From cell sorting to plasma cell identification and detection of chromosomal aberrations in multiple myeloma

Immunotherapy of cancer: from bench to bedside

Abstracts and Application Manual

3rd MYELOMA and 1st IMMUNOTHERAPY WORKSHOP

15-16/10/2008

Brno, Czech Republic

ILBIT MU Brno, Kamenice 5/A3

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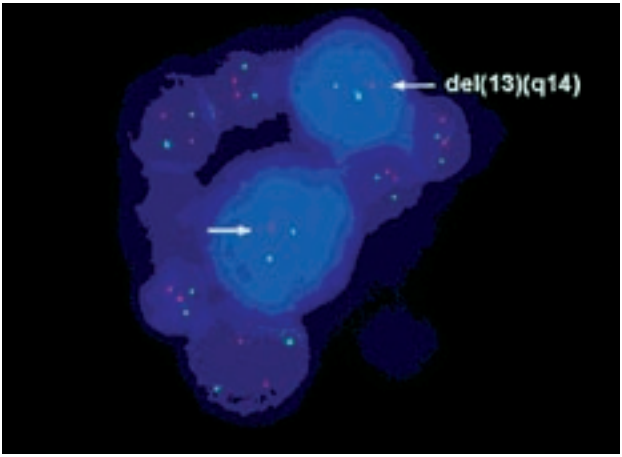

ABSTRACTS AND APPLICATION MANUAL

Dear Ladies and Gentlemen,

it is my honour and privilege to welcome you to our Third Myeloma Workshop. It is focused on FISH methods used in multiple myeloma and on separation technologies aimed at pure cell population obtaining which represents a necessary entrance point of a wide range of research applications. Cryopreservation of separated cells is also one of the crucial steps in the methodology and will be presented. I really appreciate the practical part of the workshop allowing to perform and practice respective methodologies directly in laboratory.

Second day of the workshop is focused on immunotherapy which is the second key research field of our teams sharing facilities in campus. I am very happy to see excellent agenda of this topic prepared by the team led by Prof. Jaroslav Michalek

I would like to express my thanks also to my colleagues for a very good preparation of the worskhop.

I believe that you will enjoy the stay in the areas of our new Masaryk University campus. Our research teams, Czech Myeloma Group – University Research Centre and University Cell Immunotherapy Centre has been working here already for four years. One of its main goals is, besides the research in the first instance, the transfer of techniques and support of research in the field of multiple myeloma and immunotherapy in the Central and Eastern Europe. This part of our activities is supported by the Czech Myeloma Group, the International Myeloma Foundation and the Modra Kotva Foundation.

I wish you a pleasant stay and many new scientific inspirations.

Roman Hajek
Chairman of Czech Myeloma Group (CMG)
and chairman of University Research Center- CMGr

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Scientific programme:

MACS&FISH
From cell sorting to plasma cell identification and chromosomal aberrations in multiple myeloma

15.10.2008

9:00 am – 9:10 am	**Chairman's introduction** **Roman Hajek, Brno, Czech Republic**
9:10 am – 11:00 am	**Theoretical part of workshop**
9:10 am	Cytogenetic and Proteomic Analyses in MGUS and Multiple Myeloma Patients: Role of the Microenvironment for Myeloma Pathogenesis **Johannes Drach and Christopher Gerner , Vienna, Austria**
9:40 am	Quantitative analysis of RAN, ZHX-2 and CHC1L gene expression in multiple myeloma patients **Andrea Harnicarova, Eva Bartova, Jana Krejci, Brno, Czech Republic**
9:55 am	Trends in Cryoconservation in Myeloma Bank **Eva Matejkova, Jana Muzikova, Dana Novotna, Barbara Kubesova, Jaroslav Michalek, Brno, Czech Republic**
10:10 am – 10:30 am	**Coffee break**
10:30 am	The poor prognosis associated with gain/amplification of 1q21 in relapsed multiple myeloma patients may be overcome by Velcade based regimen in contrary of thalidomide based regimen **Pavel Nemec, Henrieta Greslikova, Jan Smetana, Petr Kuglik, Roman Hajek, et al., Brno, Czech Republic**
10:45 am	Myeloma Bank – Use in Practice **Eva Matejkova, Drahomira Kyjovska, Jana Cumova, Miroslav Penka, Roman Hajek, et al., Brno, Czech Republic**
11:00 am – 12:00 am	**Practical part of workshop (part I) for registered participants only** Detection of chromosomal aberrations in multiple myeloma using simultaneous immunofluorescent labelling of malignant plasma cells and fluorescent in situ hybridization (clg FISH) **Romana Zaoralová, Henrieta Greslikova, Renata Kupska, Pavel Nemec, Petr Kuglik, Brno, Czech Republic** **Practical demonstration of magnetic separation cell on AutoMACS instrument** **Andreas Nicodemou, Biohem**
12:00 am – 13:00 pm	**Lunch break**
13:00 pm – 15:30 pm	**Practical part of workshop (part II) for registered participants only** clg FISH and image analysis using new version of LUCIA software in multiple myeloma **Dusan Kadlec, Petr Kuglik, Brno, Czech Republic**
15:30 pm	**Chairman's conclusion** **Roman Hajek, Brno, Czech Republic**

Immunotherapy of cancer: from bench to bedside

16.10.2008

9:00 am – 9:05 am	**Chairman's introduction** **Jaroslav Michalek, Brno, Czech Republic**
9:05 am – 12:10 pm	**Theoretical part of workshop**
9:05 am	Vaccination strategies in leukaemia **Katy Rezvani, London, United Kingdom**
9:25 am	RHAMM peptide vaccination in patients with multiple myeloma, myelodysplastic syndrome, acute myeloid leukemia and chronic lymphocytic leukemia **Krzysztof Giannopoulos, Jochen Greiner, Michael Schmitt, Ulm, Germany, Lublin, Poland**
9:45 am	A tool for antigen delivery and T cell immune response induction for cancer immunotherapy: The adenylate cyclase toxoid **Petr Sebo, Irena Adkins, Jana Kamanova, Marcela Simsova, Jiri Masin, Ondrej Stanek, Prague, Czech Republic**
10:05 am	Stem cell plasticity and diversity in carcinogenesis **Stanislav Filip, Jaroslav Mokry, Dennis English, Hradec Kralove, Czech Republic**
10:25 am – 10:50 am	**Coffee break**
10:50 am	Conceptual considerations and comparative evaluation of techniques for the manufacturing of dendritic cell-based cancer vaccines **Thomas Felzmann, Vienna, Austria**
11:10 am	Searching for ideal dendritic cell for cancer vaccination **Katerina Skalova, Klara Mackova, Darina Ocadlikova, Roman Hajek, Jaroslav Michalek, Brno, Czech Republic**
11:30 am	Identification and long-term monitoring of individual tumor-specific T cells **Jaroslav Michalek, Veronika Foltankova, Eva Matejkova, Vuk Fait, Roman Hajek, et.al., Brno, Czech republic**
11:50 am	Ipilimumab as anti-CTLA4 immunotherapy: preclinical and clinical data **Jaroslav Michalek, Brno, Czech Republic**
12:10 pm	**Concluding remarks** **Jaroslav Michalek, Brno, Czech Republic**
12:20 pm	**Lunch break**

ABSTRACT OF LECTURES

Cytogenetic and Proteomic Analyses in MGUS and Multiple Myeloma Patients: Role of the Microenvironment for Myeloma Pathogenesis

Drach J. [1] and Gerner Ch.[2]

[1] Dept of Medicine I, Clinical Division of Oncology, and [2] Institute of Cancer Research, Medical University of Vienna, Vienna, Austria

The development of multiple myeloma (MM) is regarded as a multistep process, with several genetic and molecular lesions leading from a normal plasma cell to the clinical characteristics of MM. However, critical events leading to the transition from MGUS to MM have still remained poorly characterized.

We first hypothesized that genetic events being associated with a poor prognosis in MM may also be important for the MGUS – MM transition. As we and others have shown, MGUS plasma cells are very similar to plasma cells from progressive MM, and in particular, abnormalities like the t(4;14), t(14;16), and deletion 13q are already present at the level of MGUS. Moreover, such primary genetic events appear to remain stable over the course of the disease.

The microenvironment of tumor cells in the bone marrow may be actively involved in the progression of malignant diseases. In MM, interactions of bone marrow stromal cells with the malignant plasma cells have gained significant importance as targets for novel therapeutic agents. Based upon these observations, we aimed at analyzing in detail the secretory capacity of bone marrow fibroblasts obtained from patients with MM in order to better understand their contribution to disease progression. We therefore analyzed the secretome of primary bone marrow fibroblasts of MM patients by proteome profiling based on highly sensitive mass spectrometry. Normal skin fibroblasts were found to secrete various extracellular matrix (ECM) proteins including fibronectin, collagens and laminins, in addition to some chemokines and cytokines including CXCL12, follistatin-like 1, insulin-like growth factor binding proteins 4, 5 and 7; and SPARC. In contrast, bone-marrow-derived fibroblasts from MM patients secreted increased amounts of ECM proteins and alpha-fetoprotein in addition to insulin-like growth factor II, stem cell growth factor and matrix metalloproteinase-2. Co-culture of primary MM cells with these fibroblasts further stimulated the secretion of ECM proteins, of cytokines such as inhibin beta A chain and growth factors such as connective tissue growth factor, which might be relevant to support the malignant clone. Analyses of the secretion capacity of bone marrow fibroblasts from patients with MGUS are in progress. Proteome profiling of secreted proteins may thus help to identify relevant tumor-associated proteins, to increase our understanding of cell cooperativity and thereby increase our understanding of progression events in monoclonal gammopathies.

Quantitative analysis of RAN, ZHX-2, and CHC1L gene expression in multiple myeloma patients

Harnicarová A., Bartova E. and Krejci J.

Institute of Biophysics, Academy of Sciences of the Czech Republic, v.v.i., Královopolská 135, CZ-612 65, Brno, Czech Republic

Real time PCR (RT-PCR) is a powerful tool for the analysis of genes involved in the pathophysiology of human diseases. Recent RT-PCR studies of RAN (6p21), ZHX-2 (8q24.3), and CHC1L (13q14.3) loci revealed that expression of these genes is important for the prognosis and progression of multiple myeloma (MM) (Armellini et al., 2008). ZHX-2 was found to be weakly expressed in high-risk disease, while increased ZHX-2 expression was associated with better responses and longer survival after high-dose chemotherapy. CHC1L was poorly expressed in hyperdiploid cells (Armellini et al. 2007). On the other hand, the RAN locus was highly transcriptionally active in symptomatic MM and myeloma cell lines (Armellini et al., 2007). ZHX-2 and CHC1L loci map closely to c-myc and Rb1, which both play an important role in tumor cell proliferation (Avet-Loiseau et al., 2001; Shaughnessy et al., 2000). Therefore, these genomic regions are important prognostic markers for many tumors.

Here, we established RT-PCR methodology that could be applied clinically for analysis of RAN, ZHX-2, and CHC1L gene expression. We searched for relevant internal controls for RT-PCR by examining expression of select genes in peripheral blood cells of healthy individuals and in myeloma cell lines. Our preliminary results show that expression of candidate genes should by analyzed in cells of newly diagnosed patients and that expression levels should be compared only in cell populations with identical infiltration of malignant clone to bone marrow. In addition, routine RT-PCR analysis of tumor-related gene expression should rely on pre-specified RNA purity requirements and RNA parameters (e.g., determined using an Agilent Bioanalyzer). Troubleshooting of gene expression analysis by clinical laboratories is also described.

