Objectives In the present study we followed activation of STAT1 and 3 during zymosan-induced arthritis (ZIA) in wildtype (wt) and IL-6^{-/-} mice. We also studied expression of the STAT inhibitors Suppressor Of Cytokine Signalling (SOCS) 1 and 3 during the arthritis.

Methods Zymosan induced Arthritis was elicited by intra-articular injection of zymosan as described in (2). Protein lysates of inflamed and control synovia were subjected to Western blotting for both total and phosphorylated STAT1 and STAT3. RT-PCR was performed for STAT3, SOCS1 and SOCS3.

Results The primary inflammation lasted for a week in both strains. In wt mice this inflammation became chronic until the end of the experiments at week 4, while it decreased rapidly in IL-6^{-/-} mice. In wt mice STAT3 became activated from day 1 until the end of the experiments. Also the level of STAT3 mRNA and protein increased. STAT1 in contrast only became activated during the chronic phase after day 7. During the arthritis there was an increase of SOCS1 and SOCS3 mRNA expression. In IL-6^{-/-} mice STAT3 only became activated at day 1. No activation of STAT1 occurred in IL-6^{-/-} mice.

Conclusion STAT3 activation occurred during both the primary and chronic inflammation in wildtype mice. During the first week IL-6^{-/-} mice also developed a primary inflammation. IL-6 is not the only cytokine that activates STAT3, explaining STAT3 activation at day 1 in IL-6^{-/-} mice. Lack of further STAT3 activation during the primary inflammation suggests a major role for IL-6 in STAT3 activation. STAT1 only became activated in the chronic phase. Arthritis increased expression of SOCS1 and 3, but this seemed not enough to inhibit STAT activation. Future research will have to address the importance of activated STAT1 and 3 for chronic synovitis and the possibility of inhibiting these signalling molecules in the inflamed synovium.

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OP0004 PIVOTAL ROLE FOR THE SMALL GTPASE RAP1 IN THE REGULATION OF INTRACELLULAR GENERATED ROS AFTER T CELL STIMULATION

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Background Oxidative stress plays a critical role in various diseases including cancer, neuronal degeneration, atherosclerosis and rheumatoid arthritis. Identifying the target proteins for redox modification is crucial in understanding how oxidants mediate the pathological processes. As previously demonstrated activation of T cells with anti CD3 antibody or with phorbol ester results in a rapid production of intracellular reactive oxygen species (ROS). In this paper we further dissect the signalling pathways leading to ROS generation and we propose a possible critical role for the small GTPase Rap1 in antagonising the intracellular generated ROS.

Objectives

Methods In our experiments we made use of the free radical dye 6-carboxy-2',7'-dichlorofluorescein DCF to measure radical generation at the single cell using flow cytometry. Transfected

Jurkat cells were incubated with the dye 20 min 37° before addition of stimuli. Readings were taken every 5-min over a 20 min period. The positive transfected Jurkat cell population was identified by detection of a co-transfected CD20 receptor.

Results T cells as well as Jurkat cells rapidly produce intracellular ROS in a time dependent manner, after stimulation with either CD3 or PMA+ionomycin. The intracellular ROS generated after T cell stimulation seem to be induced through a signalling pathway downstream of the small GTPase Ras, since Jurkat cells transfected with a constitutively active mutant of p21Ras, RasV12, produced large amounts of intracellular ROS, that could not be augmented by an additional stimulus. The observed ROS production after addition of a stimulus was suppressed by the expression of dominant negative mutant of Ras, RasN17. Jurkat cells transfected with the constitutive active mutant of another small GTPase, Rap1, failed to generate ROS after any stimulus tested. Moreover co-transfection of RapV12 and RasV12 showed that RapV12 abolishes the intracellular ROS generated by RasV12. The inhibition of ROS production appears to involve on a PI3K-dependent signalling pathway as is shown by inhibition of ROS production after Jurkat transfection with RapV12C40 (a Rap1 mutation that, analogue with RasV12C40 could affect specific binding to PI3K). Accordingly, pre-incubation with LY 294002, an inhibitor of the lipid kinase activity of PI3K, blocked the observed inhibition of ROS production by Rap1 in RasV12+RapV12 co-transfection experiments. In addition we report that Rap1 activation in T cells after activation with CD3 or PMA proved to be redox sensitive as shown by pretreatment of T cells with N-acetylcysteine and BSO. Furthermore hydrogen peroxide was capable of Rap1 activation in T cells, and pre-incubation with diphenylene iodonium (a flavoprotein inhibitor) completely blocked Rap1 activation.

