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in cooperation with the University Club Bonn on

Toll-like Receptor-based Drug Development

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Abstracts of the lectures

Scientific advisory board:
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Greetings

The first steps in tumour immunotherapy were undertaken in 1866 in Bonn (Germany). That same year, Wilhelm Busch, who held the chair for surgery at the Bonn University Clinic, observed that tumors showed transient regression during an infection. In 1866 – even before the discovery of bacteria and long before antibiotics became available – he induced an infection in the tumor area of a female patient through local injury and contamination, thereby effecting a temporary regression of the tumor. Two decades later, Sir William Coley of Rockefeller University in New York City modified this concept and tested it on a larger number of patients.

Today, 140 years after Busch's pioneering achievement, the underlying molecular and cellular mechanisms of this phenomenon are much better understood. The family of Toll-like receptors plays an important role in this respect. In 2004, the Robert Koch Prize (Germany) was awarded to three outstanding scientists for their fundamental work in this field: Professor Shizuo Akira (Japan), Professor Bruce A. Beutler (USA) and Professor Jules A. Hoffmann (France). It is a special honour for us to have won Professor Akira as a speaker for this scientific symposium, which is organized by the Paul Martini Foundation (Berlin, Germany) with the objective of promoting innovative pharmaceutical research.

Toll-like receptors play a key role in the identification of bacterial and viral infections by the immune system. Recently, it became possible to activate them in a targeted manner via molecularly defined, specific ligands. Synthetic ligands for Toll-like receptors are currently undergoing clinical development for the therapy of viral infections and tumor diseases.

The negative headlines in March 2006 regarding a disastrous clinical trial in England underscore the special due diligence and extraordinary immunological requirements for clinical studies involving immunopharmaceuticals. Professor Paul Martini has laid the basis for clinical trials of new therapeutic procedures in the 1930s, thereby establishing the field of clinical pharmacology (*Methodenlehre der therapeutischen Untersuchung* (Methods of Therapeutic Examination), Verlag Julius Springer, 1932). Just like Busch, he worked and taught in Bonn. As a result, we have come full circle, and it is an excellent turn of events that this workshop takes place in Bonn 40 years after the inception of the Paul Martini Foundation.

We hope you will enjoy an exciting and inspiring scientific workshop on Toll and its "related" receptors.

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Speaker of the Board of the Paul Martini Foundation

Prof. Dr. med. Dr. h.c. Peter C. Scriba
Scientific Advisor of the Paul Martini Foundation

Prof. Dr. med. Gunther Hartmann
Scientific Advisor of the Paul Martini Foundation for the conception of the workshop

Prof. Dr. med. Stefan Endres
Scientific Advisor of the Paul Martini Foundation for the conception of the workshop

I Introduction

Organ-specific Function of Toll-like Receptors

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Recognition of microorganisms by the host relies on expression of receptor molecules that detect conserved microbial structures and were thus termed pattern recognition receptors. Such receptors are found both, as membrane-bound receptors at the cell surface or in intracellular endosomal compartments or as intracellular receptors. Not only cells of the innate and adaptive immune system are equipped with such pattern recognition receptors but also organ-resident cells that are not derived from bone marrow, e.g. intestinal epithelial cells. Selective expression of pattern-recognition receptors on abluminal surfaces in polarized intestinal epithelial cells further allows for discrimination of colonizing from invading microbes in the gastrointestinal tract. Ligand binding to membrane-bound pattern recognition receptors elicits signal transduction, e.g. through MyD88 or Trif, which is instrumental in functional maturation of antigen presenting dendritic cells. Importantly, induction of antigen-specific T cell-mediated immunity depends on such functional maturation because co-stimulatory signals are provided only by properly activated dendritic cells.

Little is known, however, on the impact of microbial structures on local immune regulation in organs other than the gut. The liver is involved in elimination of gut-derived microbial structures from the blood without development of inflammation and is further known to be a site for induction of immune tolerance. We have investigated the role of pattern recognition receptors in controlling local immunity in the liver and concentrated on the hepatic cell population that combines extraordinary scavenger activity, antigen presenting function and immune-regulatory potential, i.e. liver sinusoidal endothelial cells (LSEC). Upon scavenging of microbial products from the blood stream LSEC are activated as a result of constitutive expression of membrane-bound toll like receptors. Interestingly, LSEC develop a refractory state upon repeated contact with LPS that is characterized by a distinct mechanism, which does not involve decreased TLR4 expression levels but rather depends on cell-autonomous control of prostanoid production. This refractory state not only controls the expression of proinflammatory mediators but also prevents development of a pro-adhesive phenotype in LSEC; thus assuring that there is no dramatic increase in leukocyte adhesion within the hepatic sinusoid and consequent microvascular perfusion failure in response to gut-derived LPS.

LSEC have many features in common with immature dendritic cells: efficient uptake of antigen, presentation of exogenous antigens on both MHC class II and MHC class I molecules to $CD4^+$ and $CD8^+$ T cells, respectively, and induction of immune tolerance in $CD8^+$ T cells. Given these functional similarities we wondered whether LSEC also mature after proper stimulation into antigen-presenting cells mediating immunity. A systematic analysis revealed that LSEC not only functionally expressed TLR4 but also TLR1/2, TLR3, TLR7 and TLR9. Stimulation with the respective ligands lead to cell activation that was characterized by increased expression and release of IL-6 as well as increased cell-surface expression levels of CD54/106. However, stimulation of LSEC with TLR-ligands did not modify their ability to cross-present $CD8^+$ T cells. More importantly, LSEC did not undergo functional maturation after stimulation with TLR-ligands, i.e. LSEC still primed naïve $CD8^+$ T cells and induced immune tolerance in $CD8^+$ T cells. Thus, in contrast to immature dendritic cells, LSEC did not show functional plasticity after stimulation with soluble TLR-ligands. These results support the notion that the two closely linked properties of LSEC, scavenger and immune regulatory function, and therefore local induction of immune tolerance in the liver are not modulated by TLR-ligand mediated stimulation.

However, LSEC not only serve as scavenger cells for bacterial products but also appear to facilitate exit of hepatotropic viruses from the bloodstream and subsequent infection of hepatocytes. Therefore, we investigated whether contact of LSEC with viruses would alter their immune-regulatory capacity. Using murine cytomegalovirus (MCMV) as a model virus we observed that LSEC rapidly internalize virus. LSEC even were infected

at low level by recombinant MCMV but did not support production of progeny virus. Importantly, expression of type I Interferon was operative in mediating protection against infection and progeny virus formation, because LSEC isolated from Interferon-receptor knockout animals were more readily infected and showed progeny virus production. Recognition of MCMV was not mediated by TLR3 or TLR9 as shown by infection of LSEC obtained from the respective knockout animals, but required viral gene expression. If virus was inactivated by UV-irradiation, little if any type I Interferon was produced by LSEC. These findings suggest that viral gene expression was required for induction of type I Interferon and that not membrane-bound but rather intracellular pattern-recognition receptors were instrumental in viral recognition. Interestingly, non-productive infection of LSEC with MCMV did not reduce their ability to cross-present and cross-prime naïve $CD8^+$ T cells. Most importantly, LSEC abortively infected with MCMV lost their ability to induce immune tolerance in naïve $CD8^+$ T cells. Using naïve T cells from two separate TCR-transgenic lines we observed that $CD8^+$ T cells primed by MCMV-infected LSEC were fully stimulated and showed immediate effector function upon TCR-specific re-stimulation. As noted above, the loss of tolerogenicity was not achieved by stimulation of membrane-bound TLR through soluble ligands but rather depended on viral gene expression, because contact of LSEC with inactivated UV-irradiated MCMV as compared to infection with intact MCMV did not result in loss of tolerogenicity. These results support the notion that an intracellular pattern-recognition receptor was operative in mediating the functional maturation of LSEC in response to viral infection. More detailed analysis further revealed that induction of tolerance or immunity required direct physical interaction of naïve $CD8^+$ T cells with antigen-presenting LSEC, thus excluding that soluble mediators amplified a local immune response. Collectively, our data demonstrate that viral infection and early viral gene expression but not stimulation via TLR resulted in functional maturation of LSEC leading to a strongly localized induction of antigen-specific $CD8^+$ T cell immunity, which probably serves as an intravascular immune surveillance system to defend the liver against viral infection. These results further support the notion that toll like receptor ligands may exert different functional effects in various organs, which critically depend on the presence of organ-specific and -resident immune cells.

