CIMT meets STRATEGIES FOR IMMUNE THERAPY

CANCERIMMUNOTHERAPY

April 12-14, 2007
Würzburg

5th ANNUAL MEETING

WWW.C-IMT.ORG

ORGANIZER:

Association for Immunotherapy of Cancer
Strategies for Immune Therapy

CIMT 2007 PROGRAM

Thursday
April 12 (8:30 - 17:30)

8:00

9:00

08:30-08:45
Welcoming Address

8:45 - 10:15
Keynote Lectures

10:00

10:15-10:45
Coffee Break

11:00

10:45 - 12:45
Session 1
„Defenses against Infectious Pathogens“

12:00

13:00

13:00 - 14:15
Lunch Break

14:00

14:15 - 15:45
Poster Session I
Guided Poster Session + Coffee

15:00

16:00

16:00 - 17:30
Session 2
„Biotech boosts Tumorimmunology“

17:00

18:00

Friday
April 13 (8:30 - 17:30)

8:00

9:00

8:30 - 11:40
Session 3
„Tumor Vaccines“

10:00

10:30 - 11:00
Coffee Break

11:00

11:40 - 13:00
Session 4
„Effector Mechanisms in Antibody Based Cancer Therapy“

12:00

13:00

13:00 - 14:00
Lunch Break

14:00

14:00 - 15:30
Poster Session II
Guided Poster Session + Coffee

15:00

16:00

15:30 - 16:50
Session 4 (cont.)
„Effector Mechanisms in Antibody Based Cancer Therapy“

17:00

16:50 - 17:30
Session 5
„Development of Immunity“

18:00

20:00 ***Dinner at Fürstensaal Residenz Würzburg***

Saturday
April 14 (8:30 - 16:45)

8:00

9:00

8:30 - 10:30
Session 5 (cont.)
„Development of Immunity“

10:00

10:30 - 11:00
Coffee Break

11:00

11:00 - 13:50
Session 6
„Host-pathogen Interaction“

12:00

13:00

14:00

13:50 - 14:30
Lunch Break

14:30 - 16:15
Satellite Session
„CIMT Monitoring Panel“

15:00

16:00

16:15 - 16:45
Round Table Discussion

17:00

18:00

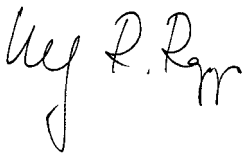
Welcoming Address

Dear Colleagues,

The organizing committee welcomes you to the CIMT meets "Strategies for Immune Therapy" Conference 2007 to be held in Würzburg (Germany) from April 12th to April 14th, 2007. After the successful meeting last year in Mainz, we have again joined efforts and resources and combined the 5th Annual Meeting of the Association for Immunotherapy of Cancer (CIMT) with the 3rd International Conference "Strategies for Immune Therapy" (SFIT).

New approaches utilizing the immune system in the fight against cancer and infections hold great promise. Many advanced clinical trials are currently underway and we will certainly hear about the latest developments during this conference. We have tried our best to bring truly outstanding international speakers covering various aspects of immunotherapy to Würzburg. In addition, we will offer again guided poster sessions to provide young talent with the opportunity to present their work and to receive fruitful feedback. We will also harbor the 4th meeting of the "CIMT Monitoring Panel" on April 14th. As last year we are going to videotape the scientific sessions in order to give all CIMT members, attendees as well as colleagues who are not able to join us the opportunity to benefit from our conference.

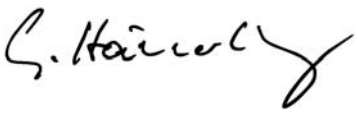
We are sure that your participation will contribute to making the meeting an exciting event and look forward to having you with us in Würzburg.



U. Rapp
(Würzburg)

Ch. Huber
(Mainz)

H.G. Rammensee
(Tübingen)



G. Hämmerling
(Heidelberg)

G. Schuler
(Erlangen)

P. Walden
(Berlin)

Th. Wölfel
(Mainz)

Program Committee and Organization

Program Committee

- Members of the Board of the CIMT consortium and T. Wölfel (Mainz)
- Members of the Board of "Strategies for Immune Therapy"

Scientific Secretary

- S. Kreiter (Mainz)
- R. Schreck (Würzburg)

Administration IKT e.V.

- E. Hauler (Mainz)
- A. Valerian (Mainz)

CIMT Office 2007

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BayGene (München)

GCWN: Deutsch-Französisches
Graduiertenkolleg 1142 “Signal
Transduction: Where Cancer and Infection
Converge” (Würzburg-Nizza)

Deutsch-Französische Hochschule
(Saarbrücken)

Graduiertenkolleg 520 “Immunomodulation”
(Würzburg)

Transregio 17

SFB 479 “Erregervariabilität und
Wirtsreaktion bei infektiösen
Krankheitsprozessen“ (Würzburg)

SFB / Transregio 34 "Pathophysiologie von
Staphylokokken" (Greifswald-Würzburg)

Scientific Program Meeting 2007
Day 1 (12th April 2007)

8:30-8:45	**Welcoming Address** *U. R. Rapp (Würzburg), T. Wölfel (Mainz)*

Keynote Lectures

Chairperson:	*U. R. Rapp (Würzburg)*
08:45-09:30	Infections and cancer: novel modes of cancer prevention *H. zur Hausen (Heidelberg)*
09:30-10:15	Bone marrow transplantation revised *F. Melchers (Berlin)*
10:15-10:45	**Coffee Break**

Session 1: Defenses against Infectious Pathogens

Chairperson:	*E. Lemichez (Nice) / J. Hacker (Würzburg)*
10:45-11:25	Helicobacter pylori: An update on vaccine development and correlates of immunity *T. Aebischer (Edinburgh)*
11:25-12:05	Synergies between V-regions and innate receptors on lymphocytes *J. Demengeot (Oeiras)*
12:05-12:45	Host microbe interactions in the gastrointestinal tract *J. Hacker (Würzburg)*
13:00-14:15	**Lunch Break**
13:15-14:00	**Press Conference**

Poster Session I

14:15-15:45	**Guided Poster Session & Coffee**

Session 2: Biotech boosts Tumorimmunology

Chairperson:	*H. Schild (Mainz) / P. Prossart (Tübingen)*
16:00-16:30	Novel antibody therapeutics for solid cancers *Ö. Türeci (Ganymed, Mainz)*
16:30-17:00	Human Monoclonal Antibodies for Therapy *M. Sproll (Morphosys, Martinsried)*
17:00-17:30	The benefits of biomedical venturing *M. Krul (ISA Pharmaceuticals, Leiden)*

Scientific Program Meeting 2007
Day 2 (13th April 2007)

Session 3:	**Tumor Vaccines**
Chairperson:	*U. Sahin (Mainz) / P. Walden (Berlin)*
08:30-09:10	Vaccination strategies in patients with renal cell carcinomas *P. Brossart (Tübingen)*
09:10-09:50	DNA fusion vaccines against prostate cancer: from the bench to the bedside *C. Ottensmeier (Southampton)*
09:50-11:00	**Coffee Break**
11:00-11:40	Listeria based vaccines for breast and cervical cancer *Y. Paterson (Philadelphia)*
Session 4:	**Effector Mechanisms in Antibody Based Cancer Therapy**
Chairperson:	*E-B. Bröcker(Würzburg), J. Fensterle (Frankfurt)*
11:40-12:20	Antibodies and their receptors: linking innate and adaptive immunity *J. V. Ravetch (New York)*
12:20-13:00	Complement and cellular cytotoxicity in antibody therapy of cancer *G. Weiner (Iowa City)*
13:00-14:00	**Lunch Break**
Poster Session II	
14:00-15.30	**Guided Poster Session & Coffee**
Session 4 (cont.):	**Effector Mechanisms in Antibody Based Cancer Therapy**
Chairperson:	*J. C. Becker (Würzburg), J. Fensterle (Frankfurt)*
15:30-16:10	Antibody-based vascular tumor targeting: from the bench to the clinic *D. Neri (Zurich)*
16:10-16:50	Antibody-cytokine fusion proteins: applications in cancer therapy *M. L. Penichet (Los Angeles)*
Session 5:	**Development of Immunity**
Chairperson:	*T. Wölfel (Mainz)*
16:50-17:30	Foxp3 positive Treg: generation, lifestyle and function *H. von Boehmer (Boston)*
20:00	**Dinner at "Fürstensaal Residenz Würzburg"**

Scientific Program Meeting 2007
Day 3 (14th April 2007)

Session 5 (cont.): Development of Immunity

Chairperson:	*T. Wölfel (Mainz)*
8:30-9:10	How promiscuity promotes tolerance *B. Kyewski (Heidelberg)*
9:10-9:50	Tumor-specific T-regulatory cells in tumor-specific vaccination strategies *S.H. van der Burg (Leiden)*
9:50-10:30	Quality strategies in self/non-self discrimination *T. Boehm (Freiburg)*
10:30-11:00	**Coffee Break**

Session 6: Host-pathogen interaction

Chairperson:	*D. Bumann (Hannover) / T. Aebischer (Edinburgh)*
11:00-11:40	Myeloid-derived suppressor cells in cancer: a novel target for therapeutic invention *V. Bronte (Padua)*
11:40-12:30	Uropathogenic E. coli CNF1 toxin molecular mechanism of action and application in vaccinology *E. Lemichez (Nice)*
12:30-13:10	System-level analysis of Salmonella-host interactions *D. Bumann (Hannover)*
13:10-13:45	**Lunch**

Satellite Session "CIMT Monitoring Panel"

Chairperson:	*S. H. van der Burg (Leiden) / C. Gouttefangeas (Tübingen)*
13:45 – 14:15	Direct access to CD4 T cells specific for defined antigens according to CD154 expression *A. Thiel (Berlin)*
14:15 – 14:45	HLA class II tetramers for enumeration and phenotyping of hepatitis C virus specific CD4+ T cells *H. M. Diepolder (Munich)*
14:45 – 15:15	Lessons learned from interlaboratory testing *C. M. Britten (Leiden)*
15:15 – 15:30	Announcement of new panel activities
15:30 – 16:00	**Round Table Discussion**

ABSTRACTS:

POSTER-

PRESENTATIONS

General Information

Abstracts that have been selected for poster presentation have been divided into six sessions:

- **Session 1:** "Vaccination Strategies"
- **Session 2:** "Monitoring of Immune Responses"
- **Session 3:** "Targets"
- **Session 4:** "Dendritic Cells"
- **Session 5:** "Antibodies"
- **Session 6:** "Adoptive Transfer, Mediators & Cytokines"

Posters will be discussed in a guided poster session. Presenting authors should be prepared to present and discuss their posters (about 5-10 minutes time).

Session	**1**	**2**	**3**	**4**	**5**	**6**
Title	Vaccination Strategies	Monitoring of Immune Responses	Targets	DC	Antibodies	Adoptive Transfer, Mediators & Cytokines
Date	12.04.07	13.04.07	13.04.07	12.04.07	13.04.07	12.04.07
Time	14:15	14:00	14:00	14:15	14:00	14:15
Chaired by	M. Schmitt (Ulm)	H. Veelken (Freiburg)	S. Stevanovic (Tübingen)	E. Schultz (Marburg)	M. Gramatzki (Kiel)	W. Herr (Mainz)
Poster Number	1	7	3	11	12	2
	5	8	4	19	18	9
	6	13	15	22	21	10
	20	16	17	25	32	14
	23	34	24	26	44	29
	33	35	28	27	57	39
	41	36	31	30		49
	47	37	38	42		51
	56	40	43	50		54
	58	46	45	52		55
		48	53			59
		61	60			63
		62				

POSTER PRESENTATIONS

P01 Upregulation of antigen processing machinery components at mRNA level in acute lymphoblastic leukemia cells after CD40 stimulation

W. Łuczyński, O. Kowalczuk, E. Iłendo, A. Stasiak-Barmuta, M. Krawczuk-Rybak

P02 First force spectroscopy measurements of physiologic VLA-4 integrin activation by the chemokine SDF-1 at the single-molecule level on a living cell

R. H. Eibl, M.D.

P03 Distorted relation between mRNA copy number and corresponding major histocompatibility complex ligand density on the cell surface

A. O. Weinzierl, C. Lemmel, O. Schoor, M. Müller, T. Krüger, D. Wernet, J. Hennenlotter, A. Stenzl, K. Klingeln, H.-G. Rammensee and S. Stevanović

P04 Melanoma-associated chondroitin sulphate proteoglycan (MCSP): a promising target antigen for cancer immunotherapy?

C. Erfurt, I. Haendle, K. Thielemans, P. van der Bruggen, G. Schuler and E. S. Schultz

P05 Regression of pancreatic carcinoma by intratumoral injection of a streptoccal lysate in mice

C. Maletzki, M. Linnebacher, B. Kreikemeyer, F. Charrier, J. Emmrich

P06 Cancer Immunotherapy based on recombinant *Salmonella enterica* serovar Typhimurium *aroA* strains secreting prostate-specific antigen

J. Fensterle, W. Goebel, C. Hotz, S. R. Meyer, U. R Rapp and I. Gentschev

P07 Cellular immune responses against the cancer testis antigen SPAN-XB in healthy donors and patients with multiple myeloma

C. Frank, M. Hundemer, A. D. Ho, H. Goldschmidt and M. Witzens-Harig

P08 High Frequency of T Regulatory Cells in Patients with B-Cell Chronic Lymphocytic Leukemia (B-CLL) Is Decreased by Thalidomide and Fludarabine Treatment

K. Giannopoulos, M. Schmitt, P. Własiuk, J. Chen, A. Bojarska-Junak, M. Kowal, J. Roliński and A. Dmoszyńska

P09 The CCR5Δ32 gene polymorphism as a predictor of survival in melanoma patients receiving immunotherapy

D. Schrama, S. Ugurel, M. Zapatka, J. C. Becker

P10 Interleukin 21-based stimulation can induce human B cells to secrete Granzyme B and gain cytotoxic potential

B. Jahrsdörfer, S. E. Blackwell and G. J. Weiner

P11 Surface expression of endocytosis receptors and endocytotic properties of monocyte derived dendritic cells are modulated by cytokine supplementation

M. Staudinger, M. Peipp, C. Kellner, M. Gramatzki, R. Repp

P12 Characterization of granulocyte-mediated antibody-dependent cytotoxicity: Reactive oxygen intermediates, perforin, caspases, cell conjugate formation and membrane lipid exchange

H. Horner, Y. Wachter, C. Frank, M. Herrmann, B. Stockmeyer

P13 Long peptide vaccine-induced migration of HPV16 specific type 1 and 2 T-cells into the lesions of VIN III patients

M. J. P. Welters, G. G. Kenter, M. J. G. Löwik, T. M. A. Berends-van der Meer, A. P. G. Vloon, J. W. Drijfhout, A. R. P. M. Valentijn, J. Oostendorp, G. J. Fleuren, R. Offringa, S. H. van der Burg and C. J. M. Melief

P14 Acute myeloid leukemia (AML)-reactive cytotoxic T lymphocyte clones rapidly expanded from CD8+ CD62L(high)+ T cells of healthy donors prevent AML engraftment in NOD/SCID IL2Rγnull mice

E. Distler, C. Wölfel, S. Köhler, M. Nonn, N. Kaus, E. Schnürer, R. G. Meyer, T. C. Wehler, C. Huber, T. Wölfel, U. F. Hartwig, W. Herr

P15 Leukemia-associated antigens (LAAs) as biomarkers of survival in acute myeloid leukemia (AML)

J. Greiner, L. Bullinger, J. Chen, K. Giannopoulos, K. Döhner, A. Schmitt, R. F. Schlenk, J. R. Pollack, H. Döhner, M. Schmitt

P16 Characterization of Myeloid suppressor cells in patients with Hepatocellular carcinoma

B. Höchst, L. Ormandy, T. Greten, F. Korangy

P17 Development of a peptide-vaccine targeting ovarian carcinomas and their vasculature

H. Hörzer, S. Stevanovic, A. Marmé, P. Altevogt, D. Wallwiener, B. Gückel

P18 Chimerization of Murine Hybridoma Antibodies by Homologous Recombination

S. Lüttgau, G. Moldenhauer, G. J Hämmerling, F. Breitling

P19 Optimized peptide delivery to dendritic cells (DCs): Increased T cell stimulatory capacity by loading DCs with complexes of cationic, antigenic peptides and Poly I/C dsRNA

H. Haenssle, P. Riedl, R. Schirmbeck, K. Zachmann, C. Neumann, J. Reimann

P20 RHAMM/CD168-R3 peptide vaccination of patients with acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), multiple myeloma (MM) and chronic lymphatic leukemia (CLL) elicits immunological and clinical responses

M. Schmitt, A. Schmitt, K. Giannopoulos, J. Chen, L. Li, P. Liebisch, M. Ringhoffer, P. Guillaume, G. Ritter, M. Rojewski, S. Gnjatic, H. Döhner, J. Greiner

P21 target cell-restricted stimulation of the CD95 (APO-1/FAs) Death receptor with various Bispecific CD20XCD95 antibodies

T. Otz, L. Große-Hovest, H.-G. Rammensee and G. Jung

P22 Development of new antigen-loading strategies of DC for vaccination

K. Birkholz, J. Dörrie, N. Schaft, M. Schwenkert, C. Kellner, E. Kämpgen, G. Fey, G. Schuler

P23 Dendritic cell therapy of patients with hormone refractory prostate cancer

R. Maier, R. de Giuli, J. Hering, M. Fopp, R. von Moos, C. Böhme, H.-P. Schmid, T. Cerny, S. Gillessen and B. Ludewig

P24 A Murine pancreatic adenocarcinoma cell line is highly immunogenic and causes cell mediated and humoral immune responses

F. Zhao, B. Vermeer, A. I. Garbe, M. P. Manns, F. Korangy, T. F. Greten

P25 Successful maturation of HM 1.24-presenting DCs in multiple myeloma patients

N. Schub, J. Catalcali, M. Staudinger, B. Gahn, A. Humpe, R. Repp, M. Gramatzki

P26 In-vitro-maturation of human monocyte-derived dendritic cells in presence of acid-treated gram-negative bacteria (R- and S-form) under serum-free conditions

B. Hildenbrand, M. Azemar, B. Sauer, M. Zimmermann, S. F. Martin, M. Huber, D. Lorenzen, C. Unger, M. A. Freudenberg and C. Galanos

