. Recent studies have shown that p53 is also induced in response to viral infections as a downstream transcriptional target of type I interferon (IFN) signalling presumably acting to increase apoptosis in infected cells and, thereby reducing the ability of the virus to replicate and spread. Moreover, many viruses, including SV40, human papilloma virus, KSHV, adenoviruses, and even RNA viruses such as polioviruses, have evolved mechanisms designated to abrogate p53 responses. We have now observed that infected mouse and human cells with functional p53 exhibited markedly decreased early viral replication even in the absence of apoptosis. This early inhibition of viral replication was mediated both in vitro and in vivo by a p53-dependent enhancement of IFN signalling, specifically of those genes containing interferon stimulated response elements (ISREs). Of note, p53 also contributed to an increase of IFN release from infected cells. We established that one component of this p53-dependent enhancement of IFN signalling involves p53-dependent transcriptional activation of a central component of the ISGF3 complex, IRF9. Our results establish a broad role of p53 in the antiviral defence, not only through increasing apoptosis in response to high viral loads, but by enhancing the IFN response to viral infection.
**Inhibition of mda-5 by the V proteins of paramyxoviruses**

*Kay Childs, University of London, UK*

Mda-5 and RIG-I are IFN-inducible RNA helicases that act as pattern recognition receptors for foreign RNA molecules such as dsRNA and 5’-triphosphate RNAs that are generated intracellularly during the course of a viral infection. Their activation leads to the upregulation of the gene encoding the antiviral cytokine IFN-β, which is a key component of the innate immune response to virus infection. Paramyxoviruses have evolved a number of mechanisms to evade the IFN response, including limiting the amount of IFN-β produced by infected cells. This is a function of the highly conserved paramyxovirus V protein, which can directly bind to mda-5 and inhibit its ability to stimulate the IFN-β promoter. We have shown that activation of both mda-5 and RIG-I by dsRNA leads to the formation of dimers, and that this is mediated through self-association of the helicase domains. The V protein targets mda-5 by binding to the helicase domain and preventing dimerisation. In contrast V is unable to bind RIG-I and hence cannot block dimerisation of the RIG-I helicase domain nor inhibit its activation.

**TRIM5, Cyclophilin A, Retroviruses and the Red Queen**

*Greg Towers, Department of Infection, UCL, London, UK*

I described our recent work on the antiviral restriction factor TRIM5 and the identification of a novel TRIMCyp fusion in rhesus macaques. TRIM5 is an antiretroviral restriction factor found in the cytoplasm of most cells. It is recruited to incoming retroviral cores and directs them to the proteasome. TRIM5 is species specific eg Many simian TRIM5 molecules block HIV-1 infectivity whereas human TRIM5 does not. The human protein blocks infection of certain mouse and horse retroviruses. The important differences between TRIM5 proteins from different species are in the B30.2 domain which is involved in virus recognition. My talk described our recent identification of a modified TRIM5 locus in rhesus macaques. A cDNA for the enzyme cyclophilin A has been inserted into a single allele of the TRIM5 gene in rhesus monkeys by L1 mediated retrotransposition. This leads to the expression of a TRIM5-CypA fusion protein referred to as TRIMCyp. The CypA domain completely replaces the virus binding B30.2 domain of TRIM5 leading to a factor that restricts viruses that recruit CypA to their incoming capsids. A variety retroviruses are sensitive to TRIMCyp including HIV-1, HIV-2 as well as lentiviruses in cats and monkeys. Remarkably this is not the first occasion on which TRIM5 has been fused to CypA. In the New World owl monkey the TRIM5 locus also contains a CypA cDNA and owl monkeys express a TRIMCyp. Differences in the CypA cDNA locations between owl monkeys and rhesus monkeys indicate that the 2 TRIMCyp genes have evolved independently. This indicates the power of the selection pressure applied on mammalian genomes by retroviruses throughout primate evolution and is an excellent example of the host virus arms race referred to as the Red Queen hypothesis.

**Factors controlling HIV latency and replication in immune cells**

*Jose Alcamí, Maria Rosa López Huertas, Mayte Coiras, Instituto de Salud Carlos III, Madrid, Spain*

Transcriptional factors NF-κB play a crucial role in immune and inflammatory responses as well as in protection against apoptosis. The NF-κB pathway also
provides an attractive target to viral pathogens. In fact, NF-κB proteins are the most important inducible elements involved in initiation of human immunodeficiency virus type 1 (HIV-1) transcription in human CD4+ T cells through binding to LTR (long terminal repeat) promoter sequences. As a consequence, an adequate control of NF-κB response is of paramount importance for both cell survival and viral spread. Its major inhibitory protein IκBα constitutes a master terminator of the NF-κB response that is complemented by degradation of p65. Both mechanisms are supposed to act synergistically to promptly terminate transcription of -κB-dependent genes. However, the contribution of both pathways may vary in different cell types.

Firstly we investigated the dynamics of the nucleocytosolic transport of NF-κB and its major inhibitor IκBα in resting CD4+ T lymphocytes to determine whether NF-κB/IκBα shuttling also exists in absence of activation. The importance of this process stays in the existence of a low level on-going control of HIV transcription that could be due to this basal shuttling. In this context, the inhibition of the nuclear export with leptomycin B in resting T cells resulted in nuclear accumulation of both IκBα and p65, as well as the formation of NF-κB/IκBα complexes. Therefore, it provided the existence of a rapid shuttling of IκBα between nucleus and cytosol even in absence of activation. Furthermore, the nuclear translocation of IκBα in resting T lymphocytes was dependent on continuous resynthesis but it was able to inhibit HIV-LTR dependent transcription.

Secondly, the function of a caspase-mediated cleaved form of p65/RelA detected in PMA or PHA-treated human peripheral blood lymphocytes (PBLs) is analyzed. Cells producing this p65/RelA cleaved form did not undergo apoptosis but showed a normal cell cycle. This cleaved form is partially amino-truncated and lacks most of DNA-binding domain although retains the dimerization domain. Consequently, it can associate with NF-κB1/p50 and IκBα but can not bind -κB consensus sites on DNA. Paradoxically, cleaved p65 increased NF-κB activity in a dose-dependent manner. Thus, its expression results in longer transactivation activity of wild-type p65 as well as in enhancement of HIV-1 replication in infected PBLs. Moreover, cleaved p65/RelA was increased in PMA or PHA-activated T cells, proving this phenomenon is related to cell activation. These data suggest the existence of a novel mechanism for maintaining NF-κB activity in human T cells through binding of cleaved p65 to IκBα, thereby protecting wild-type p65/RelA from IκBα inhibition.

