TOPIC Immunity, Autoimmune and Autoinflammatory Diseases

best oral presentation

EDGAR FERNANDEZ-MALAVÈ

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N-ras is a negative regulator of the pro-inflammatory potential of dendritic cells

Ras GTPases (H-, K-, and N-ras isoforms) are small GTP-binding proteins coupling surface receptors to intracellular signaling pathways controlling cell survival, proliferation, and differentiation. Ras isoforms are highly homologous and expressed ubiquitously, raising questions as to their functional specificity. N-ras in particular is widely expressed in both innate and adaptive immune cells, including dendritic and T cells. However, the specific roles of N-ras in these cells and its relevance to immune responses remain to be determined. Here we show that bone marrow precursors from N-ras-deficient mice differentiated efficiently into dendritic cells (DCs) upon culture with GM-CSF, and displayed higher levels of CD40 and CD86 compared to wild-type counterparts. Up-regulation of these surface molecules in response to TLR4 and TLR9 ligands (LPS and CpG-ODN, respectively) was also augmented in DCs lacking N-ras. Notably, IL-12 and IL-23 production were strongly induced by CpG-ODN, but not by LPS, in N-ras-deficient DCs compared to both wild-type and H-ras-deficient counterparts. In a L. major infection model, CpG-ODN administrated following parasite inoculation restored Th1 responses in N-ras-deficient mice, turning otherwise susceptible into resistant mice. However, in an EAE model, pro-inflammatory features of N-ras-deficient DCs were not sufficient to sustain pathogenic Th1 cell differentiation and infiltration of the central nervous system, resulting in attenuated disease in mice lacking N-ras. Taken together, our study unveil a novel and specific role for N-Ras as a negative regulator of the pro-inflammatory potential of DCs. Further, it suggests that N-ras could be a therapeutic target to modulate inflammation in the context of infection and autoimmunity.

best oral presentation

MARKUS HOFFMANN

University of Erlangen-Nuremberg

The oxidative burst mediates anti-inflammatory clearance of dead cells in a mouse model of Systemic lupus Erythematosus and inflammatory arthritis

Background and Objectives: The production of reactive oxygen species (ROS) via the oxidative burst has in the traditional view been connected with promotion of inflammation and tissue damage, but has in recent years also been implicated in regulation of inflammation and protection from autoimmune arthritis and multiple sclerosis. The aim of this project was to elucidate the impact of the oxidative burst on the development of lupus-like autoimmunity.

Methods: Lupus and arthritis (rupus) was induced by intraperitoneal injection of 0,5 ml pristane oil in BALB/c and Ncf1** mice carrying a mutation in one of the subunits of the phagocyte NADPH oxidase complex that abrogates ROS-production. The clinical course of rupus was compared by analysis of serological markers and organ involvement. Ex vivo phagocytosis assays and FACS were employed to demonstrate differential uptake and degradation of cell debris in Ncf1** and WT mice. Formation of neutrophil extracellular traps (NETs) was monitored in blood and peritonea.

Results: After pristane-injection Ncf1** mice develop strongly elevated levels of typical lupus-autoantibodies, e.g.; anti-dsDNA, anti-histone and anti-Sm/RNP and arthritis. A certain amount of spontaneously occurring autoantibodies was also apparent in Ncf1** mice. Ncf1** mice also spontaneously developed signs of glomerulonephritis and exhibited glomerular deposits of complement and immunoglobulins, further exacerbated by injection of pristane. Interestingly, in Ncf1** mice we observed a preferential uptake of dead cell material into CD11b+Ly6Chigh monocytes and a dramatically reduced ability to forms NETs. In contrast, there was no difference in uptake of inert latex beads between Ncf** and WT mice, but we could see a difference if we coated the latex beads with Immunoglobulin G.

Conclusions: Our results show that the spontaneous autoimmunity occurring in the ROS-deficient Ncf1** mouse gives rise to exacerbated Rupus. Aberrant phagocytosis in ROS-deficient animals caused by spontaneously occurring autoantibodies to surface molecules of dead cell materials could contribute to this phenotype.

oral presentation

KELLY L. HUDSPETH
Humanitas Clinical and Research Center

Homeostasis of Hepatic NK cells

The role of natural killer cells in the immune response is ever expanding. Recently several groups have reported a population of liver-resident murine hepatic NK cells that are endowed with the ability to mount antigen-specific memory responses to haptens as well as viruses. We have identified in the human liver, that the $CD56^{bright}$ and $CD56^{dim}$ subsets found in the hepatic sinusoids represent two discrete populations of NK cells. $CD56^{dim}$ NK cells present in the liver were found to be highly similar, both phenotypically as well as the transcriptionally, to its' counterpart in the peripheral blood indicating that these cells may represent a circulating subset of NK cells. In contrast, hepatic $CD56^{bright}$ NK cells displayed significant phenotypic and transcriptional differences as compared to peripheral blood $CD56^{bright}$ NK cells and express markers associated with tissue-resident lymphocytes, such as CXCR6, CD69 and CCR5, suggesting that these cells correspond to the liver-resident NK cell population described in mice. The high expression of CCR5 on these cells along with the presence of the CCR5-specific chemokines Mip1-α and RANTES in the sinusoids suggests a mechanism by which liver-resident $CD56^{bright}$ NK cells are maintained in the liver sinusoids during homeostatic conditions. Our results once again illustrate the complex role of NK cells in the immune response, specifically in the population of $CD56^{bright}$ hepatic NK cells.

oral presentation

MONIKA JURKOWSKA
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HLA-DRB1 alleles frequency in Polish patients with Mixed Connective Tissue Disease – searchfor risk and protective alleles

Mixed Connective Tissue Disease (MCTD) is a systemic autoimmune disease, originally defined as a connective tissue inflammatory syndrome with overlapping features of systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), polymosistis/dermatomyositis (PM/DM) and systemic sclerosis (SSc), characterized by the presence of antibodies against components of the U1 small nuclear ribonucleoprotein (U1snRNP). Both genetic and environmental factors affect susceptibility to and severity of MCTD.
HLA-DRB1 alleles are well established susceptibility factors in RA ('shared epitope' of DRB1*01 and DRB1*04) and SLE (DRB1*15, DRB1*03).The MHC was the first locus and HLA-DRB1*04 was the first allele demonstrated to determine the congenital susceptibility to MCTD. The aim of the study was to assess the frequency of high-resolution-typed DRB1 alleles in a cohort of Polish patients with MCTD and to compare it to the RA and SLE cohorts. Identification of potentially risk and protective variants was carried out by comparison to the DKMS Polish bone marrow donors registry (41306 alleles). The project has been accepted by Bioethics Committee of Institute of Rheumatology. Genomic DNA was isolated from blood leukocytes of 103 MCTD, 41 SLE and 184 RZS patients. The STB strategy has been used for typing. Primers complementary to DRB1 exon 2 consensus regions were designed. 7 parallel PCR reactions were carried out per patient, with subsequent Sanger sequencing in case of positivity. The IMGT Database was the source of reference alleles. The odds ratios (ORs) and 95% confidence intervals (95% CIs) associated with the different HLA-DRB1 alleles were analyzed. Statistical analysis was performed with Bonferroni correction.
Results: DRB1*15:01 was identified as a risk allele in both MCTD and SLE (Table 1) while when all DRB1*04 alleles taken into account together increased significantly only susceptibility to RA (Table 2) In the MCTD cohort it didn't reach significance after Bonferroni correction. DRB1*07:01 allele was in our cohorts protective against both MCTD (OR 0.3, p=0.0006) and RA (OR 0.3, p<0.0001).

Tab. 1 DRB1*15:01 as risk allele in MCTD and SLE

cohort	No. of alleles	OR	95 % CI	p	Bonferroni
SLE	20	2.3291	(1.4058-3.8588)	0.0010	*
MCTD	62	3.0661	(2.2747-4.1328)	< 0.0001	**
RA	23	0.5973	(0.3900-0.9149)	0.0178	ns

Tab. 2 DRB1*04 as risk alleles in RA and MCTD

cohort	No. of alleles	OR	95 % CI	p	Bonferroni
SLE	9	1.0504	(0.5252-2.1008)	0.8895	ns
MCTD	31	1.4922	(1.0175-2.1883)	0.0405	ns
RA	51	1.6972	(1.2508-2.3028)	0.0007	**

Our results confirm the modulating influence of HLA-DRB1 genotypes on development of connective tissue diseases, like RA, SLE and also MCTD. Alleles distribution underlines that MCTD, this time genetically, shares features of both RA and SLE.

best poster presentation

SHEENA BARATONO

Immunology Graduate Group of the University of Pennsylvania Children's Hospital of Philadelphia, Philadelphia

IFN and TLR9 signaling block lymphocytic differentiation of CLPs in the repeated TLR9 stimulation model of cytokine storm

Hyperinflammatory syndromes come in a variety of flavors with different immunogens and cytokines leading to variations in organ pathology. This inflammation can be driven by viral, autoimmune, or neoplastic conditions leading to profound and widespread organ pathology including hepatitis, fever, splenomegaly, cytokinemia and pan-cytopenias, including T and B lymphopenias (cite). The effect of the hyperinflammatory environment on the bone marrow results in the development of hemophagocytosis and mobilization of erythropoiesis to the spleen, but the effect of inflammation on hematopoietic precursors is less well characterized. The developing immune cells in the bone marrow are known to express a variety of cytokine and inflammatory receptors yet the effects of signaling through these receptors on hematopoiesis is incomplete. Since many infectious and autoimmune syndromes result in sustained TLR9 stimulation, understanding the effects of TLR9 driven inflammation on hematopoiesis is important for both questions of pathogenesis as well as possible therapeutic interventions that might help to restore homeostasis.

In this study we demonstrate that B lymphopoiesis is inhibited during TLR9 driven inflammation from the Ly-6D+ CLP stage onwards with different effects inhibiting development at multiple stages across B cell development. We show that TLR9 signaling can directly decrease in vitro B cell yields from CLPs, Pro and Pre B cells while increasing T cell yields. While TLR9 can inhibit B cell growth in vitro, using mixed bone marrow chimeras, we show in vivo that this TLR9 intrinsic effect is masked, likely due to other TLR9 induced inflammatory factors that also mediate decreases in B lymphopoiesis. This led us to demonstrate that IFNg also directly inhibits B cell differentiation in vitro as well as when induced by TLR9 in vivo. Microarray and RT-PCR analysis of Ly-6D- CLPs points to HOXa9 as an early B-cell directing transcription factor that is altered by TLR9 induced inflammation, as it and many of its known targets are also decreased in Ly-6D- CLPs. Additionally EBF-1, another factor essential for initiation of the B cell program was transcriptionally decreased in Ly-6D- and Ly-6D+ CLPs. Our work demonstrates both cellular and molecular targets that lead to altered B-lymphopoiesis in sustained TLR9 driven inflammation that may be relevant in a number of infectious and autoimmune/inflammatory settings.

best poster presentation

RAKESH MISHRA

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Suppressing LPS-induced fra-1 expression in macrophages by MAPK inhibitors: a critical role of MAPK-signaling

Rationale: Macrophages represent the first defense line against bacterial infection and therefore, play a crucial role in inflammatory response. MAPK signaling pathways lead to the enhanced ability of Jun and Fos family members (i.e., components of the activator protein [AP]-1 transcription factor) to activate transcription of a number of AP-1–dependent target genes involved in cell proliferation or death, differentiation, and inflammation. Recently, we have shown that the Fra-1/AP-1 transcription factor plays an important role in promoting LPS-induced acute lung injury and mortality in mice by inducing fra-1 expression. We have observed that lipopolysaccharide (LPS) induces fra-1 in MH-S cells and in this study have examined whether these responses are mediated by the mitogen-activated protein kinase (MAPK) signaling pathway. However, the molecular mechanisms by which MAPK- signaling regulate the LPS-induced fra-1 expression are not fully understoodHere, we have studied the mechanisms of Fra-1 induction by LPS in mouse alveolar macrophages and examined whether MAPK-signaling involved in this process.

Methods: A murine alveolar macrophage cell line, MH-S, was cultured and exposed to LPS (100 ng/ml) in serum-containing medium for 0 to 6 h in the presence or absence of MAPK inhibitors; (SB235699,p38 -inhibitor) or (SP600125,JNK-inhibitor) or ERK1/2 inhibitor (U0126), after which cellswere harvested for Fra-1 mRNA and protein expression was analyzed by qRT-PCR and immunoblot analysis, respectively. Fra-1 promoter activation by LPS was evaluated by transient transfection assays using Fra-1 promoter-reporter constructs. Using a Chromatin immunoprecipitation assays revealed an enhanced recruitment of Fra-1 to the endogenous fra-1 promoter upon LPS stimulation, the effects of MAPK-inhibitor in LPS-induced Fra-1 promoter activity in alveolar macrophages was evaluated. Results: LPS was found to induced fra-1 mRNA and protein expresstion in MH-S cells . LPS-induced fra-1 expression almost completely attenuated when cells were treated with of MAPK inhibitors; (SB235699,p38 -inhibitor) or (SP600125,JNK-inhibitor) or ERK1/2 inhibitor (U0126). Next, we measured fra-1 promoter activity upon LPS-stiumaltion in the presence or absence of MAPK inhibitors. LPS-induced Fra-1 promoter activity and MAPK-inhibitor markedly blocked fra-1 promoter.We next performed the ChIP assay to confirm further the mechanism by which MAPK regulates fra-1 expresstion in MH-S cells. Cells were stimulated with LPS for various time point, and the ChIP assay was performed by using ChIP –assay kit, it was found that increadsed recruited to the fra-1 promoter in response to LPS and MAPK-inhibitor suppressed the recruitment of fra-1 to the promoter.

Conclusion:Taken together, our results suggest a critical mechanistic role MAPK-sgnaling in LPS mediated fra-1 expresttion in macrophages.

best poster presentation

HALEH TALAIE

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Alteration of Serum Levels of Interlukin 1 and Tumor Necrosis Factor in Depression Independentof Treatment or Overdose of Tricyclic Antidepressants

The aim of this study was to evaluate the status of serum IL-1b and TFN-α in a depressed patients who treated or non-treated or poisoned with tricycli antidepressant (TCAs) in comparison to healthy subjects. In this prospective comparative study, patients were selected from those admitted at Loghman-Hakim Hospital from August 2007 or January 2008. Serum level of IL-1b and TFN-α were compared amog group of subjects (10 in each) of healthy subjects, TCA-poisoned patients, TCA-treated depressed patient and non-treated depressed patients. Demographic and clinical data were collected by a questioner filled out by a trained practitioner in daily clinical management. Blood was tested for liver function, blood cells, electrocardiography and arterial blood gases. Complete blood analysis and demographic data did now show significant change between groups. IL-1b level was higher among females. The group of depressed patients non-treated with TCAs showed higher serum levels of IL-1b and TFN-α that other groups. No significant difference was onserved in IL-1b and TFN-α values among healthy control, depressed TCA-treated and TCA-poisoned groups. It is concluded that depression and gender may influence the production of cytokines while neither TCAs treatment nor its overdose affect IL-1b and TFN-α.

poster presentation

ARINNA BERTONI

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Cryopyrin associated periodic syndromes (CAPS): investigations on knock-in mouse model to exploit novel approaches for the modulation of the NLRP3 inflammasome

Cryopyrin-associated periodic syndromes (CAPS) are autoinflammatory diseases characterized by recurrent episodes of fever and systemic inflammation affecting the eyes, joints, skin, and serosal surfaces. CAPS include Familial Cold Autoinflammatory Syndrome (FCAS), Muckle-Wells Syndrome (MWS), and Chronic Infantile Neurologic, Cutaneous, Articular (CINCA) syndrome, three nosological entities representing different phenotypes, from the milder to the most severe, in the context of a clinical continuum.

Cryopyrin, now renamed NLRP3, is part of the intracellular multiprotein complex inflammasome that mediates IL-1processing and secretion through caspase-1 activation. NLRP3 mutations in CAPS are gain-of-function, as they enhance inflammasome activity. The result is hypersecretion of IL-1responsible for the inflammatory clinical manifestations, as confirmed by the responsiveness of CAPS to IL-1blockade. We have generated a knock-in mouse carrying the N475K mutation into the murine NLRP3 gene. This mutation corresponds to the N477K human mutation, associated to a severe CINCA phenotype. The strategy used was that of homologous recombination in embryonic stem cells to replace the wild-type allele with a modified NLRP3 allele characterized by missense mutation chosen after in vitro trasfection studies. The N475K mutation was inserted in exon 3 by site-directed mutagenesis.

The NLRP3 knock-in mice we have obtained show hair loss, presence of skin rash and reduced survival time when compared with the respective wild type controls of the same littermate. Autopsy of knock-in mice prematurely dead revealed splenomegaly and a generalized inflammatory status.

We compared IL-1secretion by inflammatory cells from wild type (WT) and knock-in mice. Peritoneal macrophages and in vitro bone marrow derived DCs from mutant mice did not secrete mature IL-1β spontaneously. However, when stimulated with 100 ng/ml of LPS knock-in cells secreted higher levels of IL-1than myeloid cells from WT mice. The kinetics of IL-1 β secretion was much faster in knock-in cells, reaching the plateau at 3 h from exposure to LPS, thus reproducing the results we have previously obtained with monocytes from CAPS patients. Moreover, like in CAPS monocytes, brief exposure to ATP that strongly induced the secretion of IL-1by LPS-activated wild type cells failed to stimulate further IL-1 β secretion by inflammatory cells from knock-in mice.

Finally, macrophages and DCs from Knock-in mice are more sensitive to LPS than wild type cells: LPS at 0.01 ng/ml, unable to induce secretion by WT cells, triggered levels of IL-1 β similar to 100 ng/ml of LPS, thus indicating that the presence of the mutation lowers the threshold of activation in Knock-in inflammatory cells. In conclusion, the mouse model we have established recapitulates phenotype and functional characteristics of CAPS patients. Thus, this model will hopefully provide elucidations in the mechanisms underlying CAPS as well as other inflammasomepathies.

poster presentation

SONIA CARTA
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AutocrineATP signaling and cell stress dysregulate cytokine secretion by CAPS monocytes: correlation with the disease phenotype

Cryopyrin Associated Periodic Syndromes (CAPS) are a group of autoinflammatory diseases linked to gain of function mutations of the inflammasome gene NLRP3. They are characterized by systemic inflammation, urticarial rash and elevated levels of acute-phase proteins due to oversecretion of IL-1β mutated monocytes upon TLR stimulation.We have recently showed that monocytes from CAPS patients display a precarious redox equilibrium due to high levels of both Reactive Oxygen Species (ROS) and antioxidants. TLR stimulation with standard doses of LPS brakes this equilibrium leading to oxidative stress and consequently protein synthesis inhibition with strong impairment of production cytokine downstream of IL-1β, such as IL-1Ra and IL-6.
We investigated how cell stress impactsthe physiopathology of CAPS and obtained the following results:
1. The increased IL-1β secretion by CAPS monocytes following TLR stimulation is due a dramatic enhancement of ATP release mediated by the increased ROS. Blocking ROS production or the activation of the ATP receptor P2X7 results in inhibition of IL-1β secretion, indicating that the loop of ATP release, P2X7 triggering and inflammasome activation, present in normal monocytes, is functional and amplified in CAPS cells.
2. CAPS monocytes display a decrease of the threshold for activation:low doses of LPS (i.e. 0.01 ng/ml), unable to induce cytokine production in healthy monocytes, inducesecretion of IL-1β, IL-6 and IL-1Ra without inducing oxidative stress.
3. Similarly, the antioxidant DTT that prevents the oxidative stress, rescues the secretion of IL-6 and IL-1Ra suppressed by the standard doses of LPS.
4. Finally, in two kin patients with the same NLRP3 mutation but different seriousness of the clinical manifestations, monocytes from the less severely affected patient exhibited more efficient redox response and more balanced cytokine production, confirming the influence of redox response to stress on the severity of the disease.
Thus, cell stress determines a lower threshold both of activation and of oxidative stress insurgence in CAPS monocytes. The former gives reasons of the recurrent episodes of systemic inflammation in the absence of a recognizable cause, the latter is responsible for impaired production of regulatory cytokines and loss of control of the inflammatory response.

poster presentation

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Optimized “in vitro” culture conditions for human rheumatoid arthritis synovial fibroblasts

The composition of synovial fluid in rheumatoid arthritis (RA) is very complex, strongly influences the microenvironment of joints and it is an inseparable element of the disease. Currently, “in vitro” studies are performed on RA cells cultured in the presence of the recombinant proinflammatory cytokines-conditioned medium or medium alone, but these conditions do not reproduce faithfully the physiopathological situation, therefore results obtained area often biased. In this study, we evaluated the use of synovial fluid, obtained from RA patients, as optimal culture condition to perform “in vitro” studies on RA synovial fibroblasts, which play pivotal roles both in the initiation and the perpetuation of RA. Our observations demonstrate that synovial fluid is more effective in inducing DNA synthesis and proliferation in RA synovial fibroblasts with respect to TNF-alpha, or culture medium alone. Along the same line, spontaneous apoptosis observed in fibroblasts was decreased in the presence of synovial fluid. The levels of proinflammatory cytokines involved in the pathological process of RA were significantly elevated in the presence of synovial fluid with respect to those cultured in the presence of TNF-alpha or culture medium and the morphology of cells was also modified in the presence of synovial fluid. In addition, intracellular calcium dynamics involved in response to synovial fluid or TNF-alpha are significantly different both in the peak of response to ATP exposure, as well as in the timing of response onset, suggesting a role for the purinergic signalling, and in particular for the ionotropic P2X family, in the modulation of the observed effects. These results emphasize the importance of using RA synovial fluid in “in vitro” studies involving RA synovial fibroblasts, in order to ensure culture conditions physiopathologically as similar as possible to the real environmental characteristic of RA joints. This will enable to obtain a more accurate in vitro platform both to clarify the pathogenesis of rheumatoid arthritis and to evaluate the therapeutic effectiveness of new molecules.