II Preclinical Development

Toll-like Receptor-dependent and -independent Viral Recognition in Innate Immunity

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Mammalian Toll-like receptors (TLR) play a critical role in the detection of invading pathogens as well as triggering of subsequent inflammatory and immune responses. Individual TLR recognize different microbial components. Particularly, TLR3, TLR7 and TLR9 are responsible for detection of viral infection. The signaling pathways via TLR are different from each other and the difference is in part due to selective usage of adaptor molecules. MyD88 is essential for the responses to all TLR ligands except for TLR3 ligand and is involved in the induction of inflammatory cytokines through activation of IRAKs and TRAF6. In addition, the TLR3- and TLR4-mediated signaling possesses a MyD88-independent pathway, which is activated by another adapter molecule, TRIF. The TRIF-dependent pathway activates IRF3 through TBK1/IKKi, and NF-kB through recruitment of TRAF6 and RIP-1, which results in induction of type 1 interferon. Plasmacytoid dendritic cells (pDC) rapidly produce IFNα in response to viral infection or in response to TLR7-(imidazoquinolines, ssRNA) or TLR9-ligand (CpG DNA). IFNα induction by pDC involves formation of a complex consisting of MyD88, TRAF6, IRAK-1 and IRF7, in which IRAK-1 acts as IRF7 kinase. Besides TLR, two DExD/H box RNA helicase family members, retinoic acid inducible protein-I (RIG-I) and melanoma differentiation-associated gene 5 (Mda5) are involved in anti-viral responses by recognizing dRNA in the cytoplasm. These two helicases recognize different RNA viruses. RIG-I is involved in the detection of many RNA viruses including Newcastle disease virus, vesicular stomatitis virus, and Sendai virus, whereas Mda5 is involved in the detection of the picornavirus family including encephalomyocarditis virus.

We identified a novel molecule named IPS-1 that bridges between RIG-I/Mda5 and TBK-1/IKKi. IPS-1 overexpression activated the type I interferon promoter, and induced phosphorylation of IRF3 and 7. The IPS-1-dependent type I interferon promoter activation required TBK-1 and IKKi. Downregulation of IPS-1 decreased the type 1 interferon production after viral infection. Recently, we have generated IPS-1 knockout mice and demonstrated that IPS-1 is essential to both RIG-I-dependent and Mda5-dependent signaling.

We also found that intracellular injection of dsDNA induces type 1 interferon via TBK1/IKKi, indicating the presence of an intracytoplasmic detector of dsDNA.

In this symposium, I will discuss the recent progress in the TLR-dependent and independent signaling pathways which are activated after viral recognition.

Abbreviations

IFNα	interferon α
IPS-1	IFN-beta promoter stimulator-1
IRAKs	Interleukin-1 receptor-associated kinases
IRF3	interferon regulatory factor 3
Mda5	melanoma differentiation-associated gene 5
MyD88	myeloid differentiation factor 88
NF-kB	nuclear factor-kappa B
pDC	plasmacytoid dendritic cells
RIG-I	retinoic acid inducible protein-I
RIP-1	receptor interacting protein-1
ssRNA	single stranded RNA
TBK1/IKKi	TRAF family member-associated NF-κB activator-binding kinase-1 / I kappa B kinase inducible
TLR7	Toll-like receptor 7
TLR9	Toll-like receptor 9
TRAF6	tumor necrosis factor receptor-associated factor 6
TRIF	TIR domain-containing adaptor inducing IFNβ

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Toll-like Receptor Agonists: New Opportunities for Drug Development

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The recent discovery of Toll-like receptors (TLR) bestowed innate immune cells with a restricted repertoire of germline encoded receptors that recognise as ligands invariant pathogen derived constituents. There are two broad categories of TLRs, those that are expressed at the cell membrane (TLR2, TLR6, TLR4, TLR5) and those that are expressed in endosomal compartments (TLR3, -7, -8, -9). Interestingly, all TLR expressed within endosomes recognise nucleotides (TLR3 recognise double stranded RNA; TLR7 and -8 recognise single stranded (s.s.) RNA and TLR9 recognise s.s. CpG-DNA). Since structural nucleotide differences between vertebrates and pathogens are scarce, self-RNA and self-DNA recognition may drive certain autoimmune diseases. TLR promote via DCs Th1 polarisation (protective T cell immunity) and via induction of type 1 IFN innate antiviral responses. Cytosolic RNA receptors such as RIG-I and MDA-5 drive, via IRF3, Type 1 IFN production in fibroblasts and myeloid DCs, while TLR3 and TLR4 activate IRF3/7 via TRIF. In NIPs (natural interferon producing cells, also termed plasmacytoid (p) DCs) the cytosolic RNA receptors appear not to be operative. Via TLR9 and MyD88 pDCs are triggered to produce large amounts of type 1 IFNs presumably because pDCs constitutively express IRF-7.

Abbreviations

CpG-DNA	cytosine-guanosine motif containing DNA
DCs	dendritic cells
IFN	interferon
IRF3	interferon regulatory factor 3
MDA-5	melanoma differentiation antigen 5
MYD	myeloid differentiation antigen
NIPs	natural interferon producing cells
RIG-I	retinoic acid inducible gene-I
SLE	systemic lupus erythematosus
TLR	Toll-like receptor
TRAF-3	TNF-receptor associated factor
TRIF	TIR-domain containing adaptor protein inducing IFN-β

Here I will discuss recent data to show that the TLR induced and TRIF or MyD88 dependent production of α-IFNs is controlled by TRAF-3 (work in collaboration with H. Häcker and M. Karin, La Jolla, CA, USA). Thereafter I will discuss yet unpublished data according to which IRF1 controls β-interferon production in myeloid (m) DCs. The latter data describes a novel β-IFN induction pathway that operates independently of IRF-3 or IRF-7. Interferons have been implicated to drive certain auto-immune diseases such as systemic lupus erythematosus (SLE). Deciphering TLR dependent and TLR independent IFN induction signal pathway will provide fertile ground for the development of drugs that interfere with these pathways.

Toll-like Receptor-7-independent Recognition of Single-stranded RNA by a MyD88-dependent Pathway

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The innate immune system senses nucleic acid in endosomal/lysosomal compartments via a subgroup of Toll-like receptors (TLR) consisting of TLR3, -7, -8 and -9. Although the specificity of these receptors for single-stranded (ss) RNA (TLR7 and -8), double-stranded (ds) RNA (TLR3) and CpG-DNA (TLR9) has been characterized, TLR cross-reactivity has not been studied in detail. Here we report that phosphodiester ssRNA stimulates immune cells in a partially TLR7-independent, but myeloid differentiation primary response gene 88 (Myd88)-dependent manner, whereas phosphorothioate backbone stabilized ssRNA strictly depends on TLR7. Mechanisms of TLR-dependent recognition of ssRNA will be discussed.

Identification of RNA Motifs for Toll-like Receptor-7 and -8

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Cellular delivery of synthetic small interfering RNA duplexes (siRNA) or introduction of siRNA by plasmids or viral vectors is now widely used for silencing of target genes. In general, the formation of double stranded RNA (dsRNA) during viral replication is interpreted by the cell as a signal for unwanted gene activity. It is widely accepted that siRNA duplexes (generally 19-21 base pairs) are short enough to bypass non-target-related induction of type I Interferon (IFN) typical for long double-stranded RNA. We recently demonstrated that in plasmacytoid dendritic cells (PDC), an immune cell subset specialized in the detection of viral nucleic acids and production of type I IFN, some siRNA sequences independently of their GU content are potent stimuli of IFN-α production. Localization of the immunostimulatory motif on the sense strand of a potent IFN-α inducing siRNA allowed dissection of immunostimulation and target silencing. Injection into mice of immunostimulatory siRNA, when complexed with cationic liposomes, induced systemic immune responses in the same range as the Toll-like receptor-9 (TLR9) ligand CpG, including IFN-α in serum and activation of T cells and dendritic cells in spleen. Immunostimulation by siRNA was absent in TLR7-deficient mice. Thus sequence-specific TLR7-dependent immune recognition in PDC needs to be considered as an additional biological activity of siRNA, which then should be termed isRNA (immunostimulatory RNA). In addition to PDC, cells of the myeloid compartment recognize ssRNA oligonucleotides via TLR8 with a clear distinction in terms of sequence dependence. However, while the CpG motif is responsible for TLR9-mediated DNA recognition, the minimal motifs for TLR7- or TLR8-dependent RNA recognition have not been identified. Therefore we developed a systematic approach that allows a detailed analysis of sequence-dependent TLR7 and TLR8 activation by RNA oligonucleotides. Complementing our previous work, we identified additional panels of sequence motifs that contribute positively or negatively to the immunological activity of RNA oligonucleotides. This information is now integrated into an algorithm that allows to avoid the immunostimulation of siRNA used for target gene silencing or to optimize the design of isRNA for immunotherapy. Together, these insights will help to improve the application of siRNA and of isRNA as a tool in vitro and as promising drugs for cancer therapy in vivo.