Modulation of immunity by soluble cytokine receptors encoded by poxviruses and herpesviruses

Antonio Alcamí and Abel Viejo, Centro de Biologia Molecular Severo Ochoa (CSIC-UAM), Madrid, Spain

The relevant function of the chemokine system in anti-viral defense is emphasized by the identification of virus-encoded proteins that mimic chemokines and chemokine receptors, and viral secreted proteins that bind chemokines. These immune evasion strategies are employed by complex DNA viruses (poxviruses and herpesviruses) that can accommodate a large number of genes in their genomes. The chemokine binding proteins encoded by pathogens have unique amino acid sequence and structure unrelated to host seven-transmembrane chemokine receptors. The chemokine binding proteins identified so far inhibit the activity of chemokines by blocking the interaction of chemokines with their specific receptors on leukocytes or the binding of chemokines to cell surface glycosaminoglycans that present the chemokines to leukocytes.

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We have identified a novel family of chemokine binding proteins encoded by glycoprotein G from several alphaherpesviruses. Glycoprotein G is expressed at the surface of virus particles and secreted into the medium after protease cleavage of its extracellular chemokine binding domain. Glycoprotein G from alphaherpesviruses that infect several animal species, including horses, cows and cats, inhibit the ability of chemokines to induce the migration of immune cells. By contrast, glycoprotein G encoded by human herpes simplex virus 1 and 2 enhance the biological activity of chemokines. These findings uncover novel functions of the virus-encoded chemokine binding proteins and suggest that viruses have adapted the properties of these chemokine modulators to their specific needs in the infected host. These virus-encoded activities have important implications for viral pathogenesis and help us to understand the modulation of the immune response by viruses.

A critical role of the poxvirus CrmD protein, a TNF and chemokine binding protein, in immune evasion and pathogenesis


CISA (INIA), Valdeolmos, Spain, CBMSO (CSIC-UAM), Madrid, Spain, Department of Medicine, University of Cambridge, Cambridge, UK.

Poxviruses are a family of complex enveloped dsDNA viruses including several human and animal pathogens. Due to their large coding capacity, these viruses have been shown to encode a large number of genes with immunomodulatory potential. Amongst these are a number of secreted cytokine binding proteins such as the soluble IFN type I and II binding proteins, an IL-18 binding protein and the complement binding protein. A family of secreted tumour necrosis factor (TNF) receptors with similarity to the cellular TNF receptors have been identified in numerous poxviruses. We decided to address the role of these TNF receptors in vivo by using ectromelia virus (EV), a natural mouse pathogen belonging to the orthopoxvirus family. EV is the causative agent of mousepox, a severe disease of mice which was extensively studied in the 1940s and 1950s as a model for acute infections in general and smallpox in particular.

In ectromelia virus, only one predicted active TNF receptor named CrmD is found. We were able to show that this protein is able to block both TNF and chemokine activity in vitro through its N-terminal and C-terminal domains, respectively. Thus, we constructed a recombinant EV lacking the CrmD gene to determine the role of this bifunctional protein during mousepox pathogenesis. We found that in the absence of CrmD, the LD50 in susceptible Balb/c mice was elevated by at least six orders of magnitude, rendering the virus practically avirulent. Moreover, absence of CrmD was accompanied by a strong and quick inflammatory response at the initial infection site and reduced viral spread to the main target organs, spleen and liver. To determine the differential role of the TNF and chemokine binding domains, an EV recombinant expressing only the TNF binding activity of CrmD was constructed. It was found that this virus was attenuated, too, although to a lesser degree than the virus completely lacking CrmD activity. Thus, both the TNF and chemokine blockade are important for mousepox progression. In summary, we have found that the secreted EV protein CrmD, which inhibits both chemokines and TNF is an essential virulence factor in vivo.
Structural studies of vaccinia virus immunomodulatory proteins

Mohammad Bahar1, Stephen Graham1, Nicola Abrescia1, Samantha Cooray2, Ron Chen2, Daniel Whalen1, David Stuart1, Geoffrey Smith1, Jonathan Grimes1

Vaccinia virus (VACV; genus: orthopoxvirus, family: poxviridae) is a double-stranded DNA virus with a large linear genome (~200 genes). It encodes many immunomodulatory proteins, including inhibitors of apoptosis and modulators of innate immune signalling. We have solved the crystal structure of VACV N1 at 2.9Å resolution, revealing that the overall fold of N1 is strikingly similar to that of mammalian Bcl-2–like host-cell anti-apoptotic factors despite not sharing any identifiable sequence identity with this family of proteins. Guided by structural result, we performed functional experiments that showed VACV protein N1 to inhibit host cell apoptosis by binding to cellular pro-apoptotic factors Bid, Bax and Bak in a manner analogous to that of the Bcl-2–like proteins.

As a result we embarked on the systematic structural analysis of several other viral encoded proteins. We have successful elucidated the structure of B14, another immunomodulatory protein and recently the structure of A52. Protein B14 inhibits activation of the NF-kB transcriptional complex and thereby inhibits activation of pro-inflammatory cytokines that recruit lymphocytes cells to sites of infection. B14 acts by binding to IKKβ and preventing phosphorylation of the IkB complex. Surprisingly, B14 (and A52) adopt a fold similar to N1 and the mammalian Bcl-2–like family of proteins, despite not sharing significant sequence identity between themselves and with the mammalian counterparts.

Preliminary structure-based phylogenetic analysis shows that N1, B14 (and A52) are more closely related to each other than they are to any other Bcl-2–like protein. This is suggestive of a poxvirus ancestor gene acquired from the mammalian host and that has evolved by gene duplication. The corresponding Bcl-2-like fold has been retained to interfere with different signalling pathways in the host.