This work was supported by Grant No. LC06027 from the Ministry of Education, Youth, and Sports of the Czech Republic as well as by Projects Nos. AV0Z50040507 and AV0Z50040702. We thank Dr. Pavla Gajdušková for help with RT-PCR analysis.

References:

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3. Armellini A, Sarasquete ME, García-Sanz R, Chillón MC, Balanzategui A, Alcoceba M, Fuertes M, López R, Hernández JM Fernández-Calvo J, Sierra M, Megido M, Orfão A, Gutiérrez NC, González M, San Miguel JF. Low expression of ZHX2 but not RCBTB2 or RAN, is associated with poor outcome in multiple myeloma. Br J Haematol. 2008, 141(2):212-215.

Trends of Cryoconservation in Myeloma Bank

Matejkova E.[1,2,] Muzikova J.[1], Novotna D.[1], Kubesova B.[2], Michalek J.[1]

[1] *University Cell Immunotherapy Center, Masaryk University, Brno*
[2] *Tissue Bank, Faculty Hospital Brno*

Introduction:

Cryoconservation is a major method of long-term storage of unique biological material. Main advantage of this method is high viability of cells after thawing. We observed new approach of preservation using programmable freezing machine (IceCube S14, SYLAB). Thawing procedure is also very important for good cells fitness.

Material:

Mononuclear cells isolated from buffy coats from healthy donors from Transfusion Station FH Brno.

Methods:

We used conventional method of cryopreservation and compared it with four other procedures retrieved from collaborative centers. Mononuclear cells were obtained from buffy coats by gradient centrifugation. We proved three types of cryoprotective solution, two methods of freezing (programmable freezing machine and traditional ethanol container) and two methods of thawing (DMSO wash-out, leaving the cells in complete medium without DMSO wash-out). We also tested the number of cells per one cryovial.

Results:

We compared two ways of freezing – in ethanol container we reached 90,8 % (median, 56–100 %) cells after thaw compared with programmable freezing, where we got 96,8% (median, 67 – 100%). We discovered that the number of cell in one cryovial is also a limit parameter; the best ratio is about $20x10^6$. We confronted three types of cryoprotective solutions and we found out that the best results were with diluted albumin and 10% of DMSO.

For thawing the cells is better to leave them in complete medium for next 24 hours instead of wash out the DMSO immediately after thawing.

Conclusion:

We observed that the best method for success cryopreservation is programmable freezing and novel cryoprotective solution with 10% of DMSO. This high effective process has been used in Myeloma Bank from January 2008.

Supported by MSMT – NPVII 2B06058 and LC 06027.

The poor prognosis associated with gain/amplification of 1q21 in relapsed multiple myeloma patients may be overcome by Velcade based regimen in contrary of thalidomide based regimen

[1,2] Nemec P., [1,2] Greslikova H., [2] Smetana J., [1,2] Zaoralova R., [1] Kupska R., [1,2] Berankova K., [2] Filkova H., [1] Kralova D., [3] Krejci M. [3] Pour L., [3] Zahradova L., [3] Sandecka V., [3] Adam Z., [1,2] Kuglik P., [1,3] Hajek R.

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[2] Department of Genetics and Molecular Biology, Institute of Experimental Biology, Faculty of Science, Masaryk University Brno, Czech Republic
[3] Internal Haemato-oncology Clinic, Faculty Hospital Brno, Faculty of Medicine, Masaryk University, Brno, Czech Republic

Introduction

The presence of chromosomal aberrations detected by fluorescence in situ hybridisation (FISH) in plasma cells is considered to be an important prognostic factor for patients with multiple myeloma (MM). However for relapsed patients, it is unknown whether or not the negative impact of these aberrations can be eliminated by treatment based on the new drugs like Velcade or thalidomide.

Aims

This study is aimed at comparison of ability to overcome the negative prognostic impact of the most common cytogenetic aberrations by treatment regimens based on Velcade or thalidomide.

Methods

Velcade group: A total of 42 patients (median age 63.4 years; median follow-up 16.0 months; median of previous therapy lines 1 (range 0-4, 38.1% in first and 38.1% pts. in second relapse) were treated by Velcade based regimen (48% together with glucocorticoids and alkylating agens; 30% with anthracycline ± dexamethasone; 22% with dexamethasone only).

Thalidomide group: A total of 33 (median age 65.3 years; median follow-up 19.7 months; median of previous therapy lines 1 (range 0-4, 66.7% pts. in first and 15.2% in second relapse) were treated by thalidomide based regimen (94% together with dexamethasone and cyclophosphamide; 6% with dexamethasone only). Both groups were separately examined by cytoplasmic interphase FISH (clg-FISH) for presence of 1q21 gain/amplification, del13q14, del17p13, t(4;14) and hyperdiploidy/nonhyperdiploidy. Selected threshold for positivity was: 20% for gain1q21, del13q14 and nonhyperdiploidy, and 50% for del 17p13 and t(4;14).

Results for patients treated by Velcade based regimen

Gain1q21 was detected in 80% (24/30) patients, del13q14 and del17p13 were detected in 68% (27/40) and 28% (11/39) patients, respectively. Translocation t(4;14) was detected in 29% (10/35) and nonhyperdiploidy in 59% (20/34 patients. Comparison of ORR of positive vs. negative patients for any aberration was not significant. Comparison of PFS median of positive vs. negative patients for each aberration was as follows: For gain1q21 reached 8.6 vs. 7.6 months P=0.556; for del13q14 reached 6.7 vs. 8.5 months; P=0.664; for del17p13 reached 4.8 vs. 8.2 months; P=0.541; and for t(4;14) reached 8.2 vs. 7.7 months; P=0.591.

Summary/Conclusions

Both new drugs can overcome negative prognostic impact of all named chromosomal abnormalities except gain1q21. However, difference in PFS median between del 17p13 positive and negative patients is not relevant, because of presence of only 1 patient in negative group. The difference in PFS median in gain1q21 positive and negative patients treated by thalidomide based regimen suggests that thalidomide is not able to overcome negative prognostic impact of gain1q21 in contrary of treatment based on Velcade regimen, which probably may overcome poor prognosis of gain1q21. However this findings has to be confirmed on larger cohort of patients with longer follow-up.

Support

This study was supported by grant LC06027 of Masaryk University, Brno, Czech Republic, and by grants MSM0021622415 and MSM0021622434 of Ministry of Education, Czech Republic, and by IGA grant NR/9317-3 of Ministry of Medicine, Czech Republic.

Myeloma Bank – Use in Practice

Matejkova E. [1,4], Kyjovska D. [1], Cumova J. [2], Pour L. [1], Vidlakova P. [1], Moravcova J. [1], Rycova M. [1], Penka M. [1], Hajek R. [1,2,3]

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Introduction:

Long-term storage and archiving of samples in liquid nitrogen is very important for future use, especially for monitoring and precaution experiments. These samples are also considerable for diagnostic and therapeutic usage in myeloma patients. We present the current status in Myeloma Bank from the year 2001 till presence.

Methods/Patients:

We collect bone marrow samples from patients with new diagnosis of multiple myeloma (MM), with relapse, before therapy and in the phase of maximal therapeutic response. All patients confirmed the informed consent. We isolate mononuclear cells using gradient centrifugation and then we separate CD138+ (rich in myeloma cells) and CD138- cells with MACS (MAgnetic Cell Sorting, Miltenyi Biotec). We assay purity of these fractions – percentage of myeloma cells on cytospin smear and percentage of CD38+ and CD138+ cells with flow cytometry. Native cell fractions are used in following experiments or they are cryoconserved with programmable freezing and then store in liquid nitrogen.

Results:

From year 2001 till January 2008 we cryoconserved 8 549 samples from myeloma patients in form of CD138+ and CD138- cell fractions, plasma and bone marrow mononuclear cells. From peripheral blood we store serum, plasma and mononuclear cells.

Conclusion:

Separated CD138+ cells are used in cytogenetic (FISH, CGH), genomic (RT-PCR) and proteomic experiments. These cells are also exploited as a tumor antigen for vaccine preparation on the dentritic cells basis and for cultivation of myeloma specific T-lymphocytes.

Supported by MSMT – NPVII 2B06058 and LC 06027

Vaccination strategies in leukaemia

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Introduction

The graft-versus-leukaemia (GVL) effect following allogeneic stem cell transplantation is clear evidence that T lymphocytes can control and eliminate leukaemia. During a GVL response recipient malignant cells present antigens to the donor T cells and induce leukaemia-specific T-cell expansions. Some of the antigens that drive the GVL response have been characterised and can be categorised broadly into four classes: (1) neoantigens expressed as a consequence of chromosome translocations or mutations (e.g. BCR-ABL), (2) non-alleleic normal proteins that are aberrantly expressed or over-expressed in leukaemia (e.g. PR3, WT1), (3) viral antigens (e.g. EBV) , and (4) alloantigens such as minor histocompatibility antigens (mHA) (HA-1, HA-2). In the context of haematological malignancies an ideal leukaemia-associated antigen (LAA) should induce a strong cytotoxic T cell response, be expressed on leukaemic progenitors and be intrinsic to leukaemic survival, so that viable tumour escape by downregulation of the antigen cannot occur.

This review focuses on efforts to discover and use defined leukaemia protein antigens for immunotherapy, because of their potential to establish universal and practical vaccines for leukaemia. Peptides from three well characterised LAA, the BCR-ABL fusion protein of chronic myeloid leukaemia (CML), proteinase-3 (PR3) and Wilms tumour 1 (WT1) protein, serve as the basis for several clinical trials using peptide and adjuvants to treat patients with a variety of myeloid malignancies. We will therefore limit this review to data currently available on these antigens.

BCR-ABL

CML is characterised by the presence of Philadelphia (Ph) chromosome . The Ph chromosome represents a reciprocal translocation between the long arms of chromosomes 9 and 22, t(9;22)(q34;q11). The molecular consequences of this translocation are juxtaposition of the c-ABL oncogene from chromosome 9 into the breakpoint cluster region (BCR) within the BCR gene on chromosome 22, resulting in a chimeric BCR-ABL gene . Short peptides produced by cellular processing of the BCR-ABL neoantigen can be presented on the cell surface within the cleft of HLA class I and II molecules, and in this form they can be recognised by T cells . Peptides that bind with high or intermediate affinity to HLA-A3, A11, B8 and A*0201 have been identified and lysis of BCR-ABL peptide loaded target cells by CD8+ BCR-ABL peptide specific T cells in an MHC class I restricted manner has been described in humans . Mass spectrometry studies have demonstrated the presence of BCR-ABL peptide on the surface of primary CML cells in patients. In addition, these patients mounted a cytotoxic T cell response to this peptide that killed autologous CML cells , further supporting the role of BCR-ABL as a leukaemia antigen.

Proteinase 3

Proteinase 3 is a 26 kD neutral serine protease stored in azurophilic granules that is maximally expressed at the promyelocyte stage of differentiation. It is overexpressed in a variety of myeloid leukaemia cells including CML cells . PR3 itself may also be important in maintaining a leukaemia phenotype as it has been shown that PR3 antisense oligonucleotides halt cell division and induce maturation of the HL-60 promyelocytic leukaemia cell line . PR1 is an HLA-A2 restricted 9 amino acid peptide derived from PR3 and is a target epitope of CTLs that preferentially lyse CML cells . PR1 specific T-cell responses are detectable in patients with CML and AML .