P27 Highly purified R-form LPS from Salmonella in combination with IFN-γ enhance maturation and DC1-polarisation of human monocyte-derived dendritic cells under serum-free conditions

B. Hildenbrand, M. Azemar, B. Sauer, M. Zimmermann, S. F. Martin, M. Huber, D. Lorenzen, C. Unger, M. A. Freudenberg and C. Galanos

P28 Immunogenicity of FAPα-derived peptide epitopes in murine models

V. Hofmeister, H. Voigt, R. Ullrich, J. C. Becker

P29 Tumor antigen specific memory T cells in a transgenic mouse model of spontaneous melanoma

O. Abschuetz, A. Figl, W. Osen, D. Schadendorf, V. Umansky

P30 Tolerogenic dendritic cells in Ret-transgenic mouse model of spontaneous melanoma

F. Zhao, D. Schadendorf, V. Umansky

P31 Coordinated expression of clustered MAGEA and CSAG (TRAG-3) cancer/testis genes

A. Bredenbeck, V. M Hollstein, U. Trefzer, W. Sterry, P. Walden and F. O Losch

P32 Co-culture of human multicellular tumour spheroids (MCTS) and peripheral blood mononuclear cells (PBMC) with trifunctional antibodies

T. Leidig, F. Wawrsinek and W. Mueller-Klieser

P 33 DNA vaccines in B-cell lymphoma

D. Fioretti, S. Iurescia, E. Signori, G. Tonon, V. M. Fazio, M. Rinaldi

P34 Phenotypes and functions of tumor-infiltrating lymphocytes in renal cell cancer patients

S. Attig, J. Hennenlotter, A. Stenzl, S. Koch, G. Pawelec, H.-G. Rammensee, C. Gouttefangeas

P35 Characterization of tumor-specific versus virus-specific tumor-infiltrating and peripheral T cells from melanoma patients

S. Gross, U. Trefzer, W. Sterry and P. Walden

P36 Serological mini-arrays for the analysis of humoral immune response to tumor-associated antigens

Y. Shebzukhov, S. Khlgatian, A. Sazykin, P. Belousov, D. Kuprash and S. Nedospasov

P37 Analysis of regulatory T cells in patients with multiple myeloma

B. Hülsmann, U. Buttkereit, S. Ebert, K. Lennartz, M. Lindemann, W. Stellberg, D. Brandhorst, M. R. Nowrousian, P. Schütt, T. Moritz, B. Opalka

P38 Identification of a novel CTL epitope from melanoma/melanocyte-specific protein, KU-MEL-1

E. Derhovanessian, V. S. Meyer, S. Stevanovic, S. Ugurel, D. Schadendorf, G. Pawelec

P39 Uropathogenic E.coli but not commensale E.coli infection activates TLR-4 MyD independent signaling pathways in rat testicular cells

S. Bhushan, S. Tchatalbachev, J. Klug, T. Chakraborty, C. Pineau, A. Meinhardt

P40 Characterization of MUC-1 specific T-cells in breast cancer patients and healthy donors

A.-C. Bâgu, S. Kayser, S. Stevanović, A. Marmé, I. Gruber, D. Wallwiener, S. Attig, B. Gückel

P41 HSP70-antigen fusion proteins in combination with TLR ligands are efficient tumor vaccines

B. Marincek, G. J. Hämmerling, F. Momburg

P42 A comprehensive comparison of PGE2-matured and poly(I:C)-matured DC reveals differences in cytokine and IDO expression but comparable T cell-stimulating abilities

I. Möller, N. Frech, H. Veelken, A.-K. T.-Kaskel

P43 Recognition of methylated peptides by tumour-specific T cells in melanoma patients

S. Jarmalavicius and P. Walden

P44 Detection of IL2, CD25, and CD64 mRNA from spheroids co-cultured with PBMC and treated with trifunctional antibody catumaxomab

B. Rodday, T. Leidig, S. Walenta and W. Mueller-Klieser

P45 Shared MHC class I epitopes prevent T cell responses against unique tumor antigens through in situ activation of regulatory T cells in experimental antitumor vaccination

M. Buchner, M. Warncke, H. Veelken

P46 Activation of tumor specific T and B lymphocytes after radiofrequency ablation in cancer patients

M. Widenmeyer, S. P. Haen, Y. Shebzukhov, S. Clasen, D. Schmidt, A. Stenzl, H.-G. Rammensee, P. L. Pereira, C. Gouttefangeas

P47 WT-1 peptide vaccination after allogenic stem cell transplantation for patients with myeloid malignancies

C. Gentilini, A. Müßig, A. Letsch, A. Asemissen, K. Rieger, C. Scheibenbogen, E. Thiel, L. Uharek

P48 Characterisation of WT1-specific T cell responses in Peripheral Blood and Bone Marrow in leukemia patients vaccinated with WT1 peptide, GM-CSF, and KLH

A. Letsch, U. Keilholz, A. Busse, A. M. Asemissen, A. Schmittel, W.-K. Hofmann, L. Uharek, I. W. Blau, E. Thiel, C. Scheibenbogen

P49 Phase I clinical trial: Adoptive transfer of *in vitro* expanded tumor specific autologous T cells in patients with advanced ovarian cancer

A. Wiernik, C.-C. Johansson, I. Poschke, L. Adamson, K. Bergfeldt, G. Masucci, R. Kiessling

P50 CD 40 ligation during dendritic cell maturation reduces cell death and prevents IL-10 induced regression to macrophage-like monocytes

T. Buhl, H. Haenssle, S. Bram, U. Krueger, K. Reich, C. Neumann

P51 Polymorphism in genes coding for molecules associated with antitumor immune response in malignant melanoma and prostate cancer

E. Naumova, G. Gaudernack, A. Mihaylova, S. Mihaylova, M. Ivanova, E. M. Inderberg Suso, D. Baltadjieva

P52 Type I interferons are critical in the control T cell responses induced by dendritic cells activated by combined Toll-like receptor ligation

T. Warger, P. Osterloh, G. Rechtsteiner, U. Kalinke, T. Bopp, H. Schild, M P Radsak

P53 Analysis of the immunogenicity of a FLT3 internal tandem duplication (FLT3-ITD)

C. Graf, F. Heidel, S. Tenzer, M. P. Radsak, F. K. Solem, C. M. Britten, C. Huber, T. Fischer and T. Wölfel

P54 Expression and immune-inhibitory function of MIF in ovarian cancer

M. Krockenberger, Y. Dombrowski, C. Weidler, M. Ossadnik, J. Wischhusen

P55 A shift in the intestinal microflora towards pro-inflammatory bacteria and signaling via toll-like receptors 2 and 4 trigger early onset of Graft-versus-Host disease

A. Nogai, M. M. Heimesaat, S. Bereswill, E. Thiel, U. B. Göbel, L. Uharek

P56 Anti tumor vaccines based on stabilized mRNA (RNActive®)

M. Fotin-Mleczek, B. Scheel, I. Hoerr and J. Probst

P57 Generation of an improved recombinant bispecific antibody molecule and B7 fusion proteins for targeted cancer immunotherapy

D. Müller, B. Meißburger, K. Frey, A. Karle, I. Höfig, R. Stork and R. E. Kontermann

P58 The pharmaceutical formulation of peptide antigens and of toll like receptor (TLR-) agonists and the route of antigen delivery determines the strength of an antigen specific CD8+ T cell response

A. Konur, A. Graser, I. Klamp, S. Kreiter, A. Selmi, M. Diken, C. Huber, U. Sahin

P59 Cytotoxic T-cell mediated killing of cancer cells: The importance of the target epitope

R. Baek Sørensen, A. Kirkin, H. Voigt, I. M. Svane, J. C. Becker, P. thor Straten, M. H. Andersen

P60 Efficient tumor cell lysis mediated by a Bcl-X(L) specific T-cell clone isolated from a breast cancer patient

R. Baek Sørensen, S. Reker, T. Køllgaard, I. M. Svane, M. H. Andersen, P. thor Straten

P61 Spontaneous Immune Responses and Antigen expression in Patients with Acute Myeloid Leukemia

T. Køllgaard, S. R. Hadrup, O. Juul, T. Seremet, M. H. Andersen, P. thor Straten

P62 The 1 ml immunoscope: a rapid and robust FACS based assay for the identification and enumeration of lymphocyte populations

G. Ioannidis, J. Stasakova, A. Williams, C. Ottensmeier

P63 Memory $CD8^+$ T cells mediate antibacterial immunity via CCL3 activation of TNF/ROI^+ phagocytes

E. Narni-Mancinelli, L. Campisi, D. Bassand, J. Cazareth, P. Gounon, N. Glaichenhaus and G. Lauvau

Upregulation of antigen processing machinery components at mRNA level in acute lymphoblastic leukemia cells after CD40 stimulation

W. Łuczyński[1], O. Kowalczuk[2], E. Iłendo[3], A. Stasiak-Barmuta[4], M. Krawczuk-Rybak[1]

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The development of immunotherapy in hematologic malignancies has been observed in the last few years. One of the approaches is the use of cancer vaccines based on leukemia-derived dendritic cells (DC). Recent studies from our and other laboratories have shown that CD40 stimulation improves leukemia cells immunogenicity and generates an antitumor immune response. The design of future cancer vaccines requires the knowledge concerning the function of dendritic cells including antigen processing.

The aim of our present study was the assessment of antigen-processing machinery (APM) components in acute lymphoblastic leukemia (ALL) cells before and after CD40 stimulation at mRNA level.

Material and methods

Twenty five children with ALL were enrolled into the study. Leukemic cells were stimulated (or not) with CD40L and IL-4. Elements of antigen processing machinery (MB1, LMP2, LMP7, LMP10, TAP1, TAP2, calnexin, calreticulin, tapasin, ERp57, zeta, delta) were determined by real-time PCR technique. Expression of important costimulatory and adhesion molecules considered as DC markers (CD40, CD54, CD80, CD83, CD86) were determined at mRNA (PCR) and protein (flow cytometry) level.

Results:

1) we noted upregulation of all costimulatory and adhesion molecules at mRNA and protein level in ALL cells after the culture

2) the significant rise in expression of nearly all APM components after CD40 stimulation was observed.

This confirms specific stimulation of antigen processing system in ALL cells by CD40L. Future work should focus on clinical significance of these findings for immunotherapy in leukemias.

First force spectroscopy measurements of physiologic VLA-4 integrin activation by the chemokine SDF-1 at the single-molecule level on a living cell

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Recently, we became the first to quantify the rupture force of an individual pair of integrin VLA-4 / VCAM-1 adhesion receptors between two living cells [1]. Here, we further optimize this new and unique method of AFM based force spectroscopy to measure the long-time postulated and discrete activation states of the integrin VLA-4 receptor to elucidate the physiologic regulations of binding strength. Integrin VLA-4 / VCAM-1 interactions are used e.g. by circulating lymphocytes for rolling and rapid arrest within a blood vessel – an important step for migrating cells from one place to another. The chemokine SDF-1 can induce this immediate stop of rolling lymphocytes via its corresponding chemokine receptor, CXCR4. To investigate the suggested activation states of the VLA-4 receptor, increasing concentrations of the chemokine SDF-1 are used through the experiment: Physiologic concentrations of SDF-1 lead to higher rupture forces per single VLA-4 / VCAM-1 receptor-ligand pair in the physiologic environment of a living cell, whereas higher concentrations of SDF-1, i.e. an overdose leads to the striking phenomenon of 'desensitization' of the chemokine receptor CXCR4. As a control, pertussis toxin (PTX), a known inhibitor of this signaling cascade, clearly prevents the activation of the VLA-4 receptors.

In this study AFM based single-molecule force spectroscopy has been developed into a completely new pharmacological test for receptor-ligand interactions and their physiologic activation and regulation on living cells. This model appears to be extremely useful for studying known and unknown signal transduction cascades not only limited to integrins' outside-in and inside-out signaling. This technique elucidates postulated mechanisms involved in normal and pathological leukocyte homing and possibly cancer stem cell trafficking and it will be used to develop new drugs/inhibitors against organ-specific tumor (stem) cell metastasis and for immune disorders such as multiple sclerosis, rheumatoid arthritis and other diseases.

This report demonstrates how powerful a new and rather interdisciplinary approach between immunology and nanotechnology can be. To the best of our knowledge to date, the experiments presented are the first direct evidence of affinity changes and their regulation of individual integrin receptors at the single-molecule level on a living cell.

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Distorted relation between mRNA copy number and corresponding major histocompatibility complex ligand density on the cell surface

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The major histocompatibility complex (MHC) presents peptides derived from degraded cellular proteins to T-cells and is thus crucial for triggering specific immune responses against viral infections or cancer. Up to now, there has been no evidence for a correlation between levels of mRNA (the "transcriptome") and the density of MHC:peptide complexes (the "MHC ligandome") on cells. Since such dependencies are of intrinsic importance for the detailed understanding of translation efficiency and protein turnover, and thus for systems biology in general and for tumour immunotherapy in practical application, we quantitatively analysed the levels of mRNA and corresponding MHC ligand densities in samples of renal cell carcinomas and their autologous normal kidney tissues. Relative quantification was carried out by gene chip analysis and by stable isotope peptide labelling, respectively. In comparing more than 270 pairs of gene expression and corresponding peptide presentation ratios, we demonstrate that there is no clear correlation ($R = 0.32$) between mRNA levels and corresponding MHC peptide levels in RCC. A significant number of peptides presented predominantly on tumour or normal tissue showed no or only minor changes in mRNA expression levels. In several cases, peptides could even be identified in spite of the virtual absence of the respective mRNA. Thus we conclude that a majority of epitopes from tumour associated antigens will not be found in approaches based mainly on mRNA expression studies, as mRNA expression reflects a distorted picture of the situation on the cell surface as visible for T cells.

Melanoma-associated chondroitin sulfate proteoglycan (MCSP): a promising target antigen for cancer immunotherapy?

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To avoid immune escape by downregulation or loss of antigen by the tumor cells target antigens are needed which are important for the malignant phenotype and survival of the tumor. We could identify a $CD4^+$ T cell epitope derived from the human melanoma-associated chondroitin sulfate proteoglycan (MCSP) (also known as high molecular weight-melanoma-associated antigen=HMW-MAA), which is strongly expressed on > 90% of human melanoma lesions and is important for the motility and invasion of melanoma cells. However, MCSP is not strictly tumor-specific as it is also expressed in a variety of normal tissues. Therefore, self tolerance should prevent the induction of strong T cell responses against these antigens by vaccination strategies. On the other hand, breaking self tolerance to this antigen by effectively manipulating the immune system, might mediate anti-tumor responses although it would bear the risk of autoimmunity. Surprisingly, we could readily isolate $CD4^+$ T helper cells from the blood of a healthy donor recognizing peptide $MCSP_{693\text{-}709}$ on HLA-DR11 expressing melanoma cells. Broad T cell reactivity against this antigen could be detected in the peripheral blood of both, healthy donors and melanoma patients, without any apparent signs of autoimmune disease. In some patients, a decline of MCSP-specific T cell reactivity was observed upon tumor progression. Our data indicate that CD4+ T cells are capable of recognizing a membrane glycoprotein that is important in melanoma cell function, and it may be possible that the sizable reactivity to this antigen in most normal individuals contributes to immune surveillance against cancer.

Regression of pancreatic carcinoma by intratumoral injection of a streptoccal lysate in mice

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BACKGROUND & AIMS: Recently, we could show that a single intratumoral (i.t.) injection of live S. pyogenes serotype M49 resulted in complete regression of pancreatic carcinoma in a syngeneic mouse model. The antitumoral effect was mediated by both unspecific and specific immune reactions against tumor cells (Pancreas 2006; 33: 480). Hence, in this study we analyzed the potential of a streptococcal lysate to induce tumor regression without the risk of sepsis caused by live bacteria.

METHODS: Established Panc02 tumors were treated with two i.t. injections of the streptococcal lysate (day 1 and 4). Animals of the control group received equivalent volumes of vehicle only. 7, 14, 21 and 28 days after the first lysate injection animals were sacrificed and tumors were removed for histological and immunohistochemical analyses. In addition, flow cytometry was performed to detect lymphocyte subpopulations in peripheral blood and spleens. Cytotoxicity of lymphocytes against cancerous and non-cancerous cells was detected by LDH release. Supplementary, we examined apoptosis and necrosis in CFSE-stained tumor cells by flow cytometry to characterize the cytolytic activity of immunological effector cells.

RESULTS: Injection of the streptococcal lysate caused rapid tumor growth inhibition and resulted in nearly complete regression within 28 days (189.6±74.0mm³ vs. control 1369.1±426.2mm³). This antitumoral effect was accompanied by a massive infiltration of granulocytes, $CD4^+$ and $CD8^+$ T-cells into the tumor. By means of flow cytometry, numbers of granulocytes, monocytes and NK-cells in the blood were elevated during the whole observation period. Beyond it, analysis of splenocyte subsets revealed significant increases in pre B-cells (49.2±1.8% vs. control 27.0±5.7%) at day 7 as well as raised levels of activated T-cells (17.9±2.1% vs. control 8.2±0.8%) on day 21 post lysate application. Concomitantly, lytic activity of lymph node-derived lymphocytes against tumor cells was highest at day 21 (45.5±13.0% vs. control 3.5±1.8%). Continuing analyses identified the induced tumor cell death as mainly necrotic at day 14 (19.3±8.3% vs. control 3.2±1.8%) and primarily apoptotic at later time points.

CONCLUSIONS: Our data demonstrate the potential of a streptococcal lysate in the treatment of Panc02 pancreatic carcinoma. The bacterial components are able to induce immune reactions against tumor cells mediating the observed tumor regression. We conclude that this safe immunotherapy may be a promising new strategy for the treatment of pancreatic carcinoma that deserves further investigation.

Cancer Immunotherapy based on recombinant *Salmonella enterica* serovar Typhimurium *aroA* strains secreting prostate-specific antigen

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Prostate cancer is the most common malignant tumor in men and is normally associated with increased serum levels of prostate-specific antigen (PSA). Therefore, PSA is one potential target for a prostate cancer vaccine. In this study we analyzed the functionality of new bacterial PSA vaccines, expressed and secreted via the hemolysin (HlyA) secretion system of *Escherichia coli*, the prototype of the Type I secretion system (T1SS). The data demonstrate that T1SS can be successfully used for delivery of PSA via an attenuated *Salmonella enterica* serovar Typhimurium *aroA* strain as carrier to induce cytotoxic CD8 T cell responses; resulting in an efficient prevention of tumor growth in mice.