Cytomegalovirus modulation of the NK cell activation

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Human cytomegalovirus (HCMV) is a ubiquitous human herpesvirus that establishes lifelong persistent infections that must be controlled by the host immune response. Individuals with defects in NK cell function are particularly vulnerable to HCMV disease. In analysing the effect of HCMV infection on sensitivity to NK attack, laboratory strains were observed to induce less resistance than recent clinical isolates. The low passage HCMV strain Merlin was therefore cloned into a BAC vector to provide a reliable source of HCMV sequences. To characterise viral NK modulatory function, HCMV ORFs are being systematically cloned into a specially-designed, high-throughput adenovirus vector. HCMV-mediated downregulation of endogenous MHC-1 has the potential to render infected cells vulnerable to NK attack. To compensate, HCMV both encodes its own MHC-I homologue (UL18) and upregulates endogenous HLA-E. Productive HCMV infection efficiently stimulates the expression of a group of 'stress' ligands recognised by the ubiquitous activating receptor NKG2D. Virus-encoded functions (including UL16,
miR-UL122, UL142) act in concert to prevent NKG2DL reaching the cell surface. Furthermore, UL141 downregulates ligands for the NK activating receptors CD96 and CD226.

**Identification of a novel human cytomegalovirus gene that suppresses expression of the NKG2D Ligands MICA and MICB**

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Human Cytomegalovirus (HCMV) is a ubiquitous herpes virus, infecting between 60-90% of the population and causing severe morbidity and mortality in the immunocompromised host. In the immunocompetent host, Natural Killer (NK) cells play a critical role in controlling HCMV infection *in vivo*. Activation of NK cells through the activating receptor NKG2D, leads to killing of NKG2D ligand-expressing cells. Here we present data to show that HCMV encodes a novel NK evasion function that significantly reduces cell surface expression of the NKG2D ligands MICA and MICB, sequestering immature forms of these proteins in the Endoplasmic Reticulum (ER).

**Down-regulation of NKG2D ligands by the UL142 gene of human cytomegalovirus**

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Human cytomegalovirus (HCMV) infection causes up-regulation of cellular stress-molecules which are subsequently displayed at the cell surface. These bind the activating NKG2D ligand and induce natural killer (NK) cell mediated lysis. There are 7 ligands of human NKG2D: ULBP1, ULBP2, ULBP3, MICA, MICB, RAET1g and RAET1e. HCMV is known to encode proteins which down-regulate surface levels of MICA, MICB, ULBP1 and ULBP2.

UL142 is an MHC Class I-like glycoprotein which inhibits NK-mediated killing and is found in clinical strains of HCMV. We and others have shown UL142-mediated down-regulation of the rare, long MICA alleles. However, the failure to down-regulate common alleles suggested that other NKG2D ligands might be down-regulated by UL142. Flow cytometry of HCMV-infected cells showed down-regulation of surface ULBP3 and this effect was also observed following expression of UL142 in isolation. Cytotoxicity assays showed that transfection of ULBP3 increased NK-mediated killing, whilst co-transfection of UL142 protected ULBP3-transfected cells from lysis. In addition, confocal microscopy showed that UL142 caused intracellular accumulation of ULBP3 and co-localisation with golgi markers.

This shows that HCMV encodes at least 2 genes which target ligands of NKG2D and this suggests that like murine CMV, HCMV may target all ligands of NKG2D.

**Intrinsic cellular repression of cytomegalovirus gene expression is mediated through chromatin structure**

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The eukaryotic host has an arsenal of adaptive and innate anti-viral responses which must be overcome by the virus to permit efficient viral gene expression, DNA
replication and subsequent virus production and dissemination. During human cytomegalovirus (HCMV) infection, as with infection with many DNA viruses, viral genomes associate with a cellular subnuclear structure known as nuclear domain 10 (ND10) and recent studies have shown that individual protein components of these ND10, such as hDaxx or promyelocytic leukemia protein (PML), mediate an intrinsic anti-viral response against HCMV. This ND10-mediated anti-viral response appears to act by silencing expression of HCMV major immediate early (IE) gene expression, a class of viral genes essential for the subsequent activation of the full lytic cycle. We will show that cellular hDaxx in ND10 functions to repress transcription of viral IE genes immediately upon infection via the establishment of an inhibitory chromatin structure around the viral major immediate early promoter (MIEP) on the viral genome. We will also show that HCMV employs multiple mechanisms to overcome this intrinsic cellular repression of viral gene expression very early on in infection and that disruption of ND10, mediated by viral IE gene expression itself as infection proceeds, results in modification of hDaxx to an activator of the MIEP.

Characterization of proteins associated with hCLE, a factor that interacts with cellular and influenza virus RNA polymerases. Implications in viral infection

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Protein hCLE is a specific modulator of the RNA polymerase II (RNAP II) that interacts with the PA subunit of the influenza virus polymerase complex. Due to this and other described associations of influenza virus polymerase with cellular proteins involved in RNA transcription, we have characterized the effect of viral infection on the cellular transcription machinery. Influenza virus infection causes the specific degradation of the hypophosphorylated RNAP II form and concomitantly a severe cellular transcription inhibition. Besides that, hCLE silencing gives rise to a decrease in the influenza virus titer both, at high and low multiplicity of infection. To obtain more information about hCLE function, we have expressed hCLE as a recombinant protein with a TAP tag and purified the protein complexes containing hCLE both, in the nucleus and cytoplasm of the transfected cells. The obtained results clearly indicate the presence of hCLE in mRNA containing granules of different types, whose translation is detained until a particular stimulus is produced. These granules differ on whether or not they contain translation machinery, but both contain translation factors. Proteins associated with hCLE undoubtedly point out that hCLE is present in both types of granules, and that could be part of the core proteins involved in accompany certain mRNAs from their synthesis, to their transport to the cytoplasm and their translation, being involved in the "fate" of certain mRNAs.

Role of human Staufen protein in influenza virus infection

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Influenza virus genome is formed by 8 single-strand RNA segments of negative-polarity that encode 11 proteins. The NS1 protein is one of the two non-structural proteins and participates in different steps of the virus infectious cycle, including transcription, replication and virus morphogenesis. To carry out these functions, NS1 interacts with viral and cellular factors such as proteins from the nuclear pore, the interferon signalling pathway and translation-related factors. HStaufen (hStau1), a
protein present in RNA granules and involved in the transport and regulated translation of cellular mRNAs, was identified in a yeast two-hybrid test using NS1 as bait. NS1 and hStau colocalize in the polysomal fraction of influenza virus-infected cells, and coimmunoprecipitate both in virus-infected and plasmid-cotransfected cells, but there is no information about its role in influenza virus infection.