Wilms tumour

WT1 is a zinc finger transcription factor that is overexpressed in most cases of haematological malignancies. CTLs recognising HLA-A*0201 or HLA-A24 restricted epitope of WT1 selectively lyse WT1-expressing leukaemia cells whilst sparing normal progenitors . In mice, immunisation with peptide fragments of WT1 or WT1 DNA can elicit CTL responses that reject challenge from WT1 expressing tumour cells without apparent toxicity to the small number of normal tissues that express WT1, indicating that the WT1 specific CTLs generated *in vivo* in the murine model can discriminate between WT1 expressing tumour cells and physiologically WT1-expressing normal cells . In humans, WT1 has been shown to be naturally immunogenic with detectable responses in patients with leukaemia .

Peptide vaccines under clinical development for leukaemia

BCR-ABL vaccine

Previous trials of BCL-ABL vaccination composed of a pool of 6 peptide fragments showed the safety of the vaccine and adjuvant. The vaccines could elicit both humoral and T cell immune responses to BCR-ABL, however the efficacy of the vaccine was not convincingly demonstrated . In a subsequent, more stringent trial using a similar peptide combination, 10/10 patients with stable disease on imatinib showed cytogenetic improvement and 3 of 5 patients who achieved complete cytogenetic response (CCR) had undetectable BCR-ABL transcripts by nested PCR . In contrast, a recent phase I/II study from the UK (the EPIC study) reported no molecular benefit in 5 patients not in major cytogenetic response (MCR) at baseline. However, of the 14 patients in MCR at baseline, 13 developed at least 1 log fall in BCR-ABL transcripts though this occurred several months after completing vaccination . These results suggest that clinical responses to BCR-ABL peptides can be induced in patients with CML with low levels of stable disease. These rather modest results raise concerns that the method of vaccine administration or the immune status of the treated patients may be suboptimal, or that BCR-ABL does not induce sufficiently powerful CTL responses to CML.

PR1 vaccine trials

PR1 is a 9 amino-acid HLA-A*0201 restricted peptide derived from PR3, shown to elicit myeloid leukaemia-specific CTL responses that selectively kill leukaemic CD34+ cells . Highly encouraging preliminary data from a phase 1/2 study evaluating PR1 vaccination in patients with myeloid leukaemias were first presented at the Annual meeting of the American Society of Haematology (ASH) in 2004 and an update presented in 2007 . Sixty-six HLA-A*0201+ patients with acute myeloid leukaemia (AML), CML or myelodysplastic syndrome (MDS) were treated with PR1 peptide vaccine. Fifty three had active disease and 13 were in remission on entering the trial. The first 54 patient received three vaccinations, and the last 12 patients received 6 vaccinations. Patients received one of three dose levels: 0.25, 0.5 or 1.0 mg per vaccination every 3 weeks, together with Montanide adjuvant and granulocyte-monocyte colony stimulating factor (GM-CSF). Toxicity was limited to local injection site reactions only such as redness, swelling and some pain. Of the 53 with active disease, 25 (47%) had an immune response. Clinical responses ranging from improvements in blood counts to complete cytogenetic remission were observed in 9 out of 25 responders (36%) compared with 3 of the 28 non-responders (10%). Importantly, immune response to the PR1 vaccine was associated with an 8.7 month EFS compared with 2.4 months for non-responders ($p = 0.03$), with a trend towards longer OS . Among the 13 patients in remission at the start of the trial, four remained in remission for a median time of 30.5 months. Analysis of the effectiveness of this approach in a subgroup of 20 patients with myeloid leukaemia vaccinated following SCT showed a PR1 response to vaccine in 11/20 (55%) patients. Nine of 11 (82%) vaccine responders compared to 1 of 9 (11%) patients who failed to mount a response to the vaccine had clinical responses ($P = .005$). Importantly, a significant PR1 response to the vaccine was associated with significantly better clinical response and longer event-free survival (EFS) . These encouraging results have led to the initiation of several new studies with PR1 in less advanced patients.

WT1 vaccine trials

Oka et al reported the outcome of a phase I study of WT1 peptide-based immunotherapy in 26 patients with MDS, AML, breast or lung cancer . Patients received an HLA-A24 9-mer WT1 peptide in Montanide adjuvant at 2-week intervals in a dose-escalation study. The vaccine was well tolerated and the only notable side effect was profound leukopenia in two patients with hypoplastic MDS, reversed by steroid treatment, which concomitantly abrogated the WT1 T cell response. Twelve of the 20 evaluable patients had clinical responses including reductions in blood or marrow leukaemic blasts or tumour size or tumour markers. Of note increases in WT1-specific CTL frequency correlated with a clinical response.

Vaccination with a combination of PR1 and WT1 peptides

Since immune responses against leukaemia are often directed against multiple antigens there is a risk that targeting a single leukaemia antigen may result in immunologic pressure against expression of the parent protein, resulting in the selection of antigen-loss variants. We therefore used a combined PR1 and WT1 peptide vaccine approach in an attempt to improve the probability of generating a sustained immune response against MDS and leukaemia. Eight patients with myeloid malignancies received a single dose of PR1 and WT1 peptide vaccines. CD8+ T-cell responses against PR1 or WT1 were detected in all patients and the emergence of PR1 or WT1-specific CD8+ T-cells was associated with a significant reduction in leukaemia load as assessed by WT1 mRNA expression . However the responses were short lived, suggesting the need for further manipulations for a sustained response.

Clinical applications of LSA vaccines - the future

While peptide vaccination could conceivably be used to treat leukaemia, it is unlikely that it could eliminate establis hed disease unless combined with other treatments. It is likely that the immunological response induced by vaccinatior will be most effective with a lower leukaemia burden, especially as immune strategies may require a prolonged period o time for effectiveness. A logical extension of this is to vaccinate patients in a state of minimal residual disease, or patient complete remission that are at high risk to relapse as a form of prophylaxis. We are currently investigating the value o PR1 and WT1 peptide vaccination in CML patients stabilised to low levels of molecular disease by the tyrosine kinas inhibitor, imatinib (Gleevec). Within the next 5 years the benefits of novel developments in the field of immunotherapy i likely to translate into significant improvements in the outcome of vaccination in patients with cancer. The combinatior of different approaches including the use of novel adjuvants such as toll-like receptor (TLR) agonists to enhance the vaccine-induced T-cell response, the selective depletion of regulatory T cells (Tregs) prior to vaccination to remove the suppressive activity, the use of multiple peptide vaccines, in particular the inclusion of novel HLA class II epitopes to induce antigen-CD4+ T-cells to sustain the vaccine-induced CD8+ T-cell response, will all contribute to reinforce the patient immune response against leukaemia.

Reference List

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RHAMM peptide vaccination in patients with multiple myeloma, myelodysplastic syndrome, acute myeloid leukemia and chronic lymphocytic leukemia

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Introduction:

We initiated a phase I R3 peptide vaccination for HLA-A2 positive patients with acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), multiple myeloma (MM) and chronic lymphocytic leukemia (CLL) overexpressing the receptor for hyaluronic acid mediated motility (RHAMM). RHAMM is a tumor associated antigen (TAA) that is strongly expressed in several hematological malignancies and induces humoral as well as cellular immune responses.

Methods:

Twenty five patients with AML, MDS, MM or CLL were enrolled into study. RHAMM was assessed by RT-PCR and HLA-A2 expression by flow cytometry. We included only patients in early stage of the disease or with a limited tumor load or with a minimal residual disease. The first 16 (3 AML, 3 MDS, 4 MM and 6 CLL) patients were vaccinated with 300 mcg and further patients with 1000 mcg R3 peptide emulsified with the incomplete Freund's adjuvant. The vaccine was given four times at a biweekly interval and GM-CSF was added for five days each vaccination as an adjuvant. Immunological analyses were performed using enzyme linked immunospot (ELISpot) assays for interferon gamma and granzyme B as well as flow cytometry for tetramer positive CD8+ T cells.

Results:

Only mild drug-related adverse events were observed such as erythema and induration of the skin at the site of injection. We detected positive clinical effects in several patients with myeloid disorders showing a reduction of blasts in the bone marrow. One MDS patient did not need any longer erythrocyte transfusions. Two patients with MM showed a reduction of free light chain serum levels. In CLL, four of six patients showed a reduction of WBC during vaccination. We detected specific immune responses in 70% of patients. In most patients, we found an increase of CD8+/HLA-A2/RHAMM R3 tetramer+ effector T cells in flow cytometry in accordance with an increase of R3-specific CD8+ T cells in ELISpot assays.

Conclusions:

RHAMM represent a promising structure for further targeted immunotherapies in patients with a broad variety of hematological malignancies.

A tool for antigen delivery and T cell immune response induction for cancer immunotherapy: The adenylate cyclase toxoid

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Introduction:

Adenylate cyclase toxin (CyaA) targets myeloid phagocytes expressing the $\square_2$ integrin CD11b/CD18 (CR3 or Mac-1), such as the dendritic cells, and delivers into their cytosol an adenylate cyclase (AC) enzyme domain. Over the past 13 years, genetically detoxified CyaA-AC- toxoids, with the capacity to catalyze conversion of cytosolic ATP to cAMP disrupted, have been shown to efficiently deliver a broad range of passenger antigens into the cytosol of CD11b-expressing antigen presenting cells, thus allowing cytosolic processing by proteasome and presentation of antigenic peptides in complex with MHC class I molecules on APC surface, yielding induction of antigen specific $CD8^+$ cytotoxic T cell responses (CTL). In parallel the endocytosed CyaA was also shown to allow class II-restricted antigen presentation for boosting host T cell responses.

Methods:

Various antigenic epitopes (8 to 38 amino acids long) were inserted into several permissive sites in the AC domain of CyaA toxin with ablated AC activity and the recombinant toxoids were produced upon IPTG induction in liquid cultures in the presence of the activating protein CyaC by using the *E. coli strain* XL-1 Blue. After several steps of chromatographic purification CyaA toxoids with low endotoxin content were eluted with 8 M urea, 2 mM EDTA, 50 mM Tris-HCl, pH 8.0 and stored at -20°C.

Results:

Recombinant CyaA carrying only a single copy of $CD8^+$ T cell-restricted epitope from lymphocytic choriomeningitis virus (LCMV) was shown to induce specific CTLs *in vitro* as well as to provide protection against lethal challenge of mice with LCMV. Moreover, the induction of specific CTLs occurred without the need for adjuvant or help of $CD4^+$ T cells. Similarly, CyaA toxoids were shown to deliver $CD4^+$ T cell epitopes into MHC class II presentation pathway inducing specific Th1 type T cell responses *in vivo* at a 100-times-higher molar efficiency than the free peptide. Furthermore, CyaA bearing antigenic epitopes from ESAT-6 or CFP-10 proteins of *M. tuberculosis* could be used in recall response assays for diagnosis of latent tuberculosis. Moreover, CyaA toxoids have been demonstrated to elicit protective and therapeutic immunity against HPV-16-induced tumors and melanoma in mice. Since also human DC were shown to process and present CyaA-delivered melanoma epitopes to human CTL clones, phase I clinical trials for immunotherapy of cervical cancer and melanoma are currently being prepared, thus highlighting the promise of using CyaA toxoid as a tool for cancer immunotherapy.

Conclusions:

Based on the data from *in vitro* as well as *in vivo* studies recombinant CyaA carrying antigenic epitopes represents an efficient tool for immunotherapy of infectious as well as malignant diseases.