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Cellular immune responses against the cancer testis antigen SPAN-XB in healthy donors and patients with multiple myeloma

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The cancer-testis antigen SPAN-XB has been recently identified in multiple myeloma. In the present study we identified and characterized for the first time a cytotoxic cellular immune response against SPAN-XB in healthy donors and patients with multiple myeloma. Using two independent computer algorithms two SPAN-XB derived peptides (peptides 624 and 626) with predicted binding to HLA-A2 were identified. To further improve the immunogenicity of peptide 626 we designed a heteroclitic peptide (peptide 627) by modifying one amino acid on the HLA binding position 2 of peptide 626. Using an IFN-γ Elispot assay we could demonstrate the presence and functional activity of CD8 peptide specific T cells with all tested peptides. By analysis of peripheral blood of 13 healthy donors and 5 patients with multiple myeloma peptide specific T-cell precursors specifically recognizing at least one of the tested peptides could be detected and expanded in 9 of 13 of tested donors and 3 of 5 tested patients. Importantly, in two donors specific peptides could be generated against the heteroclitic peptide 627 but not against the native peptide 626. We conclude that SPAN-XB derived peptides can elicit a consistent CD8 T cell response in healthy donors and patients with multiple myeloma.

High Frequency of T Regulatory Cells in Patients with B-Cell Chronic Lymphocytic Leukemia (B-CLL) Is Decreased by Thalidomide and Fludarabine Treatment

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The control of the rather slowly progressing B-cell chronic lymphocytic leukemia (B-CLL) by the immune system is not yet fully understood. The forkhead family transcription factor FOXP-3 is critically important for the development and function of T regulatory cells (Tregs). In this study the expression of FOXP-3 was assessed in 60 previously untreated patients with B-CLL. In B-CLL patients the subpopulation of CD4+CD25^{high}FOXP3+ T cells that phenotypically correspond to Tregs was identified. The frequency of Tregs was significantly higher in B-CLL patients compared to healthy volunteers (HVs) (12.1% vs. 1.9%, p=0.0001). The progressive increase of Tregs percentages were noted in advanced stages of disease, mean: 9.5% in stage A, 13.4% in stage B and 15.4% in stage C according to the Binet classification. Immunosuppressive function of Tregs was evaluated in an enzyme-linked immunosorbent spot (ELISpot) assay to estimate T-cell immune responses against HLA-A2 restricted epitopes derived from tumor associated antigens (TAAs) of survivin, fibromodulin and RHAMM. Higher frequencies of Tregs correlated with decreased T cell responsiveness against viral and tumor antigens. In 80% CLL patients treated with thalidomide (THAL) + fludarabine (FLU) regiment significant reduction of circulating Tregs after THAL was observed. The combination with FLU resulted in further decrease of Tregs in 12 of 15 patients. After THAL therapy the mean reduction was higher in the population of Tregs compared to whole lymphocyte population (41.2% vs. 19.9%, p=0.046). Therefore the strategies combining THAL are very promising, as THAL might target not only the CLL cell compartment but also Tregs and therefore restore the CD8+ T cell function. We conclude that Tregs presented in high frequencies in B-CLL constitute the crucial immunosuppressive mechanism among the mononuclear cells of the peripheral blood.

Key words: B-cell chronic lymphocytic leukemia (B-CLL), T regulatory cell (Tregs) tumor associated antigens (TAAs), cytotoxic T lymphocytes (CTL), receptor for hyaluronic acid mediated motility (RHAMM/CD168), survivin, fibromodulin, thalidomide

The CCR5Δ32 gene polymorphism as a predictor of survival in melanoma patients receiving immunotherapy

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Purpose: Chemokines are supposed to influence both tumor progression and anti-tumoral immune responses. In this regard, a 32-bp-deletion polymorphism in the chemokine receptor 5 gene (CCR5Δ32) resulting in a non-functional receptor protein allows to study the potential impact of the CCR5 system on the clinical course and treatment outcome of malignant diseases.

Patients and Methods: CCR5 genotyping was performed by PCR on DNA extracted from serum samples of 782 cutaneous melanoma patients with known disease history and long-term clinical follow-up. Genotypes were correlated with patient survival in consideration of different therapeutic modalities.

Results: Out of 782 patients, 90 (11.5%) were heterozygous and 12 (1.5%) were homozygous for CCR5Δ32. Disease-specific survival starting from primary diagnosis was not influenced by CCR5 status. Equally, no impact of the CCR5 status could be detected on treatment outcomes of stage III patients. In 139 stage IV patients treated with immunotherapeutics, however, CCR5Δ32 was associated with a diminished survival compared to patients not carrying the Δ32 allele ($p=0.029$). Multivariate analysis revealed the CCR5 genotype as an independent factor impacting disease-specific survival in this patient group ($p=0.0084$), followed by gender ($p=0.017$), and type of primary ($p=0.029$). Vice versa, stage IV patients without immunotherapy revealed a trend towards a favorable survival when harboring CCR5Δ32 ($p=0.12$).

Conclusion: The disadvantage of CCR5Δ32-bearing stage IV melanoma patients with respect to outcome of immunotherapy indicates a strong impact of the CCR5 mediator system on anti-tumoral immune responses, and should be taken into account when choosing therapeutic modalities for these patients.

Interleukin 21-based stimulation can induce human B cells to secrete Granzyme B and gain cytotoxic potential

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B cells are not currently known to be capable of producing granzyme B or being cytotoxic. We found that human B cells activated with Interleukin 21 (IL-21) and antibodies to the B cell receptor (BCR) or immunostimulatory oligonucleotides (CpG ODN), can produce and secrete granzyme B. Various methods including granzyme B ELISpot, intracellular staining for granzyme-B, quantitative real time RT-PCR for granzyme B messenger RNA and gene expression array confirmed our finding in B cells obtained from the peripheral blood of normal individuals, various B cell lines as well as malignant B cells from patients with B chronic lymphocytic leukemia (B-CLL). This granzyme B was functional as demonstrated by cleavage of a granzyme B-sensitive colorimetric substrate. Interestingly, B-CLL cells treated with IL-21 and CpG ODN induced apoptosis of untreated bystander B-CLL cells in a granzyme B-dependent manner. We conclude that IL-21-based therapy can induce B cells to produce functional granzyme B and that at least some malignant B cells can develop into cells with cytotoxic potential. These unexpected findings could have significant implications on our understanding of the role of B cells in immune regulation and for a variety of immune phenomena including auto-, cancer and infectious immunity. Furthermore the finding that IL-21 plus CpG ODN are potent inducers of apoptosis in B-CLL cells provides rationale for evaluating this combination as a treatment for B cell malignancies, and possibly other cancers.

Surface expression of endocytosis receptors and endocytotic properties of monocyte derived dendritic cells are modulated by cytokine supplementation

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Dendritic cells (DCs) are professional antigen presenting cells, which can induce and regulate adaptive immune responses. Therefore, DCs are attractive candidates for anti-tumor vaccination strategies. DCs pulsed with antigenic protein or HLA-I haplotype restricted antigenic peptides allow for activation of either antigen specific $CD4^+$ helper T cells or $CD8^+$ cytotoxic T cells. In contrast, receptor mediated uptake of antigen would enable DCs to activate both, T cell help and cytotoxic T cells, by cross presentation of peptides in a MHC class I and II restricted manner. Moreover, this approach may open up new perspectives to finally overcome *in vitro* manipulation of monocyte derived DCs by *in vivo* application of antigens specifically directed to DCs. To establish a protocol for analysing an efficient antigen delivery to DCs through antigen capturing receptors, we comparatively investigated cells exhibiting a DC-like phenotype derived from isolated monocytes of healthy donors by cultivation using three different sets of cytokines (IL4/GM-CSF, IFNα/GM-CSF, or IFNβ/IL3). These DCs have been analysed for their surface expression levels of Fc and lectine receptors useable for antigen uptake, their endocytotic capacities and their sensitivity to maturating proinflammatory cytokines or toll-like receptor ligands. To direct antigens to specific receptors expressed on DCs for antigen loading, fusion proteins of variable regions of antibodies against different uptake receptors (FcγRI/CD64, FcαRI/CD89, Dec205/CD205, DC-Sign/CD209) and GFP as well as tumor antigens have been designed, expressed in eucaryotic cell lines and purified. Data verifying binding of these molecules to monocyte derived DCs and receptor mediated antigen loading of DCs will be presented.

Characterization of granulocyte-mediated antibody-dependent cytotoxicity: Reactive oxygen intermediates, perforin, caspases, cell conjugate formation and membrane lipid exchange

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Antibody-dependent polymorphonuclear granulocytes (PMN) -mediated cytotoxicity may play an important role in the control of malignant diseases. However, little is known which particular pathways are employed for killing of malignant cells by PMN. Production of reactive oxygen intermediates (ROI) has been observed to occur during antibody-dependent cell-mediated cytotoxicity (ADCC). However, PMN from a patient with chronic granulomatous disease showed a strong capability to perform ADCC. Recent data suggest a role for perforins in PMN-mediated cytotoxicity. However, in our assays concanamycin A - an inhibitor of perforin mediated ADCC by mononuclear cells - had no inhibitory, but even a stimulatory effect on PMN-mediated ADCC. Employing electron microscopy we observed that PMN and their target cells intimately interact with the formation of interdigitating membrane protrusions. During PMN and target cell contact fluorescent membrane lipid dyes were mutually exchanged. This was strongly increased in the presence of tumor targeting antibodies. The presence of transient PMN-tumor cell aggregates and the accumulation of PMN with tumor cell-derived membrane lipids and *vice versa* were associated with effective ADCC measured by chromium release or apoptosis induction in Raji lymphoma cells. The role of apoptosis in PMN-mediated ADCC of HER-2/neu transfected Raji cells was further investigated by inhibition of caspases. Treatment of lymphoma target cells with the pan caspase inhibitor z-VAD-fmk significantly reduced PMN-mediated HER-2/neu directed ADCC (51±3%, and 31±4% mean±SEM percent specific lysis, respectively, $p=0.00003$, $n=6$). Pre-incubation of isolated PMN with z-VAD-fmk had no effect on z-VAD-fmk treated or untreated Raji cells (28.0±3.0%, and 48.9±3.4%, respectively). Interestingly, HLA class II directed ADCC was not affected by z-VAD-fmk. As chromium release could not be blocked completely by the pan caspase inhibitor z-VAD-fmk, caspase independent apoptosis mediated by NFκB, mitochondrial release of apoptosis inducing factor or induction of necrosis may play a role in PMN mediated target cell death.

Long peptide vaccine-induced migration of HPV16 specific type 1 and 2 T-cells into the lesions of VIN III patients

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Genital infections with oncogenic types of human papillomavirus (e.g.HPV16) is very common. In general, such infections are cleared by the immune system but in a minority of subject the immune system fails to control the persistent virus and malignancies develop. Previously, we demonstrated that long overlapping HPV16 E6 and E7 long peptide vaccine in Montanide ISA 51 was safe and able to elicit strong HPV16 specific T-cell response in 25 out of 34 end-stage cervical cancer patients.

Based on these results a phase II study was started in which, 20 patients with histologically proven HPV16+ vulvar intraepithelial neoplasia (VIN) grade III are subcutaneously vaccinated 4 times at a 3-week interval. Immunological monitoring was performed at the systemic level by the analysis of blood samples, drawn before and after each vaccination, and at the local level by the analysis of HPV16-specific T-cells in tissue biopsies of the VIN lesion (before and after vaccination) as well as a biopsy from the last vaccination site.

IFNγ-ELISPOT analysis revealed that 92% of the patients mounted a T-cell response to multiple regions of HPV16 E6 and 75% of the patients against HPV16 E7, already after 2 vaccinations. This strong and broad vaccine-induced systemic T-cell response was also detected by proliferation assays, in which T-cell reactivity was associated with the production of IFNγ and IL-5 similar to the cytokine profile of the HPV16-specific memory T-cell responses observed in healthy individuals. Notably, in each patient both CD4+ and CD8+ HPV16-specific T-cells were detected after vaccination. Analysis of the local immune response demonstrated that after vaccination HPV16-specific Th1/Th2 cells infiltrated both the vaccination site and/or the VIN lesion in 6 out of 11 patients analyzed. Clinical improvements at 3 month follow-up could be measured in 7 out of 11 patients; 4 patients were without any symptoms and in 3 patients a histological complete response was observed. In 2 of these latter patients HPV16 DNA was even no longer detectable anymore.

In conclusion, our peptide-based vaccine elicits a robust and broad HPV16-specific T-cell response with the capacity to migrate into the persistently HPV16-infected lesion of patients with high grade VIN lesions. This resulted in relief of symptoms and even clinically and histologically complete responses.

Acute myeloid leukemia (AML)-reactive cytotoxic T lymphocyte clones rapidly expanded from CD8^{+} CD62L$^{(high)+}$ T cells of healthy donors prevent AML engraftment in NOD/SCID IL2Rγ^{null} mice

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Current in vitro techniques for isolating acute myeloid leukemia (AML)-reactive cytotoxic T lymphocytes (CTLs) from healthy donors are of relatively low efficiency and yield responder populations with unknown biological significance. We established an allogeneic mini-mixed lymphocyte-leukemia culture (mini-MLLC) approach by stimulating donor CD8^{+} T cells with HLA class I-matched primary AML blasts in microtiter plates. Before culture, CD8^{+} T cells were separated into CD62L$^{(high)+}$ and CD62L$^{(low)+/neg}$ subsets enriched for naive/central memory and effector memory cells, respectively. In 8 different related and unrelated donor/AML pairs, numerous CTL populations were isolated that specifically lysed myeloid leukemias in association with various HLA-A, -B, or -C alleles. These CTLs expressed T-cell receptors of single Vβ chain families, indicating their clonal origin. The majority of CTL clones were obtained from mini-MLLCs initiated with CD62L$^{(high)+}$ cells. Using antigen-specific stimulation, multiple CTL populations were amplified to 10^{8}-10^{10} cells within 6-8 weeks. Two representative CTL clones were capable of completely preventing the engraftment of human primary AML blasts in NOD/SCID IL2Rγ^{null} mice. We concluded that the mini-MLLC approach allows the efficient in vitro expansion of AML-reactive CTL clones from CD8^{+}CD62L$^{(high)+}$ precursors of healthy donors. These CTLs can inhibit AML engraftment in immunodeficient mice, suggesting their potential biological relevance.

Leukemia-associated antigens (LAAs) as biomarkers of survival in acute myeloid leukemia (AML)

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Specific T cell responses against tumor cells might play a critical role in the control of minimal residual disease (MRD) in acute myeloid leukemia (AML). It has become increasingly apparent that Leukemia-associated antigens (LAAs) play a dual role, not only as targets for immunotherapy, but also as biomarkers of disease state, stage, response to treatment and survival. Several LAAs are expressed in acute myeloid leukemia (AML) and elicit specific immune responses of CD8 positive T cells.

In this work, we investigated whether TAAs inducing specific immune responses in AML patients were associated with the clinical outcome. A DNA-microarray analysis of 116 AML samples was performed to correlate expression of TAAs to the clinical outcome. We found a significant correlation of high mRNA expression of the TAA *G250/CA9* with a longer overall survival (P=0.022), a trend for better outcome in patients with high expression levels of *PRAME* (P=0.103), and a hint for *RHAMM/HMMR*. In contrast, for other TAAs like *WT1*, *TERT*, *PRTN3*, *BCL2*, and *LAMR1* we found no correlation with clinical outcome of AML patients. Moreover, co-expression of *RHAMM/HMMR*, *PRAME* and *G250/CA9* provided a favorable prognostic effect (P=0.005). Quantitative RT-PCR based validation of our results demonstrated the power of DNA microarray technology. We detected an association between PRAME, PRTN3, LAMR1 and G250/CA9 and distinct karyotypes.

Additionally, in these AML patients specific T cell responses to TAAs were assessed by ELISPOT analysis, tetramer staining and chromium release assays. We found specific T cell responses at high frequency for these three antigens in AML patients. Positive immune reactions were detected in 8/17 (47%) AML patients for RHAMM/HMMR-R3-derived, in 7/10 (70%) for PRAME-P3-derived, and in 6/10 (60%) for newly characterized G250/CA9-G2-derived peptides. We detected a significant increased immune response of AML patients in complete remission compared to AML patients with refractory disease (P<0.001). Furthermore, we could demonstrate specific lysis of T2 cells and AML blasts presenting these epitope peptides RHAMM/HMMR-R3, PRAME-P3 and G250/CA9-G2. For the LAA PRAME, we investigated the influence of PRAME expression on cell proliferation of AML cell lines *in vitro*. As PRAME is a dominant repressor of retinoic acid receptor (RAR) signaling in the presence of retinoic acid, we detected that cell proliferation and differentiation of different AML cell lines was dependent on PRAME expression.

In conclusion, the expression of the LAAs RHAMM/HMMR, PRAME and G250/CA9 can induce strong anti-leukemic immune responses of CD8 positive T cells possibly enabling the control of MRD in AML patients. Therefore, we have initiated a polyvalent phase I peptide vaccination trial using peptides derived from these three LAAs. The LAAs RHAMM/HMMR, PRAME and G250/CA9 have been shown to play a dual role as biomarkers of AML disease and as target structures for specific immunotherapy.

Characterization of Myeloid suppressor cells in patients with Hepatocellular carcinoma

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Hepatocellular carcinoma is the fifth most common cancer worldwide with a poor prognosis and a survival rate of less than 5 %. Therapeutic options are surgery, transplantation or local therapy like transarterial chemoembolisation (TACE), but a standard chemotherapy is not established. A number of studies including our own have shown that immunotherapy might be a promising option for Hepatocellular carcinoma. We have demonstrated previously that despite tumour-specific humoral and cellular immune responses in HCC patients, the tumours still progress. Therefore, we are interested in delineating the ways that HCC tumours inhibit the host's anti-tumour immune responses.

We have seen that here is significant increase in $CD14^+$ monocytes in the peripheral blood of patients with Hepatocellular carcinoma as well as in the tumour of these patients. Phenotypic characterization of these cells has shown that they are HLA-DR^-, HLA-ABC^+, $CD80^-$ and $CD83^-$. These cells are also unable to stimulate an allogeneic T cell response and can suppress T cell proliferation. We are in the process of further characterization of these cells in patients with Hepatocellular carcinoma, which will help in enhancing the efficacy of immune based therapies in these patients.