We have purified by affinity chromatography hStau1 cytosolic complexes from HEK293T human cells that were transfected with a TAP-tagged hStau1 gene and infected with influenza virus. The presence of both viral proteins and RNAs in the purified hStau granules was analyzed. Both NS1 and NP proteins were found in the hStau complexes. Viral mRNAs and vRNAs were determined by RT-PCR. In addition, colocalization of hStaufen with NS1, NP and PA in the cytosol was shown by immunofluorescence.

To analyze the role of hStau in the infection, we have generated stable HEK293T cell lines that express a siRNA specific for hStau. Two different cell clones in which Stau expression was reduced were selected by incubation with puromycin (iStau cell lines). Single-cycle infection experiments indicated that the expression levels and nucleocytoplasmic transport of viral proteins were not affected in the silenced cell lines. The growth kinetics of influenza virus in the iStau cell lines was analyzed in low-multiplicity infections. Titration of the supernatants obtained at various times post-infection showed a 10-fold decrease in viral production in iStau clone 1-4 cells as compared to control HEK293T cells. Furthermore, the production of virus particles, determined by purification of virions from supernatants, was lower in iStau clone 1-4 cells. These results suggest a role for hStaufen during late events of influenza virus infection. As Staufen interacts with both NS1 and the RNP, it is tempting to suggest a role for this human factor during viral morphogenesis.

Recombinant SARS-CoV with attenuated phenotype as vaccine candidates

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Vaccine development requires knowledge on the molecular basis of genetic expression and attenuation of virus. Coronavirus (CoV) transcription implies a discontinuous mechanism by which the 5’-terminal leader sequence is fused to the 5’ end of the mRNA coding sequence (body). Transcription-regulating sequences (TRSs) preceding each gene include a conserved core, also found at the 3’-end of the leader, and variable 5’ and 3’ flanking sequences. Base pairing between the leader TRS (TRS-L) and the complement of the body TRS (cTRS-B) in the nascent RNA is a main determinant factor during CoV transcription. In transmissible gastroenteritis CoV, a good correlation has been observed between subgenomic mRNA levels (sgmRNA) and the G of TRS-L and cTRS-B duplex formation, with the only exception of sgmRNA N, the most abundant during viral infection in spite of its minimum G. Consequently, we postulated the presence of additional factors that regulate transcription of sgmRNA N. In fact, we have demonstrated the presence of a transcription enhancer preceding the coding sequences of N gene. These sequences have an enhancer activity similar to that described for other positive-stranded RNA virus, but not previously described within the Nidovirales order.

To generate SARS-CoV attenuated phenotypes, three defective SARS-CoV viruses were engineered in which the structural E gene (ΔE), the group specific genes 6, 7a, 7b, 8a, 8b and 9b (Δ6-9b), or E plus the group specific genes (ΔE,6-9b) were deleted using an infectious cDNA clone, indicating that none of these genes are essential for
virus replication. In monkey cells, deletion of genes 6 to 9b did not affect viral titers, in contrast, deletion of gene E caused a 20-fold reduction in virus growth. Viral particles with a morphology similar to that of the parental virus were observed in monkey cells in all cases, although the number of mature virus particles was lower for the \( \Delta E \) mutants, suggesting that E protein is more critical for virus assembly than other envelope proteins such as 6, 7a and 7b. In fact E protein affect virus pinch off. The virulence and induction of protection by the mutant viruses have been evaluated in hamsters. The \( \Delta E \) virus replicated to titers 100 to 1000-fold lower than the parental virus in the respiratory tract of hamsters and the animals showed no pulmonary inflammation, while hamsters infected with the parental virus developed pneumonitis, indicating that the \( \Delta E \) virus is attenuated in hamsters. Using the wheel activity test we have shown that the administration of SARS-CoV-\( \Delta E \) to hamsters caused no wheel activity decrease, in contrast to the administration of the parental full-length virus. Hamsters immunized with the SARS-CoV-\( \Delta E \) showed no decrease in wheel activity after challenge with the wt or SARS-CoV-\( \Delta E \). Furthermore, vaccination of hamsters with SARS-CoV-\( \Delta E \) led to the induction of neutralizing antibodies and protection against both homologous (Urbani) and heterologous (GD-03) SARS-CoV strains. The data suggest that E gene is a virulence factor, and that viruses in which this gene has been deleted are promising vaccine candidates.

**Pathogenicity of severe acute respiratory coronavirus deletion mutants in hACE-2 mice**

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Transgenic mice in which the expression of the SARS-CoV receptor, human angiotensin converting enzyme 2 (hACE-2), has been targeted to epithelial cells, have been developed. Infection in this animal model results in virus growth in lung and brain, viremia, macrophage and lymphocyte infiltration in the lungs and upregulation of proinflammatory cytokines in both the lung and the brain. Three defective SARS-CoV viruses were generated in which the structural E gene (\( \Delta E \)), the group specific genes 6, 7a, 7b, 8a, 8b and 9b (\( \Delta 6-9 \)), or E plus the group specific genes (\( \Delta E, 6-9 \)) were deleted using an infectious cDNA clone, indicating that the deleted genes were not essential for virus replication. Mutant viruses virulence and induction of protection has been studied in the transgenic hACE-2 mice described. The \( \Delta E \) virus replicated to titers 100 fold lower than the parental virus in the respiratory tract of hACE-2 mice, and the animals showed minor pulmonary inflammation, whereas animals infected with the parental virus developed pneumonitis. The \( \Delta E \) mutants did not grow in the brains of hACE-2 mice, in contrast to rSARS-CoV-\( \Delta [6-9b] \) and wt virus, indicating that gene E influences virus tropism. In contrast, no significant attenuation was observed with the \( \Delta 6-9 \) mutant in transgenic mice. Interestingly, the attenuated mutants lacking E gene provided protection from challenge with the parental virulent virus in the mice model, indicating that they are promising vaccine candidates. Two genes differentially expressed between wt and \( \Delta E \) virus-infected cells, involved in the synthesis of proinflammatory cytokines, have been identified. These genes might be responsible for the higher inflammation observed in the animals infected with the wt virus compared to those infected with the \( \Delta E \) virus.
Bunyavirus diversity and zoonotic potential