poster presentation

JENS GEGINAT
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CCR5 and PD1 identify IL-10 producing regulatory T cells that inhibit B and T cell responses

IL-10 is a potent anti-inflammatory cytokine that mediates suppression of T-cell responses by regulatory T-cells, but it is also a B-cell growth factor.
IL-10 producing regulatory T cells are difficult to identify in human tissues, and their role in B cell responses is poorly understood.
Here, we showed that IL-10 producing regulatory (Tr1-like) T-cells could be tracked by CCR5 and PD1 co-expression in lymphoid and non-lymphoid tissues in humans and mice.
CCR5+PD- 1+ Tr1-like cells produced gargantuan amounts of IL-10 ex vivo and suppressed T-cell proliferation and transfer colitis.
Moreover, they failed to up-regulate CD40L and consequently lacked B helper functions,
but conversely suppressed T-cell-dependent B-cell responses in vitro and in vivo.
Importantly, systemic lupus erythematosus patients, who harbor pathogenic autoantibodies, had enhanced frequencies of Tr1-like cells that however failed to inhibit B cell IgG production. In summary, we identified a strategy to isolate Tr1-like cells that inhibit B-cell responses directly from tissues, and showed that they are functionally impaired in patients with a breakdown of B cell tolerance.

poster presentation

DONATELLA LATTUADA
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Study of Smac 127 activity on human fibroblast like synoviocytes from rheumatoid arthritis patients

Rheumatoid arthritis (RA) is a chronic polyarticular disorder and fibroblast-like synoviocytes of RA (RAFLS) are unique cells that populate the intimal lining of the synovium. RAFLSs acquire an aggressive phenotype and mediate inflammation and destruction of the joint. Deficient apoptosis of these cells results from up-regulated anti-apoptotic molecules (IAPs) (cIAP-1, cIAP-2 and XIAP) that are implicated not only in cell death control, but also play important roles as regulators of signal transduction, events that promote inflammation and cytokines production. The anti-apoptotic activity of IAPs can be negated by the mitochondrial protein Smac (second mitochondrial activator of caspases) which is liberated into the cytoplasm in response to pro-apoptotic stimuli.Several monomeric and dimeric compounds (Smac mimetics) have been synthesized to imitate the structure of these molecules and these compounds were shown to bind specifically to XIAP, IAP1 and IAP2. The aim of this study was to investigate the pro apoptotic activity of Smac 127 in FLS isolated by synovial tissues of patient suffering from active RA. In the preliminary study we have tested the apoptotic activity of a panel of monomeric and dimeric Smac compounds with the annexin V test and the flow cytometry analysis demonstrated that Smac 127 induce a significative apoptosis in FLS of all patients analysed. This apoptotic activity was induced by a down regulation of IAPs, consequently we evaluated the levels of cIAP1, cIAP2 and XIAP on FLS extracts treated with monomeric Smac 127 compared with Smac 066 (control). Smac 127 was able to reduce the level of IAPs. The caspase activation, induced by this molecule, was confirmed by the appearance of active caspase-8 and caspase-3. In the inflammatory process cytokines have been shown to play a pivotal role. Furthermore, due to the central role of the cytokines in the
pathophysiology of inflammatory diseases , we evaluated the different effects of Smac 127 on the IL6, IL15, IL10 and TNF♋ expression on RAFLS cultured in cell culture medium or SF or TNF♋ for 7 days. Smac 127 inhibit the IL6 and IL15 and TNF♋ secretion while IL10 production was upregulated. Based on these preliminary results we can assume an active role of Smac in shutting down the inflammatory response probably becauseAPs influence a multitude of cellular processes, such as ubiquitin (Ub) dependent signalling events that regulate activation of nuclear factor κB (NFκB) transcription factors, which in turn drive the expression of genes important for inflammation.

poster presentation

ROSA LAVIERI

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TLR co-stimulation lowers the threshold of inflammatory cell activation and causes oxidative stress and cytokine storm both in vitro and in vivo

Pro- and anti-inflammatory mediators act in concert to ensure the correct outcome of the inflammation. Cytokine production is induced upon TLR stimulation and is tightly regulated by redox signaling. Loss of the cytokine network balance has detrimental effects on inflammation and is responsible for many acute and chronic human diseases.
We have investigated the effects of simultaneous stimulation of multiple TLRs in vitro, on cytokine production by primary human monocytes, and in vivo, on systemic inflammatory response in mice.
The in vitro data show that co-stimulation of TLR 2, 4 and 7/8 (with LPS, Zymosan and R848 respectively) on human monocytes strongly enhances the secretion of primary pro-inflammatory cytokines such as TNF-♋ and IL-1β, but severely inhibits production of second waves cytokines, such as IL-1Ra. This unbalance between pro-inflammatory and regulatory cytokines depends on the insurgence of oxidative stress, with different mechanisms. The early, dramatic increase of TNF-♋ in co-stimulated monocytes is due to the gene expression upregulation mediated by the enhanced production of Reactive Oxygen Species (ROS). Increased ROS levels also lead to increased externalization of ATP, that autocrinally activates the purinergic P2X7 receptor, resulting in enhanced inflammasome activation and IL-1β secretion. In the presence of high ROS levels, the antioxidant systems of monocytes fail, leading to oxidative stress a few hours from co-stimulation. As a cell response to stress, a global translational arrest occurs and causes impaired production of IL-1Ra in spite of high IL-1Ra mRNA levels. IL-1Ra secretion is restored by exogenous antioxidants that oppose oxidative stress. Secretion of IL-1Ra and other cytokines downstream of IL-1♌ is also restored by co-stimulating with lower doses of agonists, unable to induce monocyte activation if provided individually.
The in vivo data show that i.p. co-injection of the three TLR agonists synergistically induces lethal shock. Co-injected mice experienced tremor, hypothermia, weight loss and died within 48 hours, whereas mice injected with a single TLR agonist, at a dose three times as much as that used in the co-injection experiments, display >90% survival. Moreover, the levels of TNF-♋ were dramatically higher in serum of co-injected mice whereas IL-1Ra was not increased, or even decreased with respect to mice injected with LPS alone.
In summary, a massive TLR co-stimulation lowers the threshold of activation of the inflammatory response and causes oxidative stress and cytokine network dysregulation both in vitro and in vivo. Our results suggest that novel therapeutic approaches based on a combination of redox modulators and TLR or P2X7R inhibitors may reveal successful both in acute inflammatory diseases such as sepsis and in chronic pathologic conditions characterized by a dysregulated inflammatory response, including autoimmune diseases, diabetes, chronic hearth failure and cancer.

poster presentation

VIRGINIA MAINA

Humanitas Clinical and Research Center

Unravelling cellular and molecular mechanisms of skin degeneration in the hypomorphic rag2 mouse model of omenn syndrome

Several skin disorders are characterized by a strong inflammatory and autoimmune component, considered the leading cause of the disease, together with genetic and environmental factors. Skin degeneration is a common clinical sign in several PIDs. Omenn syndrome is a paradigmatic example as severe erythroderma is the clinical hallmark, and alopecia and vitiligo are reported in patients. Rag2R229Q/R229Q mouse faithfully recapitulates all the clinical and laboratory findings observed in OS. Here, we propose the Rag2R229Q/R229Q mouse as a model to study the pathogenesis of different forms of skin degeneration. Oligoclonal activated T cell infiltration and Langerhans cells' ectopically distribution and impaired migration characterize Rag2R229Q/R229Q skin. In particular, in Rag2R229Q/R229Q vitiligo skin, CD4+ and CD8+ cells are present around and within the damaged hair follicles, correlating with INF increase. By FACS analysis, CD8+ T and NK cells predominate in vitiligo whereas CD4+ T cells infiltrate psoriatic Rag2R229Q/R229Q skin. Consistently, immunostaining and real time analysis reveal the lack of tyrosinase and tyrosinase related protein in Rag2R229Q/R229Q affected skin. In Rag2R229Q/R229Q psoriatic skin, PMN and pDCs infiltration correlates with INF increase. Moreover, IL12p40 expression increases in Rag2R229Q/R229Q unaffected and vitiligo skin, while IL23p19 and IL6 increase in psoriatic skin. Reg3 is expressed only in Rag2R229Q/R229Q psoriatic skin, together with high levels of S100A8, S100A9 and IL1. Interestingly, Treg cells significantly accumulated in affected skin. Overall, these results are instrumental to understand the mechanisms underlying skin degeneration and to identify novel biological markers as useful targets in the clinical setting.

poster presentation

VIRGINIA MAINA
Humanitas Clinical and Research Center

A berrant TCR and mTORC1 signalling pathways characterize lymphopenia - induced autoimmunity in omenn syndrome

In lymphopenic conditions, compensatory homeostatic T-cell proliferation takes place to re-establish normal immune homeostasis. However, chronic recurrence of this process might generate autoimmunity. To understand the molecular pathways at the interface between immune and metabolic regulation leading to loss of self-immune tolerance, we analyzed the TCR signalling and mTOR energy-sensing pathway in the RAG2R$^{229Q/R229Q}$ mouse model of Omenn Syndrome, associating profound immunodeficiency and autoimmunity, closely recapitulating the human disease. RAG2R$^{229Q/R229Q}$ T cells have reduced early ERK phosphorylation and defective mTORC-1 activation upon anti-CD3/CD28 stimulation compared to control. Interestingly, the rapid STAT3 activation in mutant T cells correlates with the Th17-skewed phenotype. Conversely, mutant Treg cells display higher induction of ERK and STAT3 phosphorylation compared to naturally anergic control Treg. Consistently, mutant Foxp3^{+} cells display CD44^{hi}CD45RB^{lo}CD62L^{-} activated memory phenotype, similar to non-Treg population. Furthermore, stimulated RAG2R$^{229Q/R229Q}$ Treg upregulate expression of CD25 and Foxp3 markers. Remarkably, besides the major input provided by the sustained TCR signalling, a defective mTORC-1 activation is evident in mutant Tregs, correlating with their impaired suppressive function and failure to prevent gut inflammation in a Rag1$^{-/-}$ colitis model. Consistently, marked lymphocyte infiltration and pro-inflammatory cytokine production are found in the intestinal lamina propria and skin of RAG2R$^{229Q/R229Q}$ mice, in spite of the predominant presence of Tregs in these sites.
Overall, results from this study may provide mechanistic insights into the molecular and metabolic events underlying autoimmunity and lymphoproliferative disease and be therefore instrumental for the development of novel targeted immunotherapy strategies.

poster presentation

SUR MARIA LUCIA
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Inflammation in juvenile idiopathic arthritis - between hope and possibilities

Introduction. Juvenile idiopathic arthritis (JIA) is a common disorder in children that can develop with remissions and exacerbations. Inflammation in JIA is the defining element of the disease and in the same time the bridge of sighs in the treatment of JIA. The involvement of several factors in the genesi s of inflammation makes the disease to be oscillatory.
Objectives. Assessment of current diagnostic possibilities in JIA. Determine the frequency of forms of JIA.
Immunological involvement in determinism of inflammation in the three forms of JIA.
Materials and methods. The study group included 69 patients aged 2 - 18 years, admitted to Pediatric Clinic II Cluj-Napoca between 1 January 2009 and 31 December 2013. Laboratory data that we have us ed to support the diagnosis were: inflammatory syndrome sustained by values of ESR, C-reactive protein and fibrinogen. Immunological markers used were C3 complement, rheumatoid factor, antinuclear and anti - DNA ds antibodies, TNF-α, IL-1, IL-6.
Results. Olig oarticular forms are the most common (50%), followed by polyarticular forms (30%) and the systemic (20%).
Diagnostic markers show that the ESR is increased in systemic forms, followed by polyarticular form. Rheumatoid factor was present in 50% of polyartic ular forms. ANA were positive in 30% of cases with polyarticular and oligoarticular form and in 25% of cases with systemic form. C3 was positive in 7% of cases, and anti-DNA ds antibodies were positive by 10%. IL-1 has been correlated with fever and disease activity period. TNF-α and IL-6 have indicated evolution of the disease. T
he increased values of TNF-α, IL-6 and IL_1 show unfavorable evolution of inflammation. These markers have been determined in 12 cases who have had not favorable evolution under treatment, showing the progressivity of disease.
Conclusions. Immunological markers are important in supporting the diagnosis of JIA , helping to framing clinical forms and also indicating the severity of the disease.

poster presentation

JOANNA MIKULAK
Humanitas Clinical and Research Center

Full-length Soluble Urokinase Plasminogen Activator Receptor Modulates Glomerular Permeability by Reducing Nephrin Expression in Podocytes

Soluble urokinase-type plasminogen activator receptor (suPAR) represents a reliable biomarker correlating with immune activation, which increased plasma concentration, was observed in various human pathologies including kidney disorders and HIV-1 infection. Different suPAR isoforms were identified to contribute to HIV-1 pathogenesis and, in particular, lymphoid organs that showed an enhanced number of follicular dendritic cells and macrophages are an important site of production and release of suPAR comparison to those of uninfected individuals. In view of the fact that kidney diseases are important complications of HIV-1 infection we evaluate suPAR as a potential risk factor affecting kidney functionality. Using both the recombinant as well as the plasma-associated suPAR from HIV-1 infected patients we established that suPAR induces podocytes dysfunction through down-modulation of nephrin, a transmembrane protein expressed on podocytes and required for the proper functioning of the renal filtration barrier. This phenomenon was time- and dose-dependent and was associated with the suppression of Wilms' tumor 1 (WT-1) transcription factor. Furthermore, antagonist of αvβ3 integrin RGDfv reversed suPAR-induced suppression of nephrin indicating the involvement of suPAR-αvβ3 interaction in this process. These in vitro data were also confirmed in an in vivo suPAR knock out Plaur-/- mice model by demonstrating that the infusion of high dose of full-length murine suPAR inhibited expressions of nephrin and WT-1 in podocytes and enhanced glomerular permeability. These studies provide a conceptual framework for the pathogenetic contribution of suPAR in different human renal pathologies characterized by kidney disorders such as HIV-1 infection and open new therapeutic perspectives in the field.

poster presentation

ALESSIA OMENETTI
DINOGMI Department, University of Genoa, Genoa, Italy

NLRP3-Dependent IL-1Î² Secretion Is Enhanced And Correlates With Disease Activity in Pyogenic Sterile Arthritis Pyoderma Gangrenosum And Acne (PAPA) Syndrome: Study of a Single Center Cohort Including 14 Patients

Background Positional differences of Proline-serine-threonine phosphatase-interacting protein 1 (PSTPIP1) mutations may trigger opposite clinical outcomes in Pyogenic sterile Arthritis Pyoderma gangrenosum and Acne (PAPA) syndrome. Actually, complete phenotype with the typical triad may not be fully displayed, and clinical picture may vary along the disease history in the same individual. PSTPIP1-ASC-Pyrin interaction was suggested to cause NLRP3-independent interleukin (IL)-1β secretion. However, due to the disease rarity, functional studies exploring IL-1β signaling as a potential therapeutic target were mostly performed in in vitro systems or animal models, and anti-IL1 effectiveness was unveiled or refuted based on brief case reports. Hence, univocal insights are still missing.
Objectives To investigate in monocytes purified from PAPA patients whether IL-1β secretion (1) is affected, (2) is mediated by NLRP3, and (3) correlates with different PSTPIP1 mutations, disease activity and/or clinical picture.
Patients and Methods A clinically well characterized cohort including 13 genetically confirmed PAPA subjects (E250Q+ N=10, E250K+ N=1, E256G+ N=2) and 1 patient wild-type (WT) for the common PSTPIP1 mutations were evaluated and compared with 31 genetically negative healthy donors (HD). Peripheral blood monocytes were purified and studied at baseline and following LPS-induced in vitro activation. Involvement of NLRP3 was investigated by in vitro silencing. Pattern of IL-1β secretion was assessed by ELISA and findings correlated with genotype, disease activity and clinical picture. Results IL-1β secretion in PAPA cohort tent to be higher than HD, but assessments were extremely heterogeneous (P=0.059). LPS-induced IL-1β release in both patients and HD was dramatically downregulated by NLRP3 silencing, regardless the PSTPIP1 variant carried. While E250K+ patient displayed IL- 1β secretion comparable to HD, E256G+ subjects showed increased IL-1β levels. However, enormous differences in clinical picture and IL-1β release still occurred among E250Q+ subjects. Monocytes purified from patients with active lesions released much more IL-1β compared to both HD (**P<0.01) and patients displaying inactive lesions (*P<0.05). When the latter were analyzed (P=0.037) levels of IL-1β in subjects with history of cutaneous recurrences tent to be comparable to those found in HD whereas monocytes purified from patients experiencing mostly articular flares displayed IL-1β release significantly higher than those affected by recurrences of skin symptoms (*P<0.05). Consistent with these findings, long-term functional follow up of 2 patients with different genotype and phenotype unveiled that anakinra administration caused IL-1β to drop and acute phase reactants to normalized in both of them. However, while symptom-free period was gradually obtained in E250Q+ patient with articular flares, IL-1β blockage had only partial beneficial effects on cutaneous lesions in E250K+ patient but dramatically ameliorated the associated atypical manifestations such as anemia.
Conclusions NLRP3-dependent IL-1β over-secretion occurs in PAPA patients and correlates with disease activity. Patients with inactive lesions and history of articular flares display higher IL-1β levels compared to those affected by recurrences of skin symptoms. A straightforward correlation between genotype and IL-1β pathway activation was not observed suggesting that factors other than the mutation itself may play a role in regulating IL1-β signaling and response to anti-IL1-β treatment in PAPA.

poster presentation

AGNIESZKA PARADOWSKA-GORYCKA
Department of Biochemistry and Molecular Biology, Institute of Rheumatology, ul. Spartanska 1, 02-637 Warsaw, Poland

Association of single nucleotide polymorphisms in the RORC2 gene with rheumatoid arthritis

Introduction. Rheumatoid arthritis (RA) is a disease of mixed etiology, in which environmental, genetic and immune factors contribute to development and progression of its inflammatory manifestation. The association between genetic factors and pathogenesis of RA suggests that T-cells take part in the induction of the disease, which differentiation depends on a unique combination of stimulants and subsequent activation of diverse transcription factors. Humans RORC2 ((retinoic acid receptor-related orphan receptor variant 2; shorter isoform RORC gene) is a master transcriptional factor that can induced of the IL-17A, IL-17F, IL-26, TCR and CCR6 expression, initiates a wide range of phenotypic and functional programming during Th17 cells development and reduces levels of Foxp3 mRNA and proteins. The aim of the study was to identify sequence variants in the RORC2 gene and their possible association with susceptibility to and severity of RA.
Methods. A study group consisted of 276 patients with RA (259 women and 19 men), mean age of 55,8 ± 12,7 years (range 22-89 years) and of 341 healthy individuals. The single nucleotide polymorphisms (SNPs) in the RORC2 rs 12045886 (T\C), rs9017 (G\A), rs9826 (A/G) gene was investigated by TaqMan SNP genotyping assay. Serum Rorc2 levels in RA patients and controls were measured by enzyme-linked immunosorbent assay (ELISA).
Results. We observed no significant differences in genotype and allele frequencies of the rs 12045886 (T\C), rs9017 (G\A), rs9826 (A/G) variants between RA patients and controls. The genotype-phenotype analysis showed significant correlation of the RORC2 rs9017 G/A polymorphism with the longer diseases duration of RA (p=0,044), VAS score (p=0,007), mean value of CRP (p=0,0005) and mean value of creatine (p=0.018). Serum RORC2 levels were significantly higher in RA patients than in control groups (both p=0,0000).
Conclusion. Present findings indicated that RORC2 rs9017 G/A variant and RORC2 protein level may by involved, respectively, in severity of and pathogenesis of RA in the Polish population. However, RORC2 gene polymorphisms are not associated with susceptibility to RA in the investigated Polish population.

poster presentation

AGNIESZKA PARADOWSKA-GORYCKA
Department of Biochemistry and Molecular Biology, Institute of Rheumatology, ul. Spartanska 1, 02-637 Warsaw, Poland

Single nucleotide polymorphisms at the Smad3 and NFATc2 genes and their role in the Rheumatoid Arthriti

Introduction. Rheumatoid arthritis (RA) is a disease of mixed etiology. One among many factors involved in induction of RA disease are T cells, which differentiation depends on a unique combination of stimulants and subsequent activation of diverse transcription factors. Cooperative activation of NFATc2 and Smad3, through regulation of activity of Foxp3 gene enhancer, leads to the induction of CD4+CD25+ regulatory T (Treg) cells. Tregs play an essential role in preventing autoimmunity and maintaining immune homeostasis and controlling T-cell responses. The aim of the study was to identify two polymorphic variants in promoter region of Smad3 and NFATc2 gene and their possible association with susceptibility to and severity of RA.
Methods. A study group consisted of 272 patients with RA (254 women and 18 men), mean age of 55,8 ± 12,7 years (range 22-89 years) and of 321 for Smad3 and 304 for NFATc2 matching healthy individuals. The single nucleotide polymorphisms (SNPs) in the Smad3 rs6494629 C/T and rs2289263 T/G and NFATc2 rs880324 genes were investigated by PCRRFLP method and TaqMan SNP genotyping assay, respectively. Serum Smad3 and NFATc2 levels in RA patients and controls were measured by enzyme-linked immunosorbent assay (ELISA).
Results. Smad3 rs6494629 TT and rs2289263 GG genotypes were associated with significantly increased risk of RA (p=0.0138 and p=0.0102, respectively) comparing to the wild-type alleles in the tested population. The genotype-phenotype analysis showed significant correlation of the Smad3 rs6494629 C/T polymorphism with the extaraarticular manifestation (p=0,0483), longer diseases duration of RA (p=0,0168), HAQ score (p=0,0141), mean value of PLT (p=0,0448) and RF presence (p=0.0258). The Smad3 rs2289263 G variant was associated with longer disease duration in our RA patients (p=0,0019). Serum Smad3 and NFATc2 levels were significantly higher in RA patients than in control groups (both p=0,0000). Moreover, among the NFATc2 positive patients (Foxp3 concentration >5,9 pg/ml) the anti-CCP presence was significantly higher in comparision to the Foxp3 negative group of patients (p=0.0013).
Conclusion. Present findings indicated that Smad3 genetic polymorphism and Smad3 and NFATc2 protein levels may be associated with the pathogenesis of RA in the Polish population.