Lipopeptide Adjuvants

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Synthetic lipopeptides derived from bacterial lipoproteins constitute potent macrophage/monocyte activators in vitro inducing the release of various interleukins, e.g. TNFα, reactive oxygen/nitrogen intermediates and the translocation of NFκB; in vivo they are effective adjuvants acting in some cases superior to Freund's adjuvant and enhancing the immune response especially with respect to low- or non-immunogenic compounds and proteins exhibiting homology with host counterparts. Structure/adjuvanticity-related investigations of lipopeptide derivatives from lipopentapeptide and lipopeptide fatty acid libraries clearly indicated that P_3CSK_4 constitutes one of the most effective lipopeptide derivatives. This compound represents a highly efficient immunoadjuvant in conventional peptide/protein immunization (parenteral, oral, and nasal administration) either in combination with or after covalent linkage to antigen. E.g., P_3CSK_4 significantly enhanced and accelerated the humoral immune response to tetanus toxoid. The antibody response against ovalbumin and other antigens was markedly enhanced by the addition of the lipopeptides P_3CSK_4, P_3CSP_4, or Myr_3CSK_4 which was already active at a concentration of less than 1 μg/mouse/immunization. Furtheron, P_3CSK_4 could substitute up to 90% of the antigen without any loss in antigen-specific antibody level. In addition we observed that conventional immunization in the presence of lipopeptides resulted in an elongated presence of the antigen-specific immunoglobulin IgG over time. P_3CSK_4-related adjuvanticity could be further increased using lipopeptides carrying T_h cell epitopes linked to the lipopeptide backbone. According to their highly immunostimulating properties, especially with respect to low or non-immunogenic compounds, lipopeptides are most important for the generation of polyclonal and monoclonal antibodies in different species. Lipopeptide-induced antigen-specific antibodies are most suitable for the detection of drugs, toxins, antibiotics, metabolites, and other low molecular mass antigens by ELISA, radioimmunoassay, fluorescent immunoassay, or in modern biosensor monitoring. Furtheron, lipopeptides constitute significant adjuvants for the in vitro immunization of either human mononuclear cells or mouse B cells resulting in a markedly increased yield of antigen-specific antibody secreting hybridomas after fusion of the stimulated B lymphocytes with myeloma cells. We also investigated the effect of lipopeptides on the activation of human peripheral blood mononuclear cells and of dendritic cells differentiated from monocytes. We found an enhanced production of IL-12 and IFN-γ indicating the induction of preferentially a Th1 response, and the induction of the proinflammatory cytokines TNF-α, IL-6 and IL-1β. Myr_3CSK_4 was, in most cases, active at far lower concentrations than P_3CSK_4.

Investigations of the molecular mode of action showed that lipopeptide (P_3CSK_4)-induced activation of macrophages is Toll-like receptor-2 (TLR2)- and CD14-dependent but TLR4-independent; the observed activation is mediated via the MAPK signal transduction pathway resulting in NFκB-translocation and activation/repression of at least 140 genes partly involved in signal transduction and regulation of the immune response including the transcriptional activation of p53, c-rel, IκBα, iNOS, CD40-LR, ICAM-1 and IL-1/6/15. Further on, we detected significant differences with respect to lipopeptide- and LPS-induced stimulation of bone marrow-derived murine macrophages. Additional in vivo investigations with respect to the influence of lipopeptide on the Th1/Th2 bias clearly showed that, in mice, with respect to peptide/protein immunization using a wide variety of antigens and different application routes, no predictions can be made which type of immune response is favoured by lipopeptide. However, with respect to genetic immunization we monitored a shift from Th1 towards a mixed Th1/Th2-type response in the presence of lipopeptide when intramuscular application of antigen-encoding DNA is performed in the presence of lipopeptide adjuvants. Bacterial and viral vaccines based on synthetic lipopeptides, e.g. Enterobacteriaceae vaccines, vaccines against foot and mouth disease, provided protection with respect to a broad range of species; in addition, we here also showed that lipopeptides could substitute up to 90 % a of a vaccine. Besides their highly immunostimulating capacity, lipopeptides are well defined, non-toxic, non-inflammatory and can be easily synthesized in large amounts. Taken together, lipopeptides are of significant importance for further optimizing conventional and genetic immunization techniques and for the development of novel synthetic vaccines.

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The Two Faces of Nucleic Acid Recognition in Dendritic Cells

Induction of antiviral responses and autoimmunity

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Plasmacytoid dendritic cells (PDC) are major producers of type I interferons (IFN) in acute viral infection. In PDC the DNA genomes of herpes simplex virus and murine cytomegalovirus (MCMV) are recognized by Toll-like receptor (TLR) 9 and the single stranded RNAs of influenza virus, Newcastle disease virus and vesicular stomatitis virus are detected by TLR7. Triggering of TLR9 and TLR7 in PDC leads to rapid production of type I IFN and other proinflammatory cytokines and chemokines. In the MCMV infection model, the very early systemic IFNα response depends on the presence of PDC and is largely mediated by TLR9, whereas at later time points significant amounts of IFNα and -β are released in a TLR9-/MyD88-independent manner. Myeloid DC generated from bone marrow were able to produce type I IFN after infection with MCMV in the absence of MyD88 and TLR9. This response was also independent of TLR3/TRIF, TLR2, TLR4 and TLR7. Type I IFN induction in myeloid DC by MCMV was enhanced after UV-inactivation of the virus, suggesting that MCMV encoded proteins actively suppress the initial induction of type I IFN and that this novel type I IFN induction pathway triggered by MCMV does not require transcription or replication of the viral genome. Therefore TLR-dependent and TLR-independent signalling pathways triggered by viral nucleic acids are active in different DC subpopulations. The downside of the described nucleic acid recognition mechanisms is the ability of these receptors to also recognize intracellularly delivered mammalian nucleic acids, which may promote autoimmunity. We found that autoimmune complexes containing U1 small nuclear ribonucleoproteins which occur in patients with systemic lupus erythematodes triggers IFNα production in PDC in a TLR7-dependent, TLR3-independent manner. The U1snRNA component of RNP-containing autoimmune complexes acts as an endogenous ligand for TLR7 in PDC, in addition to the TLR9-mediated recognition of self DNA within autoimmune complexes. Thus, nucleic acid recognition receptors are involved in the antiviral immune response but also in autoimmune diseases.

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Sensing Viral Replication by RNA Helicases

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The cytoplasmic proteins retinoic acid-inducible gene I (RIG-I) and melanoma differentiation associated gene 5 (MDA-5) sense intracellular viral infection and trigger a signal for innate antiviral responses including the production of type I Interferon (IFN). Both proteins contain a homology domain present in members of the DExD/H-box helicase family and exhibit N-terminal caspase recruitment domains (CARD). Both proteins sense viral RNA with their helicase domain and trigger downstream signals by interaction of their CARDs with the CARD domain containing adapter MAVS (mitochondrial antiviral signaling) localized in the outer membrane of mitochondria. RIG-I and MDA-5 were found to be essential and non redundant for the defense against a variety of viruses. Infections with paramyxoviruses like Sendai virus (SV) and respiratory syncytial virus (RSV) as well as the hepatitis C virus trigger RIG-I while picorna viruses like the encephalomyocarditis virus (EMCV) trigger melanoma differentiation-associated gene 5 (MDA-5). A third member of the RNA

Abbreviations

CARD	caspase recruitment domain
EMCV	encephalomyocarditis virus
IFN	interferon
IRF	interferon regulatory factor
MAVS	mitochondrial antiviral signalling
MDA-5	melanoma differentiation associated gene 5
NF-κB	nuclear factor kappa B
RIG-I	retinoic acid-inducible gene I
RSV	respiratory syncytial virus
SV	Sendai virus

helicase family, Lgp2, shares high homology with the helicase domain of RIG-I. In contrast to RIG-I or MDA-5, Lgp2, however, lacks CARDs or any other known signaling domain. Overexpression of Lgp2 inhibits the triggering of interferon regulatory factor (IRF)- and nuclear factor kappa B (NFκB)-dependent pathways by SV and Newcastle disease virus. Like RIG-I and MDA-5 Lgp2 binds polyinosinic-polycytidylic acid, a synthetic mimic of double stranded RNA. Quantitative PCR analysis demonstrates that Lgp2 is present in unstimulated cells at a lower level than RIG-I, although both helicases are induced to similar levels after virus infection. Lgp2 therefore is supposed to act as an endogenous negative feedback regulator of antiviral signaling by sequestering viral RNA ligands away from retinoic acid inducible protein-I (RIG-I) and MDA-5.