Conclusion We define a role for ROS in T cell signalling pathways downstream from Ras and we propose a possible pivotal role for the small GTPase Rap1. Rap1 can be activated by ROS and antagonises the intracellular generated ROS in signalling pathways.

OP0005 LEFLUNOMIDE REDUCES TRANSENDOTHELIAL MIGRATION

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Background The disease modifying drug leflunomide is widely used in the therapy of rheumatoid arthritis (RA). Its active metabolite, A771726, leads to inhibition of dihydroorotate dehydrogenase, an enzyme necessary for pyrimidine de-novo synthesis. Since activated lymphocytes expand their pyrimidine pool, A771726 decreases their proliferation. A771726 also suppresses TNF mediated nuclear factor kappaB activation. We were therefore interested if A771726 also would be capable to influence transendothelial migration (TEM) of peripheral mononuclear cells (PBMC).

Objectives

Methods We investigated the TEM of PBMC, which migrated through endothelial cell monolayers in an in-vitro model. Human umbilical vein endothelial cells (EC) were cultured to confluence on collagen gels and then incubated with human PBMC of healthy blood donors. PBMC were recollected in three groups: 1) cells that did not adhere to the endothelium, 2) cells

that bound to the endothelium, 3) cells that had migrated through the endothelium, and then counted by microscope. Experiments in which EC and PBMC as well as only EC were treated with A771726 (in the absence or presence of uridine) were compared to simultaneously performed control experiments. No increased toxicity on the PBMC treated with the doses of A771726 used in our experiments, was observed. Paired T Test was used for statistical evaluations.

Results 24 h incubation of PBMC and EC with A771726 induced a significant decrease in TEM ($8 \pm 6\%$ vs. $16 \pm 12\%$ migrated cells, $p = 0.004$). Furthermore, 24 h preincubation of solely the EC with A771726 inhibited the TEM of untreated PBMC ($11 \pm 9\%$ vs. $15 \pm 8\%$ migrated cells, $p = 0.043$), indicating direct effects of A771726 on EC. Uridine significantly reversed the decrease in TEM when incubating PBMC and EC with both uridine and A771726 ($15 \pm 10\%$ vs $9 \pm 7\%$ migrated cells, $p = 0.023$).

Conclusion Our results demonstrate that leflunomide may have direct anti-inflammatory effects by inhibiting the extravasation of PBMC, possibly via downregulation of adhesion molecules on EC and mononuclear cells. These data suggest, that leflunomide, besides its influences on lymphocyte proliferation, also reduces recruitment of mononuclear cells to inflammatory sites.

OP0006 **DISTINCT MECHANISMS OF IL-1 INHIBITION REVEALED BY *IN VITRO* MODELLING OF IL-1 RECEPTOR ACCESSORY PROTEIN INTERACTIONS WITH IL-1/IL-1 RECEPTOR COMPLEX**

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Background IL-1 stimulates cellular responses by interacting with a heterodimeric receptor complex comprised of IL-1 receptor type I (IL-1RI) and IL-1 receptor accessory protein (IL-1RAcP). IL-1 first binds IL-1RI then IL-1RAcP is recruited to this complex. Inhibitors of IL-1 bioactivity may function through inhibiting any aspect of this complex formation. Understanding the mechanism of inhibition of specific inhibitors can shed light on the nature of the interfaces that define a high affinity site for IL-1RAcP binding.

Objectives To model the interaction of IL-1 Receptor Accessory Protein with IL-1 bound IL-1 Receptor using purified recombinant proteins, and assess the mechanism of antagonism for IL-1 inhibitors.

