IMMUNOTHERAPIES FOR CANCER AND INFECTIOUS DISEASES

John Hiscott

Istituto Pasteur Italia – Fondazione Cenci Bolognetti
john.hiscott@istitutopasteur.it

This research program focuses on the early events involved in the host response to RNA virus infection, with the long-term objective to utilize knowledge of the immune response against virus infection to develop novel immunotherapeutic approaches for the treatment of infectious diseases and cancer. Our goal is relevant for the translational development of novel antiviral and adjuvant compounds to augment immunity against diverse viral pathogens, including influenza, dengue, and chikungunya. This objective is also important for the development of oncolytic virus therapies for cancer, since defects in innate antiviral signaling in tumor cells contribute to the selective growth of replicating oncolytic viruses in cancer versus normal tissues. Below the main research themes of the laboratory are summarized.

1. Characterization of antiviral and adjuvant properties of RIG-I agonists

The cytosolic innate sensor RIG-I senses 5'triphosphate containing RNA to initiate a potent antiviral immune response against RNA virus infection, activating both interferon and inflammatory responses, through TBK1/IRF3 and IKK/IκBα/NF-κB pathways, respectively. RIG-I activation can also trigger apoptosis, thus limiting viral replication and spread, independently of induction of the antiviral program. Previously, our group developed and characterized a sequence- and structure-optimized RIG-I agonist (M8) that stimulated a robust innate immune response against viral infection. Furthermore, it was shown that M8 acted as a potent vaccine adjuvant against influenza, leading to high antibody titers and Th1-shift in immune responses. During the past year, we tested M8 as a cancer immunotherapeutic by taking advantage of its dual ability to induce cell death and activate innate immunity. Our results demonstrate in different cancer cell models that stimulation of the RIG-I pathway by M8 induced immunogenic cell death and maturation of dendritic cell function.

In multiple cancer cell lines, M8 treatment activated RIG-I signaling, leading to interferon and inflammatory cytokine upregulation and cancer cell apoptosis, dependent on activation of NOXA, caspase 9/3 cleavage, and Poly ADP ribose polymerase cleavage. Furthermore, M8-induced inflammatory activation in tumor cells was sufficient to mature dendritic cells (DCs) and induced chemokine synthesis. Additionally, direct effects of M8 on DCs included upregulation of costimulatory molecules (CD80, CD86),

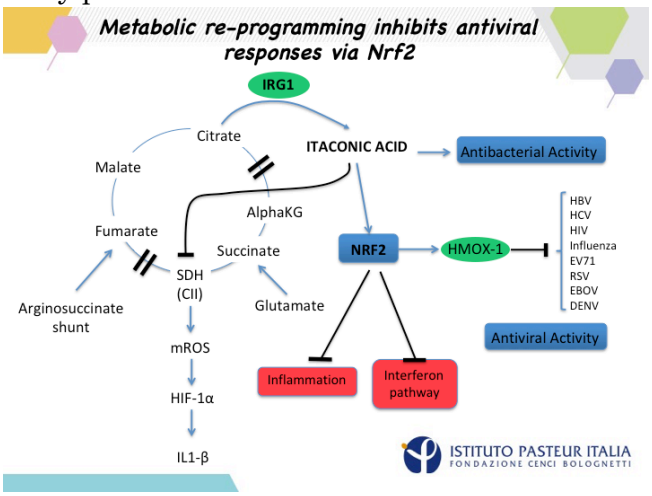
strong induction of inflammatory chemokines CXCL9 and CXCL10, and upregulation of Th1 cytokine IL-12. Th1-biasing activity was further validated with *in vitro* stimulation assay of Ag specific T cells. Next we analyzed whether M8 treated cells showed the typical markers of immunogenic cell death by flow cytometric staining of calreticulin and HMGB1 release by ELISA. M8 treated cells showed high level expression of calreticulin on cell surface, as well release of HMGB1 at a level comparable to the immunogenic cell death inducer mitoxantrone. Altogether, these results highlight the potential of the RIG-I agonist M8 in cancer immunotherapy, and as a complementary strategy in combination with immune checkpoint inhibitors. This project is progressing with the collaboration of BioNTech/TRON (Mainz) who are formulating M8-nanoparticles for delivery, and with Bristol Myers Squibb who are testing M8 in combination with immune checkpoint inhibitors to evaluate synergistic anti-tumor efforts in a variety of tumor models.