Development of a peptide-vaccine targeting ovarian carcinomas and their vasculature

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For ovarian carcinoma (OvCa) drug-resistance remains the major clinical problem, underlining the need for new therapeutical options such as immunotherapeutical approaches. Several OvCa associated antigens have been described but so far there are not many corresponding T cell epitopes known. Ideally, vaccines should be designed to target multiple and stable antigens to overcome tumor escape mechanisms. Endothelial cells stably express vascular endothelial growth factor (VEGF) and its receptor (VEGFR-2), especially under hypoxic conditions in the tumor. Recent findings showed that co-immunization against VEGF/VEGFR-2 and tumor associated antigens in mice exhibited synergistic antitumor effects [1].

The purpose of this work is to develop a multivalent peptide-vaccine targeting ovarian carcinomas as well as their vasculature. Therefore, we verified the expression of OvCa associated antigens from literature on our OvCa cell lines. In the first part of this work we focused on the L1-CAM protein, which is highly overexpressed in OvCa [2] and VEGF which is expressed in some OvCa cell lines and in the tumor vasculature. L1-CAM- and VEGF-derived and HLA-A*02 restricted peptides were predicted by the peptide motif database SYFPEITHI [3] and used for the *in vitro* stimulation of potential tumor- or HUVEC-specific CD8+ T cells. T cell stimulations were performed using peptide-pulsed autologous DC and B cells. After four stimulations, T cells were characterized phenotypically (tetramers, T cell subset markers) and functionally (cytokine expression, cytotoxicity). So far two new L1-CAM epitopes and two VEGF epitopes have been identified. Corresponding CTL-lines have been generated and demonstrated peptide-specific IFNg-release and cytotoxicity respectively. Further, IFNg treated and peptide pulsed tumor cells have been lysed specifically. In the second part of this work it will be analysed if corresponding T cells also pre-exist in ovarian carcinoma patients.

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Chimerization of Murine Hybridoma Antibodies by Homologous Recombination

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During the past three decades mouse monoclonal antibodies (mAbs) have evolved as potent tools in biomedical research and diagnostics. For therapeutic purposes, however, murine Abs are less well suited since they do not efficiently activate effector functions of the human immune system. In addition, they frequently provoke a human anti-mouse antibody response in patients. To overcome these problems molecular engineering is applied for the chimerization or humanization of therapeutic Abs.

We have developed a novel technique allowing the fast chimerization of murine hybridoma Abs. Our chimerization process is based on targeting the constant regions of the Ab genes by homologous recombination in murine hybridoma cells. Unlike other efforts in exchanging the murine variable domains against human ones we target the endogenous immunoglobulin locus by homologous recombination in the hybridoma cell without the additional introduction of any selection marker gene. We have shown that successfully recombined hybridoma cells can be isolated directly via surface displayed human Abs by Fluorescence Activated Cell Sorting (FACS), verified by ELISA and subcloned by limited dilution. Thus, isolation of cells expressing chimeric heavy and/or light chains after homologous recombination (1 in 1-3x10^8 cells) has proven feasible without prior positive or negative selection. Achieving homologous recombination while preserving Ab genes in their original chromosomal environment without modification of the surrounding genomic areas by introduction of selection marker genes or specific recombination sites guarantees stable and high Ab expression levels of the chimerized hybridoma cells. Yet two plasmids have been cloned for the chimerization of murine immunoglobulin constant heavy gamma 1 and light kappa chain loci. In principle with a limited number of plasmids all other immunoglobulin isotypes can be modified in the same way.

The feasibility of this new approach was demonstrated for the murine hybridoma cell line HEA125, which produces an anti-EpCAM Ab of IgG1 isotype with kappa light chains. In the resulting chimeric mAb chiHEA125 no more Ab heavy or light chain constant region protein of murine origin was detectable either by Western Blot, ELISA or FACS analysis. Sequencing of chiHEA125 cDNA has revealed the exact human constant region sequences spliced together with the murine HEA125 variable domains as expected after homologous recombination. During the whole chimerization process the chiHEA125 Ab has retained its high affinity to EpCAM. Preclinical testing has revealed efficient induction of both complement-mediated lysis (CDC) and activation of tumor-specific cytotoxicity by NK cells (ADCC) in vitro, both in a concentration dependant manner. Since EpCAM is overexpressed on many adenocarcinomas, the chimeric huHEA125 Ab may develop into a valuable reagent for tumour therapy e.g. in breast and ovarian cancer. As a proof of feasibility, our method was successfully applied for the chimerization of two additional murine hybridomas.

Taken together we are now able to chimerize any murine hybridoma of therapeutical interest that expresses a significant level of surface displayed immunoglobulin splice variant besides the secreted form. This holds true for 11 out of 13 randomly choosen hybridomas we tested for surface expression. The whole chimerization process with production of chimeric Ab included takes about six months with only a few days of hands on work.

Optimized peptide delivery to dendritic cells (DCs): Increased T cell stimulatory capacity by loading DCs with complexes of cationic, antigenic peptides and Poly I/C dsRNA

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In recent years dendritic cells (DCs) have become popular candidates in cancer vaccine development because of their crucial role in inducing T-cell responses. The aim of the present study is to develop a novel vaccination strategy utilizing DCs loaded with synthetic, cationic (positively charged), and antigenic peptides complexed to negatively charged Poly I/C dsRNA. The positively charged protein transduction domain of the HIV-1 TAT protein was shown to rapidly translocate across the plasma membrane of living cells. This property can be exploited for the delivery of proteins, drugs, and genes into cells. Although the mechanism of translocation is not yet understood we adapted this system by fusing an antigenic, major histocompatibility complex-class I-binding epitope (Melan-A/Mart-1 sequence: ELAGIGILTV) with a cationic sequence derived from the HIV tat transduction domain (tat49-57: RKKRRQRRR). The cationic fusion peptide compound is then mixed with a negatively charged Poly I/C dsRNA compound to quantitatively form peptide/nucleic acid complexes. We found that the loading of DCs with complexes of cationic, antigenic peptides and Poly I/C dsRNA or cationic, antigenic peptides alone did not negatively affect the DC viability. The peptide-Poly I/C complexes readily induced a full DC maturation as measured by surface expression of CD83, CD80, and HLA-DR. When using peptide-Poly I/C complex loaded DCs for two cycles of autologous T cell stimulation a quantitatively superior epitope specific IFN-gamma secretion in comparison to DCs maturated by a cocktail of cytokines and loaded with peptide could be measured in an ELISPOT assay. In conclusion, complexes of cationic, antigenic peptides and Poly I/C dsRNA might be used for a TLR-3 mediated DC maturation and intracellular peptide targeting in a single step. The improved T cell stimulatory capacity of such DCs might be due to a prolongated presentation after intracellular delivery of peptides

RHAMM/CD168-R3 peptide vaccination of patients with acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), multiple myeloma (MM) and chronic lymphatic leukemia (CLL) elicits immunological and clinical responses

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Recently, we initiated a phase I/II R3 peptide vaccination to induce immunological and hematological responses for patients with AML, MDS or MM overexpressing the receptor for hyaluronic acid mediated motility (RHAMM/CD168). RHAMM/CD168 is expressed on tumor cells of most patients with AML, MDS, MM and chronic lymphatic leukemia (CLL). We characterized RHAMM/CD168 as a leukemia-associated antigen (LAA) eliciting both humoral and cellular immune responses in patients with different hematological malignancies. CD8 positive T cells primed with the RHAMM/CD168-derived peptide R3 (ILSLELMKL) were able to lyse autologous AML blasts expressing this LAA. In this study, patients were included with positive RHAMM/CD168 expression but with a limited tumor load. At a biweekly interval, RHAMM R3 peptide (300 mcg for the first 12 patients and 1000 mcg for patients 13-24) emulsified with the incomplete Freund's adjuvant (day 3) and GM-CSF (100 mcg, days 1-5) was administrated four times subcutaneously. The primary aim of the study is safety and feasibility of this peptide vaccination, secondary aims the evaluation of a specific T cell immune response to RHAMM/CD168 R3 peptide and the assessment of the influence of the R3 peptide vaccination on the remission status. Since January 2005, 19 patients (3 AML, 8 MDS, 6 MM, 2 CLL) have been enrolled in the study. The first ten patients (2 AML, 4 MDS, 4 MM) have completed the course of four vaccinations and have been completely evaluated. Therapy related adverse events observed under R3-peptide vaccination were erythema and induration of the skin at the site of injection (CTC I°). In 5/10 patients, we detected an increase of CD8+ R3 tetramer$^+$ CD45RA$^+$CCR7$^-$CD27$^-$ CD28$^-$ effector T cells in flow cytometry in accordance with R3-specific CD8+ T cells in ELISPOT analysis. In chromium release assays specific lysis of RHAMM-positive leukemic blasts were shown for AML patients responded to peptide vaccination. 3/6 patients with myeloid disorders (1/3 AML, 2/3 MDS) achieved clinical responses: one partial and one complete remission (1 PR, 1 CR), and one hematological improvement (1 HI). One patient with MDS did not need any longer erythrocyte transfusion after four vaccinations. Two MM patients responded as assessed by free light chains, two progressed (PD).

Taken together, RHAMM/CD168 induced both immunological and clinical results and therefore constitutes a promising target antigen for immunotherapies in patients with hematological malignancies.

target cell-restricted stimulation of the CD95 (APO-1/FAs) Death receptor with various Bispecific CD20XCD95 antibodies

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It is well established that agonistic antibodies directed to the CD95 (Apo-1/Fas) death receptor induce apoptosis of CD95-positive and sensitive cell lines. However, in vivo the therapeutic use of such monoclonal CD95 antibodies is hampered due to the expression of CD95 on normal cells such as hepatocytes and the application of agonistic antibodies in mice led to fatal hepatic failure (Ogasawara et al., 1993). For in vivo application it seems to be necessary to confine CD95-mediated apoptosis to predefined target cells. This "target cell restriction" can be achieved by using bispecific antibodies with dual specificities: one directed to CD95 and the other to a target antigen on a tumor cell (Jung et al., 2001).

Here we chose the CD20 molecule as a target antigen which is a well established tumor antigen for B-cell lymphomas. We could show that chemically hybridized bispecific Fab_2 fragments consisting of the APO-1 antibody and different CD20 antibodies were able to induce in vitro an effective killing of the Fas-sensitive B-lymphoblastoid cell line SKW6.4 and to a lower extend of the less Fas-sensitive B-lymphoblastoid cell lines JY, BJAB and C1R. In addition we created an antibody in a bispecific single-chain (bs scFv) format with CD20xAPO-1 specificity. This bs scFv antibody like its bs Fab_2 counterpart was also able to kill Fas-sensitive SKW6.4 but less effectively than the bs Fab_2. Experiments in which the therapeutic activity of these various CD20xAPO-1 antibodies is evaluated are ongoing in a

Lymphoma SCID Mouse Model.

Development of new antigen-loading strategies of DC for vaccination

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Although dendritic cell (DC)-based vaccination of tumor patients has shown encouraging responses in melanoma and renal cell carcinoma, still many variables of DC vaccine generation and application have to be addressed, to further improve its clinical efficacy. In order to optimize antigen loading of DC, different methods, such as electroporation of defined tumor-antigen RNA or total tumor RNA, phagocytosis of tumor cells, direct peptide loading, or the use of antibody-antigen constructs need to be thoroughly analyzed and compared. As a model tumor antigen we chose MAGE-A3, a cancer-testis antigen expressed in many tumors including malignant melanoma and multiple myeloma. Furthermore, stimulation of $CD4^+$ or $CD8^+$ T cells electroporated with TCR recognizing certain MHC-presented MAGE-A3 epitopes was used as a functional read-out for antigen-presentation efficiency. As one possible loading strategy we applied MAGE-A3-DCLAMP RNA. The DCLAMP sequence targets the antigen to the lysosomes, and leads to MHC class II presentation of the antigen. Indeed, we saw in preliminary experiments that electroporation of mature (m)DC with MAGE-A3-DCLAMP RNA resulted in HLA-DP4 restricted presentation of the MAGE-A3 peptide KKLLTQHFVQENYLEY (KKL, aa 243-258) and induction of cytokine release (i.e. INFγ and IL-2) by autologous MAGE-A3/DP4-specific $CD4^+$ and $CD8^+$ T cells. For a different loading strategy, we cloned antibody-antigen constructs, consisting of a single-chain variable fragment (scFv) directed against DEC205, an endocytosis receptor expressed on the surface of DC, genetically linked to different parts of the MAGE-A3 antigen (i.e. KKL, EVDPIGHLY (aa 168-176), and a long peptide (aa 110-279)). The MAGE-A3 antigen should be delivered into the DC by receptor-mediated endocytosis, and subsequently presented on MHC class II molecules. We expressed these constructs under osmotic stress conditions in E.coli BL21, and could detect them in western blot analysis. Furthermore, these fusion proteins displayed specific binding to mDC and DEC205-transfected CHO cells. Our next aim is to analyze whether the antigen-loading of DC with our DEC205scFv-MAGE-A3 constructs leads to efficient T cell activation.

Taken together, we have established several tools to thoroughly compare different antigen-loading strategies of DC, which hopefully will contribute to the future generation of better DC-based anti-cancer vaccines.

Dendritic cell therapy of patients with hormone refractory prostate cancer

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The induction of a specific immune response towards tumor-associated antigens (TAA) provides a promising approach in cancer immunotherapy. Therefore autologous monocyte-derived dendritic cells, pulsed with peptides derived from multiple prostate antigens were used to vaccinate patients with hormone-refractory prostate cancer. A strict quality control concerning the expression of surface markers and the migratory capacity of the dendritic cells was performed before application. Each patient was at least vaccinated six times. Comparing the PSA-doubling time before vaccination with that obtained after the second vaccination, a prolonged doubling time in five out of six patients could be observed. The DC vaccine was well tolerated and currently we are recruiting more patients. Sensitive methods to monitor peptide-specific immune responses have been established.

A Murine pancreatic adenocarcinoma cell line is highly immunogenic and causes cell mediated and humoral immune responses

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Pancreatic cancer is one of the leading causes of cancer death worldwide. Patients diagnosed with pancreatic cancer have a 5-year survival rate of less than 5%. One promising method for a successful treatment is immunotherapy. We have generated a transgenic mouse model of TGF-alpha/*p53*$^{-/-}$ that develop spontaneous pancreatic adenocarcinoma within 120 days after birth. A tumor cell line (mPAC) has been isolated from these murine spontaneous pancreatic tumors and characterized *in vivo* and *in vitro*. After being subcutaneously (s.c.) injected into naïve wild type C57Bl/6 mice, the mPAC cell line developed solid tumours within the first seven days and then regressed. In CTL assay, only the immunization with mPAC cell line but not with an irrelevant cell line (e.g. RMA) induced a strong cytotoxic T cell response against mPAC used as targets. In addition, only mPAC vaccinated C57Bl/6 mice generated serum response to mPAC cell line. These results suggest the potential existence of a dominant antigen in the mPAC cell line, which might cause the rejection of the mPAC-derived tumor in wild type C57Bl/6 mice. To identify the antigen, the SEREX (serological analysis of recombinant cDNA expression libraries) method was performed. An expression cDNA library of the mPAC cell line was constructed and screened with sera from the mice challenged with mPAC cell line. 19 positive clones representing 12 different antigens have been identified from the mPAC cDNA library. Among them, 8 clones represent Tnks2 (tankyrase 2, TRF1-interacting ankyrin-related ADP-ribose polymerase 2), a member of the PARP family of proteins. The sera from the mice challenged with mPAC cell line and spontaneous pancreatic adenocarcinoma bearing mice have a higher response to prokaryotic expressed Tankyrase 2 protein than the sera from naive wild type mice. Tankyrase2 is a new candidate for pancreatic cancer tumor antigen.

Successful maturation of HM 1.24-presenting DCs in multiple myeloma patients

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Cellular immunotherapy based on professional tumor-antigen presenting dendritic cells (DC) and specific cytotoxic t-lymphocytes (CTL) is a promising approach in cancer therapy.

The inadequate function of dendritic cells in cancer is one of the major elements of compromised anti-tumor immune response and an important limit of cellular immunotherapy. The impaired maturation of DC in tumor patients due to inhibitory tumor environment factors is reported for lung cancer, breast cancer, renal cancer and others. Several investigations have shown that also in plasmacytoma patients tumor escape mechanisms impair the DC maturation and thereby the development of myeloma-specific CTL.

We investigated the maturation of myeloma patients' peripheral monocytes into tumor-antigen presenting dendritic cells and their functionality. For that purpose we used HM 1.24, a tumor-specific antigen that is expressed in about 70 percent of the myeloma cells. Several investigations have shown that this is a suitable target for tumor-antigen presenting cell based immunotherapeutic approaches.

In 20 myeloma-patients we generated mature DC from peripheral blood monocytes using standard plastic adherence, culture in GM-CSF and IL-4 and cytokine cocktail (IL-1β, IL-6, TNF-α and PGE_2). By flow cytometry we could show that in all patients the immunophenotype of mature DC (CD 33+, HLA-DR+, CD 14-, CD 80+, CD 83+, CD 86+) was expressed in an extent comparable to that from healthy donors.

These data show that the differentiation of monocytes from myeloma patients into phenotypically and functionally mature DC is possible in the same high extent as known from healthy donors' monocytes.

In-vitro-maturation of human monocyte-derived dendritic cells in presence of acid-treated gram-negative bacteria (R- and S-form) under serum-free conditions

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Introduction: Acid-treated gram-negative bacteria are powerful immunostimulatory agents in vivo and have been shown to enhance impaired immune functions in patients. Because they expose lipid A on their surface they are expected to mature dendritic cells most efficiently via Toll-like receptor 4. Moreover, they are non-infectious and optimal carriers for different kinds of antigens. In this work we have tested several acid-treated gram-negative bacteria (R- and S-form) for their capacity to induce maturation of human monocyte-derived dendritic cells (moDC) under serum-free conditions.

Methods: MoDC were generated in serum-free medium with GM-CSF (1000 U/ml) and IL-4 (800 U/ml) for 5 days and matured for another 24 h with acid-treated gram- negative bacteria (R- and S-form) ± IFN-γ. Matured moDC were phenotypically characterised by FACS and measured for IL-6, IL-10 and IL-12 p70 by ELISA.