poster presentation

ELENA PONTARINI
Humanitas Clinical and Research Center

Recruitment and activation of NK cells regulates early immune responses to viral infection in the salivary glands but are dispensable for the onset of autoimmune sialoadenitis in an inducible murine model of Sjogren-like disease

Background and aim: Dysregulations of Natural Killer (NK) cells have been shown to influence the development of autoimmune diseases, but their role in Sjögren's syndrome (SS) is still uknown. The main goal of our study is to investigate the recruitment and functional relevance of NK cells in the early phases of salivary gland (SG) inflammation in an inducible murine model of SS.
Methods and results: Sialoadenitis was induced in WT-C57BL/6 mice via SG retro-cannulation of a luciferase-encoding adenovirus-5 (AdV). Flow cytometric analysis of CD45pos/NK1.1pos/CD3neg NK cells in digested SG showed an early recruitment of NK cells with a peak after 4 days post-cannulation (dpc). Our experimental evidence showing that NK cells labeled with CFSE dyes and adoptively transferred in the mouse migrated and proliferated within SG after 1dpc also confirmed these results. Immunofluorescence (IF) staining for Nkp46pos/CD3neg demonstrated preferential accumulation of NK cells in association with B and T cells infiltrates. We also found an up-regulation of the activating markers NKp46 and CD69 and increased amounts of granzyme-B and Interferon-γ in NK cells migrated to SGs with a peak after 4/5dpc. Accordingly, an increased NK cell degranulation potential was observed after 5 dpc using a CD107a functional assay. The depletion of NK cells in mice affected by induced sialoadenitis significantly impaired viral control (as assessed by luciferase activity) after 3 dpc but not at later time-points, but did not affect the formation of SS-like inflammatory foci or the production of ANA or anti-AdV antibodies.
Conclusion: Taken together, our data showed that NK cells are actively recruited to the inflammatory site and are critically involved in the early immune control of AdV infection in the SG but are dispensable for the development of SS-like sialoadenitis and autoimmunity. Further studies are required to assess if the NK cell-mediated viral control in the SS-like sialoadenitis have a role in the long term in disrupting the functions of exocrine glands.

poster presentation

ANNA RADICE
Department of Sperimental and Clinical Medicine, University of Florence

One gene, two phenotypes: a familial case of ALPS

Introduction: FAS-mediated apoptosis aims at reducing activated lymphocytes and autoreactive cells in order to limit immune response and to avoid autoimmunity. When this pathway is defective, lymphocytes survive, expand, make damage.
ALPS (autoimmune lymphoproliferative syndrome) represents the best genetical and clinical model of a defective FAS mediated apoptosis. The gene encoding for FAS is the one most frequently altered; according to the new classification of ALPS, genes for FAS Ligand, FADD, caspase 8 and caspase 10 might be damaged. As a consequence of lymphocytic disregulation, ALPS patients show a peculiar clinical and serological profile. First, lymphoadenomegaly, splenomegaly, cytopenia and a higher risk of lymphoma are the main clinical features. Even if the defect is typically inherited in an autosomal dominant fashion and the relatives of the same affected family are usually heterozygous for the same mutation, their phenotype is frequently different. The hypothesis is that various parts (or domains) of the same genetic product differ among family members. Moreover, these patients exhibit interesting laboratory findings, such as double negative T cells (DNT), hypergammaglobulinemia, increased vitamin B12. The certain diagnosis requires the presence of chronic non malignant non infectious lymphoadenopathy or splenomegaly and elevated CD3-TCR$\alpha\beta$+CD4--CD8-DNT cells; moreover, the altered apoptosis has to be demonstrated.
Case presentation Here we present a familial case of ALPS. FN, 39 years old, and his son, 4 years old, share the same mutation for FAS. They both show a percentage of DNT cells >2%, and a defective in vitro lymphocytic FAS mediated apoptosis. Nevertheless, their clinical presentation is quite different. The son was initially evaluated for hepatosplenomegaly, lymphoadenopathy and microcytic anemia. FN was admitted to our ambulatories because of splenomegaly, anamnestic anemia, hypergammaglobulinemia; his laboratory analysis shown augmented vitamin B12 and IL-10 levels, hypergammaglobulinemia and a mild increase in CD8+ lymphocytes. Recently, the same FAS gene mutation was detected in other two family members: FN brother, 27 years old, reporting a history of lymphoadenopathy, and FN daughter.
Conclusion FN and his son could be considered a typical case of familial autoimmune lymphoproliferative syndrome. They both fulfill the diagnostic criteria for ALPS and as expected, they exhibit at the same time an identical gene mutation but two different phenotypes. During an initial accurated evaluation, it was found out that FN other two genetic alterations: heterozygosity for hemochromatosis (HFE gene) and a rare cardiac disorder associated to hypocholesterolemia.

poster presentation

ELISABETTA RADICE
University of Turin, Surgical Science Dept, Torino

The effects of low doses sequential - kinetic - activated cytokines on the ex vivo cytotoxicity of natural killer cells of patients with colorectal cancer

Despite the considerable advances in the treatment of colorectal cancer, substanzial changes in therapeutic strategies are required to overcome the problems of drug resistence and toxicity. Natural killer (NK) cells are innate immune system lymphocytes capable of killing tumor cells. They secrete cytokines, including interferon (IFN)-γ, which participate in shaping the initial inflammatory and downstream adaptive immune responses. Its potent immunoregulatory action means that IFN-γ might be beneficial in cases of tumor rejection, but its severe side-effects limit clinical applications. Our objective was to compare low-dose IFN-γ prepared by sequential-kinetic-activation (SKA), with standard-dose recombinant (r) IFN-γ, in terms of ex-vivo cytotoxic activity of peripheral blood (PB)-NK cells from colorectal carcinoma (CRC) patients.This was tested against the NK-sensitive K562 cell line and the less-sensitive human CRC Caco-2 and HT-29 cell lines.Methods.Twenty primitive nonmetastatic CRC patients, five metastatic CRC patients and thirteen healthy donors were enrolled. PB lymphocytes (PBL) were exposed to medium alone, SKA-IFN-γ (0.25 fg /ml) or rIFN-γ (1 ng/ml). NK cytolytic activity was examined via short-term 51Cr-release.
Results. Pretreatment of PBL from non-metastatic patients with SKA-IFN-γ caused a significant increase in NK cytotoxicity, compared to those from normal donors, although less markedly than pretreatment with rIFN-γ against all three cell lines. In contrast, PBL from metastatic CRC patients displayed significantly decreased Nk-cell activity and responsiveness to both rIFN-γ and SKA-IFN-γ treatments. Conclusions.These results demonstrate in principle the immunomodulatory capacity of low-dose SKA-IFN-γ, and might open the door to the possibility of generating a novel, safe, and feasible approach to enhancing NK-cell antitumor activity in early stage CRC patients.

poster presentation

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Wheat allergy: screening, innate immune response, autoimmunity and inflammatory reactions

Recent studies outline the new notions of innate immunity in food allergies. In the period 2012-2013, we performed the serological screening of specific allergens to wheat flour in a group of 1097 children. Immunoblot analysis showed very high antibody titer in 0.2% of the children, definite detection of antibodies in 0.3% of the children, weak antibodies in 1.2% of the children and very poor detection antibodies in 5.9% of the children. For all children found with positive wheat allergy, we explored the following: 1). innate immune response, the findings showed the average values for: eosinophils 4.85% (normal values 1-4%) basophil 1.17% (normal values 0-1%) neutrophils 35% (normal values 50-76%), lymphocytes, 51.1% (normal value 25-40%); 2). autoimmunity, with negative results for tissue transglutaminase antibodies; c). inflammatory reactions, obtaining an average value 0.43 for C-reactive protein (normal values <0.8 mg/dl). In conclusion, innate immune response to wheat allergy is defined by increased eosinophils, basophils and lymphocytes without the autoimmune manifestations related to celiac disease, and without inflammatory reactions.

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poster presentation

FRANCESCA SCHENA
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B Cell Characterization in ADA2 Deficiency Patients

Background and Objectives
ADA2 deficiency, a recently described disease, is characterized by systemic vasculopathy and episodes of strokes. The defect is due to a loss of function mutation of CECR1 gene, codifying for Adenosine Deaminase 2 protein. This protein regulates the catabolism of extracellular adenosine, which we have recently shown is an important regulator of Class Switch Recombination in B lymphocytes. Moreover ADA2 promotes T cell proliferation. Accordingly some of DADA2 patients present hypogammaglobulinemia. Therefore we decided to characterize peripheral B cell compartment of two patients to directly address if ADA2 mutation affects B cell function.
Patients and Methods
Two brothers (15 and 7 years old) carrying mutations in CECR1 were followed up from the age of two. They showed similar clinical history with livedo reticularis, fever, vasculitis and neurological symptoms caused by haemorragic strokes. Moreover both presented early hypogammaglobulinemia requiring intravenous immunoglobulin replacement therapy. Remarkably after etanercept treatment serum Igs slowly increased to a normal level. We analyzed peripheral B cell phenotype by flow cytometry, in vitro B cell proliferation and differentiation to plasmacells in response to CpG, BCR and T cell help.
The elder brother received boost of Toxoid Tetanic (TT) vaccination. We followed the kinetic of immune response to immunization: in particular the level of total and specific TT IgG in the serum has been measured and the frequency of total and specific TT antibodies secreting cells (ASC) has been evaluated after TT vaccination.
Results and Conclusions
Flow cytometer analysis showed a significant reduction of total B cells compared with age matched control. Intriguingly a defect in the memory B cell compartment (CD19+CD27+) was observed in both patients. We observed that the rate of proliferation and differentiation to immunoglobulin secreting cells (ISC) is lower as compared to normal controls and it is not supported by autologous T cells. One patient has received a TT booster immunization and immune response has been monitored: dramatically there is no appearance of circulating plasmablasts in the blood and the serum anti-TT Ig level increase slightly but rapidly decay. Therefore this patient failed to respond to TT vaccination. Our findings suggest that ADA2 defect could lead to a reduced generation of T cell dependent memory B cells.

TOPIC Innate Immunity And Viruses

best oral presentation

MONICA CAPPELLETTI

Divisions of Immunobiology Cincinnati Children's Hospital Research Foundation
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The type IFN axis regulates infection- and inflammation-induced preterm birth

Preterm birth is a leading risk factor for neonatal morbidity and mortality worldwide. Infection and inflammation are thought to play important etiological roles in a significant fraction of preterm births, however the underlying mechanisms are not well understood. Here, we show that the type I IFN receptor (IFNAR) axis regulates inflammation- and infection-induced preterm birth. In reductive mouse models, innate immune activation by TLR ligand challenge only induces premature birth via TLRs that activate type I IFNs. Of note in this regard, TLR(4) expression by maternal hematopoietic cells is necessary and sufficient to induce preterm birth in response to (LPS) challenge. Sublethal infection with influenza virus or Listeria monocytogenes, pathogens that correlate positively with pregnancy complications and with a strong type I IFN response, sensitizes mice to induction of preterm birth by doses of LPS that fail to drive preterm on their own— something that correlates with augmented proinflammatory cytokine production. Further, mice with genetic deletion of signaling intermediates critical for TLR4- and TLR3-driven type I IFN production (TRIF, IRF3, IRF7), IFN-β, or IFNAR are protected from TLR-driven induction of preterm birth, as well as infection-mediated sensitization to TLR-driven preterm birth. Conversely, pretreatment with exogenous IFN-β, which augments TLR-driven proinflammatory cytokine production in mice, rhesus macaque and humans, sensitizes mice to TLR-driven induction of preterm birth. These observations suggest that manipulation of the type I IFNs/IFNAR pathway may provide novel therapeutic targets for prevention of preterm birth.

best oral presentation

STEPHEN WAGGONER

Cincinnati Children's Hospital Medical Center

Natural killer cell cross-talk with myeloid cells in pathogenesis of chronic virus infection

Natural killer (NK) cells play a vital role in immune defense and disease during a number of virus infections. Although this putatively involves direct lysis of virus-infected cells or production of antiviral cytokines by NK cells, increasing evidence points to NK cell-mediated regulation of adaptive immunity as an important contribution to the pathogenesis of chronic viral infections. Immunoregulatory activities of NK cells include production of cytokines such as IL-10, direct lysis of virus-specific T cells, and targeting of antigen-presenting cells, including dendritic cells (DCs). Using a mouse model of persistent lymphocytic choriomeningitis virus (LCMV) infection, we previously demonstrated that NK cells facilitate persistent infection and prevent fatal immunopathology by promoting the functional exhaustion of virus-specific T cells. In the absence of NK cell-dependent changes in either the number or function of DCs, we concluded that NK cell-mediated perforin-dependent lysis of CD4 T helper cells was instrumental in fostering T cell exhaustion and viral persistence.
Nevertheless, the emergence of IL-10-expressing immunoregulatory myeloid suppressor cells is postulated to be central to immune exhaustion and LCMV persistence. The importance of myeloid cell regulation of NK cell activity during infection has been described in both humans and mice. However, the consequences of the inverse, NK cell regulation of myeloid cell differentiation and function, are less well understood. Herein we explored whether NK cell depletion had an effect on the accumulation of immunoregulatory myeloid cells during high dose persistent infection with LCMV. Consistent with previous work, a substantial fraction of lineage-negative, F4/80+CD11b+ cells demonstrated expression of IL-10 in the spleen on day 8 of LCMV infection in NK-cell sufficient mice. In contrast, there was a three-fold reduction in the number of these immunoregulatory myeloid cells in mice infected in the absence of NK cells. Likewise, myeloid-derived suppressor cell populations were expanded in the spleen and lungs of infected mice at day 10 p.i., but the number of these cells was substantially reduced in NK cell-depleted mice. Thus, NK cells contribute to the emergence of immunoregulatory myeloid cells during chronic virus infection through an as yet undefined mechanism. This relationship between NK cells and myeloid suppressor cells could potentially be targeted in order to enhance immune function in human diseases where NK-myeloid cell crosstalk has been implicated in disease pathogenesis, including HIV infection and cancer.

This work was supported by a New Scholar Award from the Ellison Medical Foundation, a National Institute of Drug Avant-Garde Award (DA038017), and startup funds from Cincinnati Children's Hospital (S.N.W.).

oral presentation

MONICA VACCARI
NIH

The decrease in the number of T Follicular Regulatory cells drives the expansion of T Follicular Helper cells in SIV infected macaques

T follicular regulatory CD4+ T cells (TFR) migrate to the germinal centers (GC) during antigen presentation where they limit the expansion of T follicular helper cells (TFH) and germinal center B cells, hence participating in the shaping of specific antibodies responses. In vivo depletion of TFR cells or blockade of their function results in TFH cell expansion, in increased GC reactions and in loss of normal proportion of IgM-switched-B cells (IgG+ and IgA+). The role of TFR cells in B cell immune dysregulation and TFH expansion occurring during chronic HIV/SIV have not been addressed.
We defined TFR cells in rhesus macaques, the preferred model of HIV infection, and we studied changes in their frequency and function during acute and chronic infection following exposure to the highly pathogenic SIVmac251.
We defined TFR in naïve macaques as FOXP3+/CD25+ expressing the CXCR5 but negative for the CCR7 chemokine receptor. TFR cells shared markers of T regulatory cells (TREGS) and of TFH cells, and their frequency was higher in lymph nodes and GALT.
Sorted TFR had similar levels of IL-10 mRNA and higher levels of TGF-β compared to TREGS. Functionally selected TFR cells, obtained by migrating sorted CD25+ CD4+ T cells to CXCL13, the ligand of CXCR5, were capable of suppressing TFH cells proliferation in vitro. Interestingly, the frequency and the number of TFR cells significantly decreased during the course of infection. This reduction was associated with the concomitant increase of TREGS cells homing to the T-cell zone (CXCR5-/CCR7+).
During infection the frequency of TFR cells was negatively associated with the frequency of IgM- switched (IgG+ and IgA+) B cells and plasmablasts, defined as CD20+/CD19+/ CD21- /Ki67+/CD38+ cells and expressing the specific plasmablasts surface marker CD39. Finally reduction of TFR was directly correlated with an increase in avidity to the gp120.
We propose a model whereas the decrease of suppressive CD4+ T cells within the germinal center of lymph nodes during chronic infection results in the unchecked expansion of TFH and, ultimately, in an augmented quantity and quality of B cells responses to HIV.

oral presentation

MELODIE WELLER
National Institutes of Health, National Institute of Dental and Craniofacial Research,
Molecular Physiology and Therapeutics Branch

Hepatitis delta virus detected in salivary glands of Sjögren's syndrome patients and induces disease phenotype in vivo

Introduction: A viral trigger has been suspected as a possible mechanism in the development of primary Sjögren's syndrome (pSS). The key element to identifying viruses capable of instigating viral-mediated autoimmunity in Sjögren's syndrome is distinguishing the normal viral signature present in healthy salivary glands and opportunistic or reactivated viral infections associated with advanced stages of the disease from those viral signatures that are able to initiate the disease phenotype in vivo.
Methods: To better understand the viral signatures present in salivary gland tissue, a viral microarray was developed to assess the viruses present in healthy salivary glands compared to patients diagnosed with primary Sjögren's syndrome. All pSS subjects studied met the American European criteria for pSS and were confirmed free of virus infections such as HCV and HIV. Salivary glands of female C57BL/6 mice were cannulated with adeno-associated viral vectors containing viral antigen expression cassettes and monitored for changes in stimulated saliva flow, lymphocytic infiltrate formation and development of autoantibodies.
Results: Using viral microarray approach, a unique viral signature was identified for hepatitis delta virus (HDV) in 50% of the primary Sjögren's Syndrome cohort. We have confirmed the presence of the viral sequence and detected viral antigens in salivary gland tissue from the pSS patient population in agreement with the initial microarray study. Expression of the viral antigens in salivary glands of female C57BL/6 mice initiated pSS-like phenotype including the reduced saliva flow, increased lymphocytic infiltrates, and development of autoantibodies. The disease profile observed in the animal model correlated with the disease parameters observed the pSS patients positive for this viral profile.
Conclusion:Identification of this novel viral signature in Sjögren's syndrome patients and induction of a Sjögren's syndrome-like disease in a mouse model expressing the HDV antigens within the salivary gland provides further support for a viral-mediated etiopathology in Sjögren's syndrome.

best poster presentation

SPELA KONJAR

Babraham Institute, Lymphocyte signalling and development laboratory, Babraham Research Campus, Cambridge, CB22 3AT, United Kingdom

CD8+ T Cells and their metabolic plasticity

It is becoming increasingly clear that T cell function and differentiation are closely connected with metabolic programs, because of that there is huge interest to develop techniques that could manipulate metabolism of immune cells for immunotherapy. During differentiation, T cells move from a nutrient sufficient environment in the secondary lymphoid organs to sites of inflamed peripheral tissues where there is a different nutritional status, low oxygen pressure and altered levels of other signals needed for immune cell metabolism. These new environmental conditions force T cells to metabolically adapt in order to survive and to perform their primary function. Here we show that $CD8^+$ memory and $CD8^+$ naïve T cells from spleen, but not $CD8^+$ T cells from the small intestine, possess substantial mitochondrial spare respiratory capacity (SRC). This indicates that $CD8^+$ T cells sourced from lymphoid organs exhibit higher reserves of energy which can be used in response to stress or inflammation. We also show that mitochondrial density in $CD8^+$ memory and $CD8^+$ naïve T cells is higher than found in $CD8^+$ and $\gamma\delta$ T cells sourced from the small intestine. Our transcriptome analysis of these subtypes of $CD8^+$ T cells highlighted gene candidates that may be able to explain why mitochondrial respiratory capacity of $CD8^+$ memory and $CD8^+$ naïve T cells sourced from spleens is higher than $CD8^+$ and $\gamma\delta$ T cells sourced from the small intestine. In conclusion, our findings give insights into how $CD8^+$ T cells adapt to different metabolic capacities depending on their functional requirements and the tissue environment they encounter.

best poster presentation

PIETRO PRESICCE

Perinatal Institute, Cincinnati Children's Hospital Medical Center, University of Cincinnati, OH 45229

Neutrophil recruitment and activation in decidua with intra-amniotic IL-1β in the preterm Rhesus macaque

Chorioamnionitis is an infection of the feto-maternal membranes frequently associated with preterm delivery. Although histologically, acute chorioamnionitis is characterized by neutrophil infiltration in the decidua, the role played by neutrophils and other leukocytes in the setting of chorioamnionitis is poorly understood. Previous studies demonstrated that intra-amniotic (IA) injection of IL-1β in the Rhesus macaque induced preterm labor. In this study, we injected IA IL-1β at ~ 80% gestation in Rhesus macaque, delivered the fetuses surgically 24h or 72h after IA injections, and investigated the role of immune cells in the chorion-amnion decidua. We hypothesized that neutrophils recruited to the decidua would be the major producers of pro-inflammatory cytokines. IA IL-1β induced a robust infiltration of neutrophils and significant increases in the expression of pro-inflammatory cytokines in the chorioamnion-decidua tissue 24h after exposure with a subsequent decrease at 72h. Neutrophils in the decidua were the major source of TNFα and IL-8. Interestingly, IA IL-1β also induced a significant increase in the expression of anti-inflammatory indoleamine 2,3-dioxygenase (IDO) in the decidua neutrophils. The frequency of T regulatory cells (Tregs) and FOXP3 mRNA expression in the decidua did not change after IA IL-1β injection, suggesting that Tregs were not involved in the attenuation of the inflammation. Collectively, our data demonstrate that during chorioamnionitis, the decidua neutrophils promote the inflammation in the gestational tissues, but also act as regulators to dampen the inflammation. These results have implications for the pathogenesis of chorioamnionitis induced preterm labor.

best poster presentation

LUCA SCHIFANELLA

Animal Models and Vaccine Section, National Cancer Institute, Bethesda MD, US

Plasmablast Phenotype and Mucosal Antibodies to V2 in Vaccine-Induced Protection Against SIVmac251

We have recently recapitulated the RV144 vaccine efficacy in a SIV^{mac251} model. In our study, rectal anti-cyclic V2 IgG antibodies correlated with a decrease risk of SIV^{mac251} acquisition (p=0.0063). Analysis of the homing markers on plasmablast (PB) resulted in a higher frequency of $\alpha4\beta7^+$ PBs in animals with higher levels of IgG and IgA to cyclic V2 in rectal mucosal. We investigated the homing potential in 3 different vaccine strategies by measuring $\alpha4\beta7$ and CXCR3 as markers for gut mucosa and inflammatory sites, respectively in a total of 63 macaques. The immunoglobulin (Ig) expression on PB and the mucosal antibody responses were assessed in all groups. We studied a cohort of macaques immunized 4 times with ALVAC-SIV and twice with gp120/alum or gp120/MF59.