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The family *Bunyaviridae* contains over 350 named isolates, classified into 5 genera: *Orthobunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus* and *Tospovirus*. The family includes significant human pathogens such as Oropouche virus, Rift Valley fever virus and Hantaan virus that cause a range of disease syndromes in humans and domestic animals including self-limiting febrile illness, encephalitis, abortion, teratogenesis and haemorrhagic fever. The viruses have a tripartite, negative-sense RNA genome. Analyses of viruses in four orthobunyavirus serogroups (Bunyamwera, California, C and Simbu) showed that the smallest (S) RNA segment encodes the nucleocapsid protein (N) and a non-structural protein called (NSs). The NSs proteins of Bunyamwera (BUNV) and La Crosse (LACV) viruses have been shown to play a role in shut-off of host cell protein synthesis in mammalian cells. This is achieved by global inhibition of RNA polymerase II-mediated transcription and thus enables the virus to overcome the host innate immune response. However, NSs is dispensable for growth in tissue culture, though NSs-deletion viruses are attenuated in cells with a competent interferon system. For BUNV, interaction of NSs with the Mediator protein Med8 is important for NSs function. No inhibition of protein shut-off is observed in BUNV or LACV-infected mosquito cells (*Aedes albopictus* C6/36 cells). Studies on Rift Valley fever phlebovirus showed that its NSs protein, which is much larger than the orthobunyavirus NSs protein and does not share sequence homology, also overcomes the host innate immune response by inhibiting general host cell transcription. Recently we have sequenced the S RNA segments of a number of divergent orthobunyaviruses and to our surprise found that some isolates do not encode an NSs protein. The implication of these findings to the zoonotic potential of orthobunyaviruses will be discussed.

NFκB-mediated activation of the CCL22 chemokine by human cytomegalovirus UL144 gene product escapes regulation by viral IE86

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The UL144 gene product of HCMV, expressed at early times post-infection, is located in the UL/b’ region of the viral genome and related to members of the TNFR superfamily, but it does not bind TNF superfamily ligands. However, UL144 does activate NFκB, resulting in the NFκB-mediated activation of the cellular chemokine CCL22. Consistent with this, isolates of HCMV lacking the UL/b’ region show no such CCL22 activation. Recently, it has been suggested that activation of NFκB is repressed by the viral IE86 gene product: IE86 appears to block NFκB binding to DNA, but not nuclear translocation of NFκB. Intriguingly, IE86 is detectable throughout virus infection, so how UL144 is able to activate NFκB in the presence of continued IE86 expression is unclear. Here we show that, although IE86 does repress the UL144-mediated activation of a synthetic NFκB-promoter, it is unable to block UL144-mediated
activation of the CCL22 promoter and this lack of responsiveness to IE86 appears to be regulated by binding of the CREB transcription factor.

**Mechanism and consequence of PI3K activation by the influenza A virus NS1 protein**

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Influenza A viruses are globally important human and animal pathogens that are responsible for both seasonal ‘flu’ outbreaks and periodic world-wide pandemics. The multifunctional NS1 protein of influenza A virus is widely regarded as a virulence factor, and contributes significantly to disease pathogenesis by modulating many virus and host-cell processes. One such function of NS1 in infected cells is the stimulation of phosphoinositide 3-kinase (PI3K), a cellular enzyme involved in the control of gene transcription, protein synthesis, cell survival, and cytokine production. During infection, NS1 binds directly to PI3K via the inter-SH2 domain of p85β, a subunit of heterodimeric (p85:p110) PI3K. Binding of NS1 to the inter-SH2 domain likely masks a specific regulatory element in p85β that normally contributes to repression of p110 catalytic activity. Thus, activation of PI3K early during infection is concomitant with NS1 expression, and has been shown by others to correlate well with delayed kinetics of virus-induced cell-death. However, it is intriguing to note that at later times during infection PI3K signalling is markedly attenuated despite high levels of NS1 expression. Here, we report on our ongoing studies concerned with the mechanism, regulation, and biological significance of NS1-mediated PI3K activation. We highlight virus strain-specific differences, and discuss the potential consequences of these for both virus replication and the host’s innate antiviral defences.

**Biochemical characterization of a novel regulator of anti-viral signalling**

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Nucleic acid is a potent activator of immune responses during viral infection. It is detected by Toll-like receptors and the cytosolic proteins RIG-I, Mda-5 and DAI. Detection of viral nucleic acid initiates a complex network of signalling pathways that culminate in the transcription of IFNβ. To counteract the host, viruses have evolved numerous mechanisms to evade the IFN response, a factor governing the development of chronic infections. The primary aim of my laboratory is to understand the processes associated with antiviral signal transduction using a combination of proteomic, cell biological and structural approaches. We are particularly interested in the multi-component protein kinase TBK1, which is essential for the transcription of IFNβ. We have recently identified a novel cellular inhibitor of this kinase called NRP. NRP is a member of the ubiquitin binding AHD-family of proteins which also includes the regulatory protein NEMO. Yeast-two-hybrid assays have demonstrated that NRP is a binding partner for the TBK1 adaptor protein TANK. Furthermore, NRP can be found in TBK1 immunoprecipitates with known TBK1 adaptor proteins. We have demonstrated that upon stimulation of cells with dsRNA, NRP is recruited to active TLR complexes and that this recruitment is dependent on binding to lysine-63 linked ubiquitin chains. Using a luciferase reporter assay that measures the activity of transcription from the IFNβ promoter, we have shown that the over-expression of NRP acts to negatively regulate TBK1 signalling. Furthermore, the negative regulation is absolutely dependent on the ubiquitin binding abilities of NRP, as a mutant that is unable to bind ubiquitin can no longer inhibit IFNβ expression. To assess the functional consequences of NRP expression for virus infection, cells were infected with Semliki Forest virus. Replication...
assays demonstrated that cells over-expressing NRP produced higher titres of virus, confirming the negative regulation role of NRP within anti-viral signalling.

Using these studies it is hoped that we can build a complete picture of how TBK1 is regulated by viral infection, which may eventually lead to the design of novel antiviral and anti-inflammatory therapeutics.