mRNA-based Vaccination against Cancer

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Injecting coding DNA and RNA into mice or rats was shown until 1990 to lead to the expression of the corresponding proteins locally at the site of injection. This resulted in substantial activities aimed at using DNA for vaccine purposes. Indeed, DNA vaccination was found to lead to antibody and CD4 and CD8 T-cell responses in mice. Trials in humans also showed immune responses. The application of RNA in vaccines, however, was not followed up for approximately 10 years, most likely because RNA is extremely short-lived in serum-containing media. On the other hand, DNA bears the intrinsic risk of integrating into the genome in an uncontrolled manner, indicating that RNA might be the better choice for vaccine development. Indeed, an important step in this development was the use of mRNA to transduce dendritic cells in vitro to be used for immunization. This led to an impressive immune response in mice and was later applied in clinical trials. RNA was then also used for the direct immunization of mice. This was indeed found to be successful; naked mRNA, as well as mRNA protected from degradation by protamin, a long-known protein stabilizing nucleic acid, led to the induction of IgG antibodies and of CD8 T cells against the corresponding protein. In the following years, our group worked on the optimization of mRNA-based vaccination. Over the last 5 years, several important findings have been made: (i) the efficiency of protein expression upon i.d. injection of coding mRNA into mice could be optimized by varying the injection solution composition, the sequences flanking the coding part of the mRNA and the purification protocol used for mRNA production; (ii) intradermal injection of mRNA into human skin was shown to lead to local expression of the coding protein; (iii) a pilot clinical trial using mRNA libraries from autologous melanoma metastases showed safety and immunogenicity of mRNA injection; and (iv) single-stranded RNA was found to activate dendritic cells as it is a ligand for Toll-like receptor-7 and -8. These developments make mRNA vaccination a promising option for future developments, especially for tumor vaccination. Because it is relatively easy (as compared with recombinant proteins) to produce defined mRNA for clinical use, it appears that the next useful step in development is to combine a number of genes known to be expressed in a certain tumor entity, for example, melanoma, and to carry out vaccination with such a cocktail of mRNA. Preparations are presently underway for such a study, using a combination of MelanA, gp100, tyrosinase, MAGE-A3, MAGE-A1 and survivin. Such combinations can be used for all melanoma patients. However, it is more desirable, and technically possible by comparative gene expression analysis, to individually analyse the patient's tumor or tumor-associated, overexpressed gene products and then put together a combination of mRNA tailored to fit the patient.

Toll-like Receptors as Therapeutic Targets in Autoimmunity

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Autoimmune diseases affect at least 5% of the population and these disorders have major consequences for individual patients, the health care system and the society. Type I diabetes, psoriasis and rheumatoid arthritis are examples of common autoimmune diseases. The prototype autoimmune disease systemic lupus erythematosus (SLE) is more rare, but characterized by a large number of autoantibodies (> 100 specificities) and inflammation in most organ systems. Many signs and symptoms seen in SLE can also be observed in other autoimmune conditions. SLE is therefore often regarded as a model for autoimmune diseases in general. Studies of the gene expression profile in SLE have revealed that a majority of patients have a dominant pattern of type I interferon (IFN)-inducible gene expression and this is consistent with earlier observations that SLE patients have increased serum levels of IFN-α [1]. Recent studies of other autoimmune diseases suggest that the type I IFN system may play a pivotal role in several of these. This is especially true for Sjögrens syndrome, dermatomyositis/polymyositis, psoriasis, systemic sclerosis, type I diabetes mellitus, rheumatoid arthritis and autoimmune hepatitis because these patients have a striking signs of IFN-α production [2]. A causative role for type I IFN in autoimmune diseases is suggested by the observation that long term IFN-α treatment of patients with malignant or viral diseases frequently results in the development of autoimmunity. Thus, a continuous exposure of the immune system of IFN-α can break the normal tolerance and promote autoimmune reactions.

The reason for the activation of the type I IFN system in SLE and other autoimmune diseases is unclear, but in SLE patients we have described the occurrence of endogenous IFN inducers consisting of immune complexes (IC) containing nucleic acid [2]. The occurrence of such interferogenic ICs is associated with active disease and these ICs activate the highly specialized natural IFN-α producing cell (NIPC), also termed plasmacytoid dendritic cell (PDC). The interferogenic ICs are internalized in NIPC/PDC via the FcγRIIa, reach the endosome and activate relevant Toll-like receptors (TLR) with subsequent induction of type I IFN production. The exact nature of the nucleic acids that trigger IFN production by ICs is unknown, but in vitro studies show that apoptotic cells generate DNA-containing material that can form interferogenic ICs [3]. Cells dying by apoptosis or necrosis also release RNA-containing material with interferogenic properties [4]. A common autoantigen in SLE, snRNP, can when complexed to autoantibodies trigger IFN-α production by NIPC/PDC [5], and highly conserved RNA sequences within the snRNP complex stimulate TLR7 [6]. DNA containing ICs on the other hand activates TLR9, provided that the DNA is delivered to the endosome [7]. Besides the NIPC/PDC, both DNA and RNA containing ICs can activate autoreactive B-cells via a sequential engagement of the B cell antigen receptor and TLR9 or TLR7, respectively [8, 9].

What are the mechanisms by which type I IFN cause autoimmune reactions? This large family of cytokines has prominent effects on key cells in the immune system that are likely to promote the activation of potential autoimmune cells. Thus, type I IFN causes maturation and activation of Dendritic cells (DC), upregulation of TLR2/3/4/7 and 8 in monocytes/macrophages, promote the Th1 pathway, increase T cytotoxic activity and enhance activation and differentiation of plasmablasts to plasma cells causing increased antibody production. In addition, type I IFN promotes the production of several cytokines important for a sustained activation of the immune system, for instance IFN-γ, IL-6 and IL-15 [10]. Type I IFN can also have direct effects on target tissues by causing expression of autoantigens [11] or up-regulate MHC class I molecules, making the cells more susceptible for autoimmune attacks [12].

Clear evidence that inhibition of the type I IFN system could be beneficial in autoimmune diseases was obtained when type I IFN receptor knock-out lupus-prone mice were noted to have a dramatically reduced SLE disease [13]. The possibility that TLR antagonists can be useful in SLE was first demonstrated when it was noted that suppressive oligodeoxynucleotides delay the onset of glomerulonephritis and prolong survival in lupus-prone NZB × NZW [14]. Recently, it was shown that TLR9 is critical for induction of anti-DNA autoantibody production, but TLR9 deficient lupus-prone mice still develop autoimmune disease and end-organ damage [15]. Whether inhibition of both TLR7 and TLR9 may improve the outcome in SLE is not known, but the possibility exist that also other sensors of nucleic acid needs to be blocked in order to obtain a good therapeutic response. For other autoimmune diseases, the role of the different TLR is unclear, but TLR9 seems to be involved in experimental autoimmune encephalomyelitis [16] and TLR3 activation may contribute to the synovitis in RA patients [17].

At the moment, we are learning more and more about the TLR and their possible role in different autoimmune diseases. Emerging data from experimental

disease models suggests that inhibition of at least some TLR may constitute a new therapeutic principle in autoimmune diseases. In some disorders, a specific TLR inhibitor may turn out to be sufficient to achieve an acceptable therapeutic effect. In other diseases, it may be necessary to administer inhibitors that block several TLR in order to obtain a clinical response. When several TLR are blocked at the same moment, the challenge is to selectively inhibit the disease-related cytokine and autoantibody production and at the same time spare the normal function of the innate and adaptive immune system.

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Toll-like Receptor-9 Ligands in Tumor Models

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We have developed an effective protocol for dendritic cell-based therapy of subcutaneous tumors generated by injection of the murine C26 colon adenocarcinoma cell line (Griswold et al. 1975). Dendritic cells are loaded in vitro with irradiated tumor cells and are activated by CpG oligonucleotides prior to administration, resulting in enhancement of the antitumor response (Brunner et al. 2000). The tumor-specific immune response, that depends on CD8 T cells, is further increased by coinjecting CpG oligonucleotides together with the dendritic cells and by a simultaneous peritumoral injection of CpG oligonucleotides (Heckelsmiller et al. 2002a; Heckelsmiller et al. 2002b).