Stem Cells and the Phenomena of Plasticity and Diversity. A limiting Property of Carcinogenesis

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Summary

The phenomenon referred to as "stem cell plasticity" brings forth a number of issues and problems related to many biological and medical disciplines. This paper reviews some important questions and opinions concerning both stem cel plasticity and processes associated with stem cells with special emphasis on "tumor stem cells" and their potential role in carcinogenesis. A considerable component of our discussion is devoted to the phenomena associated with stem cel diversity; namely, to the relationship between phenotypic diversity and plasticity. Here we attempt to formulate gene- ral definitions of plasticity and diversity and show how these properties are interrelated. To illustrate the problem, we chose a theoretical model relevant to in tissue regeneration. The results suggest some theoretical possibilities of tumo development and the reprogramming of tumor cells. It is our opinion that cell plasticity decreases with their stage o differentiation in time. However, plasticity will never be zero, since even highly differentiated cells retain a certain degree of plasticity. Generally speaking, as the number of stem cells decreases, the plasticity of the population decreases – the population has "low plasticity". If we assume that plasticity is a phenomenon applicable to all cells, including tumor cells the development of a tumor stem cell may well result in a profound increase in plasticity. This highly plastic populatior may participate in processes of tissue regeneration.

As we presume existence of a complementary relationship between the plasticity and diversity of stem cells, this highly plastic population may evoke a certain degree of diversity in all cells including tumor cells, and a population o low diversity cells may develop. This condition renders a new degree of diversity in all cells, and a new population of high diversity cells develops. On the grounds of this hypothesis, we predict that in certain circumstances, tumor stem cells may participate in tissue regeneration without initiating tumor development (low diversity cells), or with the imitation o tumor development (high diversity cells). Therefore, it may be presumed that in a certain transition period, tumor stem and progenitor cells may reprogram from the state of diversity (for example, from low → high → low) and stimulate the development of normal tissue.

Here the interrelation between the plasticity and diversity of stem cells plays an important role in determining cel fate. Since the two phenomena cannot be temporally separated from each other but are indeed mutually exclusive. We view this situation in a manner similar to the principle of indefiniteness; cells must choose either plasticity or diversity as both are not possible at the same time.

Key words:
stem cells, tumor stem cells, stem cell plasticity, transdifferentiation, cell diversity, carcinogenesis

Conceptual considerations and comparative evaluation of techniques for the manufacturing of dendritic cell-based cancer vaccines

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Introduction:

Manufacturing procedures for cellular therapies are continuously improved with particular emphasis on immune regulatory feedback loops and product safety. We previously developed a dendritic cell (DC) cancer vaccine technology platform (Trivax) that uses clinical grade lipopolysaccharide (LPS) and interferon gamma (IFN-γ) for the maturation of monocyte derived DCs. In designing the Trivax technology we aimed at accounting for the dynamic nature of DC differentiation after exposure to a maturation stimulus. As opposed to many microorganisms tumours do not represent a pathogen associated molecular pattern (PAPM) that may be identified by the DC as a danger signal. Hence an artificial danger signal needs to be supplied to the DCs in order to trick them into initiating their maturation programme and presenting tumour antigens in an immune stimulatory form. Within the first 24 hours after encountering the danger signal, DCs enter a functional window characterised by the potential to stimulate T cells. Among other factors, this time window is characterised by the secretion of IL-12. In order to prevent an immune response from running out of control, DCs enter a second functional window after 24 hours that is dominated be immune suppressive feedback loops. The tryptophan metabolising enzyme IDO was identified as a key factor in suppressing activated T cells and in inducing regulatory T cells (Tregs). Furthermore we found that γδ T cells have an immune regulatory/suppressive potential that may be relevant for anti-tumour immune therapy.

Methods:

For manufacturing of Trivax, DCs charged with autologous tumour antigens are frozen after 6 hours exposure to LPS/IFN-γ at a semi mature stage (smDCs) retaining the capacity to secret IL-12 and thus support cytolytic T cell responses, which is lost at full maturation. We compared closed systems for monocyte enrichment from leukocyte apheresis products from healthy individuals using plastic adherence, CD14 selection, or CD2/19 depletion with magnetic beads, or counter flow centrifugation (elutriation) using a clinical grade in comparison to a research grade culture medium for the following DC generation.

Results:

We found that elutriation was superior compared to the other methods showing 36±4% recovery, which was approximately 5 fold higher as the most frequently used adherence protocol (8±1%), and a very good purity (92±5%) of smDCs. Immune phenotype and IL-12 secretion (in ng/ml; adherence: 1,4±0,4; selection: 2,0±0,6; depletion: 1±0,5; elutriation: 3,6±1,5) as well as the potency of all DCs to stimulate T cells in an allogeneic mixed leukocyte reaction did not show statistically significant differences. Research grade and clinical grade DC culture media were equally potent and freezing did not impair the functions of smDCs. Finally, we assessed the functional capacity of DC cancer vaccines manufactured for three patients using this optimised procedure thereby demonstrating the feasibility of manufacturing DC cancer vaccines that secret IL-12 (9,4±6,4 ng/ml).

Conclusion:

We conclude that significant steps were taken here towards clinical grade DC cancer vaccine manufacturing. After completion of several phase I trials, currently a randomised multi-centre clinical trial is in progress to study the potency of the Trivax technology in the treatment of cancer.

Searching for ideal dendritic cell for cancer vaccines

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Introduction

Dendritic cells (DC) are antigen-presenting cells that are able to activate both helper and cytotoxic T-lymphocytes and induce antitumor immunity. That makes them a suitable source for *in vitro* cancer vaccines production development Previously, it has been demonstrated that DC can be obtained from various sources in different quality, measured by phenotypic markers as well as cytokine production profiles. While demands on maturity of DC and their immunophenotype (CD80, CD83, CD86, HLA-DR positivity and CD14 negativity) have been considered more in the past, in recent view the "ideal dendritic cell" for clinical application seems to be that one producing high level of interleukin-12 (IL-12) and low level of IL-10. IL-12 supports antitumor immunity, whereas IL-10 causes tolerance of the immune system towards the tumor antigens. We compared three different maturation cocktails used for DC-based vaccine preparation aplicable in clinical trials for cancer patients.

Methods

Peripheral blood mononuclear cells from healthy donors were isolated by gradient centrifugation. Monocytes were enriched by plastic adherence and cultured with granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-4 for 5 days to obtain immature DC. Then the cells were incubated in the presence of tumor antigen in the form of myeloma cell line ARH77 apoptotic bodies. After 2 hours three different maturation cocktails MC1, MC2 and MC3 we added. MC1 consisted of tumor necrosis factor-α (TNF-α), IL-2α, prostaglandin E2 (PGE2) and IL-6 (Jarnjak-Jankovic *et al*., BMC Cancer. 2007; 7: 119.). MC2 contained interferon-γ (IFN-γ), TNF-α, IL-1β, PGE2 and toll-like receptor 7/8 (TLR7/8) ligand (Zobywalski *et al*., J Transl Med. 2007; 5: 18.). MC3 consisted of bacterial lipopolysaccharide (LPS) and IFN-γ (Dohnal *et al*., Cytotherapy. 2007; 9: 755-70.). After 1-2 days, mature DC were phenotypically characterised by flow cytometry (FACS Canto II, Becton Dickinson) and IL-10 and IL-12 production was quantified by FACS Array (Becton Dickinson).

Results

No significant differences were noticed among the immunophenotypes (CD80, CD83, CD86, HLA-DR) of the DC cultured within various maturation cocktails. In contrast, there was wide difference in the cytokine production. The median IL-12 production was the highest using MC3 (3267,3 pg/ml), intermediate in MC2 (318,6 pg/ml) and minimal in MC1 (4,2 pg/ml). That finding corresponded with IL-12/IL-10 ratio which was high in MC3 (9,54), intermediate in MC2 (4,24) and low in MC1 (0,20).

Conclusions

Despite similar phenotypic characteristics of DC prepared under different maturation conditions, the ideal candidate for clinical grade DC preparations seems to be MC3 containing LPS and IFN-γ leading to optimal cytokine profile needed for tumor-specific T cell activation *in vivo*.

Identification and long-term monitoring of individual tumor-specific T cells

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Introduction

Relatively limited information exists about the frequency of naive T-cell precursors that are able to recognize tumo antigens.[1-8] In humans, for example, the relative frequency of cytotoxic T lymphocyte precursors (CTLp) that recognizec a MAGE-3 peptide presented by HLA-A1 was estimated to be $3x10^{-7}$ of the blood $CD8^+$ cells.[9] Assuming a mean CTLp frequency of approximately 10^{-6} of tumor antigen-specific $CD8^+$ cells, a significant increase of this frequency, for example a 40-fold increase, translates into 1/25 000 of the $CD8^+$ cells following vaccination. This is beyond the limit of detectior of all immunological methods, including tetramer, unless the frequency of antigen-specific cells is increased through an in vitro restimulation assay.[10] Direct methods can only detect substantially larger T cell populations with frequencies greater then 1/1000 of antigen-specific T cells. Such responses are observed against viral peptides and quite frequently exceed even 1/100 of antigen-specific T cells in case of cytomegalovirus or human immunodeficiency virus peptides.[11,1] In cancer immunotherapy, there is no proof that a cancer vaccine has to stimulate large number of T cells to initiate tumo rejection.[1-5] Thus, we should take into account that T cell responses to tumor antigens may be of low level and that negati- ve results obtained with most ex vivo assays may not exclude the beneficial effect of tumor-specific T cells in vivo.

Previously, we were able to identify and quantify in vivo individual alloreactive and leukemia-reactive donor T cel clones in patients undergoing allogeneic HLA-matched hematopoietic stem cell transplantation (HSCT) who developec acute graft-versus-host disease (GVHD) and demostrate their GVHD- and graft-versus-leukemia (GVL) specificity withou prior knowledge of GVHD- and GVL-specific antigens.[13-15] Using the clonotypic assay based on selection of antigen-reacti- ve T cells and further molecular analysis of their T cell receptor beta (TRB) repertoire[14,15] we were able to identify the mos immunodominant antigen-specific T cells and detect them by a clone-specific primers and probes quantitatively in vivo with a treshold frequencies of 1/100 000 T cells. Such a novel and sensitive approach to the detection of antigen-specifi T cells does not require the prior knowledge of the particular antigen and can be used for a long-term monitoring o individual tumor-specific T cells in cancer patients. Here we used the clonotypic assay in patients treated for melanoma and myeloma and correlated their clinical outcome with individual tumor-specific T cell clones monitoring.

Patients and Methods

Patient characteristics and cell collection

Patients with metastatic melanoma (American Joint Committee on Cancer stage IV) and patients with multiple myeloma were enrolled to the study and were treated according to the Czech Oncology Society guidelines at the Masaryk Memorial Oncology Institute, and the Department of Hematooncology, University Hospital Brno, both in Brno, Czech Republic since September 2004 till August 2007. Fresh peripheral blood mononuclear cells (PBMC) as well as tumor cells were obtained as a part of routine diagnostic procedures. Tumor cells were obtained from lymph node metastases at the time of lymph node biopsy or from bone marrow. Basic patient characteristics are described in Table 1. All patients provided signed informed consent prepared in agreement with the Declaration of Helsinki and approved by the local Ethical Committee. As controls, PBMC from healthy individuals were used. PBMC were isolated using gradient centrifugation on Histopaque 1077 (Sigma-Aldrich, Praha, Czech Republic) and used immediatelly or stored deeply frozen at –80°C until used.

Generation of tumor-specific T cells

PBMC were incubated in complete medium consisted of X-VIVO 10 (Bio Whittaker, Walkersville, MD, USA), 2 mM L-glutamin, 10% heat-inactivated human AB serum (Sigma-Aldrich, Prague, Czech Republic), 80 U/ml DNAse (Boehringer Mannheim, Germany) in an atmosphere of 5% CO_2 for 2 hours. Non-adherent fraction was used for the generation of cytotoxic T lymphocytes. After removing non-adherent fraction, adherent cells were then cultured in the presence of 800 U/ml GM-CSF (Schering-Plough, Kenilworth, NJ, USA) and 100 ng/ml IL-4 (Sigma-Aldrich, Prague, Czech Republic) as previously described[16] to become dendritic cells (DC). Tumor cells were irradiated (60 Gy) and served as antigen that was pulsed to immature DC at a ratio of 1:1 on day 7. DC maturation was induced on day 8 using 1000 U/ml TNF-α (Bender Medsystems Diagnostics, Vienna, Austria).

