

# IMMUNOTHERAPY OF SOLID TUMORS

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# Cancer immunotherapy

- o cancer is immunogenic (several TAA antigens are well defined, many still not known);
- o different approaches to cancer immunotherapy: monoclonal antibodies, cellular vaccines, TAA specific autologous CTLs expanded *in vitro*, TILs, LAK cells ...;
- o relatively small success in recent years;
- o still a lot of work needed due to complexity of the problem;
- o the mechanisms of immune system are as complicated to understand and to act upon as those of the human brain;
- o huge market worldwide

# The variety and complexity of tumour escape mechanisms

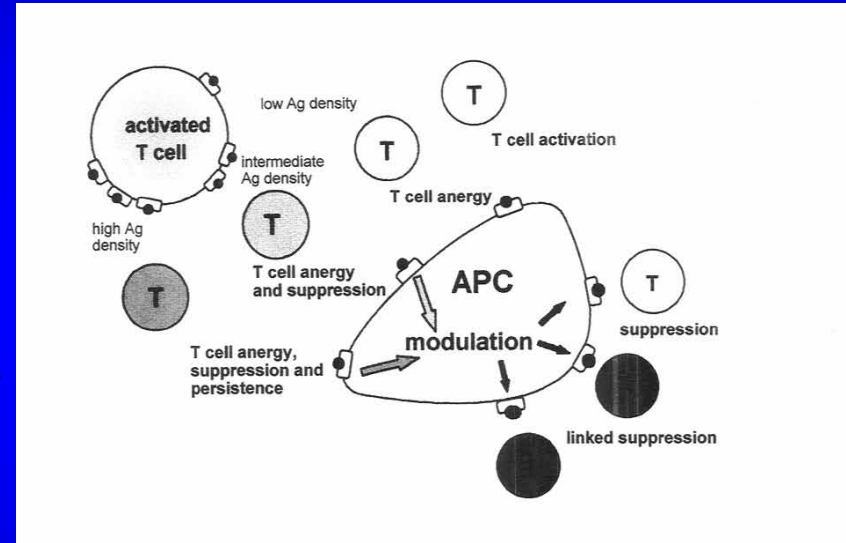
- o low immunogenicity and variable expression of TAA, rapid mutations of their encoding genes;
- o downregulation or partial/complete absence of MHC molecules on tumour cells (TC), lacking of costimulatory molecules on TC, active tumour secretion of immunoinhibitory cytokines (VEGF, TGF- $\beta$ , IL-10, IL-4, ...);
- o tumour protection oriented immunosuppression/tolerization, mediated by cancer patients' own immunoregulatory cells [subsets of T cells: Treg CD4<sup>+</sup>CD25<sup>+</sup>, Ts CD8<sup>+</sup>CD28<sup>-</sup>, macrophages (M2) and DCs (DC2)].

# Immunotherapy related problems

- o different types and properties of cancer, the stage of disease, the tumour mass;
- o the vaccine and its dose; delivery and vaccination schedules still to be optimized; intradermal or intralymphatic injections superior to intravenous ones; weekly injections with monthly boosting are mostly performed;
- o possibility of faulty Ag presentation – tolerance induction – rapid tumour progression (selective enrichment of highly aggressive TC clones);
- o how to brake tumour immune protection - the specific anti-TAA immunization is obviously not enough?
- o partial or complete loss of MHC expression;
- o as many specific targets as possible should be attacked simultaneously;
- o direct immunomodulation in vivo?