Results: Our experiments show that moDC grown in serum free medium enriched with GM-CSF and IL-4 can be matured by adding acid-treated gram-negative bacteria. Without IFN-γ the maturation with both R- and S-form acid-treated bacteria resulted in high levels of IL-10 but relatively low levels of IL-12p70 whereas addition of IFN-γ leads to significant higher levels of bioactive IL-12p70 concomitant with lower levels of IL-10. Besides, addition of IFN-γ resulted in more pronounced upregulation of CD83, CD80 and CD86.

Conclusion: All tested killed acid-treated gram-negative bacteria, known as efficient carriers for weak antigens, represent suitable agents for the ex vivo maturation of human moDC under serum-free conditions. Moreover, the combination of acid-treated bacteria and IFN-γ enhances TH1-polarisation of moDC as well as the expression of CD83 and costimulatory molecules.

Highly purified R-form LPS from Salmonella in combination with IFN-γ enhance maturation and DC1-polarisation of human monocyte-derived dendritic cells under serum-free conditions

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Introduction: Highly purified Lipopolysaccharide(LPS)-preparations from R-mutants of Salmonella readily activate mouse cells expressing the Toll-like receptor 4 in serum-free medium independent of the lipid binding proteins LBP and CD14*. Therefore, they are promising agents for maturation of human monocyte derived dendritic cells (moDC) under serum-free conditions. In this work we have tested several highly purified S- and R-form LPS-preparations from wild-type or R-mutants of *Salmonella* for their capacity to mature moDC under serum-free conditions in the presence or absence of LBP and IFN-γ.

Methods: MoDC were generated in serum-free medium with GM-CSF and IL-4 for 5 days and matured for another 24 h with S- or R-form LPS from wild-type or R-mutants of *Salmonella* ± IFN-γ. Matured moDC were phenotypically characterized and IL-6, IL-10 and IL-12 p70 were estimated in culture supernatants by ELISA.

Results: We could show that under serum-free conditions only the R-form LPS readily mature moDC, while the S-form LPS required further assistance of the LBP. Moreover, addition of IFN-γ resulted in a more pronounced upregulation of CD83, major histocompatibility complex class I and B7 molecules and led to significant higher levels of bioactive IL-12p70 concomitant with lower levels of IL-10.

Conclusion: In serum-free medium only highly purified R-form LPS, but not S-form LPS-preparations, induce a fully mature status of moDC, independently from LBP. Moreover, the addition of IFN-γ enhances both the upregulation of costimulatory molecules as well as DC_1-polarisation. Therefore, highly purified LPS-preparations from enterobacterial R-mutants represent promising candidates for the *ex vivo* maturation of human moDC under serum-free conditions.

* Huber, M et al. 2006, Eur J Immunol

Immunogenicity of FAPα-derived peptide epitopes in murine models

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The development and progression of cancer is dependent on the active contribution of the tumor stroma. Cancer-associated fibroblasts, tumor endothelial cells, and tumor-associated macrophages promote tumor development and progression by secretion of growth factors, proteolytic enzymes and angiogenic factors. In contrast to cancer cells, stroma cells are genetically more stable. Hence, focusing on the stroma for anti-tumor therapy avoids treatment resistance. In addition, the selective upregulation or induction of tumor stroma-associated antigens (TSAAs) is a universal phenomenom in carcinogenesis. Therefore, harnessing the immune system against TSAAs may help to control tumor growth and should be widely applicable for the treatment of solid tumors. A promising target for cancer immunotherapy is *fibroblast activation protein* α (FAPα), a TSAA predominantly expressed on cancer associated fibroblasts. By reverse immunology, we identified H-2k^{b}-restricted peptide epitopes derived from murine FAPα. To test the immunogenicity of these peptides in vivo, C57Bl/6J mice were immunised with peptide-loaded dendritic cells and a composite of Incomplete Freund`s Adjuvans (IFA) and peptides, respectively. At different time points, splenocytes of vaccinated mice were isolated and functionally characterized by an IFN-γ ELISPOT assay, revealing a strong immunogenicity of several FAPα-derived T cell epitopes, including the FAPα$^{289-296}$ peptide. Among splenocytes of vaccinated mice FAPα$^{289-296}$-specific CD8+ T cells were detected by H-2kb dextramer/anti-CD8 double staining. The therapeutic efficacy of these FAPα-derived peptides is currently evaluated in the spontaneous murine Grm-1 melanoma model. Together, these results will help to establish a tumor stroma directed vaccination strategy that can be transferred to the treatment of cancer patients.

Tumor antigen specific memory T cells in a transgenic mouse model of spontaneous melanoma

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Melanoma treatment needs urgently new concepts since these tumors are very often resistant to conventional therapies. Memory T cells (MTC) have been shown to react effectively to tumor antigens, to which they were previously exposed. Therefore they could be a source for the generation of therapeutic effector cells. We use a ret transgenic (Ret-tg) mouse model (C57Bl/6 background), in which about 30% of mice spontaneously develop melanomas within 3 months. These tumors express melanoma associated antigens (MAA) like tyrosinase related protein-1 (TRP-1), TRP-2, gp100 and tyrosinase and they resemble human melanomas regarding the localization, histology and genetic risk factors. Antigen unspecific activation of T cells *in vitro* by Concanavalin A or antibodies against CD3 and CD28, as well as *in vivo* immunization with ovalbumin or TRP-2, indicated that the immune system of Ret-tg mice is functionally active. Phenotypical analysis of MTC in spleen and bone marrow (BM) at different stages of melanoma development displayed that central memory T cells (CM) are enriched in these organs in Ret-tg tumor bearing mice as compared to non-transgenic littermates or tumor free Ret-tg mice. Analysis of tumors showed that effector memory T cells are enriched as compared to spleen and BM of the same mouse. Tetramer staining of CD8+ T cells from the BM and tumor of Ret-tg melanoma bearing mice revealed that 77% of mice contained MTC specific for MAA TRP-2 in BM and showed in 60% a memory phenotype (CD44+). We found in 92% of investigated tumors TRP-2 specific CD8 T cells which had in most cases (80%) also a memory phenotype. We suggest that these MAA-specific MTC may leave the BM and migrate into the tumor. In addition, we were able to reactivate MTC from the BM of Ret-tg mice to produce IFN-γ by short-term coculture *in vitro* with BM derived dendritic cells loaded with melanoma cell lysate. In conclusion, our studies established a basis for a new immunotherapeutic approach based on adoptive transfer of re-stimulated MAA specific MTC and MAA-loaded dendritic cells.

Tolerogenic dendritic cells in Ret-transgenic mouse model of spontaneous melanoma

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Dendritic cell (DC) population is heterogeneous. Some DCs can induce and maintain immune tolerance, while others are immunogenic. Immature DCs are tolerogenic because they express low cell-surface levels of MHC molecules, CD40, CD80 and CD86. However, some tolerogenic DCs can also express maturation markers but secret less IL-12 and more IL-10.

To investigate a role of tolerogenic DCs in melanoma, we here employed the Ret-transgenic mouse model (C57Bl/6 background), in which human proto-oncogene ret is overexpressed in melanocytes and which resembles human melanoma with respect to tumor genetics, histopathology and clinical development. After a short latency, around 30% of Ret-tg mice develop melanoma with metastasis in the lymph nodes, lungs and brain.

Phenotype of freshly isolated DCs from the spleen and bone marrow was analyzed by flow cytometry. We found a significant reduction in numbers of $MHCII^+CD11c^+$ DCs (total DCs) as well as $CD40^+$ DCs (mature DCs) in both organs from Ret-tg tumor-bearing mice as compared to control groups (wild type mice or Ret-tg tumor-free mice). However no accumulation of CD40- DCs (immature DCs) could be found. This indicates that in the tumor-bearing Ret-tg mouse, DCs are less efficient in T-cell priming.

We also investigated the phenotype of tumor-infiltrating DCs (TIDCs). A significant increase of $MHCII^+CD11c^+$ DCs can be detected in larger tumor (more than 400 mg of weight). DCs infiltrating larger tumors have more immature phenotype than those infiltrating smaller tumors (less than 400 mg).

To further investigate cytokines produced by DCs, we stimulated freshly isolated $CD11c^+$ splenic DCs *in vitro* with CpG for 24h. It was found that DCs from Ret-tg tumor bearing mice secreted higher amounts of IL-10 and lower amounts of IL-12p70 than DCs from age-matched wild type or Ret-tg tumor-free mice, although DCs from different groups showed similar mature phenotype after stimulation.

In conclusion, Ret-tg tumor-bearing mice have decreased numbers of DCs with less mature phenotype in the spleen and bone marrow in comparison to control groups. More DCs are infiltrating larger tumors but they are more immature. Furthermore, splenic DCs from tumor-bearing mice secrete more IL-10 and less IL-12 after CpG stimulation. These can result in the suppression of anti-tumor T-cell immune responses during melanoma progression.

Coordinated expression of clustered MAGEA and CSAG (TRAG-3) cancer/testis genes

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The twelve members of the MAGE-A subfamily of cancer/testis antigens (CTA) are expressed in cancers and testis or placenta only and, therefore, considered promising targets for cancer immunotherapy and diagnosis. As we show by RT-PCRs specific for the individual members of the MAGE-A subfamily, every melanoma sample analyzed expresses at least one of the MAGE-A genes that, thereby, appear as sensitive indicators of melanoma. The MAGE-A genes are arranged in 4 subclusters located on the X chromosome. Our analysis of the third subcluster exposes a remarkable gene organization with an inverted repeat of a triplicated and only minimally diversified couplet of a MAGE gene and the gene of another CTA family, CSAG. This entire subcluster is expressed coordinately and independent from the other MAGE-A genes suggesting epigenetic mechanisms involved in MAGE-A and CSAG gene expression.

Co-culture of human multicellular tumour spheroids (MCTS) and peripheral blood mononuclear cells (PBMC) with trifunctional antibodies

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In the past years significant progress has been made in immunological cancer therapy using therapeutic antibodies. The trifunctional antibodies catumaxomab and ertumaxomab have two different binding arms: One binds to CD3 of T cells and the other to the tumour specific antigen EpCAM (epithelial cell adhesion molecule) or to Her2 (human epidermal growth factor receptor 2) in catumaxomab or ertumaxomab, respectively. With their FC-part these antibodies have a third binding capacity to $CD16^+$ or $CD64^+$ accessory cells (e.g. NK, dendritic cells, and macrophages). This unique combination allows for the hypothetic formation of a so-called tri-cell-complex consisting of a T, cancer and accessory cell. This strategy offers an effective therapeutic tool under the exploitation of the natural immune defence.

For systematical studies we used a 3D tumour model that is well-established in our laboratory, i.e. multicellular tumour spheroids (MCTS). MCTS were cultured using the EpCAM overexpressing cell line FaDu (human head and neck squamous cell carcinoma) and the Her2 overexpressing cell line HT-29 (human colorectal adenocarcinoma) under standardised conditions (humidified atmosphere, 5% CO2, 37°C). The liquid overlay method was used for initiating spheroids in 96 well plates, and studies were done in spinner flasks in suspension culture. In our studies we focussed on dose response and interactions between tumour cells and immune cell functions.

Spheroids were co-cultured in spinner flaks under constant conditions in 20 ml growth medium with peripheral blood mononuclear cells (PBMC; $1x10^6$/ml) and different concentrations of catumaxomab or ertumaxomab (0-10 ng/ml). The effect of combined therapy was quantified by the reduction of spheroid volume and clonogenicity. In histological sections the infiltration of $CD45^+$ cells and the proportions of proliferating and apoptotic cells within the spheroid were assessed by immunostaining (Ki-67 and FragEL, respectively). Furthermore, different cytokines (IL2, IL4, IL6, IL10, TNF-α, and IFN-γ) in the spheroid culture medium were analysed with commercial ELISA tests for investigating the biological activity of the PBMC (courtesy of Dr. Lindemann, Fresenius Biotech GmbH, Munich).

Here, we demonstrate the feasibility of co-culturing MCTS from human tumour cells with human PBMC in suspension under well-defined supply conditions. Furthermore, we show that therapeutic efficiency can be quantified by various endpoints.

Our studies revealed that catumaxomab and ertumaxomab both cause a significant reduction in spheroid volume growth in a concentration-dependent manner. The ability of forming colonies was reduced as well. Therapy with clinically relevant concentrations decreased colony formation to non-detectable low levels. Spheroids were reduced in diameter through increased incidence of apoptosis. Immunohistochemical staining suggest that apoptosis was caused by infiltrated leukocytes. Measurement of cytokine concentrations and first FACS analyses suggest that these PBMC are NK and T cells. The leukocyte infiltration was strongly dependent on the antibody concentration.

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DNA vaccines in B-cell lymphoma

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The recognition of the surface immunoglobulin protein of the tumour B cells as a specific tumour antigen has prompted the development of vaccination strategies aimed at the induction of humoral and cellular antitumour responses. Results obtained in preclinical models of B lymphoproliferative diseases, as well as in initial clinical trials, have shown the immunogenic potential of the idiotype (Id).

Several reports have indicated that the immunodominant epitopes of the clone-specific Ig lie within the hypervariable regions and mainly within the CDR3 regions. Our group demonstrated that DNA immunization of outbred mice with different patient-derived V_H-CDR3 peptides elicited antibodies able to recognize native antigens on individual patient's tumor cells. Furthermore, peptides derived from the flanking framework regions (FRs) may also express cytotoxic T-cell epitopes.

These findings prompted us to investigate the antitumor response following CDR3-based DNA vaccination in a mouse model of B-cell lymphoma.

The nucleic acid sequence of the idiotypic IgM light and heavy chains from 38C13 B-cell lymphoma was analyzed and the regions belonging to the VL-CDR3 and VH-CDR3 sequences were chosen for the production of different synthetic mini-genes. The VL- sequence expresses all the CDR3 plus the cysteine 88 in FR3 and the conserved phenylalanine and glycine residues of FR4. The VH sequence was analyzed to produce two different mini-genes, encoding full-lenght VH-CDR3 or, alternatively, specifying a 8-mer "anchor-modified" epitope within the CDR3 region.

The vaccines were constructed using the bicistronic plasmid pRC110-NTS, that simultaneously co-expresses the cytokine IL-2, cloned in the second independent transcriptional cassette.

Several different approaches have been pursued to enhance the efficacy of a CDR3-based DNA vaccine.

To exploit the CDR3 epitopes in different DNA vaccine formats, we constructed several plasmid variants containing tumor epitope coupled to one or more molecular components able to improve the immunogenicity.

Therefore we have focused on the use of DNA fusion vaccines, by joining a pathogen–derived sequence to the tumor antigen. Based on the H2-Kk haplotype of C3H/HeN mice, we have selected an amino acidic region of tetanus toxin by means of epitope prediction software to overcome the immunodominance phenomenon.

Tumor peptide presentation and processing in the context of MHC I or MHC II were addressed by intracellular targeting. In particular, N-terminal signal peptide was included to target antigenic epitopes to secretory pathway through endoplasmic reticulum. The addition of an amino acid spacer sequence (Ala-Ala-Tyr) between tumor epitopes was aimed at facilitating peptides processing mediated by proteasome, resulting in increased CTL epitopes precursor frequency.

The effectiveness of the CDR3-based DNA vaccines was assessed against 38C13 tumor challenge and evaluated in terms of rate of tumor onset, tumor progression, and survival rate.

Results obtained suggest potential evaluation of these vaccines for the B-cell lymphoma immunotherapy.

Phenotypes and functions of tumor-infiltrating lymphocytes in renal cell cancer patients

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Tumor-infiltrating lymphocytes (TILs) are in intimate contact with cancer cells, but despite the presence of effector T lymphocytes, the host is commonly not protected and spontaneous tumor regression occurs only rarely. Several hypotheses have been put forward to explain this, including tumor-induced inhibition of T cells activation and function or anergy induction. These phenomena are linked to the loss of the CD3-ζ-chain, the expression of inhibitory natural killer cell receptors such as CD158b or to activation-induced cell death (AICD). Phenotypic studies have shown an increase of $CD4^+$ $CD45RO^+$ effector cells in TILs and more recently, an enhanced amount of $CD4^+$ regulatory T cells (Tregs) in peripheral blood from renal cell carcinoma (RCC) patients after treatment with high levels of IL-2 was described.

Here, we characterise the phenotype of TILs in comparison to autologous peripheral bood mononuclear cells (PBMCs) from RCC patients. Freshly prepared lymphocytes were stained with monoclonal antibodies and different T-cell subsets were analysed by polychromatic flow cytometry. An increased percentage of CD4+ and CD8+ T cells in TILs as compared to PBMCs was present, with markers of cytotoxic effector T cells such as CD57 and CD45RO found at a higher proportion than peripheral lymphocytes. In contrast, CD28 was expressed at an equivalent level in TILs and PBMCs. We also observed that the expression of CD25 by $CD4^+$ cells was reduced in TILs compared to PBMCs. This reduction of IL-2 receptor α-chain expression may be linked to cancer-derived matrix metalloproteinases (MMPs), as already reported by other groups. Preliminary results suggest only a modest increase of $CD4^+$ $CD25^{high}$ T cells inside the tumor. To verify if these are regulatory T cells, we plan to stain for intracellular Foxp3. Finally, we found no difference in the proportion either of NK cells, B cells or monocytes in PBMCs or TILs from the same patient, as judged by staining for CD56, CD19 and CD14.

Thus, we confirm that RCCs are infiltrated by activated $CD4^+$ and $CD8^+$ T cells, including cells bearing markers for cytotoxic effectors. Further experiments will show whether Tregs are increased in tumor tissue and we are starting 8 color FACS staining to analyse the cell subsets more detailed. Additionally, tumorantigen-specific T cells in TILs and PBMCs will be investigated using HLA class I tetramers refolded with relevant $CD8^+$ epitopes, and also explored on the functional level.

Characterization of tumor-specific versus virus-specific tumor-infiltrating and peripheral T cells from melanoma patients

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Successful tumor immunotherapy depends on two aspects. First, the generation of sufficient numbers of tumor-specific T cells and, second, that these cells finally "do their job". In numerous studies it has been shown, that the first part can be achieved now quite well. Following vaccination, vaccine-specific T cells can be monitored in the blood of the patients, but the clinical outcome still remains poor. In a few cases T cells have been monitored within the tumor as well. These cells are different in phenotype and functional capacity compared to those from the blood. The changes T cells are undergoing within the tumor microenvironment are still poorly understood, but remain one of the obstacles for successful immunotherapy.