**NF-κB activation upon peptidoglycan stimulation enhances HIV-1 replication in human blood T lymphocytes**

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Progressive Human immunodeficiency virus type 1 (HIV-1) disease is characterized by persistent viral replication and chronic immune activation. Paradoxically, activation of the immune system increases HIV-1 replication itself. In this context, HIV-1 co-infection with different pathogens can increase lymphocyte activation. Activation of HIV-1 gene expression is critically dependent on the activation of the nuclear factor -κB (NFκB), that binds to κB sites within the HIV-1 long terminal repeat enhancer region. NF-κB can be activated through different pathways, including Toll-like Receptors (TLR). In particular, TLR2 is essential to transduce signals elicited by peptidoglycan (PGN), the major component of the gram positive bacteria cell wall. Due to the fact that TLRs are “pattern recognition receptors” they can alert the immune system of current infections. Therefore TLRs are key components of the innate immune. In addition to innate immune cells, TLRs are also expressed in other types of cells that contribute to inflammatory responses. It has been recently reported that most human TLRs are also expressed on different types of T cells. Because TLRs regulate the activation of both innate and acquired immune system we thought about analyzing TLR-2 expression, the mechanisms involved in the subsequent NF-κB activation and the influence exerted on HIV-1 replication in human blood T lymphocytes. T cells used in all experiments were obtained from human PBMCs treated with PHA and IL-2. After depleting IL-2 from the culture medium a homogeneous T cell population, which remains at a pre-activated status, is obtained. In these T lymphocytes PGN treatment for short times increased expression of TLR2 on the cell surface. TLR2 was hold in reserve in the cytosol and therefore it was available to be quickly translocated to the cell membrane after stimulation with PGN. NF-κB activation induced by TLR2 specific stimulation occurred through both p65/RelA phosphorylation at Ser536 and degradation of its inhibitor IκBα. Interestingly, PGN stimulation increased HIV replication and disease progression through the induction of NFκB. Our results point out the importance of TLR2 on directly activating acquired immunity without previous activation of TCR or release of cytokines. These could explain the immune activation and progression to AIDS in HIV-1 infected patients in which bacterial translocation has been described.

**Evasion of the immune response by HCV**

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Hepatitis C virus (HCV) is remarkably efficient at establishing persistent infection. The current treatment with IFN-α given alone or in combination with ribavirin is ineffective at eliminating the virus in a large proportion of individuals with chronic hepatitis C. Recent data suggest that HCV blocks IFN-α signalling, an effect that facilitates viral persistence. We have shown that IFN-α-mediated STAT activation (but not IFN-γ stimulated STAT phosphorylation) is blocked HCV infected liver samples and in Huh7 cell line containing the HCV genomic replicon, while this is not observed in cells with the subgenomic replicon. In agreement with these findings, the transcriptional activity...
and the antiviral effect of IFN-α was significantly lower in cells harbouring the genomic replicon than in cells with the subgenomic replicon. These results indicate that HCV structural proteins play a key role in the escape of HCV to the interferon system. Chronic infection caused by hepatitis C virus (HCV) is characterized by weak T cell responses, recognizing very few epitopes. In contrast, viral clearance after acute infection or after interferon therapy is associated with the presence of a robust and polyclonal T cell reaction. Thus, HCV has developed efficient means to escape T cell immunity thus causing a high rate of chronic infections. The molecular mechanisms that are responsible for immune tolerance to HCV antigens remains ill understood. Indoleamine 2,3 dioxygenase (IDO) is induced by proinflammatory cytokines and by CTLA-4 expressing T cells and constitutes an important mediator of peripheral immune tolerance. In chronic hepatitis C we found upregulation of IDO expression in the liver and increased serum kynurenin/tryptophan ratio (a reflection of IDO activity). Huh7 cells supporting HCV replication expressed higher IDO mRNA than non-infected cells when stimulated with IFNγ or when co-cultured with activated T-cells. Furthermore, an increase of regulatory T cells in the liver can inhibit an immune response against HCV. We have observed that in the liver of HCV infected patients there are more regulatory T cells than in the liver of other liver pathologies and this effect can be induced directly by the virus because Huh7 HCV infected cells attract more regulatory T cells than the Huh7 cells without infection. Induction of IDO may dampen T-cell reactivity to viral antigens in chronic HCV infection. Therefore, induction of IDO and recruitment of regulatory T cells may dampen T-cell reactivity to viral antigens in chronic HCV infection.

Vaccinia virus E3 protein prevents the antiviral action of ISG15

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The ubiquitin-like ISG15 is one of the most predominant proteins induced by type I interferons (IFN). In this study using MEFs and mice lacking the gene, we demonstrate a novel role of ISG15 as a host defense molecule against vaccinia virus (VACV) infection. In MEFs, the growth of replication competent VACV (WR strain) was unaffected by the absence of ISG15, but virus lacking E3 protein (VV E3L) that is unable to grow in ISG15+/+ cells, however, it replicated in ISG15 deficient cells. Immunoprecipitation analysis revealed that E3 binds ISG15 through its C-terminal domain. In mice lacking ISG15, infection with VV E3L caused significant disease and mortality, an effect not observed after infection of ISG15+/+ mice. Pathogenesis in ISG15 deficient mice infected with VV E3L or with an E3 deletion mutant lacking the C-terminal domain, triggered an enhanced inflammatory response in the lungs compared with ISG15+/+ infected mice. These findings showed an anti-VACV function of ISG15, with the virus E3 protein suppressing the ISG15 antiviral factor.

Inhibition of immune responses by KSHV vOX2 and its cellular counterpart, CD200

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Background & Objectives: The KSHV lytic protein vOX2 shares 36% identity with human cellular CD200. An engineered soluble derivative of vOX2 inhibited neutrophil activity in vitro and had anti-inflammatory activity in vivo, suggesting the protein inhibits innate immune responses. In the present study we determined whether adaptive immune responses are modulated by native, membrane-bound vOX2 and CD200. Methods: Antigen presenting cells (APC) were engineered to express either vOX2 or CD200 on their surface by retroviral transduction and membrane expression was confirmed by flow cytometry and IFA. APC were loaded with epitope peptides and
incubated with appropriate epitope-specific CD4+ or CD8+ T cell clones. Ex-vivo restimulation of epitope-specific CD8+ T cells was performed by coculturing peripheral blood mononuclear cells (PBMC) with autologous APC engineered to express either vOX2 or CD200 on their surface. T cell function was measured by IFN-γ release.

**Results:** Expression of vOX2 or CD200 on the surface of APCs inhibited IFN-γ secretion by co-cultured CD4+ and CD8+ T cell clones up to 50% when compared to vector transduced APCs. Restimulation of PBMC was similarly inhibited. Neither HLA class I, nor CD80 and CD86 costimulatory molecule expression levels were downregulated on APCs by vOX2 or CD200 expression.