In follow-up studies, we combined an established immunotherapy protocol with two chemotherapeutic drugs currently used in the treatment of advanced colon cancer, 5-fluorouracil and irinotecan. These agents have been shown to be moderately effective for therapy

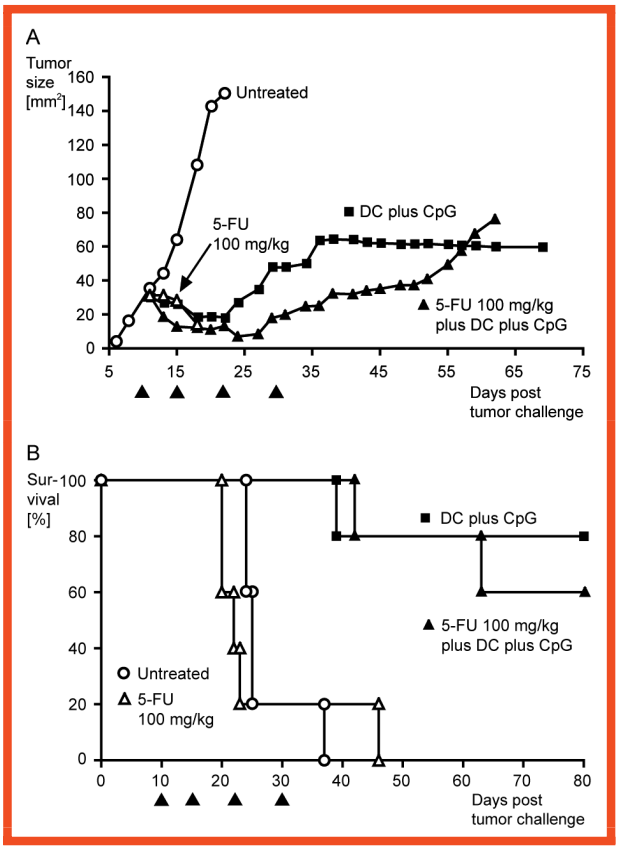

Fig. 1: Dendritic cell and CpG oligonucleotide-based immunotherapy can be combined with chemotherapy without loss in therapeutic activity (from Bourquin et al. 2006).
(A and B) Mice with established C26 tumors on one flank (mean tumor size 30 mm^2) were treated with immunotherapy (DC plus CpG) or with 100 mg/kg 5-fluorouracil with leucovorin or with both treatments simultaneously. The treatment was administered at the four time points indicated (arrows). (A) Mean tumor size of treatment groups (n = 5) is presented. Both DC plus CpG treatment and 5-FU 100 mg/kg plus DC plus CpG treatment significantly reduced tumor growth compared to untreated mice ($p < 0.02$ at all time points from day 15). (B) Survival was significantly increased in both groups receiving immunotherapy compared to untreated mice ($p = 0.002$) and to mice receiving 5-fluorouracil alone ($p = 0.006$).

of C26 tumors (Wilmanns et al. 1992; Guichard et al. 1998). We demonstrate that simultaneous administration of either 5-fluorouracil or irinotecan with dendritic cell-based immunotherapy does not affect the antitumor effect of immunotherapy.

Tumor-bearing mice were treated weekly for four weeks by this immunotherapy protocol, by 5-fluorouracil plus leucovorin or irinotecan, or by the combination of immunotherapy and chemotherapy. We observed that immunotherapy was more effective in reducing tumor growth and increasing survival than 5-fluorouracil or irinotecan (Bourquin et al. 2006). Immunotherapy was well tolerated, whereas therapeutic doses of 5-fluorouracil or irinotecan were associated with dose-limiting toxicity. Furthermore, the efficacy of immunotherapy combined with either 5-fluorouracil or irinotecan was similar to that of immunotherapy alone. Addition of immunotherapy to either 5-fluorouracil or irinotecan treatment strongly decreased the toxicity of chemotherapy. Immunotherapy both with and without chemotherapy generated a memory immune response leading to tumor rejection in mice rechallenged with C26 tumor cells up to several months after treatment. In summary, immunotherapy with a combination of dendritic cells and CpG oligonucleotides is superior to chemotherapy in the C26 tumor model. This immunotherapy protocol can be combined with current chemotherapy agents with no loss in therapeutic activity.

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Toll-like Receptor-7 Ligands for Tumor Therapy

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The immune-activating effects of Toll-like receptor (TLR) ligands have prompted their use in vaccine formulations. It is well established that binding of synthetic CpG oligodeoxynucleotides (ODN) to TLR9 both enhances the generation of an innate immune response and promotes protective Th1-type immunity in animal models. In humans, clinical studies have demonstrated a potential for CpG ODN as adjuvant in antiviral vaccination. Furthermore, CpG ODN combined with a peptide antigen promote CD8+ T cell responses to tumor antigens in melanoma patients. However, TLR9 displays a restricted expression pattern in humans, where this receptor is expressed on B cells and plasmacytoid dendritic cells (DC) but not, as is the case in mice, on professional antigen-presenting cells that are crucial for the induction of immunity to viral and tumor antigens. In contrast to TLR9, the TLRs 7 and 8 are expressed in humans on a broad range of immune cells, including professional antigen-presenting cells such as myeloid DC and monocytes. Indeed, ligands for TLR 7/8 stimulate human monocytes as well as plasmacytoid DC and B cells [1, 2]. Therefore, a ligand for TLR 7/8 may, in patients, be a promising alternative for use in vaccine formulations.

Synthetic molecules of the imidazoquinoline family such as imiquimod and its more potent analogue R848 (resiquimod) represent ligands for the TLRs 7 and 8. Stimulation with R848 induces activation of DC with increased expression of costimulatory molecules and increased synthesis of proinflammatory cytokines. As adjuvant used together with a protein vaccine, R848 increases specific antibody titers and activates virus-specific T cells [3]. Imiquimod is used in the clinic for topical therapy of several types of skin cancer.

We have previously developed an effective protocol combining CpG ODN and DC vaccination for the immunotherapy of tumors in a mouse model of colon carcinoma. In this protocol, DC are loaded in vitro with irradiated tumor cells and are activated by CpG ODN prior to administration, resulting in enhancement of the antitumor response. The tumor-specific immune response, that depends on CD8 T cells, is further increased by coinjecting CpG ODN together with the DC and by a simultaneous peritumoral injection of CpG ODN. The DC application is performed three to four times at weekly intervals and can be simultaneously combined with chemotherapy without loss of efficacy [4]. Here, in a similar protocol, we combined R848 with DC vaccination for tumor immunotherapy. Mice with established tumors were immunized weekly as in the CpG/DC protocol with tumor-loaded DC and received a simultaneous injection of R848 in the peritumoral area. In contrast to CpG-ODN-based therapy, no reduction of tumor growth was observed with R848.

Assessment of the immunostimulatory activity of R848 in vivo showed that the subcutaneous injection of 10 μg R848 induced high serum levels of the cytokines IL-12p70, IL-6 and IFN-α. While the serum cytokine levels were similar to those induced by CpG-ODN, they were of short duration, peaking at 2 h after injection. Eight hours after injection, cytokine levels had nearly returned to baseline. Activation of splenocyte populations such as T cells, B cells, NK cells, myeloid and plasmacytoid DC at levels similar to those induced by CpG ODN was observed after injection of even low amounts of R848. These results suggested that, while overall immunostimulatory activity of R848 was adequate, unfavorable pharmacokinetics might impair formation of an effective antitumor response. To counter the short duration of R848 immunostimulation, DC vaccination was performed with four subsequent injections of R848 after DC application. With this protocol, a significant reduction in tumor growth was observed [5].

We have shown that a ligand for TLRs 7 and 8 can function as adjuvant for DC vaccination in tumor immunotherapy. The development of controlled release formulations for R848 may further improve the pharmacokinetics of the compound for this application. Furthermore, the use of other TLR 7/8 ligands such as single-stranded RNA oligonucleotides [6] may provide an interesting alternative for the immunotherapy of tumors.