A second I group was immunized 4 times with NYVAC-SIV boosted twice with gp120/alum. ALVAC-SIV/gp120/alum immunized animals were protected from SIVmac251 mucosal acquisition (p=0.029). However, no protection was observed with ALVAC-SIV/gp120/MF59, NYVAC-SIV/gp120/alum regimens. ALVACSIV/gp120/alum immunizations increased the frequency of $\alpha4\beta7$ plasmablasts (p=0.015) while ALVAC-SIV/gp120/MF59, NYVAC-SIV/gp120/alum increased the frequency of $CXCR3^+$ plasmablasts (p=0.0001 and p=0.004 respectively).

Similar results were found in two human trials. We evaluated the frequency of vaccine-induced $a4b7^+$ and $CXCR3^+$ plasmablasts in the blood of vaccinees enrolled in the RV135 (ALVAC-HIV/gp120 Alum) and RV132 (ALVAC-HIV/gp120 MF59) trials. Vaccination with Alum resulted in higher frequency of $a4b7^+$ plasmablasts in blood compared to MF59, whereas the frequency of $CXCR3^+$ plasmablasts in blood did not differ. This lack of difference between macaques and humans in $CXCR3^+$ plasmablasts, may reflect the time of blood collection (two weeks and one week post last immunization in humans and macaques, respectively) that may have affected our ability to detect plasmablasts in blood.

Ongoing studies are underway in order to assess whether these results are confirmed in other vaccine strategies. Preliminary data results suggest that different vaccine modalities are able to alter the plasmablast homing and the effector functions of the antibody response at mucosal sites that may correlate with protection.

best poster presentation

OM PRAKASH SINGH

Department of Medicine, Institute of Medical Sciences, Banaras Hindu University, Varanasi (INDIA)

Leishmania specific CD4 T cells release IFNγ that limits parasite replication in patients with visceral leishmaniasis

Visceral leishmaniasis (VL) is associated with increased circulating levels of multiple pro-inflammatory cytokines and chemokines, including IL-12, IFNγ, and TNFa, and elevated expression of IFNγ mRNA in lesional tissue such as the spleen and bone marrow. However, an immunological feature of VL patients is that their peripheral blood mononuclear cells (PBMCs) typically fail to respond to stimulation with leishmanial antigen. Unexpectedly, it was recently shown that Leishmania specific IFNγ, can readily be detected when a whole blood stimulation assay (WBA) is used. We sought to define the conditions that permit whole blood cells to respond to antigen stimulation, and clarify the biological role of the IFNγ found to be released by cells from VL patients. CD4+ T cells were found to be crucial for and the main source of the IFNγ production in Leishmania stimulated whole blood (WB) cultures. Complement, antibodies and red blood cells present in whole blood do not play a significant role in the IFNγ response. The IFNγ production was reduced by blockade of human leukocyte antigen (HLA)-DR, indicating that the response to leishmanial antigens observed in WB of active VL patients is a classical HLA- T cell receptor (TCR) driven reaction. Most importantly, blockade of IFNγ in ex-vivo splenic aspirate cultures demonstrated that despite the progressive nature of their disease, the endogenous IFNγ produced in patients with active VL serves to limit parasite growth.

NICOLO' BINELLO

Animal Models and Retroviral Vaccine Section, National Cancer Institute, National Institutes of Health, Bethesda MD, USA

Pre-activation of RAS signaling in SIV vaccine strategies

Background. A prime-boost ALVAC/gp120 vaccine regimen in rhesus macaques using SIV antigens recapitulated the results observed in the RV144 trial ($p = 0.021$) and resulted in a vaccine efficacy (VE) of 44% at each challenge exposure. Systems biology techniques, including the analysis of transcriptional profiles, were performed in order to investigate the signaling pathways activated before and after immunization in all the animals. A 12-gene signature was demonstrated to positively correlate with protection, defined as delayed SIVmac251 acquisition. Strikingly, 7 out of these 12 genes were found to be hub linked to the RAS signaling pathway.
RAS is a small GTPase that plays a major role in transmitting signals within and among cells upon activation. Its overall impact on the immune system is still unclear. However, these results suggest that activation of RAS may constitute a novel approach to improve vaccine efficacy against HIV/SIV infection.
Methods. In vitro preliminary studies were performed on peripheral blood mononuclear cells (PBMCs) of one SIVmac251-infected rhesus macaque and one uninfected animal. Activation of ERK and AKT signaling were measured by flow cytometry following 5-minute and 30-minute stimulation with PDGF (Platelet-Derived Growth Factor-1) and PMA in different cell populations. Unstimulated cells were used as controls. T cells were defined as CD3+ cells expressing either CD4 or CD8 surface markers, B cells as CD20+ cells, monocytes as CD3-, CD20-, CD14+ cells, and innate immune cells as CD3-, CD20- cells. After staining, cells were acquired on LSR II Flow Cytometry (BD Biosciences) and analyzed using FlowJo (Treestar, CA).
Results. Upon stimulation with PDGF, the strongest activation of ERK, considered as a major biomarker of RAS signaling activation, was found in the innate immune cells of the protected animal, including natural killer cells and monocytes, as opposed to the infected animal where a much lower signal was detected. Monocytes of both animals showed a high expression of ERK in basal conditions as well as upon stimulation with PDGF. A 5-fold increase of ERK signal was found in CD4+ T cells upon stimulation with PDGF in both animals. Finally, expression of ERK was neither significant in B cells nor CD8+ T cells.
Conclusions. Ongoing studies seek to test the activation of RAS signaling in peripheral blood mononuclear cells of naive rhesus macaques upon administration of three different molecules able to stimulate the RAS cascade, such as IGF-1 (Insulin-like Growth Factor-1), insulin, and PDGF. After an in-depth evaluation of safety and efficacy of each molecule, we will proceed to vaccinate a cohort of rhesus macaques with ALVAC/gp120 in alum, in combination with a RAS activator, in order to assess whether this strategy affects the vaccine immunogenicity and efficacy.

ELISABETTA CACACE

Scuola Superiore Sant'Anna

Plasticity and resiliency in early T-cell development: an integrative approach

The hallmark of T-cell development is the sequential exclusion of alternative differentiative potentials: it is only after suppressing myeloid, B cell, dendritic cell and NK lineage programmes that a T-cell attains its definite commitment.
Since many of the factors needed for T-cell specification are required in other hematopoietic differentiation pathways, the ultimate specification of the T-cell fate is most likely the result of finetuned combinations of these factors, which are activated in a time- and environment-dependent fashion.
To investigate the interplay among the diverse differentiation programmes, we use a qualitative (logical) dynamical approach to model the underlying regulatory network. We mapped the regulatory network that underpins T cell differentiation until the DN4 stage, integrating information extracted from the literature along with public ChIP-seq datasets. We then used the software GINsim (Gene Interaction Network simulation: http://ginsim.org/) to evaluate the influence of different combinations of transcription factors at crucial stages of T cell development, applying logical formalism to model the interactions between network components.
We focused on two major checkpoints of T-cell specification: (i) the DN2a-DN2b transition, when a first commitment occurs due to the Bcl11b-mediated down-regulation of stem cell-associated genes, marking the beginning of TCR gene rearrangement, and (ii) the beta-selection step, where a functional and signalling pre-TCR permits the transition to the DN4 stage. In addition, we refined and enriched a previous model of the lympho-myeloid switch that governs the choice between these two alternative fates.
This model analysis particularly focuses on the interactions between PU.1 targets and Notch signalling pathway. By applying the logical formalism to these key steps of T cell development, we were able to test the influence of different combinations of transcription factors at the branching points among different lineage programmes, thereby shedding light on T-cell early plasticity and resiliency.

DANIELA ANGELA COVINO
Istituto Superiore di Sanità , Dipartimento di Ematologia, Oncologia e Medicina Molecolare

SAMHD1-independent inhibition of HIV-1 DNA accumulation by endogenous CCL2 neutralization: possible role of APOBEC3A

Macrophages are key targets of HIV-1 infection. We have previously described that the expression of CC chemokine ligand 2 (CCL2) increases during monocyte differentiation to macrophages and it is further up-modulated by HIV-1 exposure. Furthermore, CCL2 acts as an autocrine factor that promotes viral replication in infected macrophages. In this study, we dissected the molecular mechanisms by which CCL2 neutralization inhibits HIV-1 replication in monocyte-derived macrophages (MDM), and the potential involvement of the innate restriction factors protein sterile alpha motif (SAM) histidine/aspartic acid (HD) domain containing 1 (SAMHD1) and apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3 (APOBEC3) family members. We found that CCL2 neutralization potently reduced viral DNA accumulation and the number of p24 Gag+ cells during the course of either a productive or a single cycle infection with HIV-1BaL or (VSV-G) HIV-1, respectively. In contrast, CCL2 blocking did not modify entry of HIV-1 based Virus Like Particles, thus demonstrating that the restriction involves post-entry steps of the viral life cycle. Looking for correlates of HIV-1 DNA accumulation inhibition, we found that SAMHD1 expression was not affected by CCL2 neutralization. Furthermore, CCL2 blocking reduced the percentage of p24 Gag+ cells both in the absence and in the presence of exogenous dNTPs, and restricted the transduction of macrophages with Vpx+ lentiviral particles, which determined a strong down-modulation of SAMHD1 expression. These results demonstrated that altered SAMHD1 expression or function cannot account for the CCL2 neutralization-mediated restriction of HIV-1 replication in macrophages. Conversely, a strong and selective induction of APOBEC3A transcript and protein expression was associated with the inhibition of HIV-1 replication mediated by CCL2 neutralization. Interestingly, the level of CCL2 blocking induced APOBEC3A expression was comparable to those of freshly isolated monocytes. Finally, induction of A3A was type I IFN independent, since neutralizing Abs to IFN-α or IFN-β did not abolish CCL2 blocking-mediated A3A expression. Overall, these data demonstrate that neutralization of endogenous CCL2 determines a profound restriction of HIV-1 replication in primary MDM affecting post-entry steps of the viral life cycle likely involving the up-regulated expression of APOBEC3A. These results highlight a novel mechanism which may contribute to regulate the expression of innate intracellular viral antagonists in vivo. Thus, our study may potentially lead to the development of new therapeutic strategies for enhancing innate cellular defenses against HIV-1 and protecting macrophages from infection.

MANUELA DEL CORNO
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Role of TLR4 in HIV-1 gp120-induced signaling in human macrophages and hepatic stellate cells

Toll-like receptors (TLR) are the first line of the host response to pathogens and cell surface expressed TLRs, such as TLR2 and TLR4, have been shown to play an important role in human host defenses against viruses through sensing of viral structural proteins. Alterations of TLR expression and function have been described in AIDS and their capacity to modulate HIV replication has also been documented. Growing evidence points to TLR4 as a key player in chronic immune activation, HIV replication/reactivation and liver fibrosis progression suggesting that HIV triggering of this receptor may dictate some aspects of the multi-faced AIDS pathogenesis. Here we present evidence for an interaction between host TLR4 and HIV-1 gp120 in human monocyte-derived macrophages (MDM) and hepatic stellate cells (HSC). Our results unravel that gp120-induced secretion of chemokines, relevant for HIV replication and AIDS pathogenesis (CCL2, CCL4,CXCL8), as well as gp120-induced activation of p38MAPK, ERK1/2 and NF-kB involves TLR4, which is abundantly expressed on primary macrophages. Gp120-induced production of CCL2 as well as migration of hepatic stellate cells (HSC) is also strongly reduced by TLR4 blocking. Moreover, gp120 modulates the expression of inflammasome components in MDM and HSC, at least in part through activation of TLR4. Finally, our results indicate that CCR5 and TLR4 are likely part of a common receptor cluster as blocking CCR5 by specific antagonists, impairs macrophage capacity to produce chemokines in response to LPS. Chronic immune activation and liver fibrosis remain important obstacles for cART success. Thus, there is a strong need to study the mechanisms by which HIV induces immune system dysfunctions and fibrosis, to the final aim of identifying novel targets for therapeutic interventions. We believe that a comprehensive understanding of the molecular bases and functional consequences of HIV and TLR4 cross-talk is needed to guide the potential use of TLR based therapies as additional tools in the management of HIV infection and disease.

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Human CD1c+ dendritic cells secrete high levels of IL--12 and potently cross--prime cytotoxic T cell responses

Dendritic cells (DC) have the unique capacities to induce primary T cell responses. In mice, CD8α+DC are specialised to cross--prime CD8+ T-cells and produce IL-12 that promotes cytotoxicity.
Human BDCA-3+DC share several relevant characteristics with CD8α+DC, but the capacities of human DC subsets to induce CD8+ T cell responses are incompletely understood. We compared CD1c+mDC1, BDCA-3+mDC2 and plasmacytoid DC (pDC) in peripheral blood and lymphoid tissues for phenotype, cytokine production and their capacities to prime cytotoxic T cells.
mDC1 were surprisingly the only human DC that secreted high amounts of IL-12p70, but they required combinational TolI-like receptor (TLR) stimulation. mDC2 and pDC produced IFN--λ and IFN--α, respectively. Importantly, mDC1 and mDC2 required different combinations of TLR-ligands to cross-present CMV protein antigens to CD8+ T cells. pDC were inefficient, and also expressed lower levels of MHC- and co--stimulatory molecules. Nevertheless, all DC induced CD8+ memory T-cell expansions upon licensing by CD4+ T cells, and primed naive CD8+ T-cells following appropriate TLR stimulation. However, since mDC1 produced IL-12 they induced the highest levels of cytotoxic molecules. Thus, CD1c+mDC1 are the relevant source of IL-12 for naïve T cells, and are fully equipped to cross-prime cytotoxic T cell responses.

SEBASTIEN JAILLON
Humanitas Clinical Research Center

Role of the humoral pattern recognition molecule Pentraxin 3 in defence against urinary tract infections

morbidity and mortality. Immunity in the urinary tract has distinct and poorly understood pathophysiological characteristics and urinary tract infections (UTI) are important causes of morbidity and mortality. Cellular innate immune receptors and mediators play an important role in defence against UTI but the role of humoral innate immunity has not been explored. We investigated the role of the soluble pattern recognition molecule pentraxin 3 (PTX3), in UTI.
Methods: We monitored the bacterial load and the inflammatory response in kidneys and bladder of Ptx3+/+ and Ptx3–/– mice transurethrally infected with uropathogenic E.coli (UPEC). Bonemarrow chimeric mice were generated to investigate the separate contribution of PTX3 derived from hematopoietic or stromal cells. We assessed the cellular source for PTX3 production in UTI by immunohistochemistry and dissect the receptor(s) and signalling pathways responsible for PTX3 induction by RNAi-mediated gene silencing. We assessed whether PTX3 acts as an opsonin, accelerating phagocytosis and phagosome maturation. Finally, we genotyped highly selected UTI prone patients and compared their genotype frequencies in the PTX3 locus with a group of healthy patients.
Results: UTI of mice with UPEC was associated with increased levels of PTX3 in tissues and blood that correlated with the severity of the infection. PTX3-deficient mice showed a defective capacity to control UTI and increased tissue inflammation and damage. PTX3 is produced by the urothelium downstream of the protective TLR4/MyD88 pathway. Cooperative effects between PTX3-derived from stromal cells, which limited the infection, and PTX3-derived from hematopoietic cells, which protected from kidney abscess formation, were required to mount a protective immune response against UTI. PTX3 opsonised UPEC and accelerated phagocytosis and phagosome maturation by neutrophils. In human, PTX3 was detected in urine of UTI patients and amounts correlated with disease severity. Finally, human counterpart of our animal data were supported by genetic analysis of UTI-prone patients where PTX3 polymorphisms were associated with susceptibility to acute pyelonephritis and cystitis.
Conclusion and Discussion: Our results broaden the repertoire of defence effector molecules in UTI to include the humoral innate molecule PTX3 in mouse and human. Therefore, cellular and humoral arms of innate immunity exert complementary functions in UTI.

NAMAL LIYANAGE
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Natural killer cells and anti-SIV antibodies in prevention of SIVmac251 acquisition

Background: Innate immune effector cells including NK cells and innate lymphoid cells (ILCs), which reside in the peripheral blood and mucosal sites, are the first line of defense against viral infection. They play a significant role in both control of viral entry and mucosal homeostasis.
Increasing evidence suggests that NK cells and antibodies that mediate ADCC can control HIV infection. Correlate analysis of RV144 vaccine trial that showed 31% protection from HIV acquisition showed an inverse correlation between ADCC and risk of infection. We recently showed the recruitment of CD16+ (FcRγIII) NKG2A+ NK cells to the rectal mucosa during ALVAC/SIV gp120 vaccine regimen in macaques. In this study we investigated whether NK cells together with vaccine induced anti-envelop IgG at mucosal sites can protect from SIVmac251 acquisition.
Methods: IgG was purified from sera following 8 additional immunizations with ALVACSIV/gp120/Alum of 8 rhesus macaques uninfected from SIVmac251 challenge. To demonstrate whether that systemically given IgG can localize to the gut, CY5 labeled anti-human IgG was subcutaneously administered to macaques and anti-human antibodies were measured in serum and rectum by ELISA and immunohistochemistry (IHC) at 6h, 24h and weekly for 4 weeks. Eighteen macaques were vaccinated with ALVAC-SIV (SIV766 Gag-Pro gp120TM) at 0, 4, 12 and 24 weeks and ALUM was given together with the vaccine at 12 and 24 weeks. Purified IgG will be given to nine vaccinated macaques subcutaneously and intra-rectally prior to the repeated low dose challenge with SIVmac251 intra rectally in July 2014. Concurrently other nine vaccinated animals and six naïve control animals will be challenged.
Results: Subcutaneously given CY5 labeled anti-human IgG was detected in serum and rectal biopsies by ELISA and Immunohistochemistry up to 3 weeks post antibody administration. Multicolor flow cytometric analysis of rectal biopsies showed recruitment of NKG2A+NK ($p=0.03$) cells to the gut of vaccinees. Purified IgG from 8 protected animals showed 1.87% of gp120 specific activity by ELISA.
Conclusions: Our current data shows the successful delivery of antibodies to the gut mucosa by subcutaneous administration of purified IgG and recruitment of NKG2A+ NK cells to the gut mucosa following vaccination. Our hypothesis that non-neutralizing antibodies together with vaccine-mediated recruitment of NK cell to the gut can prevent SIV acquisition will be confirmed or rejected after SIVmac251 challenge at the end of July 2014.

EMILIA MARIA CRISTINA MAZZA
University of Modena and Reggio Emilia

A bioinformatics pipeline to uncover regulatory modules in human monocyte-to-macrophages differentiation and polarization

In healthy organisms, inflammation is the first defense mechanism and monocytes and macrophages are among the key players of this process. Since alterations in the activity of these cell populations are at the base of several pathological conditions, elucidating the molecular mechanisms of monocyte/macrophage activation represents a major step to study inflammatory disorders and, eventually, develop new therapeutic strategies. However, these mechanisms and their interplay during monocyte/macrophage activation still remain poorly characterized. Here, we report the setup of inflammation model, based on human primary cells, and of a bioinformatics approach that allow studying the development of the inflammatory reaction during its entire course and elucidating networks of molecular interactions which are at the basis of this process. Specifically, human blood monocytes isolated from blood of normal healthy donor have been cultured and exposed to a combination of factors reproducing acute inflammatory conditions and their gene expression profiles monitored during a time course of 48 hours. The computational pipeline starts with the identification of those genes whose expression changes during the time course and that, through enrichment analysis, appear to be involved in inflammatory reaction. These genes can be considered as controllers of the process and thus are further used to identify regulatory modules. Using these computational methods we identified regulatory genes that characterize the various steps of the inflammatory process. Finally, to gain insights into the mechanisms of transcriptional regulation during monocyte-to-macrophages differentiation and polarization, we integrated gene expression data of transcriptional modules with ChIP-seq data for several transcription factors.