We are also investigating the crosstalk between RIG-I signaling and activation of the DNA sensing cGAS-STING pathway. Both RIG and STING pathways stimulate immunogenic cells death and contribute significantly to tumor-specific cell death mechanisms, as highlighted in Zevini et al, 2017. The mechanisms of cross-talk between the innate cytosolic sensors RIG-I and STING and their relative contributions to antiviral immunity and myeloid differentiation are currently being investigated. Preliminary results from our laboratory indicate that activation of the RIG-I pathway also induces STING expression in an IRF3/NF-□B dependent manner, thus eliciting a functional amplification/cross-talk between both pathways. Our hypothesis is that STING activation is a critical component of the amplification of the host antiviral response and changes in STING expression may alter cellular function during myeloid differentiation.

2. Metabolic regulation of the innate immune response in dengue virus infection

Dengue virus (DENV) is a mosquito-borne virus that causes dramatic public health issues in more than 100 countries, with an estimate of 390 million people infected annualy. Given the elevated levels of oxidative stress markers in patients with severe dengue infection, we sought to analyze the relationship between reactive oxygen species (ROS) and DENV infection. Previously, we demonstrated a positive correlation between ROS production and viral replication in DENV-infected monocyte-derived dendritic cells (MDDCs). We now show that DENV-infected MDDCs are defective in activating the antioxidant defense system and demonstrate the transcription factor Nrf2, a master regulator of the cellular response to oxidative insults, actively translocated into the nucleus upon infection, without upregulating antioxidant gene transcription. The concomitant use of chemical NRF2 activators during infection improved DENV-induced NRF2 nuclear import, but not the expression of heme oxygenase, one of the most highly upregulated NRF2 target genes. To understand the role of NRF2, we studied DENV infection in human lung epithelial A549 cells that contain a loss-of-function mutation in KEAP1, the natural inhibitor of NRF2. Although the antioxidant response was

constitutively active in A549, DENV infection still caused ROS accumulation. Using WT, Keap1-knockout (Keap1 $^{-/-}$), and Nrf2-knockout (Nrf2 $^{-/-}$) A549 cells, DENV infection and replication were facilitated in Nrf2 $^{-/-}$ cells, whereas in Keap1 $^{-/-}$ cells, DENV infection was strongly inhibited. These results suggest that DENV infection manipulates the NRF2-dependent antioxidant response to modulate viral replication in MDDC and epithelial cells. The loss of the antioxidant gene expression during the DENV infection may provide conditions for ROS accumulation that aggravate DENV pathogenesis.



The link between the innate antiviral immune response and changes in Krebs cycle metabolism is also being investigated; it has recently been shown that the metabolic by-product itaconate induced Nrf2 expression and interfered with inflammatory and antiviral gene expression via the Nrf2 antioxidant pathway; treatment with itaconate or the chemical Nrf2 inducer sulforaphane repressed STING expression as well as other interferon stimulated genes-ISGs (see Figure 1). These preliminary studies link metabolic re-programming and Krebs cycle by products with the induction of the Nrf2 anti-oxidant pathway and inhibition of the antiviral response.

3. Combination strategies in the development of oncolytic immunotherapy

Our laboratory has for more than ten years explored a novel virus-based approach to immunotherapy of cancer, involving oncolytic viruses that replicate to high titers in tumor tissue, resulting in immune-mediated and virus-induced lysis. Although virotherapy with the prototype Vesicular Stomatitis Virus (VSV) is often effective against a variety of cancer cells, many primary tumor cells are resistant to VSV oncolysis. We were the first group to demonstrate that resistant tumor cells are sensitized to VSV-mediated killing in combination with epigenetic modulators -histone deacetylase inhibitors (HDI) - in a variety of resistant cancers. HDIs represented reversible chemical switches that dampen the innate antiviral response and improve the susceptibility of resistant cancer cells to VSV infection and spread. *(Nguyen et al, Proc. Natl. Acad. Sci. USA 2008; Samuel et al, Mol Ther. 2010; 2013; Shulak et al, J. Virol. 2014; Beljanski et al, Biol. Chem. 2015).* Despite significant progress in OV immunotherapy, the heterogeneity of the clinical response to OV-based therapies, as well as the engagement of the adaptive immune response against viral rather than tumor antigens, represent significant obstacles to the large-scale clinical implementation of