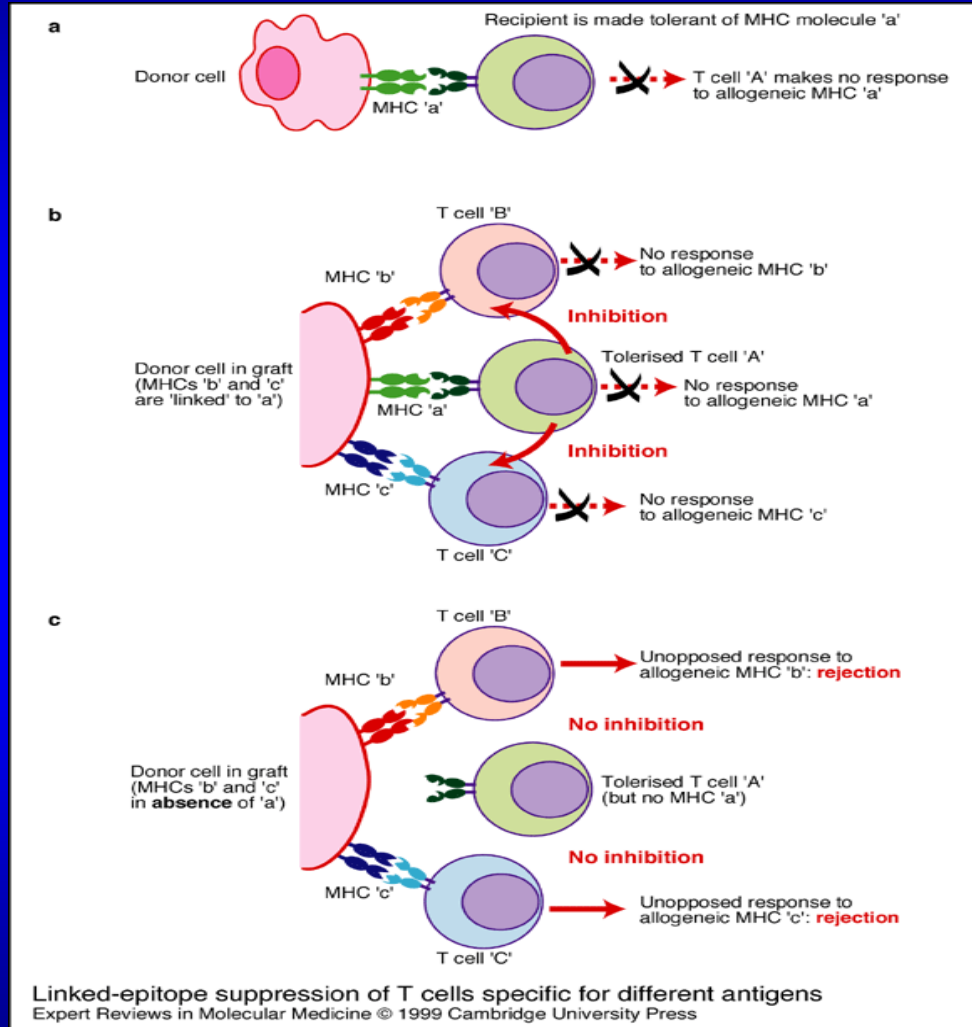
# T cell anergy and suppression

- o Ag-specific immunoregulation is achieved via APC, bringing together T cells by presenting them the specific Ag. These T-cells can then cooperate with each other directly via T cell - Tcell interactions as well as indirectly via APC. Such T cell-APC clusters can function in an activating way, mediating T cell help for CTL or in an inhibitory way via regulatory T cells suppressing the responses of other T cells within the same cluster - »linked suppression«.
- o The tolerance or nonresponsiveness can spread from T cell to T cell, a phenomenon called »infectious tolerance«, being mediated by tolerant regulatory CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Anergic T cells are able to suppress other T cells through competitive inhibitory effects, competing for the surface of APC and for the locally produced IL-2, actively suppressing T cell responses.
- o Anergic T-cells, generated through T cell - Tcell interactions are also able to modulate APC in such a way that they downregulate their T cell activating capacity. For example viral infection or CD4<sup>+</sup> T cell help can activate an APC making it »licensed« - it then functions as a temporal bridge by sequentially activating CD8<sup>+</sup> CTLs in the absence of T- cell help. Tolerogenic APCs can also function as temporal bridge between anergic regulatory T-cells and T-cells yet to be regulated.
- o Obviously anergic T cells express certain, as yet unknown surface molecule which upon interaction with its ligand on the APC mediates a dominant tolerogenic signal and turns off the APCs, even previously »licensed« ones that have already recived activation signals.