**Conclusion:** These data demonstrate that both KSHV vOX2 and cellular CD200 inhibit antigen-specific T-lymphocyte activity. They suggest vOX2 suppresses adaptive immune responses against KSHV lytically infected cells, facilitating virus replication and dissemination.

**African Swine Fever Virus Arrests Cell Cycle in G0/G1 and Targets Cdk2 to the Viral Factory**


African swine fever virus (ASFV) replication in growing Vero cells results in the inhibition of host cellular DNA synthesis and the accumulation of infected cells in the G0/G1 phase. Viral infection of Vero cells synchronized in the G0 phase of the cell cycle prevented cells from entering the S phase after serum stimulation and retinoblastoma protein (pRb) hyperphosphorylation. Nuclear staining and activity for Cdk2 was significantly reduced immediately following ASFV infection, but, interestingly, a rapid increase of the kinase activity and translocation of Cdk2 from the nucleus to the cytoplasm was observed after 16 hpi, correlating with the proteolytic cleavage of p21 and with the initiation of ASFV-induced apoptosis. Furthermore, viral early and late gene expression and DNA synthesis are impaired by roscovitine-mediated inhibition of Cdk2, thus indicating that Cdk2-specific activity is required for ASFV full viral production.

**Dendritic cells interaction with virus: PRRSV vs. influenza virus**

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Spanish pig production accounts for a 30.2% of the total national livestock production. At a world-wide level, the pig sector is expanding, particularly in Asia. Diseases are one of the limiting factors in the competitiveness of the pig sector, and among these health problems, viral infections are the main causes of economic losses. The focuses of our research are viral diseases affecting swine herds and their interactions with the immune system.

Firstly, porcine Circovirus type 2 (PCV2) interactions with the immune system were studied as it apparently modulates the immune response of the host. The implication of different components of PCV2 in the modulation of the immune response of the host were investigated by using PCV2 viral-like particles (VLPs) and 16 novel oligodeoxyribonucleotides containing CpG motifs (CpG-ODNs) based on the PCV2 genomic sequence. Interestingly, CpG-ODNs with inhibitory effect were located within the PCV2 Rep gene. Additionally, PCV2 virus was a very strong IL-12 inducer in porcine bone marrow derived dendritic cells (BMDC) cultures. Whereas IFN-
modulation on BMDC after PCV2 VLP treatment was neglectable, PCV2 VLPs were potent IL-12 inducers. Our data shows that PCV2 viral elements can distinctly regulate cytokine production depending on the cell population studied.

Secondly, studies on porcine reproductive and respiratory syndrome virus (PRRSV) were performed as it is one of the main swine diseases. *In vivo*, PRRSV replicates primarily in alveolar macrophages but has also been shown to replicate in blood monocytes and monocyte-derived macrophages *in vitro*. Our study investigated the susceptibility and the cytokine profile of porcine PBMC, porcine alveolar macrophages (PAM), porcine BMDC and PBMC purified CD172⁺ cells to sixty-three PRRSV strains isolated from 1991 to 2006 in Spain. Regarding the cytokine profiles on BMDC, our results showed that most strains were unable to induce neither IL-10 nor IFN-γ (65.8%); strains inducing IL-10 and IFN-γ were 7.3%; IL-10 only secreting strains were 22% of the total whereas IFN-γ only inducing strains were 4.8%. Our data shows that different PRRSV strains may induce different cytokine profiles in APCs and PBMCs. It seems reasonable to think that these different profiles can affect both the development of an immune response as well as on the response of immune pigs upon reinfection.

Finally, some preliminary studies on new vaccine vectors generated for influenza virus were presented and discussed with the audience. Discussions of our results with Dr. Adolfo García-Sastre lead us to invite him to visit our lab in the near future and discuss our results in more detail.

*Interaction of foot-and-mouth disease virus (FMDV) with dendritic cells: role of IL-10*

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Foot-and-mouth Disease Virus (FMDV) is the causative agent of a highly contagious vesicular disease of cloven-hoofed animals. We have previously described that FMDV serotype C infects lymphocytes *in vivo*, causing a profound lymphoid depletion. This is accompanied by a functional inactivation of antiviral T cells. Such immunosuppression occurs rapidly after infection but eventually the immune response is recovered and able to control the viral infection. The mechanism(s) that initially induces immunosuppression and leads to the loss of T-cell activation is unknown. To understand the mechanisms driving this immunosuppression, we have studied the interaction between T cells and DCs.

We have found that FMDV infects DCs *in vitro* and interferes with their maturation. More interestingly, DCs isolated ex vivo from FMDV infected pigs shown a decreased in co-stimulatory molecules. In antigen presentation assays, DCs isolated from FMDV infected swine were not able to activate T cells. The study of cytokines produced in DCs:T cells co-cultures showed a high amount of IL-10 produced. Interestingly, serum from FMDV infected swine at the peak of viremia showed an increased in IL-10. Blocking of IL-10 by anti-IL10 antibodies in DCs:T cells cocultures recovered the antigen capacity stimulation of DCs. The in vivo role of IL-10 is generally immunosuppressive although this cytokine plays also an important stimulatory role in the production of antibodies by B-lymphocytes during the development of an immune response against antigens from pathogens. Our data suggest that IL-10 is being produced in FMDV infected swine and may inhibit thymus dependent (TD) antibody responses, reflected in the observed T cell immunosuppression. By contrast, anti-FMDV thymus independent (TI) antibody response may not be affected. We are currently carrying out several experiments to rule out these possibilities.
**Effects of viral hemorrhagic septicemia virus, a fish rhabdovirus, on the RTS11 macrophage-like cell line**

Carolina Tafalla, CISA-INIA. Valdeolmos, Madrid, Spain.