oncolytic virotherapy against multiple types of cancer. We have continued to investigate the role of autophagy in regulating synergism between the HDI vorinostat and oncolytic VSV in prostate cancer, using human PC3 and DU145 cells and a murine model bearing TRAMP-C2 mouse prostate cancer cells.

To address the role of specific HDACs to impinge viral infection and output, the capacity of different HDAC inhibitors to augment VSV replication and oncolysis was evaluated in human prostate cancer (PC-3) cells that display significant resistance to VSV□51-mediated cell killing. The effect of Tubastatin A (TBSA), an HDAC6 specific inhibitor, or RGFP109, a specific HDAC 1/3 inhibitor, or Resminostat (Resm), a well-known HDAC 1/3/6 inhibitor, were compared to the ability of Vorinostat (SAHA) to potentiate VSV infectivity, as quantified GFP positive cells. HDAC6 inhibition was sufficient to enhance viral infection, as demonstrated by an increase in the number of VSV-infected cells (11 to 62% with TBSA) but did not increase cell death in the VSV infected population. In contrast, combined treatment that included SAHA or Resminostat, increased cancer cell death to 60-80%. To assess the involvement of the intrinsic apoptotic pathway in mediating VSV-induced cell death, apoptotic gene expression was evaluated in cells treated with SAHA, RESM or TBSA and infected with VSV. BH3-only pro-apoptotic genes Puma and Noxa were upregulated in HDIs pretreated cells, while a strong impairment of anti-apoptotic Mcl1 gene expression was observed in SAHA and RESM-treated infected cells, but not in TBSA-treated infected cells.

SAHA treatment induced a strong cell cycle block in PC-3 cells, accompanied by a reduction in S-phase and accumulation in G2/M-phase. To address a possible role for the cell cycle arrest in the enhanced VSVΔ51 infectivity of PC-3 cells treated with SAHA, the effect of the different HDIs on cell proliferation was determined. Comparative analysis demonstrated that RESM reduced the percentage of S-phase cells and accumulated cells in G2/M-phase; furthermore, SAHA and RESM treatments led to upregulation of p21 and p16/INK4A cyclin dependent kinases at the protein level. Conversely, TBSA treatment did not exert the same effect, suggesting that simultaneous inhibition of HDAC 1/3/6 was responsible for cell cycle arrest. SAHA and RESM strongly activated I□B□degradation, as well as enhanced autophagic flux as evidenced by turnover of p62 and increased lipidated LC3B II accumulation. Either SAHA or RESM pretreatment was able to induce the production of pro-inflammatory cytokines, such as IL6 and IL8, in VSV-infected cells. Taken together, these features are characteristic of a senescent-associated secretory (SASP) phenotype, suggesting that a SASP-like phenotype may be implicated in the increased susceptibility to VSV infection in SAHA and RESM-treated PC-3.

Using the highly metastatic Tramp-C2 cell model as a representative of aggressive prostate tumors, we analyzed efficacy of Vesicular Stomatitis Virus (VSV) with a special focus on immune activation in immunocompetent mice. VSV intratumoral injection led

to infection of both cancer and immune cells (especially monocytes and macrophages) within tumor microenvironment, as assessed by flow cytometry at 24h after injection. Upon VSV treatment, rapid immune changes were observed in the spleens of treated mice with a marked increase of myeloid subpopulations and a decrease of NK and T cells, thus highlighting a potent reshaping of immune system upon VSV treatment. Moreover, injection of VSV at $500x10^6$ PFU was sufficient to block tumor growth of subcutaneously implanted Tramp-C2 tumors. Furthermore, the block in tumor progression was associated with a strong increase of T cell tumor infiltration, particularly cytotoxic CD8+ cells, thus indicating that VSV infection stimulated an antitumor immune response. Together, these results highlight the strong immunostimulatory properties of VSV in aggressive prostate cancer.