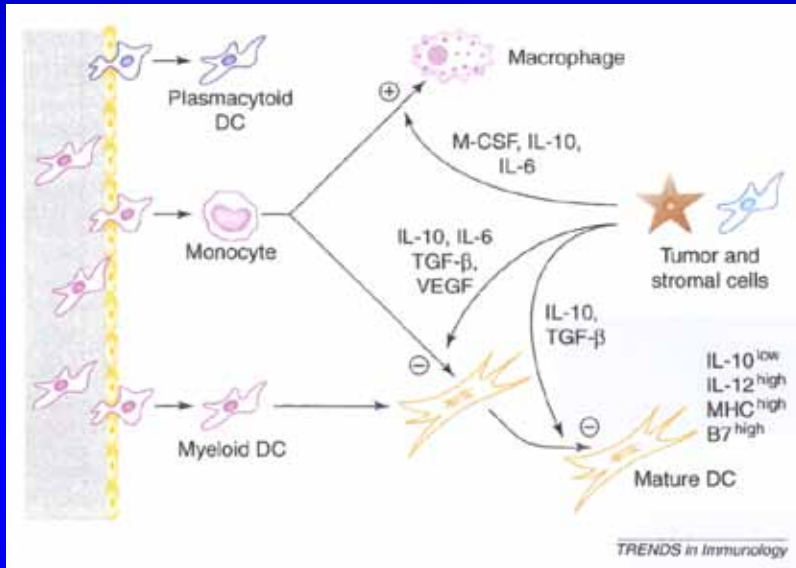


Human Immunol 2000, 61: 633 - 639

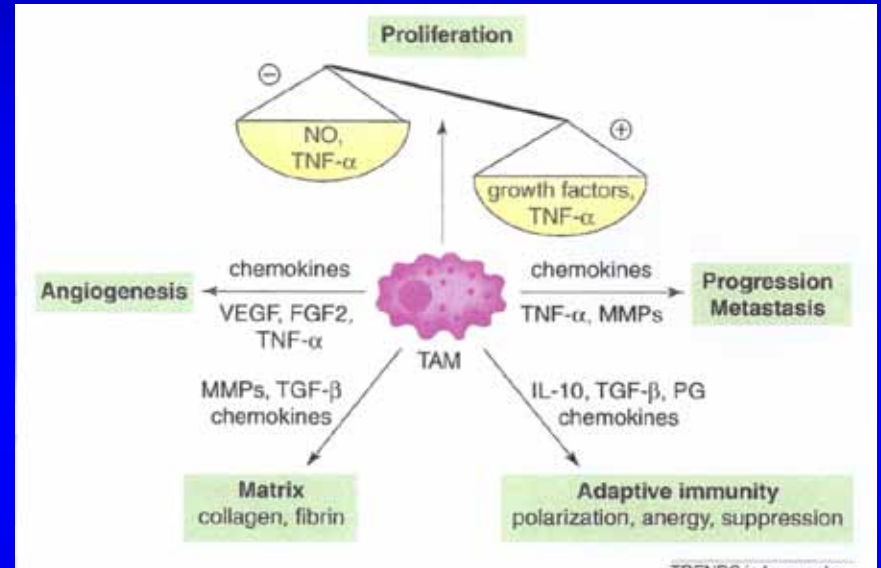
# Linked-epitope suppression of Ag specific T cells



# Tumour associated macrophages



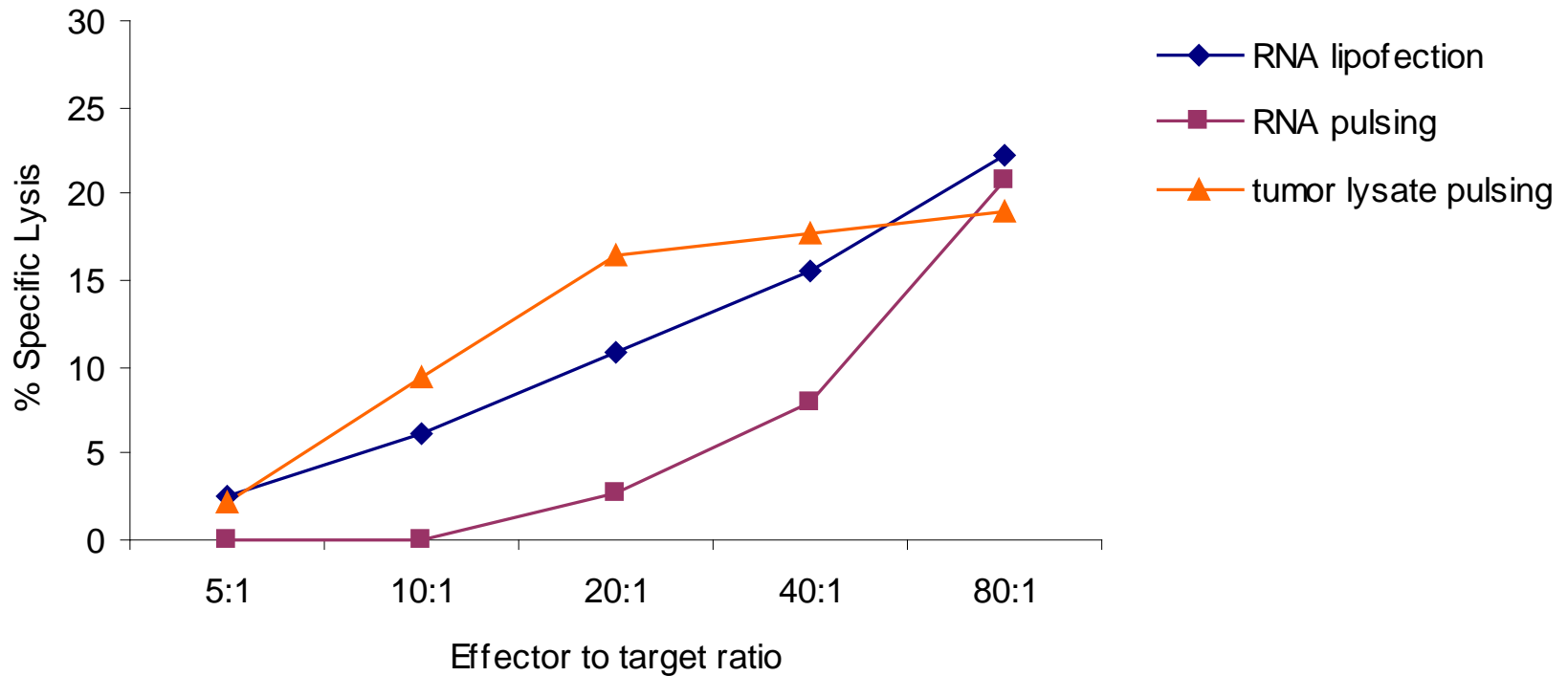
**Fig. 3.** Accumulation and differentiation of DCs in neoplastic tissues. Both myeloid and plasmacytoid DCs are present in tumors. Cytokines produced by tumor cells or stromal elements promote the differentiation of precursors into macrophages rather than DCs and block DC maturation. Abbreviations: DC, dendritic cell; IL, interleukin; M-CSF, macrophage-colony stimulating factor; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.