To elicit cytotoxic T lymphocytes (CTL), washed non-adherent PBMC fraction was resuspended in complete medium. Antigen-specific T cell priming was initiated by antigen-pulsed mature autologous DC added to primary cultures at a ratio (T cell:DC) 20:1. Restimulation with the same antigen-pulsed DC was performed at a ratio (T cell:DC) 2:1 to obtain the highest yield of tumor-reactive interferon gamma (IFNγ)-producing T cells that were harvested 24 hours after restimulation with the Cell Enrichment and Detection Kit (MACS Reagens, Miltenyi Biotec, Bergisch Gladbach, Germany) as previously described.[16]

Flow cytometric analysis

Cell surface phenotype was analyzed by flow cytometry using anti-CD4-FITC, anti-CD8-FITC, anti-CD3-APC (Immunotech, Marseille, France) and anti-IFNγ-PE monoclonal antibodies (Miltenyi Biotec). Cells were analyzed using a Cytomics™ FC 500 flow cytometer (Beckman Coulter, Miami, Florida, USA).

Tumor specific reactivity of CTL in vitro

Harvested IFNγ-positive CTLs were cultured in X-VIVO15 medium containing 2mM L-glutamin, 25mg/ml HEPES (BioWhittaker, Walkersville, MD, USA), 10% heat-inactivated human AB-serum (Sigma-Aldrich, Prague, Czech Republic) and 500 IU/ml IL-2 (Proleukin, Chiron, Amsterdam, The Netherlands) for 3-4 weeks. Phytohemaglutinin (PHA) 5μg/ml (Sigma) was added on day 1. Culture medium was replaced twice weekly, feeder cells (irradiated PBMC from healthy volunteers) were added once weekly starting the second week of expansion. CTL assay with Calcein-AM (Molecular Probes, Eugene, OR) was performed as published elsewhere.[17] This assay produces results comparable with standard Cr^{51} release assay but is nonradioactive and requires fewer cells. Allogeneic PBMC served as negative controls.

Identification of TRB CDR3 Sequences

Clonotypic assay[14,15] was used for identification of individual tumor-specific T cell clones based on analysis of unique DNA sequence of the T cell receptor beta complementarity determining region 3 (CDR3). It was performed on sorted populations of activated $IFN\gamma^+$ melanoma-reactive T cells that were further separated immunomagnetically to $CD3^+CD4^+$ and $CD3^+CD4^-$ fraction by CD4-positive selection (Miltenyi Biotec). At least 97% of the $CD3^+CD4^-$ fraction were $CD8^+$ T cells (data not shown).

In vivo quantitative monitoring of individual immunodominant T cell clones

The most immunodominant T cell clones were selected based on the frequency of their unique DNA sequence of the TRB CDR3 region (more then 10% of the same clonotype among all bacterial colonies sequenced). Clone-specific primers and probe were designed for the TRB CDR3 with Primer Express v1.5. Clonotypic quantitative (q)PCR was performed using an ABI PRISM 7700 on PBMC lysates or biopsy DNA as previously described.[15]

Statistical Analyses

Correlations were performed by Spearman's rank test, the Wilcoxon matched pairs test, and the Mann Whitney test using Prism 3.0 software.

Results

Melanoma and myeloma patients have circulating tumor-reactive $CD4^+$ and $CD8^+$ T cells

Dendritic cells loaded with irradiated autologous tumor cells were used for activation of T lymphocytes in 4 patients with metastatic melanoma and 2 patients with multiple myeloma. Patient characteristics are shown in Table 1. After 2 rounds of stimulation, low frequencies of IFNγ-producing $CD4^+$ (0.9-3.2%, median 1.3%) and $CD8^+$ (0.6-2.4%, median 1.0%) T cells were detected (Figure 1A). Melanoma-reactive IFNγ-producing T cells were immunomagnetically separated. The percentage of activated $CD3^+IFN\gamma^+$ T cells before magnetic separation was 1.8-4.4% (median 2.4%) and after the procedure reached 59.7-88.4% (median 76.2%) of all $CD3^+$ T cells (Figure 1B). Sorted $CD3^+IFN\gamma^+$ T cells (0.52-$2.39x10^6$ cells) were further expanded for 3-4 weeks until at least 10^8 viable T cells were reached. After the expansion, 96.7-99.5% (median 99.0%) of cells were $CD3^+$ T cells with the prevalence of 72.8-86.4% (median 81.1%) $CD3^+CD8^+$ cells, remaining T cells were $CD4^+$.

Expanded tumor-reactive CTLs demonstrate specific cytotoxic potential

A specificity of expanded tumor-reactive CTLs was tested against the original autologous tumor cells in 4 patients (No.1, 2, 3, and 6) with sufficient number of remaining tumor cells. Unselected T cells showed specific cytotoxicity to autologous tumor cells in all 4 individuals reaching 21-39% (median 33%) at 20:1 E/T ratio and 14-25% (median 21%) at 1:1 E/T ratio. Representative data from two independent experiments (patients No.1, and No.3) are shown at Figure 2. Negligible reactivity against autologous DC and a negative control (third-party allogeneic PBMC) was noticed (Figure 2).

Identification of immunodominant T cell clones in vitro

We next analyzed the clonal composition of the sorted IFNγ^+ tumor-reactive T cells and defined them by the unique sequences of their TRB CDR3 region in patients No.1-6. TRB loci of the sorted cells were amplified by template switch anchored RT-PCR as described in the Methods to ensure that all tumor-reactive TRB sequences were amplified without bias to particular TRBV families. Thus the tumor-reactive clonotypes were represented in the anchored RT-PCR product with a relative frequency reflecting that in the original sorted cell population. Sequencing of at least 50 bacterial colonies containing TRB CDR3 from each sort was performed to identify the number and frequency of individual T cell clones in each sorted population. We have previously demonstrated the specificity and sensitivity of this approach to the analysis of clonality of HIV-, GHVD- and GVL-specific T cells.[15,17] We have also demonstrated that only highly immunodominant T cell clones (forming at least 10% of all in vitro identified clonotypes) are also detectable in vivo.[15,17] Table 2 shows the number of successfully sequenced bacterial colonies and the number of different tumor-reactive T cell clones identified for each patient. The frequency and aminoacid sequences of CDR3 regions which occurred in more than 10% of bacterial colonies in vitro are also shown. These are likely to represent the most 'dominant' T cell clones and their presence in peripheral blood of melanoma patients were further studied in vivo. We observed considerable heterogeneity in the clonality of melanoma-reactive T cells among the patients. Notably, melanoma-reactive CD8$^+$ T cells from patients No.3-4 who died due to rapid cancer progression were highly polyclonal, comprising more individual clonotypes, whereas melanoma-reactive CD8$^+$ T cells from patients with at least some measurable clinical response were far more oligoclonal, comprising less individual clonotypes. Indeed, within these oligoclonal repertoires, only one or two CD8$^+$ clones stood out as being clearly dominant in frequency for each patient with at least some treatment response. Melanoma-reactive CD4$^+$ T cells were rather polyclonal in all patients regardless their clinical response, comprising between 15 and 32 individual clonotypes with no clearly dominant clones (Table 2A). In two patients with myeloma, both CD4$^+$ and CD8$^+$ clones were identified.

Long-term persistence of immunodominant CD8$^+$ T cell clones correlates with better clinical outcome

Having identified individual immunodominant CD8$^+$ T cell clones in vitro we next quantified the dominant clones at multiple time points after the patient was diagnosed with metastatic melanoma in peripheral blood. We did not test the myeloma patients due to the lack of follow-up samples. We designed clone-specific PCR primers and probes specific for the TRB CDR3 region of each dominant melanoma-reactive clone whose sequence had been detected in at least 10% of bacterial colonies from the anchored RT-PCR product (Table 2B). We performed clonotypic quantitative real-time PCR (qPCR) on each sample from each recipient. Because a single T cell contains one productively rearranged TRB locus of a particular sequence, the copy number detected in each PCR reaction was directly equivalent to the absolute number of melanoma-reactive clone cells present in the sample. Figure 3 shows the levels of each immunodominant clone in peripheral blood of 4 patients characterized and numbered as shown in Table 1. Notably, subjects 1 and 2 who are long term survivors of metastatic melanoma have demonstrated a long term persistence of the most dominant CD8$^+$ T cell clones in vivo in their peripheral blood. On the other hand, subjects 3 and 4 had only a temporary detectable immunodominant CD8$^+$ T cell clones in their peripheral blood. Quantification of absolute numbers of melanoma-reactive CD8$^+$ T cell clones showed in general some variability reaching from the treshold levels of qPCR $1/10^6$ to $1/10^3$ of CD8$^+$ T cells in peripheral blood. It revealed minimal if any expansion in long-term survivors but disappeared shortly after diagnosis of MM in non-survivors.

Discussion

By using dendritic cells loaded with irradiated autologous tumor cells we were able to identify circulating tumor-reactive T cells in patients with metastatic melanoma and multiple myeloma. Both populations of CD4$^+$ and CD8$^+$ tumor-reactive T cells were present. Such cells can be enriched and further expanded in vitro.[16,19] Despite the advanced stage of tumor spread, we were able to expand mostly CD8$^+$ CTLs that demonstrated tumor-specific cytotoxic effect in vitro. This finding confirms an important role of CD8$^+$ CTLs in specific cytotoxicity which is in agreement with studies using a defined melanoma-specific peptide HLA class I-restricted antigens.[9,10]

In this study we identified in vitro, and quantified in vivo, individual immunodominant tumor-reactive CD8$^+$ T cell clones by developing an approach that combines in vitro cell culture, immunomagnetic sorting of activated IFNγ^+ T cells, TRB locus anchored RT-PCR, and clonotypic qPCR. In published studies, the identification of clonal T cell responses has been accomplished by flow cytometry using monoclonal antibodies directed against TRBV regions, by CDR3 spectratyping, or by microplate hybridization assay.[20-22] These studies were able to demonstrate that TRB repertoire is highly variable, ie. monoclonal, oligoclonal or polyclonal CTL responses can be induced depending on tumor antigen used.[20-22] Interestingly, either monoclonal or polyclonal responses can be elicited by the same peptide derived from a MAGE-3.A1 tumor-specific antigen in different individuals.[21,22] Yet, using these techniques does not enable detailed analysis of individual T cell clones since, in the case of flow cytometry, the series of antibodies are not complete enough to analyze all TRBV region families, in case of spectratyping and microplate hybridization assay, TRBV region families but not individual T cell clones within these families can be identified.[23-25] Our approach obviated skewing of the clonotype population by prolonged stimulation and propagation. Furthermore, the anchored RT-PCR step used to amplify TRB CDR3 regions of all T cell clones present in the sorted population avoided the incomplete coverage of, and bias to, particular TRBV families conferred by using TRBV-specific PCR primers. This approach required no prior knowledge of either the specific target antigen or the MHC restricting element, and when combined with qPCR, it allowed for the sensitive and specific quantification of melanoma-reactive T cell clones in the patient at any point and in any blood or tissue sample. In agreement with spectratyping and microplate hybridization studies[23-25] we were able to detect oligoclonal or monoclonal expansions of tumor-reactive T cell clones. The sensitivity of clone-specific qPCR reached the treshold of $1x10^{-6}$, thus allowing to monitor low-frequency clones.