Methods The extracellular domain of IL-1RAcP was expressed and purified. The interaction of IL-1RAcP with IL-1RI, IL-1 and IL-1RI/IL-1 complex was explored in binding assays. Antibodies to IL-1 and IL-1RI have been used to investigate the roles of individual components of the complex. IL-1 bioassays have been developed to validate the *in vitro* findings.

Results As expected, IL-1RAcP did not interact with either IL-1 or IL-1RI alone, even at high concentrations (100 nM), however, binding to complex was measured to be high affinity (~ 1 nM). Furthermore, purified IL-1RAcP enhanced binding of IL-1 to receptor. Antibodies to IL-1b have been identified which block binding of IL-1/IL-1RI to IL-1RAcP but fail to influence IL-1 binding to receptor.

Conclusion The observation that anti-IL-1beta antibodies can selectively block binding of IL-1RAcP demonstrates a novel mechanism of IL-1 inhibition and supports the hypothesis that IL-1 makes direct contacts with IL-1RAcP. Since IL-1 does not

demonstrate any measurable affinity for IL-1RAcP when not bound to IL-1RI, it is likely that additional elements of the binding site are comprised of IL-1RI residues. Future studies mapping the elements of IL-1RAcP involved in the contact points will further define the binding interface.

OP0126 **CD28 DIRECTLY INITIATES IL-4 GENE TRANSCRIPTION AND INDUCES TCR INDEPENDENT TH2 CELL DIFFERENTIATION IN HUMAN MEMORY CD4 T CELLS**

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Background Evidence suggests that human autoimmune diseases might be driven by pro-inflammatory Th1 cells whereas immunomodulatory Th2 cells are rarely found. Th1 dominated immunity might result from disordered regulation of memory T cell differentiation with insufficient generation of Th2 cells to down-modulate inflammation.

Objectives To gain insights into the mechanisms regulating human Th2 effector differentiation, we investigated Th2 differentiation in isolated human CD4^{pos} naive and memory T cells *in vitro*.

Methods A cell culture system was employed that permitted Th2 cell differentiation after short term priming. The phenotype of freshly isolated and primed T cells was determined by cytometric analysis of intracellular cytokines. Analysis of mRNA was performed using semiquantitative RT-PCR.

Results Th2 cells could be generated from resting cord blood naive CD4^{pos} T cells by priming with anti-CD3 and anti-CD28. Exogenous IL-4 was not required for Th2 cell differentiation from naive T cells and did only marginally increase Th2 priming efficiency. In striking contrast, co-stimulation of memory T cells through CD3 and CD28 increased Th2 cell frequencies only minimally if at all. When recombinant IL-4 was added during priming, an increase in Th2 effectors was noted. Of marked interest, however, significant Th2 cell differentiation was induced by priming of CD4^{pos} memory T cells with anti-CD28 in the absence of TCR mediated signals. CD28 induced Th2 cell differentiation was dependent on IL-4 as neutralising of IL-4 inhibited the generation of Th2 effectors. As no exogenous IL-4 was required for CD28 mediated Th2 cell differentiation, the impact of CD28 ligation on IL-4 gene transcription in memory T cells was evaluated. IL-4 mRNA levels increased after CD28 engagement. To distinguish whether the increase in IL-4 mRNA levels after anti-CD28 stimulation resulted from initiation of transcription or from stabilisation of preformed mRNA, purified CD4^{pos} memory T cells were stimulated with anti-CD28 in the presence or absence of actinomycin D. Actinomycin D completely inhibited the CD28 induced increase of IL-4 mRNA. Furthermore, when actinomycin was added when IL-4 mRNA levels were already increased, IL-4 mRNA levels declined markedly with a half-life of app. 60 min, indicating that the increase in IL-4 mRNA after CD28 engagement is caused to a significant extent by the induction of IL-4 gene transcription.

Conclusion CD28 ligation directly initiated IL-4 gene transcription and induced Th2 differentiation in a TCR independent manner in memory but not in naive T cells. TCR independent generation of Th2 effectors might provide a way to control Th1 dominated cellular inflammation.