SILVIA PICONESE
"Sapienza" University of Rome, Italy

IFN-α antagonizes regulatory T cell-mediated suppression and favors their plasticity into Th1-like cells

Regulatory T cells (CD127lowFOXP3+) (Treg) are classically viewed as immune suppressive cells, tipping the balance between host defense from pathogens and prevention of excessive immunity. However, under inflammatory conditions, Treg can be de-programed into cytokine-producing cells. The purpose of this study was to characterize the functional changes induced in Treg upon exposure to IFN-α. Recombinant IFN-α exerted an anti-proliferative effect against both conventional T cells (Tconv) and Treg in vitro, without affecting the Tregintrinsic suppressive function in Tconv-Treg cocultures. We tested IFN-α effects in vivo in patients with chronic HCV infection submitted to peginterferon/ribavirin therapy, before and 2 days after the starting of therapy. In this setting, IFN-α de-creased the frequency of Treg, and especially of the most suppressive Treg subset (stable CD45RA–FOXP3highHelioshigh Treg), mostly by enhancing apoptosis.
On the other hand, IFN-α boosted the polarization of IFN-γ-producing, so called "Th1-like", unstable (plastic) Treg both in vitro and in vivo, by directly inducing phosphorylation of STAT1 and STAT4. Of note, IFN-γ secretion by Treg and Tconv subsets showed a linear correlation in vivo, suggesting that Tconv-derived IFN-γ might play a relevant role in Treg diversion into Th1-like cells.
Our data indicate that IFN-α may amplify Th1-type inflammation by limiting the pool of fully suppressive Treg and concomitantly fostering Treg deprogramming into Th1-like cells.

HALEH TALAIE
Toxicological Research Center, Loghman Hakim Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Prevalence of Class 2 Integrons in Multidrug-Resistant Acinetobacter Baumannii in Toxicological ICU Patients in Tehran

Background: Acinetobacter baumannii is an important opportunistic pathogen which causes complications in hospitalized patients, especially those in ICU. The aim of this study was to determine the frequency of class 1 and 2 integrons in multi-drug resistance A. baumannii and to investigate the association between the presence of integrons and antibiotic resistance patterns.
Methods: A total of 40 A. baumannii strains were isolated from 372 ICU patients from June to Oct 2012. A. baumannii was detected in 50% of tracheal cultures, 15% in blood, 15% in urine samples, and 22.5% in other locations. In accordance with CLSI 2011, 12 antibiotics were used through disc diffusion method. Existence of integron classes was investigated by PCR assay with the amplification of integrase genes.
Results: The most effective antibiotic against Acinetobacter baumannii was polymyxin B with 100% susceptibility, followed by meropenem, piperacillin, cotrimoxazole, ceftazidime with 100% resistance; this was followed by ciprofloxacin 97.5%, tetracycline, 92.5%, imipenem 62.5%, and gentamicin 60% resistance. The presence of integron class 1 was 7.5%, class 2 was 67.5%, and non-integron was 20%.
Conclusion: The association between multidrug resistance and class 2 integron was not statistically significant. Other factors accounting for the lack of significance of the findings may be the impact of other resistance determinants such as transposons or plasmids, not investigated in the current study. Considering the increasing trend of MDR infections among ICU patients with critical problems in follow up, the use of appropriate infection control strategy and a regular surveillance system is necessary in our hospital.
Keywords: Acinetobacter Baumannii, Class 1 Integron, Class 2 Integron, Multidrug Resistance, PCR Assay.

STEPHEN WAGGONER
Cincinnati Children's Hospital Medical Center

Natural killer cells suppress humoral immunity and development of neutralizing antibodies

Pathogen-specific neutralizing antibodies (nAbs) are a key component of protective immunity after infection or vaccination. However, broadly-specific nAbs that can prevent infections with highly mutable viruses like HIV are rarely seen in infected patients and are poorly elicited by current vaccines. Therefore, new vaccine strategies are needed to enhance the induction of nAbs, potentially by augmenting the germinal center (GC) reactions that facilitate somatic hypermutation, affinity maturation and development of high-affinity nAbs. Here, we demonstrate that natural killer (NK) cells impair humoral immunity by contributing to a weak GC response and subdued generation of virus-specific nAbs after acute lymphocytic choriomeningitis virus (LCMV) infection of mice. The magnitude and duration of the LCMV-induced GC response was substantially reduced by NK cells, which constricted the number of both follicular helper T cells (TFH) and GC B cells present in lymphoid tissues. NK cell-mediated suppression of GC responses was also observed in different strains of mice and after infection with a variety of dissimilar pathogens, suggesting that NK cell suppression of humoral immunity is a universal feature of infection. Notably, NK cell inhibition of the GC response during LCMV infection resulted in a markedly delayed and relatively weak nAb response, as well as in reduced frequencies of LCMV-specific antibody-secreting cells in the bone marrow as early as one month and as late as three months post-infection. Thus, targeting of immunosuppressive NK cells could represent a revolutionary method to enhance the efficacy of future vaccine regimens.

MAILI ZIMMERMANN
University of Verona, Verona, Italy

Cell-Specific Chromatin Landscapes Drive Differential IL-6 Gene Expression in Human Neutrophils and Monocytes

IL-6 is a pleiotropic cytokine with a broad range of pro- and antiinflammatory functions, currently representing a potential therapeutic target in various inflammatory diseases. While monocytes are major source of IL-6, whether human neutrophils produce this cytokine remains still controversial. Herein, we performed new studies, also at epigenetic level, to clarify whether TLR-activated neutrophils express and produce IL-6. Human neutrophils and CD14+-monocytes, isolated from buffy coats by immunomagnetic beads at a purity of 99.7 ± 0.2 % and 98 ± 1 % respectively, were stimulated with TLR4 and TLR8 agonists, as well as with other ligands for up to 20 h. IL-6 mRNA expression and production were then measured by RT-qPCR and ELISA, respectively, while analysis of the chromatin status at the IL-6 genomic locus was investigated by chromatin immunoprecipitation assays for histone modifications or transcription factor binding.
Our data prove that highly purified neutrophils display the capacity to produce biologically active quantities of IL-6, but only after a prolonged incubation with R848 or, less efficiently, very elevated doses of LPS. Data also uncover that IL-6-induction in neutrophils by R848 is preceded by PU.1 recruitment, H3 methylation (e.g., H3K4me1 and H3K4me3) and H3 and H4 acetylation (e.g., H3K27Ac and H4Ac) at the IL-6 locus. Analysis of the distribution of histone modifications within 64 kilobases upstream of the IL-6 transcription start site also revealed that neutrophils and monocytes utilize both common and cell-specific enhancer sites. In consideration that TLR8 recognizes single strand RNA from viruses, our observation that neutrophils treated with TLR8 agonists produce IL-6 is consistent with a role of these cells in the context of antiviral responses, as recently highlighted by our previous findings. Data clarify that, at least under TLR-stimulation, neutrophils may generate IL- 6 by activating a cascade of events controlled at the epigenetic level.

TOPIC Innate Immunity, Inflammation and Cancer

best oral presentation

MARCO MACAGNO

University of Turin - Molecular Biotechnology Center

Role of Perforin+ Cells in the Microenvironment of ErbB2+ Salivary and Mammary Carcinomas in Transgenic Male Mice

Background: Tumor immune surveillance rests on T cell and NK cells. In both cases perforin (pfp) may be a major effector molecule. Our previous works showed that pfp is involved in the immune surveillance of ErbB-2-mammary carcinogenesis in BALB/c female mice transgenic for the transforming rat ErbB-2(neu) oncogene under the control of the mouse mammary tumor virus promoter (BALB-neuT mice) (Street et al, 2007; Cancer Res 67:5454). Here we evaluate the unique role of pfp in male mammary cancer immune surveillance.
Methods: While BALB-neuT male mice rarely develop mammary carcinomas, all BALB-neuT females develop multiple, fast-growing, invasive carcinomas in all mammary glands. When we back-crossed BALB-neuT mice with pfpKO BALB/c mice, tumor onset in male and female BALB-neuT and BALB-neuT/pfpKO mice was evaluated.
Results: An accelerated onset of mammary carcinomas and a higher number of carcinomas per mouse were found in BALB-neuT/pfpKO female mice. Moreover, while BALB-neuT male mice rarely develop mammary carcinomas, surprisingly, the great majority of BALB-neuTpfpKO male mice develops them. BALB-neuT/pfpKO male mice display impaired readsorption of mammary gland ductules in the last period of fetal life and in the newborn mice. Mammary cancer develops from rudimental ductules expressing the ErbB-2 oncogene and persisting in adult BALB-neuT/pfpKO male mice.
Conclusion: Present data show that pfp-mediated mechanisms play crucial roles in mammary gland morphogenesis and in the immune surveillance of ErbB-2+ carcinomas. Altered readsorption of mammary ductules allows ErbB-2+ cells to persist in adult male mice and give rise to carcinomas. All together these findings provide further proof of the multiple roles that pfp-mediated mechanisms perform in cancer control and may be important for the development of NK cell-based therapeutic strategies..

best oral presentation

ELEONORA TIMPERI

Dipartimento di Medicina Interna e Specialità Mediche, Policlinico Umberto I, Roma

Regulatory T cells expressing Helios, OX40 and CD39 in human colorectal cancers

Regulatory T cells (Treg), or better Treg subsets, may play dual roles in the development of human colorectal cancer (CRC).
Indeed, Treg may not only suppress the anti-tumor immunity that is responsible for keeping in check tumor onset, but also inhibit the inflammatory response that rather fosters tumor progression.
Our previous data unraveled a peculiar role for the transcription factor Helios in distinguishing committed Treg subsets, expressing receptors such as OX40 and CD39, especially in tumor microenvironment, that can influence the suppressive function, the stability and the activation of Treg fostering the progression of tumor development.
The goal of this study was to characterize the frequency and the phenotype of different Treg subsets, at the tumor site (T) or in the normal mucosa (N) of patients with CRC at different progression stages.
To characterize the infiltrating Treg, the mononuclear cells were extracted from T and N (and from peripheral blood as control) and then analyzed by multiparameter surface and nuclear flow cytometry.
Our results indicate that patients with CRC showed Treg accumulation in N an T, compared to PBMC. The analysis of different Treg subsets showed the increase of both Helios+ and Helios- Treg in N and T especially Helios+ in T. Helios + Treg may represent the subset preferentially devoted to induce tumor progression.
Supporting this idea we found higher Helios + Treg frequency in N of stage III/IV patients, compared to stage I/II patients. Helios + Treg contained higher proportion of Treg expressing CD39, a molecule responsible for Treg suppression and known to be polymorphic in the polulation. In our cohort, we noticed that the subgroup of patients with a CD39 high profile was enriched in patients with more advanced disease. Both Helios+ and Helios–, but especially CD39+ Treg, expressed high OX40 level not only in N but even more in T, compared to PBMC, indicating their strong activation state. Overall our data indicate that specific Treg subsets can accumulate and be activated at the tumor site possibly fostering CRC progression.

oral presentation

VALENTINO MARIO LE NOCI
Dipartimento di Scienze Biomediche per la Salute , Università degli Studi di Milano, Italia

Combined aerosolization of TLR9 and TLR3 agonists in the treatment of lung metastases

Introduction The immunostimulatory ability of synthetic oligonucleotides containing CpG motifs (CpG-ODN), agonists of Toll-like receptor 9 (TLR9), can be harnessed to promote antitumor immunity by their application at the tumor site to stimulate local activation of innate immunity. However, tumor microenvironment is a critical factor for successful of these immunotherapeutics, since tumor-associated immunosuppression can subvert such antitumor innate immune responses and foster immunological anergy. Molecules able to induce a shift of immune cells with suppressive function to cells with tumoricidal function, such as TLR3 agonists Poly(IC), that promote the conversion of tumor associated macrophages (TAM) from M2 to M1 phenotype, could increase the antitumor activity of CpG-ODN. We evaluated the antitumor efficacy of locoregional administration by aerosol of CpG-ODN combined to Poly(IC) to simultaneously blocking TAM-induced immunosuppression while activating a large number of innate immune cells, in the treatment of experimental B16 melanoma lung metastases, a low immunogenic tumor able to selectively recruit M2 macrophages. Moreover, aerosolization with the two agonists was evaluated as adjuvant therapy with Dacarbazine (DTIC), an alkylating agent used as therapeutic standard in patients with inoperable metastatic melanoma, able to exert immunostimulatory effects through the upregulation of NKG2D ligands on tumor cell.
Methods C57BL/6 mice were i.v. injected with 5×105 B16 cells and treated starting 4 days after injection with CpG-ODN 1826 and Poly(IC) by aerosol (1.5 mg and 15 mg, respectively, to treat 10 mice in the aerosol box) twice/week; DTIC (75 mg/Kg) was administered intraperitoneally (i.p.) 5 days/week. Expansion of different immune cell populations was analysed by flow cytometry in digested lungs, while the frequency of M2 macrophages in the tumor was assessed by immunofluorescence on sections of lung tissues stained for CD68 and arginase/ IL-10.
Results Combined aerosolized CpG-ODN and Poly(IC) revealed no overt signs of toxicity, such as weight loss, hunching, ruffled fur or difficulty breathing, and the absence of injury of the bronchial-bronchiolar structures and of the alveolar walls. As expected, histopathological evaluation of lung sections from aerosolized Poly(I:C)/CpG-ODN-treated mice revealed areas of mononuclear and granulocytic infiltrate. Immunofluorescence analysis to test the effect of aerosolized poly(I:C) on the frequency of M2 macrophages showed a reduced number of M2 phenotype-associated arginase-producing macrophages in lung of mice bearing B16 metastases treated with TLR3 agonist, as compared to untreated mice. Concominant aerosolization of the two agonists induced a greater reduction in the number of metastases in lungs of mice 3 weeks after i.v. injection of B16 melanoma cells, as compared to mice treated with each agonist alone. Moreover, combined aerosolization significantly improved the efficacy of chemotherapeutic treatment with DTIC in mice i.v. injected with B16 cells, inducing 3 out of 8 mice completely cured at the end of the experiment.
Conclusions Our data support the efficacy of combined CpG-ODN and Poly(IC) aerosolization as a simple approach for repeated applications of immunostimulants directly at the tumor site and point to its value as adiuvant therapy in the treatment of lung metastases.

oral presentation

CHIARA PORTA
University of Piemonte Orientale

The p50 NF-κB subunit shapes pro-tumor inflammation supporting colorectal cancer development

Macrophage plasticity is a key determinant of tissue homeostasis, in both steady state and pathological conditions, including infections and cancers. We previously demonstrated that in response to bacterial-derived lipopolysaccharide nuclear accumulation of p50 NF-κB drives both M2-polarization and macrophage tolerance towards subsequent bacterial challenges. This latter may be relevant in the gut, where exposure to pathogenic microorganisms and commensal flora requires a tight control of tissue homeostasis, whose alteration is associated with inflammation and colorectal cancer (CRC) development. The present study demonstrates that, in a murine model of colitis-associated CRC, the anti-inflammatory role of p50 NF-κB leads to divergent effects on colitis versus CRC development. Indeed, whereas progression from colitis to cancer was associated with up-regulation of a selected cluster of M2-related genes, ablation of p50 NF-κB correlated with exacerbated colitis score and reduced CRC development.
Interestingly, lack of p50 NF-κB in tumor bearing mice instated reduced number of both lamina propria and tumor-associated macrophages (TAM), along with increased number of cytotoxic cells (NK, NKT, CD8+ T cells) and apoptotic cancer cells. This anti-tumor immune infiltrate was associated with enhanced expression of an M1/Th1 cytokine profile, including IL-12 and CXCL10, which was confirmed in primary tumors from CRC patients with favorable clinical outcome. Accordingly, administration of either IL-12 or CXCL10 was able to restrain CAC development in wt mice. Our study identifies p50 as key controller of intestinal inflammation and provides first evidence that M1/Th1 cytokines should be considered both as prognostic indicators and immunotherapeutic agents in CRC.

best poster presentation

ANTONINO BRUNO
Oncology Research, Polo Scientifico e Tecnologico, IRCCS MultiMedica, Milano, Italy

Tumor infiltrating Natural Killer cells (TINKs): a new player in the inflammatory orchestration of cancer progression

Immune cells infiltrating tumors often show a polarized phenotype that reflects attenuation of anti-tumor activity and enhancement of pro-tumor activities, including angiogenesis. Natural Killer (NK) cells are effectors lymphocytes of innate immunity that can potentially control tumors by their cytotoxic activity. Recent studies suggest that tumor infiltrating NK cells are able to acquire a modified phenotype potentially supporting tumor progression and angiogenesis. In Non Small Cell Lung Cancer (NSCLC) samples, the CD56brightCD16-NK phenotype, associated with cytokine production, predominates in the tumor samples while the CD56dimCD16+ cytotoxic phenotype dominates in adjacent normal tissues. In contrast, the CD56brightCD16- NK cells predominate in both the tumors and adjacent tissues derived from colo-rectal carcinoma (CRC) samples. We found that NSCLC as well as CRC CD56brightCD16- infiltrating NKs are able to release substantial amounts of pro--angiogenic factors, including VEGF, PIGF and IL-8, they exert low cytotoxic activities, and induce migration of and capillary- like structure formation of endothelial cells in vitro. TGFβ1 strongly up--regulates VEGF and PIGF release by peripheral blood NK cells from healthy age-matched donors, suggesting that this may be one mechanism driving tumor NK cell polarization. Taken together, the altered phenotype and function of tumor infiltrating NK cells has a broad implications in the immune response to tumors, ranging from a deficient control of cancer to an altered crosstalk with other relevant players of the immune response. This places NK cells as a new player in inflammation and pro-angiogenic activity for tumor development and progression.

best poster presentation

ELENA CIAGLIA
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Enrichment of CD56dimKIR+CD57+ highly cytotoxic NK cells in tumor infiltrated lymph nodes of melanoma patients

NK cells contribute to melanoma cell recognition and anti-tumor immunity, which is traditionally analyzed using human peripheral blood NK cells. An important checkpoint in the progression of melanoma is the metastasis to lymph nodes. To investigate the role of lymph node NK cells in disease progression, we analyze frequency, phenotype and functions of NK cells from tumor-infiltrated (TILN), tumor-free ipsilateral lymph nodes (TFLN) and peripheral blood (PBL) in a cohort of stage III-IV melanoma patients. The NK cells in healthy lymph nodes are predominantly CD56bright. The comparative analysis of the lymphocytes subsets associated with lymph nodes cell suspensions and autologous peripheral blood lymphocytes reveal a perturbation of NK cell subpopulation frequencies in the TILN where an expansion of CD56dimCD57dimCD69+CCR7+KIR+ NK cells is observed. Here, despite the presence of tolerogenic Treg cells, TILN associated NK subset is functionally active and display robust cytotoxic activity against autologous melanoma cells. This peculiar phenotype is suggestive of a their potential role in clinical outcome as a high proportion of CD56dimCD57+in the infiltrated lymph nodes is associated with improved patients' survival. Moreover in the blood of metastatic melanoma patients the frequency of NK cells expressing the CXCL8 receptor is increased compared to healthy subjects, and blood NK cells also express the receptors for CCL2 and IL6. These factors are produced in high amount in TILN and are able in vitro to switch the phenotype of blood NK cells from healthy donors to the phenotype associated with TILN. In conclusion our data suggest that the microenvironment of TILN generates and/or recruits a particularly effective CD56dimCD57+CD69+CCR7+KIR+ NK cell subset that should be considered as interesting candidate for the design of new adoptive immunotherapy protocols. Lymph nodes explanted during common clinical practice may be a good source of proliferating and highly cytotoxic NK cells that can be expanded ex vivo and subsequently administered to patients.

best poster presentation

TANIA LAHERA
Laboratory of Basic Research and Immunology, National Institute of Oncology, Havana, Cuba

Tumor-infiltrating lymphocytes and macrophages in the prognosis of patients with colon cancer

Introduction: Colon cancer (CC) is one of the leading causes of cancer death in both developed and developing countries. Until now, the anatomic extent of tumor (TNM classification) has been the most important factor to estimate the prognosis of CC patients. However, in recent years, some studies have demonstrated that the immune contexture in the tumors can be an essential prognostic factor.
Aim: To assess the prognostic role of tumor-infiltrating lymphocytes and macrophages, and to evaluate its relation with clinicopathological features in patients with CC. Methods: Paraffin-embedded specimens were retrospectively collected from 50 patients who underwent resection for CC at National Institute of Oncology, Havana, between 2004 and 2008 years. The density of cells was determined by immunohistochemistry technique and its relation with survival and clinicopathologic features was evaluated. Lymphocytes positive for CD3 (T cell), CD45RO (memory marker), CD8 and granzime B (T cell cytotoxic) were assessed according to intraepithelial and stromal location in the tumor. Macrophage infiltration along the tumor front was also evaluated using CD68.
Results: Patients with poor grade of differentiation, mucinous type and clinical stage IV showed low infiltration of immunologic cells. The 5-years overall survival for patients who had high-density of cells were better compared with patients who had low-density, with significant differences statistical for intraepithelial CD8 (p=0,035) as well as stromal CD3 (p=0,001), CD8 (p=0,041), CD45RO (p=0,007) and CD68 (p=0,041). In univariate survival analysis, the high expression of CD3 (p=0,006), CD45RO (p=0,011) and CD68 (p=0,042) at stromal level and CD8 intratumoral (p=0,014) were significant prognostic factors for overall survival. Among these variables, level of CD45RO (HR: 0,187; CI95%: 0,060-0,578; p=0,004) was independent prognostic factor on multivariate analysis, by Cox regression.
Conclusions: This study is the first approach, in our country, on the prognostic significance of tumor-infiltrating lymphocytes and macrophages in CC. This assessment, mainly CD45RO TIL, might be used in the prognostic estimate of CC, although further studies will be required to validate these findings.

best poster presentation

SOFIA XANTHOULEA
Department of Plastic Surgery, NUTRIM School for Nutrition, Toxicology & Metabolism, Maastricht University Medical Centre, Maastricht, The Netherlands

Wound administration of M2-polarized macrophages does not improve murine cutaneous healing responses

Macrophages play a crucial role in all stages of cutaneous wound healing responses and dysregulation of macrophage function can result in derailed wound repair. The phenotype of macrophages is influenced by the wound microenvironment and evolves during healing from a more pro-inflammatory (M1) profile in early stages, to a less inflammatory pro-healing (M2) phenotype in later stages of repair. The aim of the current study was to investigate the potential of exogenous administration of M2 macrophages to promote wound healing in an experimental mouse model of cutaneous injury. Bone marrow derived macrophages were stimulated in-vitro with IL-4 or IL-10 to obtain two different subsets of M2-polarized cells, M2a or M2c respectively. Polarized macrophages were injected into full-thickness excisional skin wounds of either C57BL/6 or diabetic db/db mice. Control groups were injected with non-polarized (M0) macrophages or saline. Our data indicate that despite M2 macrophages exhibit an anti-inflammatory phenotype in-vitro, they do not improve wound closure in wild type mice while they delay healing in diabetic mice. Examination of wounds on day 15 postinjury indicated delayed re-epithelialization and persistence of neutrophils in M2 macrophage treated diabetic wounds. Therefore, topical application of ex-vivo generated M2 macrophages is not beneficial and contraindicated for cell therapy of skin wounds.