It has been shown that the frequency of naive T cells recognizing melanoma-specific peptide antigens such as MAGE-A3$_{168-176}$, gp100, or MAGE-A10 exists in vivo with the frequencies bellow $1x10^{-6.10}$ The Melan-A/MART-1$_{28-36}$ is a remarkable exception with a very high naive T cell frequency of about $5x10^{-4}$ of $CD8^+$ T cells.$_{26}$ Recently, Speiser et al. demonstrated that human $CD8^+$ T cell clone specific to the Melan-A/MART-1 persisted for more then 1 year in a patient with melanoma reaching up to 2.5% of the circulating $CD8^+$ T cells.[27] At the time of melanoma-specific T cell clonal expansion the disease stabilized, but subsequently progressed with loss of Melan-A-specific T cell clone.[27] In agreement with that study, we were able to demonstrate that the immunodominant $CD8^+$ T cell clones in 2 patients with progressive disease were only temporarily present in the circulation and these patients subsequently died of their disease. On the other hand, both melanoma patients with objective responses had melanoma-reactive $CD8^+$ T cells present in the circulation for 18-60 months with frequencies of 10^{-6} to 10^{-3}. These low-frequency $CD8^+$ T cell clones can be probably expanded as a response to tumor grow or as a response to melanoma treatment. Such observation further confirms the importance of individual tumor-reactive $CD8^+$ T cell clones in cancer survival. Thus, prolonged persistence of melanoma-reactive $CD8^+$ T cell clones is associated with better survival.

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Table 1:

Characteristics of patients with metastatic melanoma (No.1-4) and multiple myeloma (No.5-6)

Patient no.	Sex+	Age at diagnosis [years]	No. of metastatic sites	No. of therapeutic regimens	Treatment response*/ survival [months]
1	M	46	1	1	SD / 60
2	F	67	2	2	PR / 22
3	F	76	2	1	PD / 12 - died
4	F	64	5	2	PD / 4 - died
5	F	66	-	3	SD / 15
6	M	58	-	2	SD / 13

+M=male, F=female;
*SD=stable disease, PR=partial response, PD=progressive disease, MR=mixed response.

Table 2:

Characteristics of immunodominant T cell clones
A) Individual T cell clonotypes

Patient no./ clinical response	$CD3^+CD8^+$ T cells		$CD3^+CD4^+$ T cells	
	Number of individual clonotypes	Number of dominant clones+	Number of individual clonotypes	Number of dominant clones+
1 / SD	9	2	16	0
2 / PR	5	3	15	0
3 / PD	28	4	32	0
4 / PD	35	2	19	0
5 / SD	8	2	12	2
6 / SD	6	1	23	2

SD=stable disease, PR=partial response, PD=progressive disease;
+The dominant T cell clones were defined as more then 10% frequency of their unique DNA sequence of the TRB CDR3 region among all bacterial colonies sequenced.

B) Immunodominant T cell clones

Patient no.	Clone	Clone frequency+	TRB V region*	Aminoacid sequence of the TRB CDR3 region	TRB J region*
1	1A	21/54	V6-1	EAGRY	J1-4
	1B	6/54	V20-1	GGEHQN	J2-7
2	2A	24/51	V12-4	LKTPSSYN	J1-1
	2B	13/51	V11-1	DTRRKSSP	J1-3
	2C	9/51	V6-2	QAGTDT	J2-1
3	3A	9/55	V4-1	EAPAGYNLSSGN	J1-5
	3B	7/55	V5-5	TRRNQP	J1-1
	3C	6/55	V5-1	GASPYNN	J1-2
	3B	6/55	V20-1	GVPSSYNT	J2-1
4	4A	7/51	V17	VRWTRG	J1-6
	4B	6/51	V3-1	NPGLAIY	J2-6
5	5A	14/50	V2-1	RGEGA	J2-5
	5B	8/50	V6-3	TRDRGVD	J1-1
	5C	7/50	V10-1	SFL	J1-5
6	6A	17/53	V22-1	RER	J2-4
	6B	11/53	V2-3	ASD	J1-2
	6C	6/53	V11-2	DWASGGN	J1-6

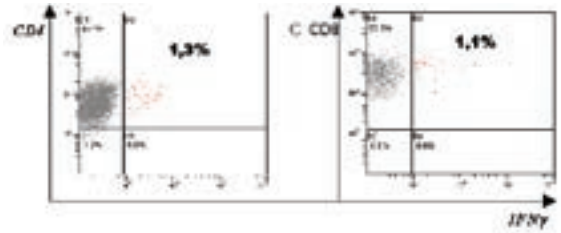
+ Clone frequency represents the frequency of unique DNA sequence of the TRB CDR3 region among all bacterial colonies sequenced;
*according to the IMGT classification of TRB (http://imgt.cines.fr).

Figure 1:

Sorting melanoma-reactive IFN-γ^+ T cells

Tumor-reactive IFN-γ^+ T cells were sorted immunomagnetically. Representative data from patient No.3 are shown. Panel A demonstrates pre-sort cell population (gated on $CD3^+$ cells) with the presence of both $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells. Panel B demonstrates enrichment of CD3+IFN-γ^+ T cells.

A)



B)

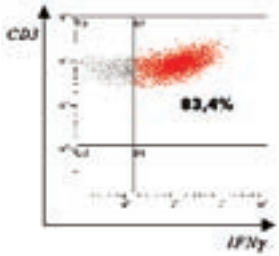


Figure 2:

CTL assay

Expanded tumor-reactive T cells demonstrate specific cytotoxic effect to autologous tumor cells (Melanoma). Negligible response against autologous dendritic cells (DC only) and no response to irrelevant antigens such as third-party allogeneic PBMC (alloPBMC) was noted. Representative data from 2 different patients (No.1 and No. 3 from Table 1) are shown.

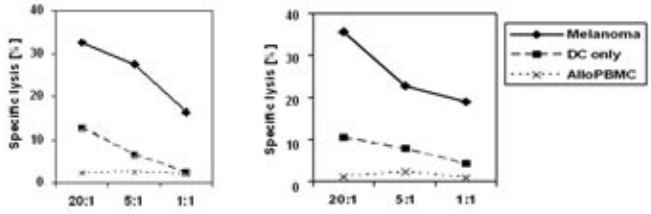
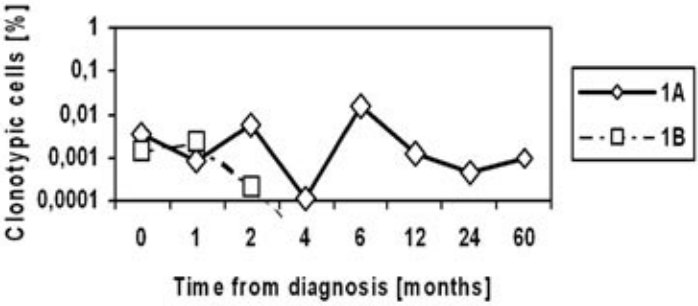


Figure 3:

Long-term in vivo monitoring of immunodominant T cell clones

Individual immunodominant melanoma-reactive T cell clones identified in vitro at the time of metastatic dissemination (Time 0) were quantitatively monitored in vivo in peripheral blood from patients No.1-4. Number of clonotypic T cells is expressed as a percentage of all $CD3^{+}CD8^{+}$ T cells at the time of examination. The detection treshold for the clonotypic assay was $10^{-40\%}$.

No.1



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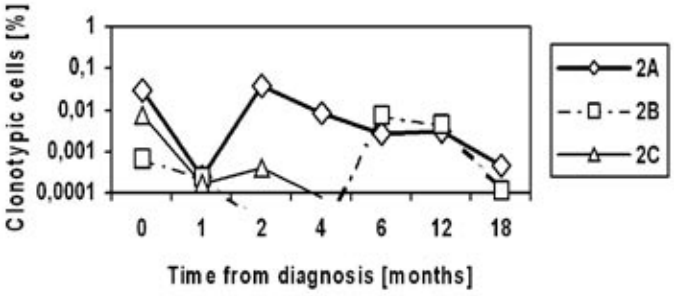

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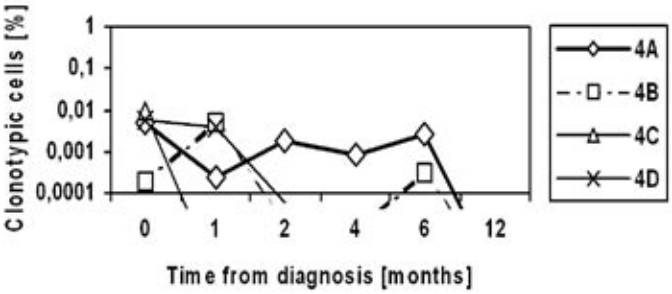
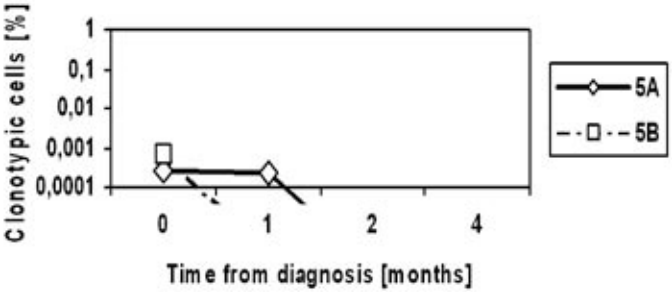

Clonotypic cells [%]
1
0,1
0,01
0,001
0,0001
0
1
2
4
6
12
Time from diagnosis [months]
4A
4B
4C
4D

No.4


Clonotypic cells [%]
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0,1
0,01
0,001
0,0001
0
1
2
4
Time from diagnosis [months]
5A
5B

Ipilimumab as anti-CTLA4 immunotherapy: preclinical and clinical data

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Background

The immune system recognizes and responds to numerous antigens and has evolved a system of regulation to prevent autoimmunity. Many tumor cells produce antigens that are self-antigens and therefore are not effectively destroyed by the host immune system. The mechanisms involved in tolerance to self-antigens are numerous. Signaling from the T-cell receptor (TCR) alone is not sufficient to evoke full immune responses, and a second co-stimulatory molekule is needed to enhance TCR signaling and overcome the threshold for a T-cell response to occur[1]. TCR enhancement primarily occurs via CD28 on T-cells, which is activated by B7 expressed on antigen-presenting cells. Upon activation, T-cells express another receptor, the cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4), which also binds B7 molecules, but with higher afinity than CD28. CTLA-4 inhibits T-cell responses, resulting in blockade of the immune response. Studies in CTLA-4 knockout mice have shown that CTLA-4 is essential for maintenance of tolerance as these mice develop at age of 3-4 weeks fatal lymphoproliferative disorder leading to lymphocytic infiltration and destruction of major organs[2].

Introduction to anti-CTLA-4 therapy

Researchers have now begun using an alternative approach in cancer immunotherapy in which the immune system is targeted as a whole together with its interaction with the tumor. It has been shown that CTLA-4 blockade can be used alone or in conjunction with a tumor vaccine to potentially enhance antitumor responses. Coupling of anti-CTLA-4 antibodies with antitumor vaccines could direct immune responses toward target antigens and result in effective antitumor responses. This approach seems to be very promising as many cancer vaccines used in monotherapy including several large trials have reported disappointing results[3]. Thus, it appears that modifications in the current approaches to cancer immunotherapy are needed[4].