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Preclinical Development of Melanoma Vaccines with Toll-like Receptor Ligands in a Novel Genetic Mouse Model

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Malignant melanoma is an attractive model disease for the development of antigen-specific immunotherapy because many antigens recognized by tumor-specific T cells have been identified. In C57BL/6 mice, genetic immunization with recombinant adenovirus encoding xenogeneic human tyrosinase-related protein 2 (Ad-hTRP2) induces protective but not therapeutic cellular immunity against growth of transplanted B16 melanoma cells. Here we additionally applied synthetic CpG DNA and double-stranded RNA (polyI:C) which activate the innate immune system via toll-like receptors (TLR). Both adenoviral vaccination and peritumoral injections of TLR agonists were required for rejection of established B16 melanoma in the skin. To more closely mimic the expected clinical situation in patients with melanoma, we developed a novel genetic mouse model which recapitulates the histopathology and molecular pathogenesis observed in the human disease. Hepatocyte growth factor transgenic mice (HGF) were crossed with mice carrying an oncogenic germline mutation in the cyclin dependent kinase 4 (CDK4R24C) gene. Overexpression of HGF promotes melanocyte growth via enhanced receptor tyrosine kinase signalling and is associated with a dark skin phenotype in C57BL/6 mice. Mutated CDK4R24C can not be inhibited by p16/INK4a leading to impaired cell cycle control. Neonatal treatment of HGF x CDK4R24C mice with the carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) induces multiple rapidly growing autochthonous melanomas in the skin which spontaneously metastasize to lymph nodes and lung. Therapeutic vaccination of carcinogen-treated HGF x CDK4R24C mice bearing established cutaneous melanomas with Ad-hTRP2 followed by injections of CpG DNA and polyI:C resulted in delayed tumor growth and a reduction in the number of spontaneous lung metastases. However, tumor regression and autoimmune destruction of melanocytes were not observed. Importantly, carcinogen-treated HGF x CDK4R24C mice bearing multiple autochthonous melanomas were unable to reject concomitantly transplanted B16 melanoma even when treated with Ad-hTRP2 and TLR agonists. This result suggests a state of profound tumor-induced immune tolerance. Further investigations in our novel genetic melanoma model may help to identify the relevant tolerance mechanisms involved and provide a scientific basis for improved treatment strategies.

III Clinical Development

Cancer Immunotherapy: TLRs, NLRs, and RLRs

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Cancers arise in the setting of chronic inflammation [1–3], in adults but not in children [4]. We retrospectively studied 27 pediatric and 13 adult cancers at first diagnosis by immuno-histochemistry and identified inflammatory cells and their location within the tumor. Most tumor-associated leukocytes (TAL) were found at the periphery of tumor islands except in pediatric tumors where they were scattered throughout the malignant tumors. Pediatric tumors were infiltrated predominantly by macrophages that accumulated in areas of necrosis, with a virtual absence of dendritic cells (DC). Intratumoral DC in pediatric samples was 4.1 %; whereas in adult tumors, they formed 36.9 % of TAL within tumors and 25.1 % around the tumors. These findings provide a major nosologic difference, reclassifying pediatric and adult tumors based on inflammatory etiology. What recruits these inflammatory cells has been an area of interest for our laboratory and clinical group. We hypothesize that the major cause of inflammatory cell recruitment is unscheduled cell death and release of damage-associated molecular pattern molecules (DAMP), promoting recruitment of inflammatory cells and resultant reactive angio- and stromagenesis.

Prototypic of the DAMP is the nuclear factor [3], high mobility group box 1 (HMGB1) which is secreted as a late mediator of septic death, promoting leukocyte recruitment and inflammation following injury and released during chronic inflammatory states such as arthritis and cancer. Ischemia-reperfusion, frequently found within hypoxic and necrotic regions within tumors, drives Toll-like receptor-4 (TLR4)-dependent recruitment of inflammatory cells which we have shown also promotes further hepatocyte damage [5, 6]. Conversely, preconditioning with HMGB1 results in liver protection associated with a higher expression of IL-1R-associated kinase-M, a negative regulator of TLR4 signaling, critically activated through HMGB1-induced TLR4 signaling [7]. Recently, we have also demonstrated in preliminary experiments, that TLR2 and TLR4 deficient animals have decreased tumor growth when compared with their wild type counterparts, suggesting a critical role for inflammatory pathways mediated by these signaling molecules in tumor progression.

Tumor immune responses, including most immunotherapy strategies, fail to control tumor progression either because of lack of tumor immunogenicity, inability to deliver or sustain immune effectors within tumor, or release of suppressive cytokine by tumors or myeloid DC found frequently at such tumor sites in adults. Typically Th1 cytokines are induced by HMGB1 release, important for initiating an effective immune response. Conversely, the Th3 cytokine, Interleukin-10 (IL-10) promotes development of tolerogenic DC in an antigen-specific manner [8]. IL-10 is likely secreted by chronically HMGB1-recruited myeloid cells, found abundantly within the tumor microenvironment. Tumor cell death mediated by application of chemotherapeutic agents in most tumor cell lines is associated with release of HMGB1, even when lysed by activated NK cells or tumor-specific T-cells (Ito, N., Kalinski, P., Zeh, H. J., Lotze, M. T., in preparation). In contrast, platinum treatment of cells causes HMGB1 sequestration, similar to what is observed following apoptotic death in programmed death-competent cells [9]. Furthermore, recombinant HMGB1 which matures myeloid DC, inhibits responsiveness of plasmacytoid DC to TLR9 agonists such as CpG oligodeoxynucleotides [10]. Thus we are developing a sense that persistent release of DAMP is the proximate cause of much of the unusual tumor cell biology associated with immunosuppression in the tumor microenvironment. Means to promote apoptotic death or limit the release of DAMP and their

Abbreviations

bFGF	basic fibroblast growth factor
CXC	cysteine-x-cysteine (where x is another amino acid)
DAMP	damage-associated molecular pattern
DC	dendritic cells
HGF	hepatocyte growth factor
HMGB1	high mobility group box 1
IL	interleukin
miRNA	micro-RNA
NK cells	natural killer cells
NLR	NACHT–like receptors
RLR	RIG-I-like receptors
TAL	tumor-associated leukocytes
TLR	Toll-like receptors
VEGF	vascular endothelial growth factor

recruitment of immunosuppressive cells is a major programmatic goal.

Recently, IL-23 has been identified as an important cytokine, promoting tumor growth and escape from control [11]. Signalling through similar pathways as IL-12, a closely related family member [12], IL-23 is found at increasing quantities in progression of gastrointestinal neoplasms. Which regulatory factors, either at the transcriptional level or at the level of miRNA promoting individual developmental or inflammatory pathways is as yet an "unknown unknown" [13]. One of the major cytokines induced by IL-23 is the cytokine IL-17, released by a novel subset of CD4+ T-cells, which we have termed Th4 cells [and others, Th17 cells]. IL-17 enhances the growth of vascular endothelial cells induced by basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) [14]. Similarly, in chronic inflammation in the gut associated with IL-2 deficient mice, we have found large numbers of cells secreting IL-17 in association with small bowel pathology (Hoffman, R., in preparation). We have also examined the biological action of IL-17 on human tumors and demonstrated that IL-17 selectively augments secretion of an array of angiogenic cysteine-x-cysteine (CXC) chemokines, including CXCL1, CXCL5, CXCL6, and CXCL8 but not angiostatic chemokines [12]. Transfection with IL-17 into murine tumors in immuno-competent mice or human xenografts, transplanted in SCID mice promoted tumor growth, associated with increased tumor vascularity. In primary tumors, IL-17 expression is frequently detected in infiltrating inflammatory cells, associated with increased tumor vascularity. The role of DAMP ligands and conversely TLR, NLR, or RLR [16] in promoting effective Th1 immunity or largely ineffective, Th2-4 immunity, is an important and understudied aspect of tumor biology. We hypothesize that unscheduled cell death within which typically reduced molecules are found within the cell, meet up with the oxidative environment outside the cell, that biology is indeed turned upside down [17]. Targeting TLR ligands or the receptors themselves should prove to be a rich area for further development in the treatment of patients with cancer.

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Challenging and Promising Aspects of CpGs as Vaccine Adjuvants

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Vaccination is the most powerful strategy to prevent infectious disease, and thus contributes considerably to public health. Biomedical research has focused strongly on improving existing vaccines and developing new ones. Nevertheless, many features of underlying biological mechanisms responsible for immune protection still remain poorly understood.

The two A's

Synthetic vaccines are composed of (at least) two basic components: Antigen and adjuvants (the two A's). Adjuvants are immune stimulating agents: they are essential components because immune responses remain poor when antigens are administered alone. For many years, adjuvants have been developed empirically, without significant progress in understanding their molecular nature. The discovery of dendritic cells (DC), and of their central role to link innate with adaptive immune responses was key for progress. Besides regulating central mechanisms of the innate immune system, DC are the most effective antigen presenting cells for enabling antigen specific T and B cell responses. But how are they put in action? Only about one decade ago it was discovered that DC become activated due to triggering of their pathogen recognition receptors (PRR). These receptors enable the innate immune system to sense microbes. The most prominent family of PRRs are the Toll-like receptors (TLR) that bind microbial products [1–4].