CRISTINA BELGIOVINE
Humanitas Clinical And Research Institute

Human and mouse mononuclear phagocytes are the only leucocyte subset expressing functional death receptors for Trail

Targeting of Tumor-Associated Macrophages (TAM) is considered a promising therapeutic strategy as these myeloid cells are known to have several pro-tumor functions. We recently reported that Trabectedin, a marine anti-tumor drug, is able to impair tumor growth by targeting monocytes/macrophages via activation of TRAIL receptors (TRAILRs). In this study we performed an in-depth analysis of the expression and function of TRAIL-Rs in leukocytes. We evaluate expression and modulation of death receptors and activation of caspase-8 in human and mouse leucocytes by FACS analysis and immunofluorescence while macrophage targeting in vivo was tested in tumor-bearing mice treated with TRAIL.
We found that human blood monocytes and tissue macrophages express discrete levels of signalling TRAILRs (DR4, DR5) and lack the non-signaling decoy receptor DcR1. In contrast, neutrophils and lymphocytes mostly express DcR1. Accordingly, TRAIL-ligand induced caspase-8 activation exclusively in monocytes/ macrophages. Different natural compounds and selected cytokines were able to modulate TRAILRs in monocytes. Murine blood monocytes and macrophages, including TAM, express TRAILR DR5. In vivo treatment of mice bearing a TRAIL-resistant fibrosarcoma with TRAIL ligand decreased monocyte number and resulted in tumor reduction.
It was assumed that the apoptotic cytokine TRAIL spares normal cells and selectively kills neoplastic cells. Our results demonstrate that human and murine monocytes/macrophages are susceptible to TRAIL and undergo apoptosis in a caspase-8 dependent manner, in vitro and in vivo. Of note, monocytes/macrophages are the only leukocyte subset expressing functional TRAILRs. This differential susceptibility to TRAIL-induced apoptosis could be exploited to selectively target monocytes/macrophages in the tumors.

FRANCESCA BERGOMAS
Humanitas Clinical And Research Institute

Heterogeneity of the immune infiltrate in colorectal cancer: tumor infiltrating lymphocytes and tertiary lymphoid tissue

Tumor-infiltrating T cells (TILs) have been shown to have a protective role in human colo-rectal cancer (CRC), being significantly associated with better clinical outcome. We have recently demonstrated that tertiary lymphoid tissue (TLT), a lymph-node-like reaction which develops at sites of chronic inflammation, plays a key role in recruitment of TILs to CRC and that they cooperate in an anti-tumor immune response and in predicting better patient's outcome. Both T and B cells are key components of TLT. However, while T cell role in cancer has been addressed, the role of B cells in tumor progression and their clinical relevance have not been elucidated. In a murine model of colitis-associated CRC (AOM/DSS), a quantitative evaluation of B cells indicated that they increase in the tumor mucosa as compared to controls. Notably, B cells localized in two different histological compartments, either scattered at the tumor invasive margin as interspersed cells (B-TILs) or within TLT (B-TLT). To better investigate the duality of the two B cell components in colon cancer, we evaluated tumor growth in B cell-genetically deficient mice (μMT) in a subcutaneous CRC model (CMT93), which did not induce formation of TLT in its microenvironment. We found that growth of CMT93 cells was reduced in μMT mice compared to controls, thus suggesting that interspersed B-TILs might have a protumoral role. On the other hand, in the AOM/DSS model, including TLT, genetic depletion of B cells did not affect TLT occurrence, further suggesting that B-TILs and B-TLT are distinct immunological compartments. Similarly to murine colon mucosa, within human CRC tissue, CD20+ tumor infiltrating B cells localized both in tertiary lymphoid tissue in the stromal compartment (CD20-TLT) and scattered around the tumor margin (CD20-TILs). The density of CD20-TLT linearly correlated with the density of CD20-TILs ($r=0.18$; $P=0.006$, $N=204$), consistent with the ability of TLT to mediate recruitment of lymphocytes. Future plans include a deeper analysis of B cell function in preclinical models of CRC and the definition of their clinical relevance in human CRC.
Results might suggest TLT as a new therapeutic target to manipulate lymphocyte recruitment at the tumor site and induce an antitumor immune response in CRC.

FRANCISCO MIGUEL ANGEL BIANCHETTO AGUILERA
Department of Pathology and Diagnostics, Division of General Pathology, University of Verona, Verona, Italy

Genome-wide patterns of PU.1 and H3K4me1 localization in human neutrophils and monocytes

Neutrophils and monocytes are the two most represented population of phagocytes in the human bloodstream. Neutrophils are integrated in the activation, regulation and effector mechanisms of the innate and adaptive immune systems. Beyond their well-known role in acute inflammatory responses and resistance to extracellular pathogens, neutrophils also function as major players of diverse pathologies via the release of de novo formed mediators. However, the mechanisms that in neutrophils control transcription are still very poorly studied, mainly because neutrophils are seen as short-lived effectors cells. Multiple knockout models have demonstrated the importance of PU.1 in the differentiation of myeloid cells. Indeed, this pioneer TF, that acts as a transcriptional master regulator, initiates nucleosome remodelling followed by histone modifications depositions at large numbers of genomic regions. These changes in the cell chromatin landscape serve as beacons for the recruitment of additional factors, which drive both cell-specific gene expression and signaldependent responses. Here, by performing chromatin immunoprecipitation (ChIP) followed by high-throughput sequencing in freshly isolated neutrophils and monocytes, we globally mapped the distribution of the enhancer mark histone H3 lysine 4 monomethylation (H3K4me1) and the master regulator PU.1. The numbers of PU.1-bound genomic sites were 63,461 in neutrophils and 49,041 in monocytes, whereas the H3K4me1 peaks were 54,269 in neutrophils and 63,661 in monocytes.
While most of the PU.1 peaks localized in promoter-proximal regions showed similar occupancy levels in both neutrophils and monocytes, cell-specific PU.1 recruitment were predominantly located in enhancer-distal regions for both type of phagocytes. Analysis of the genomic distribution of the H3K4me1 along a definite region of approximately 3,000 bp from the centre of enhancerdistal PU.1 peaks showed a typical bimodal distribution. In addition, we found that PU.1 recruitment at cell-specific enhancer sites strictly correlates with distal cell-specific H3K4me1 also in human neutrophils. Moreover, by performing microarray gene expression profiling in freshly isolated neutrophils and monocytes, we identified either common or cell-specific transcripts. By integrating the transcriptomic and epigenetic signatures, we observed stringent correlation between cell-specific transcript expression and H3K4me1 and PU.1 deposition at specific enhancer regions of the related gene. Together, these findings suggest that histone modifications and PU.1 recruitment at cell-specific genomic regions are likely responsible for the peculiar biological differences between the cells types.

EDUARDO BONAVITA
Humanitas Clinical And Research Institute

The long pentraxin ptx3 is a novel player regulating cancerassociated inflammation, tumor development and progression

Pentraxins (PTXs) are conserved prototypic components of the humoral arm of innate immunity. The long pentraxin PTX3 plays a fundamental role in innate immunity, in regulation of the inflammatory response and in tissue remodelling, through different molecular mechanisms, including Complement regulation and P-selectin-dependent leukocyte recruitment. PTX3 is produced by diverse cell types present in the tumor stroma, such as leukocytes, endothelial cells and fibroblasts.
To define the role of PTX3 in tumor-associated inflammation, we investigated the susceptibility to 3-MCA induced sarcoma and to DMBA/TPA-induced skin carcinogenesis of PTX3-/- and wild type mice.
In these models, PTX3-deficiency was associated with increased tumor incidence, fast tumor growth and higher inflammatory response (e.g. leukocyte recruitment, inflammatory cytokines). The analysis of tumors revealed increased complement C3 and reduced Factor H deposition in PTX3-deficient mice, in agreement with the complement-regulatory function of PTX3. In an effort to define the molecular mechanisms underlying this phenotype, we observed that C3-deficiency and inhibition of macrophage recruitment reverted the susceptibility of PTX3-/- mice to 3-MCA. The genetic analysis of “hot” genes Tp53 and K-ras, classically mutated by 3-MCA, showed that PTX3-deficiency was associated with higher mutation frequency. Finally, PTX3 expression was epigenetically regulated in human epithelial cancers by methylation of two CpG islands located in regulatory regions, thus suggesting the relevance in human of data obtained in mice. All together, these results are consistent with the hypothesis that PTX3 is a regulatory protein of cancer-associated inflammation playing an oncosuppressor function.

GIOVANNI FRANCESCO CASTINO
Humanitas Clinical And Research Institute

Tertiary lymphoid tissue, a novel player in the immune microenvironment of pancreatic ductal adenocarcinoma

Objective. Recent data from our group point to the occurrence and clinical relevance of organized ectopic (or tertiary) lymphoid tissue (TLT) at the tumor site, which act together with tumour infiltrating lymphocytes (TILs) to coordinate an antitumour immune response. We are currently investigating the occurrence of Tertiary Lymphoid Tissue in human Pancreatic Ductal Adenocarcinoma (PDAC), and its relationship to other leukocyte populations. Moreover we are studying TLT formation and function in a preclinical model of immunotherapy.
Methods. The occurrence and characterization of TLT and other immune populations have been evaluated by immunohistochemistry in PDAC tissue specimens from a cohort of 86 consecutive PDAC patients who underwent surgical resection at the Humanitas Clinical and Research Centre. The percentage of immune reactive area (IRA%) of CD20+TLT and CD8+ T cells at the tumor margin has been evaluated by computer assisted image analysis. A dendritic-cell based vaccine was designed, by administering weekly injections of dendritic cells previously cultured with apoptotic/necrotic Panc02 cells or DT6606 murine cells. All samples have been analyzed by immunohistochemistry to assess the presence and features of TLT.
Results. In human PDAC tissue specimens, we identified organized lymphoid tissue, including compartmentalized T and B cell areas, dendritic cells and high endothelial venules (HEV). TLT occurred preferentially in the stromal compartment, rich in mesenchymal cells. The density of TLT correlated positively to the density of intra tumor CD8 T cells. In a preclinical model of PDAC, DC-based vaccination sporadically induced formation of B and T cells aggregates, resembling the ones occurring in human PDAC. Evaluation of tumor infiltrating leukocytes into vaccinated mice evidenced that TLT occurrence coordinates with other immune cells, including CD3+ T cells and B cells, suggesting that they are an important player in the tumor microenvironment.
Conclusions. Immunotherapeutic strategies inducing TLT might represent a therapeutic approach to target the tumor stroma, by creating a lymphoid like microenvironment, to increase the recruitment and activation of T cells. More data is required to understand the prognostic significance of TLT in the context of PDAC.

MARCO ERRENI
Humanitas Clinical And Research Institute

Fractalkine ligand - receptor axis dictates prognostic outcome in human colorectal cancer

Colorectal cancer (CRC) is a frequent malignancy and still a major cause of cancerrelated death. Even if advances have defined the genetic alterations and molecular pathways involved in tumor progression, better understanding of the biological diversity of this neoplasia and the relationship to clinical outcome are needed. In this study, we examined the prognostic value of the chemokine CX3CL1 and its specific receptor CX3CR1 by tumor cells in CRC patients. We analysed CX3CL1 and CX3CR1 expression in tumor cells, by immunohistochemistry, 100 primary CRC, 33 lymph node and 10 liver metastases; immune-reactive scores were correlated to pathological features and patients' follow-up. Tumor cells expressed higher levels of CX3CL1 and CX3CR1 in comparison to healthy mucosa. CX3CL1 was up-regulated in early stage tumors, but decreased in advanced stage and in lymph node and liver metastases; CX3CR1 was up-regulated in primary tumors and decreased in metastases. Concomitant low expression of CX3CL1 and CX3CR1 (axis-negative tumors) was associated with reduced patients' survival (DFS p=0.0002, DSS p=0.0003) and 3.4-fold increased risk of worse prognosis (DFS p=0.006, DSS p=0.03). In particular, axis-negative tumors showed an increased likelihood of developing metachronous distant-organ metastasis post-surgery ($p<0.0001$).
Since CX3CL1 is a trans-membrane molecule, we hypothesized that the concomitant expression of ligand and receptor could increase the adherence among cancer cells, thus preventing tumor dissemination. By using mixed populations of CX3CL1- or CX3CR1- transduced tumor cells, we observed that ligand-induced engagement of receptor-positive tumor cells resulted in enhanced adhesive activity. Most importantly, in a mouse spleenliver model of metastasis, tumors not expressing the CX3CL1-CX3CR1 axis had higher metastatic potential. In conclusion, our preclinical data are consistent with the view that the trans-membrane chemokine CX3CL1 and its specific receptor engages in a relatively stable adhesion loop that prevents tumor cell dissemination by holding CX3CR1+ cells locally. In human CRC, low tumoral expression of CX3CL1 and CX3CR1 identifies a patients' subset at higher risk of tumor recurrence with distant metastasis, needing closer post-surgical surveillance.

FRANCESCA FAGGIOLI
Milan, UOS, IRGB, CNR and Humanitas Clinical and Research Center

The role of cellular senescence in the progression from chronic liver inflammation to cancer

Purpose: Cellular senescence, as a state of irreversible proliferative arrest, is the first-line defense against the malignant progression of tumoral cells1. However, their ability to secrete a unique inflammatory repertoire (inflammasome) paradoxically turns senescent cells in promoters of neoplastic advance2. In several liver chronic inflammatory conditions, often progressing into hepatocellular carcinoma (HCC), senescent cells tend to accumulate; however it still unclear whether they act as a tumoral barrier or contribute to the progression of malignancy, exhibiting immunoregulatory properties3. We therefore interrogated the phenotype and function of liver senescent cells in the Mdr2-/- mouse, a model of chronic cholangitis progressing into HCC4.
Methods: Paraffin included liver samples from knockout mice and relative controls (n=8) were analyzed using immunostaining technique at different disease stages. Fresh isolated liver lymphocytes from ko and wt mice were characterized by FACS analysis; for immune functional assays, ex vivo isolated senescent cells have been co-cultured in plate-bound anti-CD3ε with wt splenic CD4+T cells; cytokine secretion was analyzed by ELISA.
Results: Senescent hepatocytes accumulate at the level of portal spaces, where the process of fibrosis begins, and increase in number with the progression of the pathology in ko livers. In Mdr2-/- mouse, the parenchymal liver cells modulate the proliferation and the cytokine profile of CD4 naïve T cells.
Conclusions: The phenotypical and functional alterations of wt lymphocytes observed in the co-culture with ko hepatocytes suggest a role played by hepatocytes in triggering either tumor immunity or tumor tolerance in a setting of chronic inflammation. Further experiments are needed to establish whether the escape from parenchymal cells surveillance and progression to HCC, at later stages of the disease, can be directly ascribed to immunoregulatory properties of senescent cells.

MARIA ROSARIA GALDIERO
Humanitas Clinical And Research Institute

Prognostic and predictive significance of tumor infiltrating neutrophils in patients with colorectal cancer

Background: Neutrophils have emerged as candidate cells that may modulate the tumorigenic process and the antitumor immunity. Epidemiological studies suggest that tumor-infiltrating neutrophils may be associated with patient outcome.
Objective: To investigate the clinical significance of tumor-infiltrating neutrophils in 128 primary Stage I-Stage IV colorectal cancer (CRC) patients and their role in predicting chemotherapy response in 179 Stage III CRC patients.
Method: CRC histological sections were immunohistochemically treated with monoclonal antibodies against CD66b. For each section, intratumoral (IT) and invasive margin (IM) neutrophil densities were calculated using a computer-aided image analysis system and expressed as the mean immunoreactive area (CD66b+ IRA) of three randomly selected and non-contiguous fields.
Results: Higher percentage of IT and IM CD66b+ IRA was associated with better Disease Specific Survival (DSS) (p=0.01 and p=0.0016) and Disease Free Survival (DFS) (p=0.02 and p=0.02). Multivariate analysis demonstrated that high IT CD66b+ IRA was an independent prognostic factor for better DSS (HR 0.48; 95% CI: 0.25-0.92; p= 0.03). IT CD66b+IRA was also found to be predictive for 5-FluoroUracyle chemotherapy response in Stage III mismatch repair proficient CRCs (p=0.04). Moreover, through an in vitro system we found that CRC cell lines produce soluble factors, which promote neutrophil chemotaxis and survival and that, in turn, neutrophils exert cytotoxic activity against CRC cell lines.
Conclusions: Our findings suggest that neutrophil infiltration has a good prognostic and predictive significance for 5-FluoroUracyle based chemotherapy in patients with CRC.

FABIO GRIZZI
Humanitas Clinical and Research Center

High frequency of HLA class I antigen processing machinery (APM) component up-regulation in primary hepatocellular carcinoma tumors

Malignant transformation of cells is associated with down-regulation of HLA class I APM components in most of the tumors. These defects are clinically relevant, since they are frequently associated with the clinical course of the disease.
Only in a few tumors malignancy is associated with the up-regulation of HLA class I antigens. Among them is hepatocellular carcinoma (HCC). The frequency of HLA class I APM component up-regulation and its clinical significance in HCC are not known. These topics were investigated in the present study, since the resulting information may contribute to assess the therapeutic efficacy of T cell-based immunotherapy for the treatment of HCC. Twenty-one surgically resected primary HCC tumors and autologous adjacent non-malignant tissues were stained with a unique panel of monoclonal antibodies which recognize HLA class I APM components. The staining patterns of the malignant tumors were compared to those of the autologous non-malignant tissues.
To assess the functional significance of changes in HLA class I APM component expression in HCC tumors, the results of immunohistochemical staining were correlated with the extent of CD8+ cytotoxic T-lymphocyte infiltrate, quantified with a computer-aided image analysis system. In all the HCC tumors, malignant hepatocytes expressed high levels of HLA class I APM components. In contrast these molecules were not detected in normal hepatocytes, although they displayed a low expression in some apparently normal hepatocytes adjacent to the HCC tumor. The HLA class I APM component up-regulation in HCC was associated with the extent of CD8+ T cell infiltrate, although this association did not reach the level of statistical significance.
Our results corroborate the information in the literature about the lack of HLA class I antigen expression in hepatocytes. Furthermore our study shows for the first time that APM components are also not detectable in normal hepatocytes. Lastly our study shows that HLA class I APM component up-regulation is very frequent in HCC. Its association with T-cell infiltrate, although not statistically significant, is compatible with the possibility that HCC cells are recognized by CD8+ T lymphocytes. If so, HCC should represent an attractive target to apply T cell-based immunotherapy.

EWELINA GRYWALSKA
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Plasma concentration and intracellular expression of interferon gamma are low in patients with chronic lymphocytic leukemia with detectable levels of Epstein-Barr virus DNA

Introduction: Chronic lymphocytic leukemia (CLL) is characterized by highly heterogeneous clinical manifestation. Although treatment, including various combinations of cytostatic and biological agents, induces remission, a recurrence is eventually observed in most cases, making CLL an incurable condition. Pathogenesis of CLL is associated with an array of not completely understood disorders of cell- and humoral-mediated immunity. Interferon (IFN)-gamma is a crucial regulatory cytokine in cellular immunity that is important in immune surveillance of EBV.
The aim of the study was an assessment of plasma concentration and intracellular expression of IFN-gamma, and its relationship with Epstein-Barr virus (EBV) DNA copy number, and selected clinical parameters in patients with CLL and healthy controls.
Material and methods: The study included samples of peripheral blood from 110 untreated patients with CLL. The control group comprised of 40 persons who were age and sex matched healthy individuals. The EBV-DNA copy number in peripheral blood mononuclear cells (PBMCs) was analyzed with EBV PCR kit (a single gene encoding EBV nuclear antigen 1 was amplified). The immunophenotype of PBMCs, including intracellular expression of IFN-gamma, was determined with flow cytometer. Concentration of INF-gamma in plasma was determined by ELISA.
Results: Presence of EBV DNA in PBMCs was found in more than one half of the studied patients with CLL (59 persons). There was a lack of detectable amounts of viral genetic material in healthy individuals. Decreased number of lymphocytes showing intracellular expression of IFN-gamma (CD3+/CD4+/IFN-gamma+ T cells, $p=0.0027$; CD3+/CD8+/IFN-gamma+ T cells, $p=0.0044$; CD19+/IFN-gamma+ B cells, $p=0.0089$), and low plasma concentration ($p<0.0001$) of this cytokine in CLL EBV(+) patients, as compared to EBV(-) subjects and healthy controls, reflect the suppression of cell-mediated immune response and non-reactivity of lymphocytes to the studied virus. The most important factors shortening the survival of CLL patients and time to progression were: EBV-DNA copy number (>17 copies/ug DNA) and low plasma concentration of IFN-gamma (<5.25 pg/ml).
Conclusion: These data show IFN-gamma deficiency in some patients with CLL and suggest that normal T- and B-cell response is crucial for favorable prognosis in this disease.