Ipilimumab

Ipilimumab is fully human IgG1 anti-CTLA-4 MAb ipilimumab binds to human CTLA-4 and prevents the binding of B7-1 Ig and B7-2 Ig expressed on antigen-presenting cells. This enables B7 molecules to continue to enhance TCR signaling, thereby propagating an ongoing immune response. Ipilimumab has exhibited particular clinical promise, with excellent results obtained when administered as monotherapy and in combination with anticancer vaccines in patients with melanoma, lymphoma and prostate, ovarian and renal cancer[5]. This lecture should describe ipililumab´s mechanism of action (effect on activated T-cells and T-regs expressing CTLA-4 constitutively), preclinical data as well as clinical efficacy and safety data from several phase II trials, with main focus on metastatic melanoma[6].

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METHODOLOGY

Protocol for the identification of malignant plasma cells in bone marrow samples using simultaneous staining of cytoplasmic immunoglobulin with FISH (cIg FISH)

Ahmann, G.J., Syed, M.J. at al.: A Novel Three-Color, Clone-Specific Fluorescence *In situ* Hybridization Procedure for Monoclonal Gammopathies. Cancer Genet. Cytogenet. 101, 7-11, 1998.
Johannes Drach and Jutta Ackermann – personal communication, modification Petr Kuglik

Reagents and equipment:

Solutions:

3 : 1 methanol-acetic acid fix, 96 % ethanol fix, 10 mM citrate buffer - citric acid (1g/500ml H_2O) / natrium citrat (1,45g/500ml H_2O) mix 1:1; pH 6,0, PBS solution, ethanol (70 %, 80%, 96 %), 0,5 x SSC, antifade solution (0,01 % p-phenylendiammine dissolved in glycerol/PBS buffer pH=8,0, 1:1)

Antibodies used (Vector Laboratories)

- Amca Anti-Human Kappa Chain, Catalog No. CI-3060, 1 : 10 (diluted in PBS)
- Amca Anti-Human Lambda Chain, Catalog No. CI-3070, 1 : 10 (diluted in PBS)
- Amca Anti-Goat IgG (H + L), Catalog No. CI-5000, 1 : 20 (diluted in PBS)

DNA Probe used:

- del(13q14)/monosomy 13 (Vysis-Abbott LSI RB1 DNA Probe/ Vysis – Abbott LSI 13q34 DNA Probe)

Digital Image Analysis

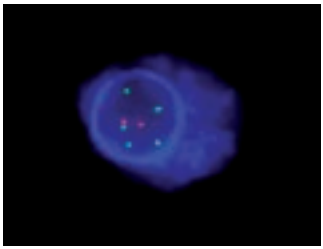
- Fluorescence Microscope Olympus BX-61
- CCD Camera Vosskuhler 1300D
- **Software:** Lucia 4.80 – KARYO/FISH/CGH, Laboratory Imaging, s.r.o., Prague, Czech Republic

Method:
(this protocol is for use with Abbott Vysis DNA probes)

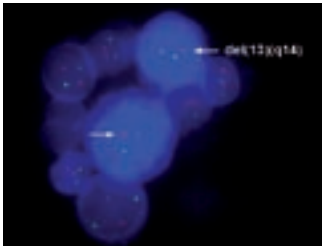
cIg FISH is carried out on bone marrow (BM) aspirate. BM samples are cultured in Panserin 441 medium for 24 hours at 37 ^{0}C in 5% CO2. The cells are harvested using hypotonic potassium chloride, fixed by methanol/glacial acetic acid (3:1), and then stored at -20^{0}C.

1. Collects the fixed bone marrow cells by centrifugation at 1500 rpm for 10 min
2. Remove the supernatant, and add 8 ml of 96 % ethanol. Resuspend sediment and incubate 15 min at room temperature
3. Repeat step 1 and 2
4. Resuspend the pellet in 96 % ethanol and place a drop of fixed cells onto a cleaned microscope slide. Let slides air dry, and examine under phase contrast microscope to check cell density
5. Incubate the slides 10 min in glass Coplin jar with 10 mM citrate buffer prewarmed at 95 ^{0} C (water bath)
6. After incubation, remove Coplin jar from water bath and leave cool at room temperature for 30 min
7. Wash slides twice for 2 min in PBS solution (at room temperature)
8. Apply 150 µl of diluted Amca Anti-Human Kappa Antibody or Amca Anti-Human Lambda Antibody onto each slide, and incubate under plastic cover slip in humified chamber for 1 hour (1 – 1,5 hour)
9. Wash slides twice for 2 min in PBS solution (at room temperature)
10. Apply 150 µl of diluted Amca Anti-Goat Antibody and incubate under glass cover slip in wet chamber for 1 hour (1 – 1,5 hour)
11. Wash slides twice for 2 min in PBS solution (at room temperature)
12. View slides under fluorescent microscope to check staining of cytoplasmic immunoglobulin
13. Dehydrate slides by serial ethanol washing (70 %, 80 %, 96 %) for 2 min
14. DNA Probe Preparation: denatured probe - manufacturer ´s protocol is recommended
15. Hybridisation: add 10 µl of DNA Probe per 22 x 22 mm slide area. Apply glass cover slip and seal with rubber cement. Incubate 16 hour in a humified chamber.
16. Post-hybridisation wash: wash slides in 0,5 x SSC for 4 min at 72 ^{0}C, and leave slides in the dark to dry
17. Pipette 10 µl of antifade solution onto each slide, overlay with cover slip and analyze under fluorescent microscope
18. Score 100 Amca positive plasma cells for each patients

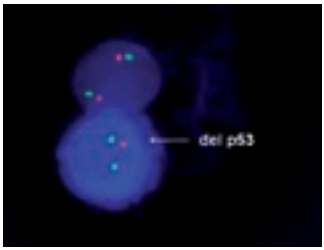
Examples of clg - FISH



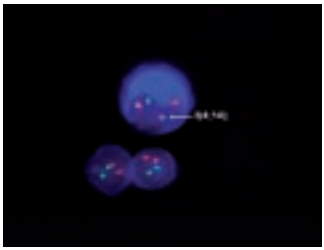
Abnormal malignant plasma cell hybridized with LSI 1q21 (green) / LSI 1p36 (red) probes. The cell shows amplification of 1q21 (Amp(1q21)) as 5 copies of the 1q21 region indicated by five single green signals. Red signals enable to distinguish Amp(1q21) from trisomy or tetrasomy of chromosome 1.



Abnormal malignant plasma cell hybridized with the LSI 13q14 (red)/13q34 (green) probes.The cell shows deletions of one copy of the 13q14 regions of chromosome 13 as indicated by the single red signal.Green signals enable the differentiation of the interstitial deletions from loss of the entire q arms.



Abnormal malignant plasma cell hybridized with LSI 17p13 (red) / CEP17 (green) probes. The cell shows deletion of p53 gene (del 17p13) as one copy of the 17p13 region indicated by one single red signal. Green signals enable to distinguish deletion of 17p13 from monosomy of chromosome 17.



An abnormal interphase cell hybridized with the LSI IgH / FGFR3 Dual Color, Dual Fusion Translocation Probe. The cell shows the one red (FGFR3), one green (IgH), two fusion (der (4) and der (14)) signal pattern indicative of a t(4;14).

Cryoconservation of bone marrow sample for subsequent molecular genetic and cytogenetic analyses

Material

5 ml of bone marrow heparinized

Solutions

- **Hanks' Balanced Salt solution**, SIGMA, No. H-6648, 500 ml, store at laboratory temperature until expiration date
- **Albumin human 20%**, Grifols, No. 59/361/91-C, 100 ml, store at 2 – 8 °C until expiration date
- **Histopaque-1077** (density 1,077 $g.ml^{-1}$), SIGMA, No. H-8899, 500 ml, endotoxin tested, store at 2 – 8 °C until expiration date
- **Ethanol 70%**, 250 ml, store at laboratory temperature
- **Carnoy's solution (fixative)**: Acetic acid 99% : methanol, 1:3 vol.

EQUIPMENT

- Refrigerated centrifuge
- Haematological analyzer or Bürker cell
- Laminar box
- Automatic pipettes
- Syringes 20 ml

EXPENDABLE SUPPLIES

- Sterile syringes, 20 ml and 10 ml
- Pasteur pipette sterile, 3 ml
- Sterile tubes, 50 ml
- Laboratory gloves
- Sterile tips for pipettes – 10-200 μl, 200-1000 μl, 1-5 ml

A. ISOLATION OF MONONUCLEAR CELLS FROM BONE MARROW (MNC)

Work sterile in a laminar box.

1) Put the bone marrow into sterile tube and add the same amount of Hanks' solution. Mix thoroughly.
2) Gradient density centrifugation:
   - Layer using 20 ml syringe with needle:

1. Histopaque
2. Histopaque: sample, proportion 2 :1 vol.
   - Max. volume in one 50 ml tube is 30 ml of sample and 15 ml of Histopaque.
   - Centrifugation 400G / 35 min/ 4 °C or 200 G / 45 min / laboratory temperature

3) After centrifugation aspire the layer containing the MNC (above the Histopaque medium) by the Pasteur pipette and put it into a new sterile tube. Note: The layer could not be visible.
4) Add a redundant amount of the Hanks' solution to the tube.
5) Centrifugation 300 G / 10 min / 4 °C or 400 G / 8 min / laboratory temperature.
6) After centrifugation waste the supernatant and resuspend the sediment in Hanks' solution. Final volume should be about 5 ml. Measure the cellularity.

B. THE TREATMENT OF MNC FOR FISH

1. Aliquote the isolated mononuclear cells:

• 1×10^6 MNC for DNA analysis	1 tube	- 80 °C
• 1×10^6 MNC for RNA analysis	1 tube	- 80 °C
• 2×10^6 MNC in Carnoy's fixative for FISH	1 tube	- 20 °C

2. Process the aliquots:

- **MNC for DNA analysis:**
  Centrifuge 500 G / 10 min / laboratory temperature
  Waste supernatant
  Label the cryotube by the code due to the evidence paper
  Freeze the sediment in cryotube to – 80 °C
  Store in an appropriate box

- **MNC for RNA analysis:**
  Centrifuge 500 G / 10 min / laboratory temperature
  Waste supernatant
  Label the cryotube by the code due to the evidence paper
  Freeze the sediment in cryotube to – 80 °C
  Store in an appropriate box

- **MNC in Carnoy's fixative for FISH:**
  Centrifuge 500 G / 10 min / laboratory temperature
  Waste supernatant
  Add a redundant amount of the Carnoy's solution
  Resuspend thoroughly
  Incubate for 1 hour
  Centrifuge 500 G / 10 min / laboratory temperature
  Waste supernatant
  Add the Carnoy's solution 1,5 – 2 ml
  Resuspend thoroughly
  Label the cryotube by the code due to the evidence paper
  Freeze to – 20 °C
  Store in an appropriate box

EUROPEAN MYELOMA NETWORK RECOMMENDATIONS FOR FISH IN MYELOMA 2007

Ross FM (1), Avet-Loiseau H (2), Drach J (3), Hernandez Rivas JM (4), and Liebisch P (5) on behalf of the European Myeloma Network FISH Working Party

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A workshop to discuss the problems particular to FISH in myeloma was held at the Royal Marsden Hospital, London on 11 March 2005 and attended by representatives of 31 European laboratories. The following recommendations are the result of agreement at the time combined with updates resulting from e-mail discussion between the participants in May 2007. The recommendations apply only to newly diagnosed or relapsed myeloma; monitoring of disease or testing of plasma cell dyscrasias with very low levels of marrow involvement may require different criteria. **Results found by applying the recommendations in this document should not yet be used to make treatment decisions except in the context of a clinical trial.**

1. It is not acceptable to report FISH results in myeloma without either concentrating the plasma cells or employing some means of plasma cell identification so that only these cells are scored. Haemodilution is a universally reported serious problem in myeloma. Clinicians should be encouraged to send part of the first draw of the aspirate for FISH, and further aspiration should involve repositioning of the needle.