To address the question whether or not these changes within the tumor microenvironment are antigen-specific, we evaluated the phenotypes of tumor-specific tumor-infiltrating T cells (TIL) and peripheral T cells in melanoma patients in comparison to T cells specific for immunodominant viral antigens.

We used 12 color flow cytometry to analyze Dextramer stained cells *ex vivo*. In total 76 samples (54 tumors and 22 PBMC) of 49 patiens and 3 healthy donors were analyzed. Tumor specific (Tyrosinase, Telomerase and gp100) Dextramer positive cells were compared to virus specific (CMV and EBV) Dextramer positive cells. These cells were phenotyped for the expression of proteins that indicate their activation status and their differentiation into memory or effector cells. In addition, cells were characterized for the expression of two receptors which have been shown to negatively regulate T cell receptor signaling, programmed death 1 (PD-1) and CTLA-4.

In the tumor, we found both, tumor-specific and virus-specific T cells. The percentages were comparable to those found in PBMCs. However, the tumor specific T cells showed a different expression pattern for PD-1 and CTLA-4 than the virus specific T cells.

From these results it can be concluded that the infiltration of T cells into the tumor does not depend on their specificity. Nonetheless, some of the phenotypic changes T cells undergo within the tumor microenvironment, do seem to depend on the recognition of their cognate epitope on the tumor cells.

Serological mini-arrays for the analysis of humoral immune response to tumor-associated antigens

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Recognition of tumor antigens by autoantibodies of cancer patients provides attractive opportunities for cancer diagnostics and monitoring, but limited by insufficient reactivity of individual antigens. In order to develop an applicable serological tool selected tumor-associated antigens should be combined in the array format. We recloned and expressed as $(His)_6$-tagged proteins the sets of antigens identified by immunoscreening of expression libraries (SEREX), as well as a set of serological cancer markers, discovered by other approaches (ex. p53, Survivin, Cyclin B1). We also included to our mini-arrays several candidate antigens overexpressed in lung and renal tumors and showing promising serological reactivity with cancer sera.

We included to our mini-arrays positive controls: antibodies against human IgG (reflecting quality of sera samples) and total human IgG (controlling secondary reagents). Our low-cost mini-arrays demonstrate promising sensitivity and specificity, long shelf-life at ambient conditions and provided additional opportunities for standardization (using antibodies to $(His)_6$-tag) and normalization (using positive controls) for subsequent analysis of serological data obtained in independent experiments (performed in different locations, explored different sera collections etc.).

Analysis of regulatory T cells in patients with multiple myeloma

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Vaccination with antigen-pulsed dendritic cells is a promising novel therapeutic strategy for treatment of malignant diseases. So far, however, clinical response rates are limited. Consequently, more efforts are needed to develop optimized vaccines and to understand mechanisms of the immune responses in the immunocompromized tumor-bearing host. Among other mechanisms of immunotolerance, $CD4^+CD25^+$ regulatory T cells (T_{reg}) have been considered as key players in suppression of tumor-specific effector cells of different lineages, e.g. cytotoxic T cells, NK cells, and others. Elevated numbers of T_{reg} have been found in lymphocytic tumor infiltrates as well as in the peripheral blood of cancer patients, and these have been related to a poor prognosis in e.g. ovarian cancer. In certain types of lymphoma, however, T_{reg} expansion may be indicative for a favorable prognosis. In multiple myeloma (MM) divergent results were reported with respect to numbers and functional integrity of T_{reg} so that further investigations are warranted to elucidate the role of T_{reg} in this malignancy. As a contribution to this we are currently investigating T_{reg} in MM patients undergoing conventional and high dose chemotherapy as well as treatment with drugs like thalidomide and bortezomib. Antibody panels were established to investigate $CD4^+CD25^{high}$ T cells as well as $CD4^+CD25^{low\text{-}intermediate}$ T cells, CD8 T cells, and to discriminate naïve and memory cells among them. FOXP3 can be measured intracellularly and the recently suggested T_{reg}-specific marker $CD127^{low}$ has been added to our marker panel recently. Isolation of T_{reg} was performed using commercially available kits and cell sorting by flow cytometry. Cell sorting worked best in our hands a yield of 60 % in preliminary experiments with normal donor cells from a buffy coat. Functional assays to measure suppression of T cell proliferation by $CD4^+CD25^{high}$ T cells are established and potential T_{reg} have been expanded in culture up to tenfold. Until now, in an ongoing study peripheral blood and/or bone marrow samples from 40 MM patients were investigated as well as samples from 11 normal donors and 12 patients with solid tumors (lung cancer). All in all, individual MM patients showed a slight tendency for an increase in T_{reg} numbers. For example, in 17 patients 0.9 - 23.7 % of $CD4^+CD25^{high}$ cells were detected, 8 / 17 patients having > 5% of these cells. A detailed analysis of the data with respect to disease status, clinical parameters, and therapy is currently under way. Moreover, MM patients eligible for immune therapy are recruited for a phase I trial for vaccination with paraprotein-pulsed dendritic cells, and the influence of this treatment on the T_{reg} compartment will be investigated.

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Identification of a novel CTL epitope from melanoma/melanocyte-specific protein, KU-MEL-1

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The melanoma/melanocyte-specific protein, KU-MEL-1 was originally identified by SEREX using serum from a melanoma patient with widespread vitiligo and a good prognosis. The KU-MEL-1 antigen is widely expressed in most melanoma and cultured melanocytes and weakly expressed in other cancer cell lines and normal testis. Moreover, strong humoral responses against this antigen have been reported in patients with metastatic melanoma as well as the autoimmune syndrome Vogt-Koyanagi-Harada (VKH) disease. Here we describe direct elution of an HLA-B*1402-restricted epitope from this protein from a melanoma cell line, Ma-Mel8a. MHC class-I-peptide complexes were isolated from Ma-Mel8a lysates using affinity chromatography with the pan-HLA-class-I monoclonal antibody W6/32. The eluted MHC-peptide complexes were separated by ultrafiltration and the peptide flow-through sequenced using mass spectrometry. The immunogenicity of a synthetic peptide (DRLKAFLL) corresponding to the eluted peptide was tested in HLA-B1402+ healthy individuals *in vitro* using autologous dendritic cells as potent antigen presenting cells. T cell lines from one of 5 donors tested specifically recognised the peptide. Moreover, the tumor cell line from which the peptide was eluted as well as another HLA-B1402+ melanoma cell line was also recognised. Because of its high expression levels in melanoma cell lines and induction of a strong humoral response in melanoma patients, the KU-MEL-1 protein may be an attractive target for immunotherapy in melanoma. Although, our results suggest that only a minority of healthy individuals may be able to recognise the particular epitope investigated here, peptide modification, use of different epitopes, or other manipulations may increase the frequency of KU-MEL-1 responders.

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Uropathogenic E.coli but not commensale E.coli infection activates TLR-4 MyD independent signaling pathways in rat testicular cells

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Immunological infertility due to infection, inflammation or autoimmunity accounts for at least 12-13% of all cases of male infertility. *Uropathogenic Escherichia coli (UPEC)* is the most frequent pathogen causing acute and chronic bacterial genital tract infections in men, however, the testicular defence to bacterial infection is poorly investigated. Therefore, we investigated the basal mRNA expression pattern of Toll-like receptors (TLRs) 2-10 in isolated testicular cells and peritoneal macrophages (PM) because TLRs function as sensors for conserved pathogen-related molecular patterns (PRMPs) such as LPS and peptidoglycan. Using RT-PCR all somatic and germ cell types as well as PM expressed mRNAs for at least two TLRs. Expression of TLR4 protein, that is recognizing LPS as PRMP, was induced 2h and 6h after UPEC infection in primary TM and Sertoli cells (SC) or peritubular cells (PTC), respectively, whereas after infection with non-pathogenic commensale E.coli (NPEC) no TLR4 was detectable. TLR4 induction coincided with already macroscopically visible massive cell death due to apoptosis. When tracing the TLR signaling pathways activation of either p38, JNK and/or ERK1/2 was observed in all three cell types and PM, but only PTC and PM reacted with degradation of IkBa. Infection with UPEC induced expression of MCP-1, IP-10 and IFNα/β in all cell types except TM indicating the induction of MyD88 independent signaling pathways in SC and PTC. Surprisingly, the proinflammatory cytokines IL-1 and IL-6 and TNFα were induced in PM only by LPS and NPEC, but not by UPEC. TNFα induction by LPS or NPEC could be abolished by co-infection with UPEC. We conclude from our results that UPEC but not NPEC infection is sensed by TLR4 in testicular cells. Moreover, UPEC infection seems to activate MyD88-independent signaling pathways.

Characterization of MUC-1 specific T-cells in breast cancer patients and healthy donors

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MUC-1 is a well characterized tumor-associated antigen (TAA) overexpressed in many epithelial tumors – including breast and ovarian cancer). MUC-1 is expressed in >90% of all breast adenocarcinomas and might prove as a target for humoral and cellular immune responses.

We demonstrated that MUC-1 specific T-cells could be detected frequently in breast cancer patients (BCP) as well as in healthy donors (HD). In patients, pre-existing MUC-1 specific T-lymphocytes have been detected predominantly in early stage disease prior adjuvant therapy. However, little is understood about the nature of TAA-reactive T-cells. The purpose of our work was to analyse differences in potentially dysfunctional MUC-1-specific T-cells in BCP and HD.

Using PBMC of HD we were able to generate MUC-1-specific T-cell lines by *in vitro* priming using peptide-pulsed autologous APCs (dendritic cells and B-cells) as stimulator cells. Best results were obtained with the modified peptide sequence MUC-$1_{\mathbf{950\text{-}958L}}$, which demonstrated improved HLA-A*02-binding. Peptide-specificity was shown by tetramer-staining. However, MUC-$1_{\mathbf{950\text{-}958L}}$-specific T-cells did not show interferon (IFN)-□-production after stimulation. In addition, we monitored T-cell responses in BCP during a clinical trial using peptide-pulsed autologous DCs as vaccine. In post vaccination PBMC, a significant enhancement of MUC-1 related T-cell responses was detected by IFN-□-specific quantitative RT-PCR.

To further analyse the functional state of MUC-1 specific T-cells we will use tetramers in combination with several lymphocyte-subset markers to distinguish i.e. between naive, effector and memory T cells.

HSP70-antigen fusion proteins in combination with TLR ligands are efficient tumor vaccines

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Heat shock proteins (HSPs) are known to induce cross-presentation of antigens by dendritic cells (DC) as well as DC maturation. These properties make HSP antigen complexes good candidates to prime CD8 T cell responses against tumor-associated antigens. There exist around 20 different HSPs, but little is known about their relative capacity to cross-present antigen. In this study, we employed HSPs of the HSP70 family fused to a fragment of ovalbumin (OVA) as a model tumor antigen. We investigated the efficiency of recombinant *M. tuberculosis* Hsp70, murine (m) Hsp70, mHsc70 and mHsp70-L1 OVA fusion proteins to prime *in vivo* OVA-specific CD8 T cells from a TCR transgenic repertoire or a polyclonal CD8 T cell repertoire. *M. tub.* Hsp70-OVA was found to be superior to all other HSP70-OVA fusion proteins in its capacity to prime CD8 T cell responses. The vaccination with HSP70-OVA was dependent on DCs, as depletion of DCs in CD11c.DTR mice resulted in a lack of CD8 T cell responses.

To determine the effect of the TLR4-binding component LPS on OVA-specific immune responses, we carefully removed LPS from the *E.coli*-derived recombinant HSP-OVA fusion protein. LPS removal resulted in a strongly reduced efficiency of HSP fusion proteins to stimulate CD8 T cell responses, and a single immunization did not reduce the growth of OVA-expressing B16 melanoma cells *in vivo*. However, multiple immunizations with LPS-depleted *M. tub.* Hsp70-OVA efficiently primed cytotoxic T cell responses and significantly reduced the growth of OVA-expressing B16 melanoma cells *in vivo*. When this experiment was repeated in TLR4-deficient mice, no reduced tumor growth was observed. These observations suggest that HSP70-antigen fusion proteins require TLR4 for their immunostimulatory capacity, either through direct binding to TLR4 or indirectly through minute LPS contamination of the recombinant HSPs.

Interestingly, a combination of LPS-depleted *M. tub.* Hsp70-OVA and the TLR9 ligand CpG oligodeoxynucleotide resulted in efficient B16/OVA tumor rejection and tumor-specific memory using a single prophylactic immunization. Preliminary data indicate that this combination is also a promising therapeutic regimen. Our results suggest that *M. tub.* Hsp70 fused to a tumor antigen in combination with a strong TLR ligand are potent tumor vaccines.

A comprehensive comparison of PGE2-matured and poly(I:C)-matured DC reveals differences in cytokine and IDO expression but comparable T cell-stimulating abilities

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Mature dendritic cells (DC) are one of the most commonly used platforms for cancer immunotherapy. A component of the most frequently used DC maturation protocol, PGE2, has been shown to inhibit IL-12 secretion, possibly interfering with the Th1-polarizing capacities of DC. In contrast, poly(I:C) has been shown to be one of the strongest IL-12-inducing signals. We therefore compared two maturation cocktails containing PGE2 and poly(I:C), respectively and comprehensively studied the resulting DC phenotype, cytokine and chemokine secretion, IDO expression and functional properties.

Monocyte-derived DC were made from buffy coats of healthy donors and matured with IL-1β, IL-6, TNFα and PGE2 („PGE2-based") or IL-1 β, TNFα, IFNα, IFNγ and polyI(I:C) („poly(I:C)-based") for two days. Mature DC were then co-cultured with hCD40L-transfected K562 cells for 24h. Phenotype was analysed by FACS. Cytokine and chemokine secretion and IDO expression were assessed by RNA microarray and on a protein level by ELISA and Western Blot. In two donors, T cells were stimulated three times in vitro with MART-1 peptide-loaded autologous DC and individual wells were subjected to IFNγ ELISPOT and pentamer analysis.

Both methods yielded phenotypically mature DC with similar expression of CCR7. In RNA microarray analysis, the most dominant finding was the upregulation of the T-cell attractants CXCL9, 10 and 11 by poly(I:C)-matured compared to PGE2-stimulated DC. For CXCL9 and CXCL11, this was confirmed by ELISA, whereas the secretion of CXCL10 varied between donors. IL-12 secretion by PGE2-based DC was absent or low, while poly(I:C) caused high levels of IL-12 secretion. IL-10 secretion was also low or undetectable after PGE2-based maturation, whereas poly(I:C)-based DC secreted IL-10, albeit less than IL-12. Cytokine secretion after maturation with the poly(I:C)-containing cocktail was reduced when omitting poly(I:C), but increased after subsequent CD40 ligation in 4/5 donors, especially when IFNα was also absent during maturation. The omittance of PGE2 from its respective cocktail did not change the levels of IL-12 and IL-10 secretion, but rendered DC responsive to CD40L stimulation which induced the secretion of IL-12 and low levels of IL-10. This was independent of the presence of IFNγ during maturation. The expression of functional IDO protein in poly(I:C)-based DC exceeded that of PGE-based DC. The expression of IDO was strongest when both Poly(I:C) and IFNα, or PGE2 and IFNγ were present in the respective cocktails, whereas when both stimuli were absent, there was no expression of IDO. The same was observed regarding the expression of CCR7. After in vitro priming with MART-1-peptide loaded DC, IFNγ secreting T cells were detected in 3/8 and 4/8 wells after stimulation with poly(I:C)-based DC and in 1/16 and 1/10 wells after stimulation with PGE2-cocktail-matured DC. Pentamer binding was detected in 4 and 5 out of 30 wells after poly(I:C)-based DC stimulation and in 2 and 7 after PGE2-based DC stimulation.

In summary, we could show that poly(I:C) maturation induces IL-12- and IL-10- secretion and IDO expression and that although PGE2-matured DC do not secrete IL-12 and express IDO, they are as potent as poly(I:C)-matured DC for T cell priming.

Recognition of methylated peptides by tumour-specific T cells in melanoma patients

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The analysis of the HLA peptidomes of tumour cells reveals the immunological face of these cells and can lead to the identification of target antigens for cancer immunotherapy. Using affinity chromatography we isolated HLA class I-peptide complexes of melanoma cell lines newly established from patients. The peptides were extracted from these complexes, separated by 2-dimensional HPLC and subjected to mass spectrometry. The MS/MS spectra of the peptides were analysed by *de novo* sequencing as well as database-based sequencing procedures to identify modified peptides utilising the Sequit software developed in our lab and on-line MASCOT tools, respectively. Of one of the peptides three variants were isolated, one was unmodified, the second monomethylated and the third asymmetrically dimethylated on arginine. The modifications were identified by the specific fragmentation patterns of the methylated side chains and confirmed with synthetic peptides with the thus predicted sequences including mono- and dimethylated arginine. These three forms of the peptide were tested by *ex vivo* priming assays combined with ELISpot read-outs for interferon γ with peripheral white blood $CD8^+$ T cells of healthy donors for their immunological activities. The unmodified peptide was active but none the other two. In direct *ex vivo* ELISpot analyses with peripheral blood mononuclear cells of melanoma patients the monomethylated peptide was very potent in inducing T cell responses. The responses induced with the di- and the non-methylated forms of the peptide were far weaker. These results indicate that the monomethylation of the peptide plays a specific role in anti-melanoma T cell-mediated immune responses and suggest that posttranslationally modified peptides may be targets for cancer immunotherapy.

Detection of IL2, CD25, and CD64 mRNA from spheroids co-cultured with PBMC and treated with trifunctional antibody catumaxomab

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Purpose: The trifunctional antibody catumaxomab is assumed to bind to CD3 (T cells), tumour specific antigen EpCAM (epithelial cell adhesion molecule), and CD16 or CD64 (accessory cells) simultaneously, forming the so-called tri-cell-complex. This leads to enhanced therapeutic effects, e.g. increased transcription of IL2 and IL2□ receptor alpha (CD25).

In this study the IL2 and CD25 mRNA-levels were analysed in a co-culture system of MCTS (multicellular tumour spheroids) and activated PBMC (peripheral blood mononuclear cells). The rate of macrophages and/or dendritic cells infiltrating MCTS was detected by measuring CD64 mRNA.

In a separate experiment, isolated PBMC cultured under standardised conditions were activated with catumaxomab, and IL2 transcription level was recorded as a function of time using semi-quantitative RT-PCR.