The effect of viral hemorrhagic septicemia virus (VHSV) was studied on the established rainbow trout (*Oncorhynchus mykiss*) monocyte/macrophage-like cell line RTS11. The virus was not able to complete its replication cycle as infectious viral particles were not released from the cells. However, in RTS11, the virus was capable of producing mRNA from at least its N and G genes. At the protein level, only N protein was detected two days post-infection, whereas a faint band corresponding to the G protein was also observed after 5 days post-infection. These results suggest an interruption of viral protein translation at some point. The expression of N mRNA was significantly inhibited in cells pre-treated with Poly I:C, but not affected by 2-aminopurine (2-AP), an inhibitor of the dsRNA-dependent protein kinase (PKR), thus indicating that PKR has no effect on mRNA expression directly. However, when cells were preincubated with Poly I:C in the presence of 2-AP, the levels of N mRNA were restored suggesting that Poly I:C can limit viral transcription through an antiviral mechanism dependent of PKR. The effect of VHSV on the expression of transcripts for different immune genes was determined, but significant induction was found only for genes related to the type I interferon (IFN) response, such as IFN-1 and -2 and the three Mx isoforms. Heat-inactivated virus failed to induce IFN-1 and -2, suggesting that early events in the VHSV life cycle were necessary for the type I IFN response. Poly I:C alone also induced transcripts for the antiviral Mx proteins. Prior exposure of RTS11 to VHSV did not prevent Poly I:C from inducing transcripts for Mx1, Mx2 and Mx3. Perhaps the failure of VHSV to disable antiviral mechanisms in RTS11 accounts for the aborted infections.

**Fish innate immune responses and inflammation against virus**

Beatriz Novoa, Instituto de Investigaciones Marinas (CSIC) Vigo, Spain.

Our group focuses on the study of the interactions of virus and fish. These pathogens cause important mortalities in the aquacultured populations, with the associated economical losses. The infectious pancreatic necrosis virus (IPNV), the infectious hematopoietic necrosis virus (IHNV), the viral hemorrhagic septicemia virus (VHSV) and nodavirus are the most important viruses affecting to freshwater and marine aquaculture fish species.

We have studied the effect of these viruses on the fish innate immune system mechanisms and in the later years, we have began studies of the molecular basis of these responses.

The lack of reagents and gene sequences data have made this task difficult at the begging, however, now, the new genomic tools such as the construction of cDNA libraries, the use of microarrays or the application of zebrafish (Danio rerio) as a model species may facilitate this research field.

**Sequestration of small RNAs by the silencing suppressor P1b from the ipomovirus Cucumber vein yellowing virus**

Adrian Valli, Centro Nacional de Biotecnología-CSIC, Madrid, Spain.

RNA silencing plays an important antiviral role in eukaryotes. To counteract this defense barrier, most plant viruses of the *Potyviridae* family express the silencing
suppressor HCPro. Recently, we have shown that a member of the family Potyviridae, the ipomovirus Cucumber vein yellowing virus (CVYV) that lacks HCPro1 has a duplicated P1 coding sequence2, and the downstream P1 copy, named P1b, has RNA silencing suppression activity3. We have suggested that CVYV P1b suppresses RNA silencing by siRNA interaction, in similar manner than HCPro4. EMSA experiments revealed that CVYV P1b binds double stranded, but not single stranded, siRNAs. Moreover, by competition assays we found that CVYV P1b is similar to the potyviral HCPro protein in preferring 21-nt duplexes rather than 24-nt or 26-nt duplexes. However, whereas HCPro has been described to require 2-nt overhangs for siRNA binding, CVYV P1b binds a blunted 19 nt duplex with high efficiency. In vivo binding of CVYV P1b to Nicotiana benthamiana siRNAs was demonstrated by co-purification experiments. Small RNAs interacting with CVYV P1b are being analyzed by high-throughput sequencing. Preliminary results confirmed the preference of CVYV P1b for siRNAs of 21 nt.

A mutational analysis has localized a siRNA-binding domain of CVYV P1b in a conserved basic motif placed just upstream from a putative zinc finger that is required for correct oligomerization of the protein.

In spite of the absence of any appreciable sequence similarity between CVYV P1b and potyviral HCPro, these proteins appear to be functionally equivalent since Plum pox potyvirus (PPV) chimeras whose HCPro or P1-HCPro coding sequences were replaced by that of CVYV P1b were viable and infected with varied degrees of efficiency different PPV hosts.


Arbovirus interference with mosquito innate immunity- the Semliki Forest virus model

Alain Kohl, Centre for Infectious Diseases, University of Edinburgh, Edinburgh, UK

Arboviruses is a generic name for a large number of viruses from various families transmitted to humans or animals by arthropods (mosquitoes, ticks). They can cause mild to severe disease (encephalitis, hemorrhagic fever etc.) in vertebrates. The most important members of these virus families are the Flaviridae (Dengue, Yellow fever), Togaviridae (Semliki Forest virus, Chikungunya) and the Bunyaviridae (Rift Valley fever etc.), with some viruses from other (mostly RNA) virus families also represented. One of the characteristics of these viruses is that they cause little or no disease in arthropods by which they are transmitted. While we have good understanding of innate immune response pathways in vertebrates, we know little about arthropod antiviral immunity. It seems that some pathways (STAT etc.) are conserved throughout evolution but how viruses manage to persistently infect arthropods and how arthropods deal with arboviruses is the subject of my lab’s research. We work mainly on Semliki Forest virus (SFV), a prototype alphavirus from the Togaviridae family. I will present some of our results on how arboviruses interact with immune signaling pathways in mosquito cells, and which cellular signalling pathways interact in turn with virus replication. We use a variety of reporter systems to study these mechanisms, as well as direct analysis of SFV replication in mosquito cells. These results contribute to our general understanding of arbovirus biology, and allow us to compare the biology of infection in very different lineages of viral hosts.
8. Evaluation questionnaire

(VER DOCUMENTO ADJUNTO)

9. General comments

Very professional and helpful service provided by the co-ordinator, Belén Fortea. This sort of opportunity for young scientists to present their work & meet with & discuss their science with leaders in the field in Spain and Britain. Is very noble & worthwhile. A privilege participating. (Christine Ferguson)

A really enjoyable meeting- well done! A good balance of numbers of participants & informality promoted interaction. I expect to collaborate as a result of this meeting. (David Blackbourn)

I have met many good virologists, now they know what I'm doing. I know what they are doing so if I need them it should be easier to collaborate. And I have a better knowledge about virus and immune response (Rafael Aldabe)

In my opinion the subjects exposed in the meeting were too diverse. Probably, restriction of presentations to a few numbers of viruses sharing common characteristics (involved in human disease and mammalian viruses...) would result in a more comprehensive meeting. (Pepe Alcami)

A very productive and enjoyable meeting. (Greg Towers)

This has been one of the best meetings I've attended. I liked very much that we had an afternoon for an external activity; it was a perfect set up for informal discussion. My only suggestion would be to encourage the British Council and CSIC to repeat this experience more frequently.

10. Acknowledgements

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