In part from above studies on DENV pathogenesis, we sought to evaluate the efficacy of small molecule regulators of the anti-oxidant response in oncolytic virotherapy, since manipulation of the anti-oxidant network via transcription factor Nrf2 augmented VSV replication and sensitized cancer cells to viral oncolysis. Activation of Nrf2 signaling by the antioxidant compound sulforaphane (SFN) enhanced VSV spread in OV-resistant prostate cancer cells and improved the therapeutic outcome in different murine syngeneic and xenograft tumor models. Furthermore, chemoresistant A549 lung cancer cells that display a constitutive dominant hyperactivation of Nrf2 signaling were highly susceptible to VSV oncolysis. Mechanistically, enhanced Nrf2 expression and signaling stimulated viral replication in cancer cells and disrupted the type I IFN response via increased autophagy. This study revealed a previously unappreciated role for Nrf2 in the regulation of the innate antiviral response that complements the therapeutic potential of VSV-directed oncolysis against multiple types of OV-resistant or chemoresistant cancer.

2017 PUBLICATIONS

Vijayan M, Xia C, Song YE, Studstill CJ, Johnson M, Baldwin M, **Hiscott J**, Kester M, Alexander S, Hahm B. Sphingosine 1-phosphate lyase enhances the activation of IKKε to promote the antiviral type I interferon response. J. Immunol. 99:677-687 (2017)

Olagnier D, Lababidi R, Bel Hadj S, Goulet ML, Chiang C, Sze A, Liu Y, Knatko E, Dinkova-Kostova A, Lin R, **Hiscott J.** Re-programming the oxidative stress response by manipulation of Nrf2 enhances viral oncolysis. Mol. Therapy 25:1900-1916 (2017).

Pelliccia S, Wu Y-H, Coluccia A, La Regina G, Tseng C-Km Famiglini V, **Hiscott J**, Lee J-C, Silvestri R. Inhibition of dengue virus multiplication by novel inhibitors of RNA-dependent RNA polymerase and protease activities. J Enzyme Inhib Med Chem. 32: 1091-1101 (2017).

Olagnier D, Chiang C, **Hiscott J**. Evaluation of innate immune gene expression following HDAC inhibitor treatment by high throughput qPCR and PhosFlow cytometry. Methods Mol Biol.1510:245-255 (2017).

Metcalf TU, Wilkinson PA, Cameron MJ, Ghneim K, Chiang C, Wertheimer AM, **Hiscott J**, Nikolich-Zugich J, Haddad EK. Human monocyte subsets are transcriptionally and functionally altered in ageing in response to pattern recognition receptor agonists. J Immunol. 199:1405-1417 (2017).

Zevini A, Olagnier D, **Hiscott J.** Cross-talk between cytosolic RIG-I and STING innate sensing pathways. Trends in Immunology 38:194-205 (2017).

Di Nicola M, Apetoh L, Bellone M, Colombo MP, Dotti G, Ferrone S, Muscolini M, **Hiscott J**, Anichini A, Pupa SM, Braud F, Del Vecchio M. Innovative therapy, monoclonal antibodies and beyond. Cytokine Growth Factor Rev. 38:1-9 (2017).

Lab Members

Luciano Castiello, PhD
Michela Muscolini PhD
Alessandra Zevini PhD
Matteo Ferrari MSc
Enrico Palermo MSc
Dominga Lovechia BSc
Fani Souvalidou BSc

International Student Exchanges

Shirley Mann (MSc Student, Leiden University Medical Center)
Giselle Rangel (PhD student, University of Madrid, Spain)
Apurwa Trivedi (MSc Student, Pierre & Marie Curie University, Paris France)

Collaborators

David Olagnier/Martin Jakobsen/Soren Paludan – Aarhus University
Bernadette van den Hoogen/Ron Fouchier - Erasmus University
Marjolein Kikkert/ Eric Snijder - Leiden University Medical Center
Frank van Kuppeveld - Utrecht University
Luke O Neill/Andrew Bowie - Trinity College Dublin
Romano Silvestri/Francesca Cutruzzola – Sapienza University
Marco Sgarbant/Angela Battistini/Enrico Proietti - ISS