LUIS FELIPE INGRASSIA CAMPESATO
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Investigation of the role of sigirr/tir8, a new upregulated gene in her2 - positive breast tumors, and its association with the immune response

The oncogene ErbB2/HER2 is overexpressed in roughly 30% of all breast tumors and is directly associated with rapid disease progression and poor prognosis, although its molecular mechanisms remain to be better understood. Transcriptional changes associated with overexpression of HER2 in human breast tumors were investigated using an immortalized mammary epithelial cell (HB4a) and its HER2 overexpressing variant (HB4a-C5.2). Global analysis of gene expression identified SIGIRR/TIR8, a negative modulator of pro-inflammatory signals triggered by IL-1R and TLRs, as an upregulated gene in the C5.2 variant. Given the dual role of inflammation in tumor initiation and progression, we hypothesize that TIR8 overexpression in breast tumors may fine-tune inflammation and attenuate the antitumoral adaptive response. We validated the correlation between HER2 and TIR8 by bioinformatic analysis of public microarray and RNAseq data and by real-time PCR of different HER2+ tumors and breast tumor cell lines. Other preliminary data from our group indicate that the knockdown (KD) of TIR8 expression in tumor cells increases 2 to 3-fold the activation of NFkB pathway and different pro-inflammatory cytokines expression, such as IL6, IL8, TNF and IFNbeta, and the chemokines CCL2, CCL5, CSF2 and CSF3. Conditioned medium derived from KD tumor cells increases neutrophil recruitment, skew polarization of macrophages towards a M1 phenotype. Besides, co-culture of TIR8-KD cells with NK increases its IFNy secretion. In vivo studies are now been conducted to address the role of SIGIRR in breast tumor progression and its association with the immune infiltrate.

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Phenotyping TILs in situ: Automated Enumeration of FOXP3+ Regulatory T Cells in Follicular Lymphoma

Background: In many cancers, tumor-infiltrating lymphocytes (TILs) indicate levels of tumor immunogenicity and predict survival. In particular, increased levels of regulatory T cells (Tregs) are associated with poorer prognosis, whilst CD69+ T-cells may also be prognostic. Understanding the phenotype and pattern of TILs in situ within tumors would be advantageous. However, visual TIL assessment cannot easily determine the type of lymphocyte in situ and multimarker quantitation is difficult with standard methods. We present a multi-marker, computer-aided event-counting method for determining the phenotypes of lymphocytes in follicular lymphoma using a multispectral imaging (MSI) automated tissue segmentation and counting approach.
Material and methods: A tissue microarray containing follicular lymphoma (FL) cores from 70 patients was chromogenically immunostained for CD3, CD69 and FOXP3, counterstained with hematoxylin, of which 40 cores were informative for both triplex staining and clinical follow-up. Each core was imaged using MSI and the individual staining of each marker separated from each other using spectral unmixing. Images were analyzed using software trained to recognize different tissue compartments based on morphology, specifically based on CD3 rich (extra-follicular) and poor (intra-follicular) areas. The FOXP3 or CD69 status of each CD3+ TIL was then determined and number Treg (FOXP3+/CD3+) and CD69+ T-cells counted in the intra- and extra-follicular areas.
Results: The intra-follicular (CD3 poor) and extra-follicular (CD3 rich) regions were accurately recognized within each core, based on abundance of CD3 cells. MSI enabled the accurate quantitation of CD3, CD69 and FOXP3 without crosstalk. The number of FOXP3+/CD3+ Tregs and CD69+ T-cells were counted in each core and used in Kaplan-Meier survival analysis, which demonstrated association of FOXP3+/CD3+ Tregs with favourable outcome in both the intra- (p=0.0173) and extra-follicular (p=0.0173) areas, as well as CD69+ T-cells in intra-follicular (p=0.0175) areas; CD69+ T-cells were not prognostic in extra-follicular areas (p=4509).
Conclusions:This study demonstrates use of an automated method for counting Tregs in follicular lymphoma, showing association of FOXP3+ Tregs with good outcome. Given the generic nature of the method automated multiplexed tissue cytometry analyses are feasible for routine clinical studies and work with many multiplexed IHC staining methodologies, of importance for translational cancer studies in general.

OLIVIA MARINI
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Identification and characterization of candidate myeloid derived suppressor cells in the mononuclear fraction of peripheral blood samples from patients affected by nonhodgkin lymphoma (nhl), hodgkin lymphoma (hl), and multiple myeloma (mm)

Myeloid derived suppressor cells (MDSCs) constitute a heterogeneous population of myeloid cells characterized by the ability to suppress T lymphocytes' immune responses. MDSC include two major subsets (i.e. monocytic and granulocytic), defined by the expression of distinctive antigens (CD11b+CD14+HLA-DRlow/– and CD11b+CD33+CD15+), and by the content of specific immune suppressive molecules. Currently, a large number of MDSC phenotypes have been described in different human diseases, including solid tumors, infections and inflammatory diseases. However, the existence of MDSCs in human hematological malignancies remains debated and to date myeloid CD11b+CD14+HLADRlow/– or CD11b+CD33+CD15+ cell populations with putative immunosuppressive functions have been described only by two studies conducted in a NHL and in a MM series, respectively.
Aim of our study was to identify candidate MDSCs in the peripheral blood mononuclear fraction (PBMC) of peripheral blood samples obtained from patients affected by NHL, HL and MM. Upon informed consent, 107 newly diagnosed patients affected by NHL (60), HL (21), MM (26), and 39 age- sexmatched healthy donors were enrolled in the study. Based on the analysis of CD11b, CD66b, CD33, CD16, CD14 and CD45 by flow-cytometry, we evaluated within the PBMC fraction of each sample the frequency and the absolute number of total myeloid (CD11b+), monocytic (CD11b+ SSClow CD33++), and granulocytic (CD11b+ SSChigh CD66b+) cells. Noteworthy, the latter are supposed to sediment in the mononuclear layer upon activation-induced degranulation. Our data revealed that the frequency of total myeloid cells (mostly composed by CD11b+ SSChigh CD66b+) was significantly increased in PBMCs of patients as compared to healthy donors. Of interest the frequency of total CD11b+ and SSChigh CD66b+ cells resulted significantly reduced in PBMCs of patients in complete remission as compared to the same patients at the diagnosis. Moreover, preliminary in vitro experiments have shown that the depletion of CD11b+ elements from patients' PBMCs restored the proliferation of autologous T lymphocytes, thus indicating a suppressive activity of myeloid cells.
Our data suggest the presence of candidate CD11b+ MDSCs in the PBMC fraction of patients affected by NHL, HL and MM. Further in vitro studies are ongoing in order to specifically assess the immunosuppressive activity of SSChigh CD66b+ cells within the CD11b+ component of PBMCs.

IRENE MATTIOLA
Humanitas Clinical and Research Center

M1 macrophages enhance NK cell anti-tumoral activities via multiple cytokine networks

PURPOSE: Macrophages are endowed with a variety of microenvironmental molecules promoting distinct polarized phenotypes ranging from classic (M1) to alternative (M2) activation. The tumor microenvironment induces an M2-like pro-tumoral phenotype and also suppresses the innate cytotoxic activity of tumor-infiltrating natural killer (NK) cells. We have investigated the reciprocal functional influence of these two key cell types with respect to their potential impact on tumor biology.
METHODS: Autologous human NK cells and monocyte-derived macrophages were obtained from buffy coats of healthy donors. Using an in vitro reconstituted tumoral microenvironment, we evaluated the effect of direct interactions or soluble factors derived from polarized macrophages on NK cell activities.
RESULTS: We did not detect significant effects of M2 on NK cell functions. Conversely, M1 induced IFN-γ production, degranulation and CD69 expression by resting NK cells. M1-secreted IL-1β and IFN-β respectively induced NKp44 and increased NKG2D expression on NK cells, thereby enhancing NK cell degranulation. Importantly, M1 secretion of IFN-β triggered expression of IL-15 and IL-15Rα on NK cells, inducing a mechanism of cis-presentation. It strongly enhanced IFN-γ secretion, further sustained by 2B4-CD48 interactions. On the other hand, IL-15 membrane trans-presentation mediated by M1 amplified NKG2D-dependent degranulation of NK cells.
CONCLUSIONS: Our results reveal a complex M1-dependent cytokine network which sustains NK cell cytotoxicity (via an IL-1β or IFN-β-dependent effect on NKp44 or NKG2D expression respectively, or IL-15 trans-presentation) and promotes production of IFN-γ by NK cells (via IL-15 cis-presentation). Considering that NK cell-derived IFN-γ may repolarize tumor-associated macrophages to M1 and that M1 support NK cell cytotoxicity, we conclude that the macrophage/NK cell cross-talk may set in motion a virtuous circle, suggesting new approaches to impact on tumor biology.

MICHELA MIRENDA
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Protein tyrosine phosphatase, receptor type, g (PTPRG) is a negative regulator of JAK PTKs and dependent LFA-1 affinity triggering by chemoattractants

LORENZO MORTARA
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Natural killer cells from malignant pleural effusions are characterized by a CD56brightCD16- pro-angiogenic phenotype

Natural killer (NKs) cells are crucial effector cells of innate immunity able to kill tumor or virus-infected cells and able to regulate adaptive immune cells by cytokine release. Their activities depend on the fine balance between activating and inhibitory surface receptors. However, in tumor-bearing host the anti-tumor function of NKs are largely impaired. Interestingly, in the early phase of pregnancy, a leading role of a CD56superbrightCD16- NK cell subset (decidual NKs) with a potent pro-angiogenic feature, releasing VEGF, IL-8, and SDF-1, has been described. Recently, we have found that tumor infiltrating NKs in non-small cell lung cancer (NSCLC) are enriched in the CD56brightCD16- NK cell subset characterized by a pro-angiogenic phenotype and function. We have now characterized fresh and 3 day in vitro-cultured NKs derived from peripheral blood (PB) and pleural effusions (PE) of patients with primary or metastatic tumors of different origin, including mesothelioma, lung, breast and epatocarcinoma. We investigated several surface markers by flow cytometry, such as: NKGD2, NKp30, NKp44, NKG2A, pan-KIR, and two NK decidual-like markers: CD9 and CD49a. We also performed several functional assays: cytokine production upon PMA and ionomycin stimulation; degranulation assay against the classic K562 cell target, and chemotaxis and morphogenesis assays using NK supernatants on endothelial cells. We observed that the percentage of NKs from PB and PE are similar but that the CD56brightCD16- NK cell subset predominates in PE. Moreover ex vivo phenotype of NKs from PE are positive for CD9 and CD49a decidual markers. Moreover NKs from PE are able to generate a complex pro-angiogenic cytokine response, much higher than their counterpart in PB,
namely VEGF, IL-8, SDF-1, Osteopontin (OPN), and moderate/low levels of IFNγ. Further, in vitro degranulation assays of patients' NKs showed that this crucial effector function is present, albeit at low level (10-15%) for both ex vivo NKs from PE and PB, but this activity could be enhanced after short (3 days) culture with IL-2 cytokine, and only partly enhanced with IL-2 plus TGFβ. However, both in vitro cytokine treatments (IL-2 or IL-2 plus TGFβ) induced an enhancement in the releasing of pro-angiogenic factors by the NK cells, in both PE and PB compartments.
In conclusion, our findings highlight the fact that NKs from patients with malignant pleural effusions either from PB and PE, are not anergic and that they could be rescued by a short IL-2 in vitro culture. However these NKs are skewed towards a potent pro-angiogenic phenotype and that this feature is reinforced by a TGFβ in vitro culture, a cytokine frequently associated with tumor progression.

ANDREA PONZETTA
Humanitas Clinical and Research Center

Multiple Myeloma growth differently affects natural killer cell subset trafficking to bone marrow

Natural killer (NK) cells are innate lymphoid cells that play critical roles in several hematologic malignancies, and are particularly effective against Multiple Myeloma (MM) cells.
On NK cells, the ability to extravasate and reach the inflamed tissue is significantly influenced by their chemokine receptor expression, which determines NK cell responsiveness to different chemotactic stimuli. Here, using a murine model of MM we performed an in vivo analysis of NK cell trafficking capacities at various stages of tumor growth in the bone marrow (BM), where MM cells reside.
Initially, we highlighted a differential distribution of NK cell subsets, which were defined according to the expression of the maturation markers CD11b and KLRG1: at an earlier stage of tumor growth, the number of KLRG1- NK cells selectively decreased, while the KLRG1+ fraction remained intact.
At the same time, the expression of the chemokine receptor CXCR3 on BM NK cells was down---modulated, while its ligands CXCL9 and CXCL10 were found increased within BM supernatants. Interestingly, the key factor for NK cell BM retention CXCL12 was significantly reduced in the tumor microenvironment.
With a first set of adoptive transfer experiments we showed that KLRG1 - NK cell homing to BM in tumor-bearing mice was impaired, while no differences were observed for the KLRG1+ subset. In addition, CXCR3 expression analysis on transferred NK cells in MM BM revealed the same down---modulation that occurred on endogenous cells, suggesting either the NK cell recruitment to BM through CXCR3, followed by ligand-induced receptor internalization, or alternatively, the reduced migration of CXCR3+ NK cells to BM of tumor-bearing mice.
In order to test whether CXCR3 axis could regulate NK cell homing to BM, we performed competitive adoptive transfer experiments using WT and CXCR3-deficient splenocytes: our data indicate that CXCR3 does not promote total NK cell homing to BM, neither in control nor in tumor-bearing mice. On the whole, these results point out that during tumor growth KLRG1+ NK cell maintenance in BM is favored, while CXCR3 - expressing KLRG1 - NK cells are driven to other tissues.

SABRINA REMELLI
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A SRC/ABL/SOS1/RAC1 signaling pathway regulate podosome formation in murine macrophages

PURPOSE: Activation of tyrosine kinases of the Abl family is a key step in signal transduction to the cytoskeleton in both hematopoietic e non hematopoietic cells. Myeloid leukocytes form actin-based plasma membrane protrusions, called podosomes, that are implicated in their recruitment into tissues and cell migration within the interstitium.
Mechanisms of formation of podosomes and invadopodia have been elucidated only in part. We implicated Abl and Src kinases in macrophage migration and podosome formation (1, 2) and here we identified Sos1 as a downstream target of this Src/Abl signaling pathway.
METHODS: Bone marrow-derived murine macrophages (BMDM) were assayed for cell migration, gelatin degradation and podosome formation by standard assays. Macrophages were silenced for Sos1 expression by siRNA. For confocal analysis, images were collected using the SP5 confocal microscope from Leica Microsystems.
RESULTS: The tyrosine kinase Abl phosphorylates Sos1 and co-immunoprecipitates with it in COS-7 and Baf3 cells. We also found that Sos1 is a component of podosome rosettes and its expression is indispensable for podosome rosette formation and gelatin degradation. Additionally, Abs recognizing total Rac1 or GTP-loaded Rac1 stained podosomes and in Sos1 silenced BMDMs we detected a much lower GTP-loaded Rac1 signal. DISCUSSION: We propose Sos1 as a specific guanine exchange factor through which Abl regulates Rac1 activation and podosome formation. In fact, Sos1 phosphorylation was mediated by Abl and Sos1 silencing in BMDMs led to a morphologic and functional phenotype that mimics the Abl silenced cell one..

FEDERICA RIVA
University of Milan

TIR8/SIGIRR-deficiency increases susceptibility to develop B-cell lymphomas in Lpr mice

The association among autoimmunity, chronic inflammation and lymphoma development has been described and confirmed by epidemiological studies. In these conditions, early events include persistent chronic antigenic stimulation of microbial or self origin, clonal expansions of B cells and the acquisition of genetic aberrations leading to constitutive B-cell or T-cell activation. A major role in these events is played by constitutive activation of the NF-kB pathway, which controls cell survival and proliferation. Constitutive NF-kB activation can result from mutations within the B cell receptor pathway, the Toll-like receptor pathway (e.g. MYD88) and the NF-kB pathway itself.
In this study, we investigated the role of Tir8/SIGIRR gene, known to be associated with autoimmunity, in the development of lymphoma. Indeed, the ability to dampen signaling from IL-1R and TLR family members confers TIR8/SIGIRR the ability to act as regulator of inflammation, cancer-related inflammation and autoimmunity. To this aim, Tir8-/- mice on an lpr background and their control (B6lpr/lpr/Tir8-/- and B6lpr/lpr mice) were followed up to 16 month of age, and histopathological and immunohistochemical analysis were performed on different tissues and organs to investigate lymphoma development. Aged B6lpr/lpr/Tir8-/- mice developed Diffuse Large B-Cell Lymphoma (DLBCL) with significantly higher frequency then B6lpr/lpr mice. DLBCL occurred earlier and were more aggressive, causing significantly higher mortality in B6lpr/lpr/Tir8-/- mice than in B6lpr/lpr mice. Histopathology and immunohistochemistry of spleen and lymph nodes of B6lpr/lpr/Tir8-/- mice documented clear-cut DLBCL areas arising within a context of atypical lymphoproliferative disorder.
IgH gene rearrangement was investigated by Southern blot and PCR on genomic DNA from different organs showing clonal or oligoclonal B-cell expansions. Transplantation experiments of cells from B6lpr/lpr and B6lpr/lpr/Tir8-/- mice confirmed the clonality and malignant nature of lesions of Tir8-/- mice. These observations unveil a role for TIR8 in the occurrence and development of DLBCL, and suggest that TIR8 is a novel potential therapeutic target. Moreover, this study suggests that B6lpr/lpr/Tir8-/- mice could be exploited as model of DLBCL potentially useful to establish new therapeutic protocols.

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Inflammation triggered by oncogenic KRAS^{G12V} in pancreatic tumor cells

INTRODUCTION: Pancreatic ductal adenocarcinoma (PDAC) has one of the worst prognoses among all cancers and current therapeutic approaches are limited and largely ineffective. PDAC is almost invariably associated with mutations in the KRas gene, which is essential for its maintenance. However, how KRas mutations promote pancreatic carcinogenesis is not fully understood. KRas drives cancer-associated inflammation and its contribution to tumor development and possibly to Epithelial to Mesenchymal transition (EMT) needs to be elucidated.
AIMS AND METHODS: The AIM of this study is to define the perturbation in inflammatory mediators caused by over-expression of mutated KRas and to establish a possible link between KRas-induced inflammation & Epithelial to Mesenchymal Transition. METHODS: HPDE clones transduced with oncogenic KRas-G12V were screened for inflammatory mediators, gene expressions, KRas-activity and invasion ability. NSG (NOD/SCID/IL-2gr-/-) mice were injected with KRas-transduced clones to monitor tumor growth in-vivo, and treated with IL-1R antagonist (Anakinra).
RESULTS: KRasG12V transduced-HPDE clones exhibit distinct epithelial or EMT/Mesenchymal phenotype. EMT clones were found to exhibit high KRas activity and higher invasive ability as compared to epithelial clones. Significant amounts of proinflammatory cytokines (IL-6 and IL-8) are secreted from all clones, whereas IL-1β and Pentraxin-3 are produced more in mesenchymal clones. Treatment in-vitro with IL-1 R antagonist, inhibited the production of inflammatory mediators, but suppression of IL-6, IL-8 and PTX-3 was more marked in epithelial than in mesenchymal clones. Similarly, in-vivo growth in NSG mice of tumors derived from an epithelial clone was reduced by administration of IL-1 RA, while growth of EMT clones was unaffected.
CONCLUSION: KRasG12V-pancreatic clones with different cellular phenotypes “epithelial” or “mesenchymal” showed different susceptibility to IL-1-mediated regulation. EMT clones exhibited less suppression in the production of inflammatory mediators in-vitro as well as in-vivo when the IL-1 signaling was blocked. This suggests that KRas-expressing pancreatic cells with EMT phenotype are less dependent on IL-1 for production of inflammatory mediators. Our results provide a link between oncogenic KRas-induced inflammation and IL-1, which is bypassed during mesenchymal transition.