Abbreviations

CpG	deoxycytidylate-phosphate-deoxyguanylate
DC	dendritic cell
pDC	plasmacytoid dendritic cell
HLA-A2	human leukocyte antigen A2
IFA	Incomplete Freund's Adjuvant
IFN-γ	interferon-γ
MART-1	melanoma antigen recognized by T cells-1
Melan-A	melanoma antigen-A (identical to MART-1)
MPL	monophosphoryl lipid A
QS-21	a natural saponin
PRR	pathogen recognition receptors
TLR-9	Toll-like receptor-9
TNF-α	tumor necrosis factor-α

CpG oligodeoxynucleotides (CpG ODN)

TLR-9 is triggered by unmethylated DNA with "CpG motifs" (GTCGTT for humans; GACGTT for mice [5, 6]). These motifs are characteristic for a large number of microbes. In 1995, it was found that oligodeoxynucleotides (ODN) with CpG motifs stimulate immune cells. Due to the powerful and promising effects observed in animal models, CpG ODN (hereafter referred to as CpG) were applied and successful in clinical trials. Today, there is already a large knowledge both of basic biological aspects as well as therapeutic approaches of CpG. Nevertheless, much more needs to be learned in order to fully identify the mechanisms of action, and make optimal use for novel therapies.

As outlined in the literature and at this workshop on TLR, CpG can be used for prevention and treatment of infection, allergy and cancer [5]. There are numerous researchers and several companies currently developing new therapies taking advantage of CpG, either as single agent or in combination with other drugs. Here I focus on the fact that CpG are promising vaccine adjuvants.

CpG as vaccine adjuvant for antibody responses

Adding CpG to vaccine formulations may result in strongly improved strength and kinetics of antibody responses, as demonstrated for example for human vaccination against hepatitis B [7]. This has central importance, since the number of booster vaccines can be reduced, and the problem of vaccine non-responders is resolved. Indeed, CpG should soon become available for routine vaccination. CpG used as B cell adjuvants are described extensively in the literature [5, 6]; for reasons of limited space this subject is not further discussed here.

CpG as vaccine adjuvant for T cell responses against cancer

There is a relatively large consensus that immune protection against malignant disease requires antigen specific (adaptive) immune responses including T cells.

Some experts argue that stimulation of the innate immune system alone may be sufficient to generate tumor specific immunity, since cancer tissue often produces tumor antigen allowing low level activation of antigen specific immune responses. Therefore, an increasing number of novel immunotherapies are developed without taking advantage of (synthetic or recombinant) tumor antigens, probably also because this "antigenless approach" simplifies drug production and application. Our experience is different, because we repetitively found that endogenously expressed tumor antigen is not present at the appropriate time, quantity and/or location. Consequently, immune responses generated around naturally expressed antigen are not sufficiently timed, strong and/or anatomically focused to protect from tumor progression. In addition, immunotherapy without antigen often requires high and in part toxic drug doses, in contrast to vaccines containing synthetic antigens that can have powerful effects already at low doses. For these reasons we propose that synthetic cancer vaccines should contain tumor antigens.

A rational development of novel therapies requires detailed revelation of mechanisms of action. The postulated mechanisms should not only be revealed in basic (animal) models. Similarly to what is progressively installed e.g. in AIDS and hepatitis research, in depth investigation in cancer patients is necessary to improve our insight in biological mechanisms of novel therapies. Early phase (I) clinical studies are well suited to check whether those mechanisms that were established in basic models are actually functional in humans.

Of course this is much easier said than done. For example, DC are difficult to assess, because they are infrequent, and it remains very difficult to trace activated DC in vivo. Hopefully, future technologies will enable to identify DC and to characterize how they react to various stimuli, and how they subsequently impact on immune responses. By contrast, a field where great progress has already been made during the last decade is for investigation of T cells.

What is the benefit of analyzing T cells in patients?

T cells are highly "sensitive detectors" of immune stimulation. Upon natural infection with a virus, antigen specific naïve T cells expand during the first 1–2 weeks to reach high T cell frequencies, with up to 10^5 fold expansion [8, 9] thus exceeding the proliferative potential of most other cells in the body! In contrast, ineffective T cell triggering leads to much lower numbers of T cells, which are less likely to protect from disease. Besides these quantitative aspects, the quality of T cell responses is likely to be important. For example, recent data suggest that T cell responses should include both effector and memory T cells [10]. Therefore, novel immunotherapies should elicit substantial numbers of T cells, and profoundly impact on T cell differentiation such that effector cells are capable to destroy tumor cells, and that memory cells assure long term maintenance of responses.

Ex vivo assessment of T cell responses

To obtain precise information on quantity and quality of immune responses, T cells should be investigated directly ex vivo. By doing so, we have performed a number of clinical studies with a rational design to develop CD8 T cell based immunotherapy in melanoma patients. These studies were based on the HLA-A2/Melan-A^{MART-1} antigenic system, which constitutes a well defined model situation for studies of CD8 T cell responses in humans [11–13]. In untreated melanoma patients, Melan-A specific (multimer+) CD8 T cells can regularly be identified at relatively high frequencies in melanoma metastases (1–15 % of CD8 T cells). In peripheral blood of untreated patients, Melan-A specific T cells are rarely increased in frequency, but a significant fraction is antigen-experienced, in contrast to the exclusively naïve cells found in healthy controls.

Development of a T cell vaccine against melanoma

For the identification of optimal adjuvants, it is necessary to perform clinical vaccination studies with direct comparison between interesting candidates of vaccine formulations. With this strategy, we have treated stage III/IV melanoma patients with different vaccines all containing the Melan-A analog peptide ELAGIGILTV, and measured Melan-A specific CD8 T cell frequencies directly ex vivo in circulating blood [14]. After vaccination with peptide in saline, T cell frequencies were comparable to healthy donors and thus not increased. After vaccination with peptide mixed together with QS-21 and MPL, only 1 of 12 patients showed an increased frequency of T cells. In contrast, vaccination with peptide in Incomplete Freund's Adjuvant (IFA; Montanide ISA-51; provided by Seppic) leads to increased frequencies in about half of vaccinated patients [15]. Much more strikingly, the addition of the synthetic CpG oligodeoxynucleotide 7909 (provided by Coley Pharmaceutical Group and Pfizer) gave strongly and consistently increased frequencies in all patients, reaching a mean of > 1 % of Melan-A specific cells among circulating CD8 T cells [16]. Besides triggering quite high T cell frequencies, vaccination with CpG, IFA and peptide was efficient to promote effector cell differentiation, such that effector functions reached high levels comparable to what is found in EBV and CMV specific T cells [17]. In conclusion, the best "conventional" and widely available adjuvant is IFA (Montanide ISA-51), and the addition of CpG 7909 leads to roughly tenfold higher T cell frequencies [16], indicating that CpG in conjunction with antigen, are the strongest known stimulators for induction of human CD8 T cell responses.

Reviewing the literature reveals that various other vaccine types induce lower T cell frequencies. This accounts for many different approaches such as peptide pulsed DC [18], recombinant vaccinia viruses expressing peptides and B7 [19] or multiple T cell antigens [20], peptide with IL-12 [21], or peptides with IFA and anti-CTLA-4 mAb [22].

Comparable conclusions can be drawn from similar studies with gp100 analog peptides, albeit no results are yet reported using CpG in conjunction with gp100 antigen. Similar to the studies with Melan-A, various studies also suggest that IFA leads to better T cell activation as opposed to other vaccine formulations [23–25]. In our studies, we used low doses of CpG, IFA and peptide, which was necessary since it was a first-time application of this combination in humans. Meanwhile, it became clear that one may apply higher doses. With regard to the peptide/IFA mix, higher doses can indeed induce higher T cell frequencies [25]. Possibly, higher doses of CpG, IFA and peptide will finally be capable to reach the high levels of CD8 T cell frequencies, and strong effector function, as observed in acute viral diseases, representing benchmarks in the development of synthetic T cell vaccines.

Why are CpG so effective as adjuvants?

How do CpG promote T cell responses in vivo? It is likely that multiple mechanisms are involved. CpG primarily trigger plasmacytoid dendritic cells (pDC) which are inefficient in antigen presentation but release large amounts of type I interferons that activate NK cells and T cells [26–30]. Besides pDCs and B cells, CpG may perhaps also directly activate NK cells which respond by IFN-γ and TNF-α secretion. All this promotes activation of myeloid DC which then leads to activation of antigen specific cytotoxic CD8 T cells.

Triggering multiple DC and/or TLR

Besides TLR-9 mediated activation, triggering of other TLR may be beneficial for the generation of T cell responses [10, 31]. Since pre-clinical data are promising, such new TLR ligands should also be tested as vaccine adjuvants in humans. Recent animal studies suggest that multiple TLR need to be triggered in order to obtain robust and thus protective immune responses [10]. Since immune protection may require not only effector but also memory T cell subsets, future research should focus on the question of how memory cells are induced, and whether this involves different DC and/or triggering of further TLR.