2. It is strongly recommended that bone marrow aspirates are not performed on Friday.
Processing for FISH is time-consuming and results depend on good viability plasma cells.

3. Purification and simultaneous immunostaining and FISH (cIgFISH) are equally valid methods.
Choice of method depends on individual laboratory requirements. In general, the expense of purification is best justified in the context of a cell bank. Differences in purification methods should not affect FISH results.

4. Purified plasma cells must be checked for the proportion of plasma cells (by morphology or immunostaining)

5. Purified plasma cells should be fixed in 3:1 methanol:acetic acid. The choice of doing this to the cell suspension (with or without prior hypotonic treatment) or to cytospin slides is up to the individual laboratory. Both slides and suspensions can be successfully stored at -20°C for prolonged periods.

6. Immunostaining for light chains is recommended for cIgFISH. This gives a stronger result that CD138.

7. Cells for cIgFISH should be subjected to red cell lysis or density gradient centrifugation and fixed in 3:1 methanol:acetic acid. Slides can be made directly or the suspension stored at -20°C. Bone marrow smears can be used for cIgFISH but only when they are very fresh.

8. Cut off levels should be 10% for dual fusion and break-apart probes, and 20% for numerical abnormalities and single fusion results with dual fusion probes. These are conservative figures based on mean+3SD results o 5-10 controls in several laboratories but it is recognised that suitable control material is difficult to obtain and myeloma cells are prone to artefacts, thought to be due to the paraprotein levels. Any laboratory setting up myeloma FISH should ensure that their results are compatible with these levels. Laboratories with low mean+3SD for deletions may wish to consider results in the 10 – 20% range to be borderline for their own records but they should not be reported to clinicians as positive.

9. The 2005 recommendation that control probes should be used for all deletion probes is no longer considered essential. This recognises that each probe behaves in a unique way and that experience of the probes helps interpretation. In practice many laboratories prefer to use a control probe. In 2005 it was recommended that 13q and p53 should not be used together because of the frequency of double deletions. However, this objection has also been withdrawn.

10. A single experienced analyst is considered enough for reporting all abnormalities in the majority of cells from good preparations. However, results should always be checked where there is an equivocal signal pattern, where there are few plasma cells for clg FISH or where purified plasma cells make up <30% of the total. Smaller labs are recommended to use 2 analysts with a third to check any results with a discrepancy of <5%.

11. Wherever possible 100 cells should be scored. Results are only acceptable from <50 cells where all cells are identical or >75% have the abnormality.

12. p53, t(4;14) and 13q should be tested in all cases. The t(14;16) also has h*IgH* priority and many still consider t(11;14) worth doing. Where all these tests are performed there will be extremely few 'normal' 13 results reported incorrectly due to unsuspected near-tetraploidy. However, if an *IgH* break-apart strategy is used to decide which cases to test for fusion genes then some *IgH* negative near-tetraploid cases will be missed. The Vysis 5/9/15 probe is recommended to avoid this problem. Alternatively reports should be qualified with regard to the 13q and p53 results.

13. Probes to use: **All commonly used probes in 13q14 are acceptable** for 13q deletion which is usually of the whole chromosome.
Fusion strategies must usual dual fusion probes that cover a large enough area on the donor chromosome to encompass all breakpoints and allow detection of unbalanced translocations (eg loss of der(14) in t(4;14)). For the t(11;14), the difference between the Vysis standard and Tx probes was not thought to be significant
17p probes should be specifically for p53.

14. Reporting results.
The method of plasma cell identification should be reported.
The proportion of affected plasma cells should be reported. It is not clear what is the cut-off level for clinical significance. It is suggested that abnormalities in <40% cells should be emphasised in the text as being at a low level (although a few laboratories prefer not to report these).
All abnormalities should be expressed as clearly as possible. Thus the workshop did not endorse the use of ISCN.
Fusion probe results (especially for the t(4;14)) should record whether there was a dual or single fusion.

These recommendations should be reviewed yearly by email, with further workshops being held if there is significant controversy.
Anyone wishing to be included in the discussions should contact fiona.ross@salisbury.nhs.uk

A quality assurance scheme has been set up by Hervé Avet-Loiseau. The first round is underway but anyone wishing to be included in future rounds should contact herve.avetloiseau@chu-nantes.fr

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CRYOCONSERVATION IN MYELOMA BANK IN FACULTY HOSPITAL BRNO

Introduction

To ensure reproducible results and continuity in research and biomedical processes, today's scientists are faced with the task of genetically stabilizing living cells. Serial subculturing is time consuming and can lead to contamination or genetic drift as smaller and smaller portions of a population are selected. However, a population of cells can be stabilized by subjecting them to

cryogenic temperatures which, for all practical purposes, stops time.

Since water is the major component of all living cells and must be available for the chemical processes of life to occur cellular metabolism stops when all water in the system is converted to ice. Ice forms at different rates during the cooling process. During slow cooling, freezing occurs external to the cell before intracellular ice begins to form. As ice forms water is removed from the extracellular environment and an osmotic imbalance occurs across the cell membrane leading to water migration out of the cell. The increase in solute concentration outside the cell, as well as

intracellularly, can be detrimental to cell survival. If too much water remains inside the cell, damage due to ice crystal formation and re-crystallization during warming can occur.

Cryoprotective Agents

Cryoprotective agents serve several functions during the freezing process. Freezing point depression is observed when DMSO is used which serves to encourage greater dehydration of the cells prior to intracellular freezing.

We dilute DMSO to the desired concentration in cooled human albumin prior to adding it to the cell suspension. This minimizes the potentially deleterious effects of chemical reactions such as generation of heat, and assures a more uniform exposure to the cryoprotective agent when it is added to the cell suspension, reducing potential toxic effects. We use DMSO in concentrations ranging 10%.

Preparation of Cells

Several factors must be considered when preparing cells for cryopreservation. These include the type of cell, cell viability, growth conditions, physiological state of the cells, the number of cells, and how the cells are handled. We observed that the most effective is about 20x106 cells per vial, not less than 15x106 and not more than 30x106. It is necessary to examine the culture for possible contaminating microorganisms.

The cell suspension should initially be prepared at a concentration that desired for preservation so we prepare an equal volume of Dilute (33 % 20%Human Albumin + 33% Hanks Balanced Solution) with cells and then Freezer mix (10% DMSO + 23% Hanks Balanced Solution) is added. Both Dilute and Freezer mix must be pre-cooled. Gentle handling during cell harvesting and concentration procedures will ensure healthy cells prior to subjecting them to cold stress. Vigorous pipeting and high-speed centrifugation should be avoided if possible.

Rate of Cooling

Once the cells and the cryoprotectant have been combined and dispensed into vials, the next step is to cool the suspension. The rate of cooling is important since it affects the rate of formation and size of ice crystals, as well as the solution effects that occur during freezing. Different types of cells may require different cooling rates, however a uniform cooling rate of 1°C per minute from 4°C as starting temperature is effective for a wide variety of cells. Despite the control applied to the cooling of cells, most of the water present will freeze at approximately -2°C to -5°C. The change in state from liquid to crystalline form results in the release of energy in the form of heat; this is known as the latent heat of fusion. Warming of the sample occurs until the equilibrium freezing point is reached, at which temperature ice continues to form. To minimize the detrimental effects of this phenomenon, undercooling must be minimized by artificially inducing the formation of ice.

To achieve uniform, controlled cooling rates, it is better to use a programmable- rate cell freezing apparatus. Simple units allow only the selection of a single cooling rate for the entire temperature range. More sophisticated units, however, allow a selection of variable rates for different portions of the cooling curve. Less costly and easier-to-use systems are available for simulating a controlled-rate cooling process by placing the vials in a mechanical freezer at -80°C. In order to accomplish a uniform rate of cooling, the vials must be placed in specially designed containers.

We use an alcohol bath, which provides a simple-to-use system designed to achieve a rate of cooling very close to 1°C per minute.

Storage

The temperature at which frozen preparations are stored affects the length of time after which cells can be recovered. The lower the storage temperature, the longer the viable storage period. For ultimate security, living cells should be stored at liquid nitrogen temperatures. Liquid nitrogen units that provide all-vapor storage are ideal as long as the working temperature at the opening of the unit remains below -180°C. This working temperature can be attained in most liquid nitrogen freezers. Improper handling of material maintained at cryogenic temperatures can have a detrimental effect on the viability of frozen cells. Each time a frozen vial is exposed to a warmer environment, even briefly, it experiences a change in temperature.

To maximize the available space in liquid nitrogen freezers and minimize exposure of material during retrieval, use small storage boxes in stainless racks. Each rack has ten positions which can be easily identified in cryobank protocol.

Reconstitution (Thawing)

For most cells, warming from the frozen state should occur as rapidly as possible until complete thawing is achieved. To achieve rapid warming, we place the frozen vial into a 37°C water bath. Sometimes gentle agitation of the vial during warming will accelerate the thawing process. As soon as the contents of the vial have been thawed, we remove the vial from the water bath. To minimize the risk of contamination during reconstitution, we disinfect the external surface of the vial by wiping with alcohol-soaked gauze prior to opening.

Immediately we transfer the contents of the vial to fresh growth medium following thawing to minimize exposure to the cryoprotective agent. We incubate these cells 24 hours in 37°C to remove residual cryoprotective agents.

Determination of Recovered Cells

Methods used to estimate the number of viable cells recovered following freezing depend on the type of material preserved. We make visual inspection by Methylen Blue Staining and following counting in Burker chamber.

3rd MYELOMA and 1st IMMUNOTHERAPY WORKSHOP, Brno 2008

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Notes:

Notes:

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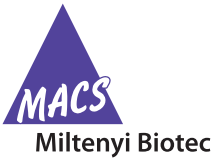

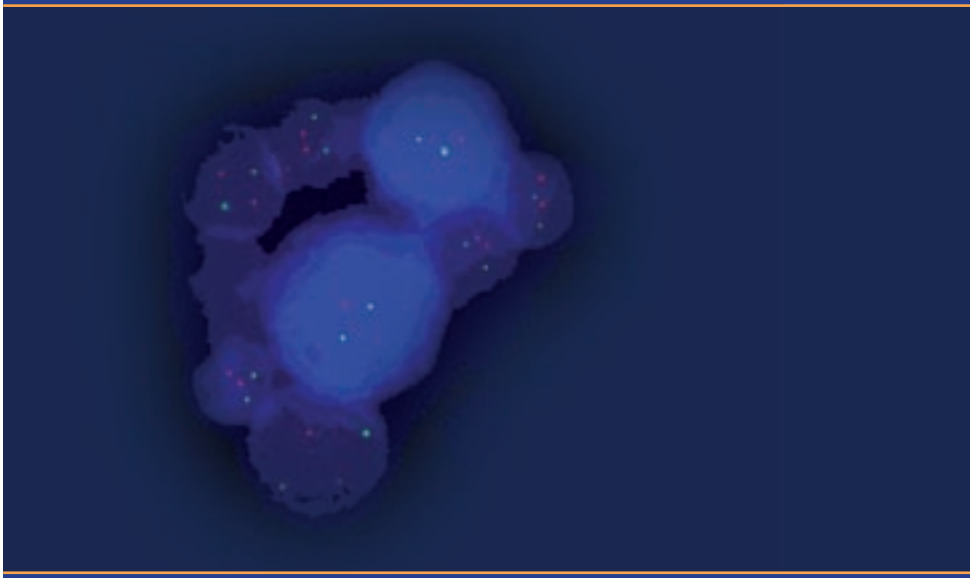

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