Methods: MCTS from FaDu cells (human head and neck squamous cell carcinoma) were cultured in spinner flasks with supplemented DMEM under standardised conditions. Spheroids were co-cultured with (1) PBMC or (2) with PBMC plus catumaxomab (PBMC: $1x10^6$/ml; catumaxomab: 2.5 ng/ml). Untreated MCTS were used as control (3). PBMC were obtained from buffy coats by density gradient centrifugation. After 5 days in co-culture, total RNA was extracted from sedimented spheroids using a commercial RNA extraction kit. The expression levels of IL2, CD25, and CD64 were analysed by RT-PCR.

Additionally, the kinetic of IL2 transcription was measured in PBMC activated with catumaxomab (2.5 ng/ml). For this purpose the PBMC were maintained in standard culture flasks with complete RPMI medium. Samples were removed after 6, 12, 18, 24, 48, 72, 96, 120, and 144 h for RNA-extraction. IL2 transcription levels were analysed by semi-quantitative RT-PCR. Non-activated PBMC served as control.

Results and Conclusion: In the MCTS co-cultured with catumaxomab, activated PBMC, IL2, CD25, and CD64 mRNA was detectable for up to 120 h. Since RNA was extracted exclusively from spheroids, PBMC producing this mRNAs must have infiltrated MCTS (T and accessory cells, respectively). CD64 mRNA was also found in MCTS in co-culture with unactivated PBMC. Donor-dependent transcription of IL2 and CD64 was measured which may be due to variable pre-activation of PBMC in individual buffy coats.

The data suggest that catumaxomab may induce IL2 transcription in PBMC after penetration into MCTS for a relatively long period of time. Considering the importance of IL2 in the human immune defence, the data indicate that catumaxomab enhances the effectiveness of the human T cell immune response. Furthermore, the antibody promotes penetration of macrophages and/or dendritic cells into spheroids.

Experiments with catumaxomab activated PBMC in standard culture flasks has shown a transient transcription boost of IL2 mRNA with a maximum at 6 h. Subsequently, a continuous decrease occurred in mRNA-levels which were no longer detectable after 48 h. One-time transcription and following cytosolic RNA degradation could be responsible.

Since MCTS closely mirror the *in vivo* situation of tumours, the present data support efforts for the introduction of catumaxomab to clinical immunotherapy of cancer.

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Shared MHC class I epitopes prevent T cell responses against unique tumor antigens through in situ activation of regulatory T cells in experimental antitumor vaccination

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The monoclonal antigen receptor of of B-cell lymphomas ("idiotype") constitutes a specific tumor antigen. Immunization with idiotype may induce specific, MHC-restricted T cell responses. The precise localization of the immunogenic epitopes within the idiotype has remained controversial: After immunization of B-lymphoma patients with recombinant idiotype, we have found predominant recognition of individual CDR-derived peptides by IFN-□ ELISPOT analysis (Bertinetti et al., Cancer Res. 2006). In contrast, other groups have generated cytotoxic T cell lines which predominantly appear to recognize shared framework-derived epitopes in in vitro stimulation experiments (Trojan et al., Nat Med. 2000). To address this issue in an animal model, we immunized BALB/c mice with dendritic cells (DC) generated under serum-free conditions (Warncke et al., JIM 2006) and loaded with peptides derived from the idiotype of the A20 lymphoma. In this model, a shared J-region-derived peptide was described as the immunodominant target of a specific T cell response (Armstrong et al., JI 2002). We now performed comparative immunizations against the J peptide and a individual heteroclitic CDR3-derived peptide with similar MHC binding properties. Only T cells with specificity against the CDR3 peptide were detected in vivo during a primary immune response by IFN-g and pentamer staining. These cells were able to efficiently kill target cells presenting the heteroclitic or the native peptide in both in vivo and in vitro assays. None of these assays detected specific immune response after J peptide vaccination. After in vitro restimulation of splenocytes from immunized animals, up to 5% of CD8+ T cells specifically recognized the respective immunization antigen as determined by intracellular IFN-□ staining and cytotoxicity assays.These data indicate a strong in vivo tolerance against the J peptide. The shared J but not the unique tumor antigen led to a potent activation of polyclonal regulatory T cells as indicated by proliferation of CD4+Foxp3+ cells, suppression of peptide-specific CD8+ T cells, and IL-10 secretion. Primary CD8+ T cell responses against highly immunogenic antigens were efficiently blocked by co-immunization with the shared J peptide. This inhibiton was absent in CD25-depleted animals. Our data suggests that epitopes unique to the tumor may represent the superior antigen for efficient anti-tumor immunization and that shared tumor epitopes should be avoided in tumor vaccines in order to prevent regulatory T cell activation and therefore induce efficient immune responses.

Activation of tumor specific T and B lymphocytes after radiofrequency ablation in cancer patients

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Percutaneous radiofrequency (RF) ablation is a safe, effective and therefore one of the most popular minimally invasive techniques used for the treatment of unresectable primary and secondary liver tumors. It destroys tumor tissues generating a thermal coagulation necrosis by using an electromagnetic wave with RF energy. Since necrotic cells have been shown to activate professional antigen presenting cells after releasing danger signals, such as heat shock proteins, it can be hypothesized that RF ablation leads to an activation of the immune system against the tumor, including stimulation of tumor-specific effector lymphocytes. We investigated the effects of RF ablation on the T-cell ($CD8^+$ cytotoxic effectors) and B-cell (antibody production) repertoires in treated patients.

Blood and serum samples from patients with hepatocellular carcinoma or with liver metastasis of colorectal or breast cancer were obtained at defined time points before and after RF ablation. Peripheral T lymphocytes of selected patients were stimulated in vitro with synthetic peptides derived from tumor antigen-specific T-cell epitopes. T-cell activation was analyzed in an IFN-gamma linked immunospot (ELISPOT) assay and by intracellular IFN-gamma staining. For assessing the tumor-specific antibodies in the patient's sera, recombinant tumor antigens were produced and dotted onto nitrocellulose membranes. These miniarrays were incubated with the sera samples and the reactions were revealed using secondary enzyme-conjugated anti-human antibodies.

A clear induction of tumor antigen-specific $CD8^+$ T lymphocytes could be measured in several patients after RF ablation. This activity was observed between 3 to 7 months after treatment, but was not present before treatment. Also, antibodies recognizing tumor antigens were clearly induced in some donors several weeks after RF ablation. Further patients are being tested for evaluating the amplitude and the duration of this immune response.

These results suggest that in addition to the destruction of malignant tissue, RF ablation can generate the local conditions for activating a specific immune response against the patient's tumor, at least in a fraction of treated patients.

WT-1 peptide vaccination after allogenic stem cell transplantation for patients with myeloid malignancies

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Purpose of our study was to generate a WT-1 specific immune response through peptide vaccination after HSCT in patients with high risk leukemias in order to enhance the GvL effect and control the minimal residual disease. In a phase I study six HLA A*0201 positive patients with high-risk AML (median age 63 yrs, range 33-67) were vaccinated with the WT-1 derived peptide RMFPNAPYL beginning at day 21 after HSCT. Four patients had been vaccinated previously with WT-1, relapsed and underwent HSCT. The vaccination protocol consisted in four biweekly vaccinations with the peptide administered with keyhole limpet hemocyanin as adjuvant. Concomitantly, patients received daily doses of GM-CSF for four days beginning two days before vaccination. After the first four cycles, vaccine was administered monthly. When patients showed signs of GvHD or infection vaccination was discontinued. No severe acute toxicity attributable to the vaccination was observed. Four patients achieved a complete remission of the AML after transplantation and did not relapse. One patient developed an intraneural relapse, was successfully treated by irradiation and maintains a full donor chimerism 600 days after HSCT. One patient relapsed and died four months after transplantation.

In five patients responses were monitored in peripheral blood using tetramer analysis and IFN-gamma ELISPOT. Prior to transplantation two patients showed a WT-1 tetramer positive $CD8^+$ T-cells (5,31% and 4,84%,respectively) but no response in the ELISPOT. In all five patients specific T cells were detected after one (3/5) or two vaccinations (2/5). Two patients showed a positive response in the ELISPOT after the 6^{th} and the 2^{nd} vaccination cycle, respectively. In the patient who relapsed WT-1 specific T cells were found in the peripheral blood after vaccination (0,17% $WT1^+/CD8^+$ cells), but disappeared three months after HSCT, when she relapsed.

Two patients developed a grade I GvHD shortly after the first vaccination. Therefore vaccination was discontinued and resumed two weeks after resolution of the GvHD. No GvHD signs were observed after the subsequent vaccination cycles. One patient developed a grade III GvHD and one patient developed a grade IV GvHD.

We conclude that WT1 vaccination could contribute to the maintenance of a complete remission in patients with high-risk AML after HSCT. However, it may enhance GvH reactions because of the adjuvants used. Therefore further clinical observations in a larger number of patients are needed.

Characterisation of WT1-specific T cell responses in Peripheral Blood and Bone Marrow in leukemia patients vaccinated with WT1 peptide, GM-CSF, and KLH

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Purpose: The transcription factor Wilms tumor protein (WT) 1 belongs to a new generation of tumor antigens, which is essential for tumor cell proliferation and is expressed in many hematologic and solid malignancies. We have performed a phase II vaccination trial with the HLA-A2-restricted WT1.126-134 peptide in patients (pts) with WT1 overexpressing AML and MDS with immune response as the primary objective.

Methods: Pts received intra/subcut. vaccinations with 0.2 mg WT1.126-134 peptide (day 3), 62.5 mcg GM-CSF (days 1-4) as DC-stimulant and 1 mg keyhole limpet hemocyanin (day 3) as T helper protein. Vaccination was given biweekly x 4 followed by 4-weekly in the first 13 pts and continuously biweekly in the subsequent 13. WT1-specific T cell responses were quantitated and characterized by tetramer and cytokine flow cytometry in peripheral blood (PB) and bone marrow (BM).

Results: Of 29 pts enrolled 25 received at least 4 vaccinations and were evaluable for immunological response (AML n=23, MDS n=2). A median of 11 (range 4 – 25) vaccinations was administered with 3 pts still on treatment. Serial analyses of WT1 tetramer specific T cells showed an increase of the percentage of patients with WT1+ CD8+ T cells in PB from 28% prior to vaccination to 52% at week 10. In the pts without a preexisting response the median frequency of WT1 tetramer+ CD8+ T cells increased from 0.11% (range 0%-0.19%) prior to vaccination to 0.25% (range 0-0.54%) at week 10 ($p<0.01$) whereas in the 7 patients with preexisting responses these could not be boosted (0.48% prior to vaccination, 0.54% at week 10). In 19 out of 25 pts T cell responses could be analysed in parallel in PB and BM at week 18, showing significantly higher frequencies of WT1 tetramer+ CD8+ T cells in BM (0.36%, range 0.04-1.35%) than in PB (0.26%, range 0.06-1.95%, $p=0.046$). The percentage of pts who had a WT1 peptide specific cytokine response in PB increased from 18% at week 0 to 48% at week 10 with 7 pts producing IFN□, 10 producing TNF□, and 4 of these producing both cytokines. WT1-specific cytokine production in BM was confirmed in selected patients. As observed for the tetramer responses in none of the pts with preexisting WT1-specific cytokine producing T cells these could be boosted. No significant quantitative or functional difference in T cell responses was found among the two vaccination schedules. Further characterization of vaccine-induced WT1-specific T cells in selected patients revealed their differentiation into both effector and memory subsets as well as cytotoxic and proliferative capacity and specific cytokine production in response to autologous leukemic blasts.

Conclusions: These results show that WT1 vaccination generates functional T cell responses, which infiltrate leukemic bone marrow and warrant future trials in patients with hematologic and solid malignancies.

Phase I clinical trial: Adoptive transfer of *in vitro* expanded tumor specific autologous T cells in patients with advanced ovarian cancer

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Ovarian cancer represents the third most common cause of cancer related mortality in Sweden. The majority of patients are usually diagnosed with advanced disease (stage III-IV). Current therapeutic modalities could only achieve a modest increase in survival rates in the last decades, thus indicating that novel therapies are urgently needed.

A phase I clinical trial on adoptive transfer of *in vitro* expanded autologous T cells in patients with advanced ovarian cancer is currently being approved. Patients with stage III or IV epithelial ovarian carcinoma (EOC) that failed to respond to current therapy will undergo laparocentesis. Tumor cells as well as T lymphocytes will be isolated from ascitic fluid by elutriation. Monocytes and additional T lymphocytes from the same patient will subsequently be purified from peripheral blood mononuclear cells (PBMC) after leukopheresis. *In vitro* matured monocyte-derived dendritic cells (DCs) loaded with EOC tumor cell lysate or tumor derived apoptotic bodies will be used to stimulate T cells. These T cells will be tested for tumor reactivity and transferred back to the patient via a combination of intra-venous and intra-peritoneal injection.

In preliminary experiments we are exploring whether DCs more efficiently stimulate T cell expansion when pulsed with tumor cell lysate or apoptotic bodies.

The ability of T cells expanded by one of these methods to recognize, proliferate and/or kill in response to alternatively pulsed DCs as well as to autologous tumor cells will be evaluated. As repeated stimulations will be necessary to generate specific T cells and achieve sufficient number for repeated transfer to the patient, we will compare if restimulations with the same type of loaded DC are superior over restimulations with PBMC or alternatively loaded DCs.

Since T cells isolated from ascitic fluid might be suppressed by cells and soluble factors present in the tumor environment we will investigate if it is feasible to generate large numbers of functional and tumor reactive T cells from ascites as compared to peripheral blood lymphocytes.

We will also monitor the phenotype of the T cell cultures expanded according to the different stimulation protocols with regard to accumulation of regulatory T cells and CD4+ or CD8+ T cells of different activation status.

Administration to different sites will increase the possibility of transferred tumor-reactive T cells to encounter the appropriate signals to engage in anti-tumor immune responses.

After adoptive transfer we will follow the T cells and investigate their antigen specificity if clinical responses can be observed.

CD 40 ligation during dendritic cell maturation reduces cell death and prevents IL-10 induced regression to macrophage-like monocytes

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Dendritic cells (DCs) have become popular candidates in cancer vaccination because of their crucial role in inducing T-cell responses. However, clinical studies greatly differ in their protocols for generating DCs and the efficacy in treating established tumors needs to be improved. We systematically analyzed DCs maturated by five different protocols for surface markers, the allo-proliferative T cell response, the DC survival after cytokine deprivation, the stability of surface markers under the influence of interleukin-10 (IL-10), and the DC cytokine secretion pattern. Monocyte derived DCs were maturated by CD40-ligand (CD40-L), unmethylated CpG-oligodinucleotides (CpG-ODN), an inflammatory cytokine cocktail (ICC), a combination of ICC and CD40-L, or ICC, CD40-L and CpG-ODN.

A high co-expression of DC maturation and costimulation markers was found after treatment with ICC plus CD40-L (69.3% ± 9.6% CD83/CD80 double positive staining) and correlated with a significantly increased cell survival, a high expression of the antiapoptotic factor bcl-xL, a stable CD83high/CD14low expression under the influence of IL-10, and a strong allo-proliferative T cell response. In conclusion, our data support the use of maturation protocols containing ICC plus CD40-L in order to generate highly mature, phenotypically stable, cell-death resistant, and T cell stimulatory DCs for clinical application in cancer patients.

Polymorphism in genes coding for molecules associated with antitumor immune response in malignant melanoma and prostate cancer

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The elucidation of many of the genetic events that underlie the cancer progress will contribute to the development of novel diagnostic and prognostic approaches for improvement of patient survival. The objective is to identify immunogenetic factors involved in malignancies and to study their relevance for the outcome of the immunotherapy. Samples from 35 Norwegian patients (20 with advanced malignant melanoma - MM; 15 with prostate cancer - PC) were evaluated for KIR, MIC A and B gene polymorphisms within ENACT project. Ten MM patients were treated with vaccine therapy with dendritic cells (DCs) transfected with autologous mRNA and 10 patients with chemotherapy (Temodal) combined with telomerase vaccine. Prostate cancer patients were vaccinated with mRNA transfected DCs. MIC genotyping was performed by PCR-SBT method and KIR genotyping by PCR-SSP method (PEL-FREEZ®KIR Genotyping Kit)**.** Compared to healthy controls MIC A*00801, *00702, *019 alleles were more frequent, while alleles *00201, *00901 were in lower frequencies in cancer patients. The comparative analysis between different cancer groups (MM and PC) showed increased frequency of MIC A*004, *005, *01201, *042 alleles in malignant melanoma and MIC A*007, *019 alleles in prostate cancer. KIR genotyping revealed that the distribution of inhibitory KIR alleles was similar in both MM and PC patient groups. Statistically significant (p=0.02) decreased frequency of activating *KIR2DS3* was observed in malignant melanoma compared to prostate cancer patients. Similarly, this activating *KIR* was found to be decreased in melanoma patients compared to healthy individuals. The analysis of KIR haplotypes (A and B) revealed 11 KIR haplotypes in melanoma and 9 in prostate cancer patients. In both groups the diversity was more pronounced in B haplotypes. Additional haplotypes that could not be defined according to the used classification were also observed. KIR A1 haplotype homozygosity was more prevalent in cancer samples, particularly in prostate cancer compared to healthy controls. Longitudinal clinical follow up of the patients will allow the assessment of the relevance of these polymorphisms for therapy outcome.

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Type I interferons are critical in the control T cell responses induced by dendritic cells activated by combined Toll-like receptor ligation

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Toll-like receptors (TLR) recognize conserved microbial or viral structures and mediate activation of innate immune cells such as dendritic cells (DC) that control the induction of adaptive immunity.

Here we report a synergy for DC activation upon triggering with selected combinations of TLR agonists. We show that the simultaneous ligation of a MyD88 with a TRIF-dependent TLR results in the synergistic production of type I interferon (IFN-β) mRNA as well as the chemokine RANTES. This result was unexpected since it was reported TLR2 signalling could not induce IFN-β at all, nor would it synergize in type I interferon expression with another TLR ligand. This translates into the adaptive immune response: vaccination with these selected TLR ligands or combinations thereof revealed a synergistic potential to initiate CTL activation. As one possible mechanism by which DCs stimulated with a certain combination of TLR agonists are able to induce superior T cell priming, we identify the feature that they render $CD4^+$ and $CD8^+$ T cells less susceptible to regulatory T cell mediated suppression. We have gained strong evidence, that soluble factor(s) secreted by simultaneously TLR3+TLR7 -stimulated DC are responsible for the observed unresponsiveness on the T cell side towards inhibition by regulatory T cells. At last we identify that this TLR "superstimulation" confers a fast induction of cellular death by apoptsis of the DCs as compared to single TLR ligation. We thus hypothesize that TLR mediated "super-stimulation" also leads to an early DC death by an apoptotic mechanism, thus resulting in the reduction of mature DCs' life span.