ROSALINDA SORRENTINO
University of Salerno

Human Cancerous Plasmacytoid Dendritic Cells are responsive to PolydA:dT, AIM2 inflammasome ligand

Lung cancer masses, are populated by plasmacytoid dendritic cells (pDCs), which presence is strictly correlated to bad prognosis. However, their role in the tumor mass is still controversial according to mouse and human evidence. Therefore, the aim of our study was to understand the role of human tumor-associated pDCs (TApDCs) in tumor cell proliferation. Samples of lung obtained from non-small cell lung cancer (NSLC) patients undergoing thoracic surgery were used to isolate pDCs identified as B220+CD19-BDCA-2+CD123+ cells. Tumor masses presented higher percentage of pDCs than healthy (non-cancerous) tissues and they were in their immunesuppressive phenotype as determined by the higher levels of CD33, PD-L1. Although HLA-A and HLA-D were increased, TApDCs were not able to exert cytotoxic activity against tumor cells, but instead promoted their proliferation as well as T cell proliferation. In this scenario, cancerous pDCs were able to produce high levels of type IFN, but also of IL-1α under PolydA:dT, AIM2 inflammasome agonist. This effect was reduced after the blockade of IFN receptor (IFNAR) and LL-37 addition, but surprisingly, even after the addition of mitochondrial oxidative stress-derived oxygen species (ROS) sequesters. Our data demonstrate that the activation of AIM2 by the autocrine activity of type I IFN plays a crucial role for the immunesuppressive activity of TApDCs and tumor progression. Therefore, strategies aiming at modulating AIM2 activity in TApDC in the tumor site might prove to be novel and effective to limit tumor cell proliferation.

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MicroRNA-135b at the crossroad of macrophage polarized activation

Background: MicroRNAs (miRNAs) are small non - coding RNAs with a post - transcriptional effect. Recent evidence indicates that miRNAs are involved in the inflammatory response, but their role in the activation of macrophages in response to different stimuli is not well defined. The aim o f this work was to typify the miRNome of human polarized macrophages and to define its relevance for the polarization process.
Methods and results: Human monocyte-derived macrophages were polarized to classic (M1; LPS + INFγ) or alternative (M2; IL-4) cells and 733 miRNAs were profiled using TaqMan MicroRNA Arrays.
Out of 733 miRNAs investigated, 69 resulted differentially expressed among the polarized macrophages (59 restricted to M1 and 10 to M2 macrophages). Among these, MiR-135b was strongly associated with M1 polarization. Furthermore, its expression profile were intimately linked to macrophage polarity as its expression levels were decreased in M1 re-polarized to M2 and enhanced in M2 re-polarized to M1.
In silico analysis using the Ingenuity Pathway Analysis (IPA) software identified miR-135b as a potential modulator of the alternative activation pathway through direct targeting of the key transcriptional factors c-Myc and KLF4.
These targets were validated by luciferase assay and Western blot analysis. Consistent with these findings, miR-135b overexpression led to a significant reduction in the production of M2-associated chemokines (CCL18 and CCL22).
Conclusions: We show that macrophage phenotypes are characte rized by differential expression of miRNA profiles and identified miR-135b as an M1-associated miRNA targeting the transcription factors c-Myc and KLF4, thus suppressing M2 polarization in favor of the M1 phenotype. Altogether these data indicate miR-135b as a pro-inflammatory miRNA possibly involved in the maintenance of macrophage classical activation.

CARLOTTA TACCONI
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Modulation of the VEGFC/VEGFR3 pathway alters VE-Cadherin distribution on intestinal lymphatic vessels: implications for colorectal cancer metastatic dissemination

Background: Colorectal cancer (CRC) is one of the most common causes of cancer-related deaths in the Western countries. Metastatic cells can spread directly into local tissue or invade via blood and lymphatic vessels (LVs) distant organs. Angiogenesis plays an important role in of tumor development and progression. However, the role of tumor lymphangiogenesis is less clear and only recently its significance has been described in cancer. VEGFC is a key player in lymphangiogenesis that signals through its cognate receptor VEGFR3 and its blockade has been used in several models of metastatic cancer. In CRC, high VEGFC levels have been described to promote tumor lymphangiogenesis and metastasis. However, very little is known about the role played by adherens' junction proteins, such as Vascular Endothelial (VE)-Cadherin on the lymphatic endothelium during the metastatic process. Nevertheless, no data is available on the direct interaction between the VEGFC/VEGFR3 pathway, VE-Cadherin and the lymphatic metastatic process. We hypothesized that lymphatics-targeted therapy may be effective in abate metastasis dissemination by maintaining LV junctions' integrity.
Methods: Balbc/nude mice were orthotopically injected with mCherry CT26 colon cancer cells. Modulation of lymphangiogenesis was obtained by systemic inhibition of VEGFR3 or adenoviral overexpression of VEGFC. Tumor size was monitored by in vivo imaging and measured at the end of the protocol. Liver, lung and draining lymphnodes were processed for metastasis quantification by FACS analysis. Whole mounts of colons were stained to analyze dimension of LVs within peritumoral and tumoral regions and immunofluorescence was used to evaluate LVs density. Moreover, VE-Cadherin distribution and expression were studied in vivo on LVs and in vitro on human intestinal lymphatic endothelial cells (HILEC) stimulated with VEGFC or with a VEGFR3 inhibitor.
Results: Systemic inhibition of VEGFR3 inhibited lymphangiogenesis in peritumoral and tumoral regions, reducing both area density and LVs dimension. In addition, even if it did not affect tumor size, blocking VEGFR3 abated metastatic dissemination, when compared to untreated animals. In contrast, metastasis formation was enhanced by VEGFC, which in turn increased lymphangiogenesis within the same regions. Whole mount staining showed that VEGFC altered VE-Cadherin expression, whereas VEGFR3 inhibitor kept endothelial junction integrity, thus linking the VEGFC/VEGFR3 pathway to VE-Cadherin-dependent metastasis dissemination. HILEC experiments revealed that VEGFC-dependent VEGFR3 activation led to the disruption of the VE-Cadherin-β-catenin complex resulting in the phosphorylation of both proteins. Moreover, the activation of the VEGFR3 signaling was related to VE-Cadherin internalization and β-catenin cytoplasmic release, thus leading to impaired lymphatic barrier integrity. Finally, we found that VEGFC significantly enhanced CT26 transendothelial migration through HILEC monolayer, thus directly linking this pathway to metastatic dissemination.
Conclusions: Our findings demonstrate for the first time that VEGFC/VEGFR3-dependent lymphangiogenesis plays a key role CRC metastatic dissemination. Our study reveals a novel mechanism for controlling lymphatic junction integrity. Overall, blocking VEGFR3 activation could represent a novel therapeutic tool for CRC metastasis by inhibition of tumor lymphangiogenesis and by maintenance of lymphatic endothelial barrier integrity.

TOPIC Innate Immunity and Inflammatory Bowel Diseases

best oral presentation

LISA KORN

University of Pennsylvania, MD/PhD student

Regulatory T cells occupy an isolated niche in the intestine that is antigen-independent

Regulatory T cells (Tregs) are CD4+ T cells that are critical in maintaining immune homeostasis and preventing autoimmunity.
Like all CD4+ T cells, Tregs require antigen - specific signals via T cell receptor - major histocompatibility complex class II (TCR-MHCII) interactions for their development and homeostasis. However, the requirement for MHCII in Treg homeostasis in tissues such as intestinal lamina propria (LP) is unknown. Tregs in the LP are important in preventing inflammatory bowel disease and in preventing excessive inflammation after infection. We examined LP Treg homeostasis in a transgenic mouse model that lack speripheral TCR-MHCII interactions and generation of extra-thymic Tregs (iTregs). Thymically-generated Tregs (nTregs) entered the LP of weanlings and proliferated independently of MHCII to fill the compartment.
Strikingly, the adult LP was a closed niche; newly generated thymic Tregs were excluded and Tregs in parabiotic pairs were LP - resident. This isolated niche was independent of IL-2 but dependent on commensal bacteria.
Thus, an LP Treg niche can be filled, isolated, and maintained independently of antigen signals and iTregs. This niche may represent a tissue-specific mechanism to maintain immune tolerance.

best oral presentation

RAJAT MADAN

Division of Infectious Diseases, University of Virginia, Charlottesville VA,
Division of Infectious Diseases, University of Cincinnati, Cincinnati OH

Leptin signaling alters susceptibility to Clostridium difficile by controlling host inflammation

Clostridium difficile is the leading source of healthcare-associated diarrhea and is classified as an “urgent” public-health threat by the CDC. Most of the current therapies target the bacterium and are clearly inadequate as evidenced by ~10-15% mortality and high recurrence rates. Notably, vigor of host immune responses, and persistence of inflammation are believed to be key determinants of disease outcome. A better understanding of host factors that control inflammation and immunity during C. difficile colitis could thus lead to novel, host-targeted therapies. Towards this goal, we have explored the role of leptin and polymorphisms in the leptin receptor (LEPR) in regulating mucosal immunity during C. difficile infection. Leptin is an adipose tissue-derived cytokine with functions in nutrition, metabolism and immunity. Leptin regulates food intake, metabolism, is pro-inflammatory and augments the host defense during infections, presumably by enhancing immune responses.

We examined the role of leptin signaling pathway during gastro-intestinal infections. We showed that a common mutation in leptin receptor (LEPR), associated with diminished leptin-STAT3 signaling, is also associated with increased susceptibility to C. difficile (OR 3.03, $p = 0.015$; patients at University of Virginia Medical Center), and worse outcomes after Entamoeba histolytica infections (children in Bangladesh). We investigated the underlying mechanisms of LEPR polymorphism-mediated actions using murine models. Mice homozygous for the human LEPR mutation (RR mice), and sex- matched, littermate wildtype control mice were challenged with purified C. difficile toxin A by intra-cecal injection. RR mice had less inflammation ($p = 0.026$) and decreased neutrophil recruitment ($p = 0.048$). Further, neutrophil surface expression of the common receptor for neutrophil-recruiting chemokines (CXCL1 and CXCL2), i.e. CXCR2, was lower in RR mice ($p = 0.014$). The same phenotype: decreased inflammation, lower neutrophils and lower CXCR2 expression, was replicated in RR mice after intra-cecal challenge with E. histolytica.

Conclusion: Our studies bridge metabolism with infection and inflammation. We have identified a novel host mechanism, LEPR signaling-mediated control of neutrophilic inflammation and chemokine receptor expression, that could explain the increased susceptibility of humans with LEPR mutation (RR genotype) to C. difficile and E. histolytica infections.

oral presentation

SILVIA D'ALESSIO
Humanitas Clinical and Research Center

Conditional deletion of Prep1 in the intestinal epithelium alters epithelial homeostasis, intestinal development, and controls colitis susceptibility

Background: Balanced and dynamic interactions among mucus layers, intestinal epithelial cells, and microbiota, are essential for the maintenance of the intestinal mucosal homeostasis. The disruption of this balance leads to a defective mucus barrier with increased permeability that results in intestinal inflammation. The homeodomain transcription factor, Prep1, is expressed in the post-mitotic differentiated intestinal epithelial cells and is essential in embryonic development. Here we report for the first time the involvement of Prep1 in intestinal epithelial homeostasis, and its functional role in experimental colitis.
Methods: Mice lacking Prep1 in intestinal epithelium were generated by mating Prep1$^{flox/flox}$ mice with VillinCRE animals. Goblet cells staining was carried out by Alcian Blue and Periodic Acid-Schiff (PAS) staining, while mucins were quantified by real-time PCR and immunohistochemistry. The dextran sulfate sodium (DSS) model of colitis was used in VillinCRE/Prep1$^{flox/flox}$ and wild-type (wt) mice. Moreover, Prep1 expression was evaluated in the intestinal epithelium of surgical specimens from normal individuals and patients with IBD, by Western Blot. Cross-linked, sonicated chromatin from human intestinal epithelial cells, was then immunoprecipitated with an anti-Prep1 antibody and the precipitated DNA analyzed by real-time PCR with a set of primers within the MUC1, MUC2, and MUC13 promoters (ChiP).
Results: Mice with conditional ablation of the Prep1 gene from the intestinal epithelium were viable. However, transgenic animals appeared smaller than wt, with a defective development of both the small and large intestine. VillinCRE/Prep1$^{flox/flox}$ mice showed an altered Goblet cell maturation and differentiation, resulting in MUC2 (soluble mucin) overproduction and MUC1/MUC13 (membrane-bound mucins) overexpression. Mutant mice were protected from DSS-induced colitis, as assessed by body weight loss, colitis activity Index, and histological scores. This correlated with reduced intestinal permeability ($p<0,001$), and bacterial penetration ($p<0,05$) within the inner mucus layer. Finally, Prep1 was find to be up-regulated in the intestinal epithelium of IBD patients, when compared to controls, and ChiP assay revealed that Prep1 modulate the transcription of the human MUC2, MUC1 and MUC13 genes, by directly binding to their promoters and acting as a repressor.
Conclusion: Our findings demonstrate an essential role for Prep1 in intestinal development, epithelial homeostasis and maintenance of the mucosal barrier. Its manipulation, could indeed represent a novel therapeutic approach for the treatment of IBD, possibly restoring the mucin barrier.

oral presentation

ROSITA RIGONI
Humanitas Research Center

Microbial signals control inflammation and autoimmunity induced by hypomorphic RAG defects

Hypomorphic mutations in the RAG genes result in profound lymphopenia associated with multisystem autoimmune manifestations in humans and mice. The role of gut homeostasis and microbial signals in the immune dysfunctions and disease pathogenesis is still debated. The Rag2R229Q/R229Q mutant mice developed an inflammatory bowel disease involving the small intestines, characterized by marked infiltration of CD4+T cells and, intriguingly, also of Treg cells, in the lamina propria compartment. Increased expression of the gut homing receptors CCR9 and $\alpha4\beta7$ on peripheral CD4+ T cells confirmed the abnormal lymphocyte trafficking to this environmental interface. A pro-inflammatory profile, characterized by a Th1/Th17 skewing, distinguished the intestinal immune responses in the Rag2R229Q/R229Q mice. Remarkably, similar pattern was also evident in the periphery. On the contrary, B cells were poorly present in the gut of mutant mice and the fecal level of IgA was obviously reduced. Interestingly, these findings correlated with augmented intestinal permeability and enhanced epithelial innate responses. Metagenomic analysis revealed substantial changes in the composition of the gut microbial communities in the mutant mice, with an overall reduced bacterial biodiversity. Importantly, decreasing microbial load with antibiotic treatment significantly limited the lymphocytic infiltration, as demonstrated by the reduction in the frequency of peripheral CCR9+ T cells, and ameliorated both the intestinal and systemic inflammation by dampening pro-inflammatory Th1/Th17 immune responses. Overall, these results suggest that microbial factors may play a substantial role in the pathogenesis of human autoimmune disease associated with hypomorphic RAG defects.

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Expression pattern of DNAM-1 family co-receptors (DNAM-1 and Tigit) on peripheral and mucosal T cell subsets in paediatric individuals and Inflammatory Bowel Diseases patients

The mucosal immune system of the gastrointestinal tract plays a crucial role in the surveillance and protection against pathogens, as well as in maintaining the tolerance against non pathogenic microbial species and dietary antigens. The dysregulated crosstalk between mucosal T cell populations and the stroma, together with the disbiosis of the commensal flora, are all recognized factors in the pathogenesis of inflammatory bowel diseases (IBD).
Effector T cell functional capability and survival is regulated by innate immunity receptors, such as NKG2D, CD161 and DNAM-1 family members, that positively or negatively fine-tune their responsivity, upon interaction with specific cell surface ligands. Collectively taken, these coreceptors regulate cell-cell communication within the immune system and with tissue cells, and may be involved with a crucial role in the correct tuning of effector lymphocyte activity at mucosal sites, and in IBD immune-mediated tissue damage.
We evaluated the expression pattern and functional role of DNAM-1 family co-receptors on mucosal and peripheral blood T lymphocyte (conventional and "innate-like") subsets of pediatric IBD patients and age-matched controls.
Our findings show that Tigit and DNAM-1 are expressed on a sizeable portion of the major peripheral blood T cell subsets of pediatric individuals: DNAM-1 is differently expressed on "innate-like" peripheral T cells in respect of conventional ones, and receptor distribution is not altered in IBD patients.Tigit doesn't show a significantly different pattern between innate-like and conventional T cells, nevertheless its distribution among different subsets is altered in IBD patients.
Moreover, the expression patterns of DNAM-1 and Tigit on T cells strikingly differs between peripheral and mucosal compartments: DNAM-1+ cells are strongly reduced within mucosal T pupolations, whereas Tigit+ cells are augmented in CD4+ and DN, but not on CD8+, T cell subsets. Interestingly, the active phase of the disease seems to correlate with a reduced expression intensity of the two co-receptors in selected T cell subsets.
We also have evaluated the possible functional implication of co-receptor expression on peripheral blood T cells. In this context, co-receptor(+) cells, within each T cell subset, show a higher capability of producing IFN γ, as compared with co-receptor(-) ones.
The results of our study could contribute to a deeper knowledge of mucosal T cell behaviour and developmental dynamics. Furthermore, they may add novel information to the definition of T cell dysregulation in IBD pathogenesis, in the privileged setting of the paediatric condition.

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Stimulation of intestinal epithelial cells with the TLR8 agonist R848 affects the differentiation/functions of human monocytes

This study aimed at investigating the role of the Toll-like receptor (TLR) 8 agonist R848/resiquimod in shaping intestinal epithelium microenvironment and in regulating immune cell functions.
To this aim, polarized intestinal epithelial cells (IEC), obtained by culturing Caco-2 cells on polycarbonate-coated transwell chambers in high glucose, were stimulated with R848 at their apical side and conditioned medium (CM) was collected from the basolateral side after 24 h.
We report that CM from R848 conditioned IEC drives monocyte differentiation toward a CD14+ cell population expressing mannose receptor, DC-SIGN and HLA-DR. These cells overexpress TLR2 and TLR4 while exhibiting a reduced response to the specific ligands. Co-culture experiments in which R848-shaped cells were used as antigen presenting cells demonstrated their ability to inhibit IFN production in both allogeneic CD4+ naïve T cells and autologous T lymphocytes, thus suggesting a role in the maintenance of tolerance. However, the CD14+ cell population is quite heterogeneous as it expresses both regulatory (IL10, IL6) and inflammatory (TNF , IL1 , CCL2) immune mediators, and includes a subpopulation of CD14highCD16+ cells with potential inflammatory features. The role of the different cell populations as well as the mechanisms through which R848 exerts its immunomodulatory functions are under investigation.
These results suggest that exposure of intestinal epithelium to R848/resiquimod may regulate the equilibrium between tolerance and inflammation. They also highlight novel mechanisms regulating the cross-talk between intestinal epithelium and innate immunity cells and provide new information on the use of the immune response modifier resiquimod as vaccine adjuvant.

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Stimulation of lymphatic function and macrophage polarization via VEGFR3 as a novel therapy for Inflammatory Bowel Disease

Background: In both forms of inflammatory bowel disease (IBD), such as Crohn's disease (CD) and ulcerative colitis (UC), submucosal edema, extensive dilation of lacteals, and lymphocyte aggregates often containing granulomas suggest the existence of poor lymph drainage secondary to either lymphatic obstruction or impaired contractile function of the mesenteric lymphatic vessels. In addition, increased lymphangiogenesis has been described in IBD. However, whether this intense lymphangiogenesis is initially an appropriate and beneficial adaptation, or is abnormal from the onset of disease remains to be established. Together with LV dysfunction, macrophages (MΦ) may also have a fundamental contribution to IBD pathogenesis. The factors that modulate MΦ polarization could indeed affect the severity of human and experimental colitis. In the current study we examined the effect of stimulating lymphatic function and adaptive immune response via the VEGF-C/VEGFR3 signaling on the severity of intestinal inflammation, on lymphatic drainage as well as bacterial antigen clearance and MΦ activation during inflammatory conditions. Furthermore, we evaluated the mechanism through which this pathway acts on experimental disease progression.
Methods: We addressed these issues in vivo by systemic inhibition of VEGFR-3 and delivery of the lymphangiogenic factor VEGF-C in the dextran sulfate sodium (DSS) model of colitis by a blocking antibody or adenovirus transfer respectively. Whole mount of proximal and distal colons were stained during acute and chronic colitis with antibodies against LYVE-1 and CD31 to measure area density and dimension of lymphatic vessels. Lymphatic drainage was assessed by intramucosal injection of Evans blue dye. Moreover, to determine whether the protection seen in VEGF-C-treated mice during disease progression was at least in part the result of VEGF-C-induced MΦ activation, clinical parameters, lymph flow, and edema formation were monitored after treatment with Clodronate-containing liposomes. Finally, adoptive transfer of VEGF-C-differentiated resolving MΦ in colitic mice, was used to study the putative mechanism by which VEGF-C exerts its protective role during chronic intestinal inflammation.
Results: Despite the enhanced lymphangiogenesis, lymph flow was highly reduced during DSS-induced colitis, particularly in chronic DSS colitis. Systemic inhibition of VEGFR-3 blocked lymphangiogenesis, reducing area density and lymphatic vessel dimension, while significantly increased inflammatory edema
formation and inhibited disease resolution, assessed as body weight loss, colitis activity Index and histological score. Vice versa, although lymphatic drainage was inhibited in the chronic phase of colitis, it was enhanced by systemic delivery of the lymphangiogenic factor VEGF-C, which in turn significantly improved colitis both clinically and histologically. This was observed in combination with increased inflammatory cell mobilization and bacterial antigen clearance from the inflamed colon to the draining lymph nodes. Moreover, we report that the VEGF-C/VEGFR3 pathway regulates MΦ plasticity and activation both in vivo and in vitro, imparting an hybrid M1/M2 phenotype and that the so called "resolving" MΦ mediates the protective role of VEGF-C during chronic experimental colitis in a STAT-6-depedent manner.
Conclusion: Our study provides the first proof of concept that it may be possible to treat chronic gastrointestinal inflammatory disorders by stimulating lymphatic vessel functions to promote drainage and bacterial antigen clearance, together with adaptive immunity, effects achievable through the modulation of the VEGF-C/VEGFR3 pathway.