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Illuminating the Mode of Action of Toll-like Receptor-7 and -8 Agonists in Dermatology

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Toll-like receptor-7 and -8 agonists represent a new group of immune response modifiers including imiquimod and resiquimod (R-848). Imiquimod is used for topical treatment of genital warts induced by human papillomavirus. In addition, imiquimod was also successfully applied for the treatment of several benign and malignant skin tumors including, verruca vulgaris, actinic keratosis, extragenital Bowen's disease and basal cell carcinomas. In the majority of cases the regression of epithelial lesions under imiquimod treament is associated with a local inflammatory response, usually presenting as erythema.

Treatment of genital warts resulted in an increase of mRNA levels of several cytokines like interferon-α (IFNα), IFNβ, IFNγ, tumor necrosis factor-α (TNFα) and interleukin (IL) 12p40. In addition transcript levels for 2',5'oligoadenylate synthetase, CD4 and CD8 were also increased while CD1a was decreased. In vitro studies have also shown that imiquimod is able to induce several cytokines including IFNα, TNFα, IL6, IL8 and IL1 receptor antagonists. Cytokine induction has been demonstrated in different cells, involved in immune responses, but the strongest effects were found in monocytes and macrophages. Although imiquimod does not induce T-cell proliferation or expression of IL2 and IL2R directly, T-helper cytokine production (IFNγ) may be induced indirectly via IL12. Since IL4 and IL5 production in peripheral blood mononuclear cells is repressed by imiquimod, the induced T-cell response is of the Th1 type. Based on these findings the clinical regression of epithelial lesions under imiquimod treatment most likely involves the elimination of virus infected or dysplastic/neoplastic cells by induction of both innate and cellular immune responses.

Remission of clinical lesions, however, is not always associated with signs of local inflammation suggesting the possible induction of other mechanisms that may be involved in the resolution of epithelial lesions during imiquimod treatment. A possible mechanism by which cells were removed without inflammatory response is represented by apoptosis.

CpG Oligodeoxynucleotides in Malignant Glioblastoma

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Glioblastoma (GBM) is the most frequent malignant glioma in adults. Despite surgical resection and radiotherapy (RT), the prognosis in these patients remains poor, with a median survival around 12 months. When recurrence occurs, the efficacy of chemotherapy is limited and the median survival is around 6 months.

Synthetic phosphorothioate oligodeoxynucleotides (ODNs) containing unmethylated CpG dinucleotides (CpG ODNs) are strong activator of both innate and adaptive immunity, and drive the immune response towards the Th1 phenotype. In cancer, the identification of tumor antigens is a limiting step for the design of therapeutic vaccines. To overcome this problem, CpG ODNs alone can be directly injected into the tumor, expecting that the immune system will select the most relevant antigens. In addition, CpG ODNs activate innate immunity (natural killer cells and macrophages) which can directly kill tumor cells. The validity of such

approach was shown in a several animal models, including malignant glioma.

On the basis of our preclinical data, we initiated clinical trials to assess the feasibility and safety of local injections of CpG ODNs in patients with recurrent glioblastoma. Direct infusion of a phosphorothioate CpG ODNs (CpG-28, Oligovax) into recurrent GBM was achieved by implanted catheters and high-flow microinfusion. This technique allows fluids to be distributed by bulk flow (convection) through the interstitial spaces and spread throughout distant areas of the brain. Catheters were placed using stereotactic guidance and targeted the contrast-enhanced areas. Three to six patients were treated with escalating doses of CpG-28 (0.5 to 20 mg), and patients were observed for at least 4 months.

Twenty-four patients entered the trial. All patients had previously been treated with radiotherapy and most patients had received one or several types of chemotherapy. Median age was 58 years (25–73) and the median Karnowski performance status (KPS) was 80 % (60–100 %).

Adverse effects possibly or probably related to the studied drug were moderate. There were no treatment-related deaths, and we found no evidence for drug-induced edema, or demyelinizating disease after administration of CpG-28. Transient neurological worsening was the most significant toxicity observed in our patients treated at the 10 or 20 mg dose level. The mechanism underlying this worsening is unclear and was not related to increased edema. This worsening regressed spontaneously, and was severe enough to be considered as a potential dose-limiting toxicity (DLT) in only one patient. In the month following administration of CpG-28, short partial seizures possibly related to the treatment occurred in 2 patients. Increased body temperature (> 38 °C) was noted in 5 patients (1 patient in level 3, 2 patients in level 5 and 2 patients in level 6). Fever peaked on day 3 (max. 39.3 °C in one patient), was well tolerated, and disappeared within 5 days without antibiotics. Seven patients experienced grade 3 lymphopenia (< 500/mm^3), 3 of whom had already a grade 2 lymphopenia at the time of inclusion. This lymphopenia recovered in all cases on day 60, and was not associated with any infectious diseases. The relationship with the studied drug is possible, although no dose relationship was found.

Efficacy was not the primary objective of this phase 1 trial, conducted on previously heavily treated patients and with escalating doses. Median survival was 7.2 months, and 1 year survival was 29 %, in this population mostly treated at time of second or third recurrence. These figures compare favorably with previous trials using temozolomide at first recurrence in glioblastomas, in which median survival was around 6 months, and 1 year survival was under 15 %. These numbers also compare favorably with our institution's database, in which the median survival of glioblastoma patients with good performance status (KPS > 60 %) was 6.5 months at time of first recurrence and 4.6 months at time of second recurrence. Preliminary evidence of activity is further suggested by the minor radiological response observed in 2 patients, although radiographic responses may not be an adequate tool for evaluation of therapies based on local immunostimulation because worsening in contrast enhancement may be secondary to local inflammation rather than tumor progression.

In summary, local treatment with CpG ODNs in recurrent glioblastoma patients is feasible and well tolerated at doses up to 20 mg. Main side effects were limited to transient worsening of neurological condition and fever. Further improvements can be suggested for next trials. Convection-enhanced delivery is subjected to a number of variables, such as back-flow along the catheters or marked heterogeneity of drug distribution within tumor. Administration by more than 2 catheters might improve CpG ODN distribution, but it should be underlined that complete coverage of the tumor mass is not theoretically needed in a regimen that aims to trigger an immune response. A phase II trial is currently on-going.

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Clinical Development of Toll-like Receptor-9 Agonists

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The clinical development of CpG Toll-like receptor-9 (TLR9) agonists has advanced significantly in recent years after the immunstimulatory effects of CpG containing oligonucleotides were discovered in the early 90's by Arthur M. Krieg, then at the University of Iowa, Iowa City, USA. A status of the various development programs will be given in this presentation.

Hitherto 10 TLR have been identified in humans and each of them recognizes a unique molecular pattern of foreign pathogens. One of the TLR, TLR9, detects pathogen DNA, which can be mimicked by synthetic oligonucleotides containing specific CpG motifs. In contrast to most other TLR, TLR9 is highly restricted in its expression to B cells and plasmacytoid dendritic cells, which appears to be crucial for the high efficacy and good tolerability of synthetic CPG TLR9 agonists as they have been designed and are being developed by Coley Pharmaceutical. Administration of CPG TLR9 agonists induces a comprehensive immune response by activating both, a rapid innate immune response and a longer term adaptive response. In recent years we have discovered several distinct classes of CPG TLR9 agonists, which are designed for targeted, disease specific responses. Only two classes are currently tested in humans and both have demonstrated clinical activity in various areas including cancers and hepatitis C virus infection while being generally well-tolerated.

In cancer PF-3512676 (also known as CPG7909 or Promune) is in pivotal phase III registration trials in first-line advanced lung cancer performed by our partner Pfizer after having achieved highly encouraging results in Coley's randomized multinational phase II trial in first-line non-small cell lung cancer and objective responses in other advanced tumors as well. PF-3512676 belongs to the so called CpG B-class and was well tolerated at active dose levels.

In infectious diseases Actilon or CPG10101 is currently in phase II combination trials in the treatment of chronic hepatitis C virus infection based on positive activity observed both as single-agent and in combination therapy of HCV. Actilon was designed to induce a broad anti-HCV activity and as a TLR9 agonist falls into the CPG C-class. Actilon recently received fast track designation by the FDA for treatment in refractory patients. Actilon is generally well-tolerated alone and in combination with interferon and ribavirin.

VaxImmune, Coley's third drug candidate is in clinical development with several external partners as GlaxoSmithKline and Novartis as an adjuvant for vaccine development in infectious diseases and cancers.

Together with our partner sanofi-aventis we are designing and developing specific CPG TLR9 agonists in the area of asthma, allergic rhinitis and COPD.

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