Analysis of the immunogenicity of a FLT3 internal tandem duplication (FLT3-ITD)

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During hematopoiesis the fibroblast-macrophage stimulating factor receptor (FMS)-like tyrosine kinase receptor 3 (FLT3) plays an important role for the proliferation, survival and differentiation of early hematopoietic progenitor cells. FLT3 is also relevant in leukemogenesis. It represents the most common mutated gene in AML. The two predominant types of mutation are internal tandem duplications (FLT3-ITDs) within the juxtamembrane domain and point mutations within the kinase domain. FLT3-ITDs occur in 25–30% of AMLs and lead to leukemic transformation by constitutive phosphorylation and uncontrolled activation of the tyrosine kinase. Although the length of those vary enormously from 3 to over 400 bp, the initiation site for that alteration is mostly limited to codons 591-601 due to the palindromic sequence in that sequence range. In addition, FLT3-ITDs are in frame and may be combined with insertions located in between the duplicated sequences.

We hypothesized that duplication regions encode immunogenic neoepitopes restricted by individual HLA alleles, and that FLT3-ITD-positive AML cells might then be specifically targeted by the T-cell system.

Using a reverse-immunology approach, we analyzed the FLT3-ITD of an AML patient for encoding HLA class I-restricted, immunogenic peptides. The stimulation with one of the chosen peptides (YVDFREYEYY) induced *in vitro* autologous T-cell responses restricted by HLA-A*0101. Immune responses against this peptide were also detectable using *ex vivo* $CD8^+$ lymphocytes. The peptide-reactive T cells recognized targets transfected with the patient's FLT3-ITD, but not wild-type FLT3, and recognized the patient´s AML cells. Our data show that AML leukemic blasts can in principal process and present immunogenic FLT3-ITD neoepitopes. Therefore, FLT3-ITD represents a potential candidate target antigen for the immunotherapy of AML.

In a next step, we are about to study a cohort of FLT3-ITD-positive patients for anti-FLT3-ITD T-cell responses and to include $CD8^+$ as well as $CD4^+$ T-cell responses via all individual HLA-I and -II alleles.

Expression and immune-inhibitory function of MIF in ovarian cancer

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Epithelial ovarian carcinoma (OvCA) is the most common cause of death from gynecological malignancy. Even with extended surgery and chemotherapy, 5-year-survival rates do not exceed 20-40%. There is a strong correlation between favourable immunological parameters and long-term survival indicating that immunotherapy might have considerable impact in this type of cancer that is characterized by apparently paradoxical immunological properties: While cytolytic anti-tumor immune responses are strongly suppressed by OvCA-derived factors, inflammation is required for malignant transformation and, later on, maintained in the tumor microenvironment.

The pro-inflammatory cytokine macrophage migration inhibitory factor (MIF) is known to stimulate tumor cell proliferation, migration and metastasis, to promote tumor angiogenesis and to suppress p53-mediated apoptosis. We have now investigated the expression of MIF in ovarian cancer and found that MIF expression correlates with malignancy and the presence of ascites. On a functional level, MIF compromises anti-tumor immunity by transcriptionally repressing the activating immunoreceptor NKG2D. Inhibitors of MIF that are being developed for the treatment of inflammatory disorders may therefore also hold potential for cancer therapy.

A shift in the intestinal microflora towards pro-inflammatory bacteria and signaling via toll-like receptors 2 and 4 trigger early onset of Graft-versus-Host disease

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Pattern recognition receptors such as Toll-like-receptors (TLR) and NOD proteins bind specifically to microbial components and activate innate immune responses. Reducing the bacterial load by antibiotics was shown to ameliorate the severity of graft-versus-host-disease after allogenic stem cell transplantations. We hypothesize that sensing of intestinal bacteria by TLRs could trigger acute GvHD. Because TLR 2 and 4 represent the major receptors for Gram-positive and Gram-negative bacteria, respectively, we investigated their impact on the induction of T-cell-alloreactivity and GvHD in a murine transplantation model.

Wild-type (wt) mice and TLR2-/-, TLR4-/-, TLR2/4-/- mice, which served as recipients of MHC-mismatched Balb/c grafts after conditioning with treosulfan and cyclophosphamide. The onset of GvHD was delayed in recipients lacking both TLR 2 and 4 ($p<0.05$). On day 20 after stem cell transplantation, 40% of TLR2/4-/- recipients but only 7% of wt mice were still alive. Moreover, GvHD scoring and histological data confirmed the protective effect of the respective TLR deficiencies. However, long-term survival until day 50 did not differ between wt and TLR2/4-/- recipients.

In addition, analyses of the gut microbiota in fecal samples showed a pronounced increase of the luminal E. coli load within 11 days after stem cell transplantation. This increase correlated with the severity of graft-versus-host-disease.

Taken together, these results support potential roles of TLRs 2 and 4 and bacterial components in the induction and early onset of GvHD, first described nearly 50 years ago. In expansion of these early findings, our data elucidate the role of the innate immune system and its receptors for the induction of alloreactive T-cell responses, providing evidence that the modulation of the intestinal gut flora might prevent or reduce GvHD in humans.

Anti tumor vaccines based on stabilized mRNA (RNActive®)

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In view of the lack of efficient therapies for the treatment of many tumor diseases the development of novel anti-cancer strategies remains still very important. In the field of anti-tumor immunotherapy a stabilized messenger RNA (RNActive®) based vaccination represents a very promising contribution.

As we could previously demonstrate, the injection of protamine condensed RNActive® leads to the development of antibodies and cytotoxic T-cells directed against the protein encoded by the nucleic acid. The major advantage of RNA-based vaccines, compared with DNA vaccines is the lack of potential toxicity associated with the latter (integration into the genome or induction of anti-DNA auto-antibodies). In order to improve the bioavailability of injected mRNA molecules different parameters have been studied, addressing mainly two points: injection conditions and molecular format of the messenger RNA.

Besides an optimized injection buffer ("RNActive® transfer"), a chromatographical purification method (PUREmessenger®) and modifications within untranslated region of the vaccine we could demonstrate that also sequence modifications within the coding region of RNActive® molecules have a significant effect on the level of translated protein. By maximizing the GC content of the mRNA according to the degenerated genetic code, the efficiency of the RNActive® transfer could be elevated by a factor of 10 compared with the wild type sequence.

The increased expression level of protein encoded by GC-rich mRNA correlated with the significant enhancement of immune responses achieved in the mice vaccinated with GC-rich RNActive® molecules. Vaccination with GC-rich variant of mRNA, coding for model antigen ovalbumin, yielded significantly higher antibody titers compared with the wild type mRNA. Moreover mice vaccinated with sequence-optimized mRNA were widely protected against the subsequent challenge with tumor cells expressing model antigen. The protective effect of GC-rich mRNA was not only restricted to the prophylactic vaccination but persisted also under therapeutic conditions.

Taken together we show that injection of stabilized and sequence-optimized antigen encoding mRNA induces potent adaptive immune responses that inhibit tumor growth *in vivo*. Our data demonstrate an enormous therapeutic potential of RNActive™ -based strategy, which due to its flexibility for different cancer diseases represents a highly attractive option in the development of tumor vaccines.

Generation of an improved recombinant bispecific antibody molecule and B7 fusion proteins for targeted cancer immunotherapy

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The recombinant bispecific antibody format single-chain diabody (scDb) has shown to be able to retarget T lymphocytes to tumor cells, leading to their destruction. However, therapeutic efficacy is hampered by the short serum half-life of this small molecule (55 kDa). Thus, improvement of the pharmacokinetic properties of small bispecific antibody formats is required to enhance efficacy *in vivo*. We have generated a fusion protein of single chain diabody and human serum albumin (scDb-HSA) and analyzed this molecule for biological activity and pharmacokinetic properties. The scDb-HSA, which is directed against the tumor antigen carcinoembryonic antigen (CEA) and the T cell receptor complex molecule CD3, retained full binding capacity to both antigens and showed strong increase in circulation time compared to the unfused scDb molecule. In order to provide a tumor target specific costimulatory signal, fusion proteins of the extracellular domain of B7.2 (CD86) and single chain Fv or diabody against CEA were generated. This constructs showed specific binding to CEA and CD28/CTLA-4. Costimulatory properties were assayed in combination with the scDb (providing the first stimulatory signal) by monitoring IL-2 release after incubation with PBMCs. Here, B7-Db showed to be superior to B7-scFv. Thus, in combination with B7-Db an enhancement of tumor antigen-specific retargeting and activation of T cells could be achieved for scDb and scDb-HSA. In summary, combining recombinant bispecific antibodies with improved pharmacokinetic properties and tumor directed costimulatory fusion proteins might be a promising approach for efficient retargeting and activation of cytotoxic T lymphocytes in cancer immunotherapy.

The pharmaceutical formulation of peptide antigens and of toll like receptor (TLR-) agonists and the route of antigen delivery determines the strength of an antigen specific CD8+ T cell response

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The induction of a strong and specific T cell response is a major challenge in immuno-therapeutical approaches to fight cancer. Not only specificity of a T cell response which is induced and controlled by the use of specific tumor antigen(s) in what form soever but also the usage of immune response modulating adjuvants are mandatory for an efficient vaccine formulation. Using a tyrosinase-related protein 2 (TRP2) derived epitope as an antigen for immunization we could show that synthetic peptides encapsulated as a liposomal formulation (AVE3) are much more effective in the induction of an antigen-specific CD8+ T cell response than vaccination of mice with free TRP2 peptides. Besides their depot forming property AVE3 liposomes bind specifically to $CD11c^+$ Dendritic cells (DC) but not to T- and B cells. Furthermore, we showed that a 10 fold lower TRP2 peptide concentration is sufficient to induce a comparable T cell response when administered as a liposomal formulation as compared to the administration of the free form. Importantly, our studies demonstrated that AVE3/TRP2 liposomes do not exhibit an inherent adjuvant effect and at least one adjuvant has to be added to induce a T cell response. The adjuvants monophosphoryl lipid A (MPLA), the lipopeptide Pam_3Cys and oligodeoxynucleotides containing unmethylated CpG motifs (CpG-ODN) were used as a free and/or liposomal formulation. A synergistic adjuvant effect was observed when 1826 CpG-ODN was mixed with liposomal MPLA and Pam_3Cys, respectively but not with their free form. Moreover, we found that the route of vaccination has a great impact on the overall number of Ag-specific T cells. In this regard, injection of vaccine formulations into the footpad was clearly more effective than intradermal immunization into the flank. In summary our data demonstrate that liposomes as carriers for peptide-antigen and adjuvant induce a strong antigen-specific T cell response and are superior over free vaccine formulations.

Cytotoxic T-cell mediated killing of cancer cells: The importance of the target epitope

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A large number of therapeutic vaccination trials against cancer have been conducted over the last decade. However, until recently only limited attention has been focused on the impact of the targets for induction of clinically relevant anti-cancer immune responses for these trials. In the present study we isolated and expanded tumor specific cytotoxic T lymphocytes (CTL) clones from the peripheral blood of cancer patients. We established CTL clones reacting against the melanoma differentiation antigens MART-1 and gp100 as well as against the regulators of apoptosis proteins survivin and Bcl-2. As expected the anti-apoptotic protein specific CTL clones efficiently lysed tumor cells of different origin, i.e. breast cancer, colon cancer, and melanoma cells, whereas the MART-1 and gp100 specific CTL clones specific only killed melanoma cells. However, surprisingly, even melanoma cells were killed much more efficiently by the anti-apoptotic protein specific CTL clones than by the clones specific for MART-1 or gp100, although the T-cell avidity of the latter was higher for their respective targets. Notably, the resistance to killing by the MART-1 specific T cell clones could be overcome by pulsing the melanoma cells with the respective T-cell epitopes. Our data emphasize that the selected tumor antigens and/or epitopes are critical for the outcome of anti-cancer immunotherapy.

Efficient tumor cell lysis mediated by a Bcl-X(L) specific T-cell clone isolated from a breast cancer patient

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Based on the detection of spontaneous immune responses in cancer patients with cancer of different origin, Bcl-X(L) was recently described as a highly interesting tumor antigen recognized by $CD8^+$ cytotoxic T lymphocytes. To further characterize Bcl-X(L) as a tumor antigen we isolated and expanded Bcl-X(L) specific T cells from the peripheral blood of a breast cancer patient hosting a strong Bcl-X(L) specific T cell response. We describe that HLA-A2 restricted Bcl-X(L) specific T cell clones very efficiently lyse peptide pulsed T2 cells. Furthermore, tumor cell lines of different origin, i.e., breast cancer, colon cancer, and melanoma, are efficiently lysed in an HLA-dependent manner. Finally, *ex vivo*-isolated leukemia cells, but not non-malignant B and T cells are killed by Bcl-X(L) specific T cells. Our data underline Bcl-X(L) as an universal tumor antigen widely applicable in specific anticancer immunotherapy.

Spontaneous Immune Responses and Antigen expression in Patients with Acute Myeloid Leukemia

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Despite the increased knowledge on the molecular biology of acute myeloid leukemia (AML) the treatment of AML patients still remains challenging. Between 20%-30% of AML patients receiving chemotherapy achieve long-term survival (1).

In this study, we analyzed the expression (real-time RT-PCR) of the potential tumor-antigens; Survivin and Proteinase 3 in the bone marrow of 20 AML patients. The expression of both antigens in bone marrow (BM) samples was significantly higher than in peripheral blood samples from healthy donors. Generally, the expression of Proteinase 3 in BM was higher than that of Survivin (10-100 fold). No correlation was found between the expression of Proteinase 3 and Survivin and the presence of spontaneous T-cell responses against these antigens in bone marrow as detected in ELISPOT analyses. Furthermore, ELISPOT assays demonstrated spontaneous T-cell responses towards HLA-A2 restricted peptides from Survivin (2/10 patients), Proteinase 3 (3/9 patients) and Wilms tumor antigen (WT1) (3/8 patients) in the peripheral blood before chemotherapy. Patient blood samples collected after chemotherapy are currently being analyzed to further investigate the impact of chemotherapy on T-cell responses in the periphery. Preliminary results will be presented.

(1) Tallman MS, Gilliland DG, Rowe JM. Drug therapy for acute myeloid leukemia. Blood 2005 Aug 15; 06(4): 154-63

The 1 ml immunoscope: a rapid and robust FACS based assay for the identification and enumeration of lymphocyte populations

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BACKGROUND AND OBJECTIVE: Various lymphocyte subsets have been proposed as possible surrogate markers for the immune status in cancer and the effects of treatment. We have developed a FACS based assay, which allows the robust and sensitive detection of a wide range of lymphocyte populations in the blood and further either as a snapshot analysis or over time. We have then used this assay to investigate the kinetics of lymphocyte populations in the peripheral blood of patients with advanced-stage solid malignancies during the course of platinum-based chemotherapy.

METHODS AND PATIENTS: A FACS Canto 6 colour machine was employed in this study. For the characterization and enumeration of lymphocyte populations three distinct multicolour flow cytometry assays were developed and validated in 18 healthy volunteers: one for the identification of B-, T-, and NK-cells, including the CD4+, CD8+ and TNK subsets; a second for the phenotyping of memory T-cell subpopulations including naïve, central memory and effector memory cells; lastly, an intracellular staining procedure for Foxp3+ T-regulatory cells (Tregs). All three methods required only 0.5 ml of fresh lysed whole blood. CD45 was used for the accurate gating of the total lymphocyte population and absolute numbers of populations were calculated from the FBC taken at the same time. Both the percentages and absolute counts of the various subsets were determined at baseline and during one treatment cycle with a standard platinum-based chemotherapeutic regimen. Of the 13 patients enrolled so far, data are available for seven with the following characteristics: 6 male- 1 female, mean age: 67 years, 5 with non-small cell lung cancer, one with small cell lung cancer and one with malignant melanoma. A control cohort of six age-matched healthy volunteers (3 male- 3 female, mean age: 67 years) was evaluated in parallel.

RESULTS: In two out of seven patients the baseline levels of total lymphocytes were below normal. Absolute B-lymphopenia was revealed in three cases and reversal of the CD4/CD8 ratio in two, without reverting to normal after one cycle of treatment. Among the six healthy controls only one exhibited baseline total lymphopenia, while another one had both B-lymphopenia and CD4/CD8 ratio reversal persisting over follow-up. Overall, no statistical significant difference was observed at baseline between the patients and healthy controls for any of the cell subpopulations. The alterations in the percentages and absolute numbers of the lymphocyte subsets following one cycle of chemotherapy did not reach statistical significance.

CONCLUSIONS: Even a small blood sample of less than 1 ml is sufficient for the detailed and robust analysis of lymphocyte subsets in the blood of patients with solid tumours. Further clarification on the biological significance of quantitative abnormalities detected at baseline is warranted. Although still preliminary, our results indicate that after recovery from the treatment-induced nadir platinum-based chemotherapy does not appear to affect the distribution of lymphocytes in the peripheral blood, including T-regulatory cells.

Memory CD8$^+$ T cells mediate antibacterial immunity via CCL3 activation of TNF/ROI$^+$ phagocytes

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Cytolysis, IFN-γ and TNF-α secretion are major effector mechanisms of memory CD8$^+$ T cells and are believed to be sufficient for immunological protection *in vivo*. By using mutants of the intracellular bacterium *Listeria monocytogenes*, we show that memory CD8$^+$ T cells need to cooperate with innate inflammatory mononuclear phagocytic cells (MPCs) to orchestrate protective responses against secondary *L. monocytogenes* infection. Upon reactivation, bacteria-specific memory CD8$^+$ T cells secrete CCL3 that induces rapid TNF-α secretion by MPCs. TNF-α further promotes the production of Radical Oxygen Intermediates by both MPCs and neutrophils leading to subsequent bacterial killing. These results uncover two levels of regulation of the antibacterial secondary protective response: (i) memory CD8$^+$ T cells control the activation of the innate immune system and (ii) MPCs coordinate innate immunity and promote the bactericidal effector activities.

Announcement CIMT 2008

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