**Fig. 4.** A simplified view of the role of TAMs in the immunobiology of tumors. Abbreviations: FGF, fibroblast growth factor; IL, interleukin; MMP, matrix metalloproteinase; NO, nitric oxide; PG, prostaglandin; TAM, tumor-associated macrophage; TNF, tumor necrosis factor; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

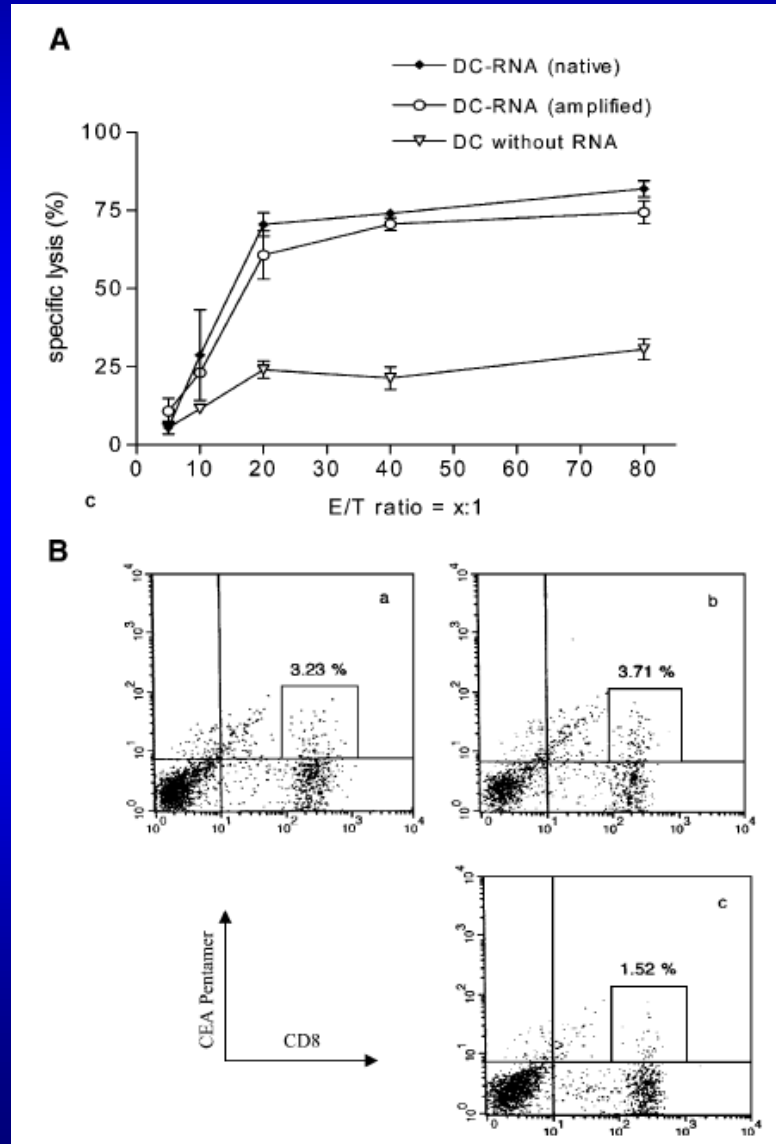
Trends in Immunology 2002, 23: 549-555

# TAA presenting capacity of DCs



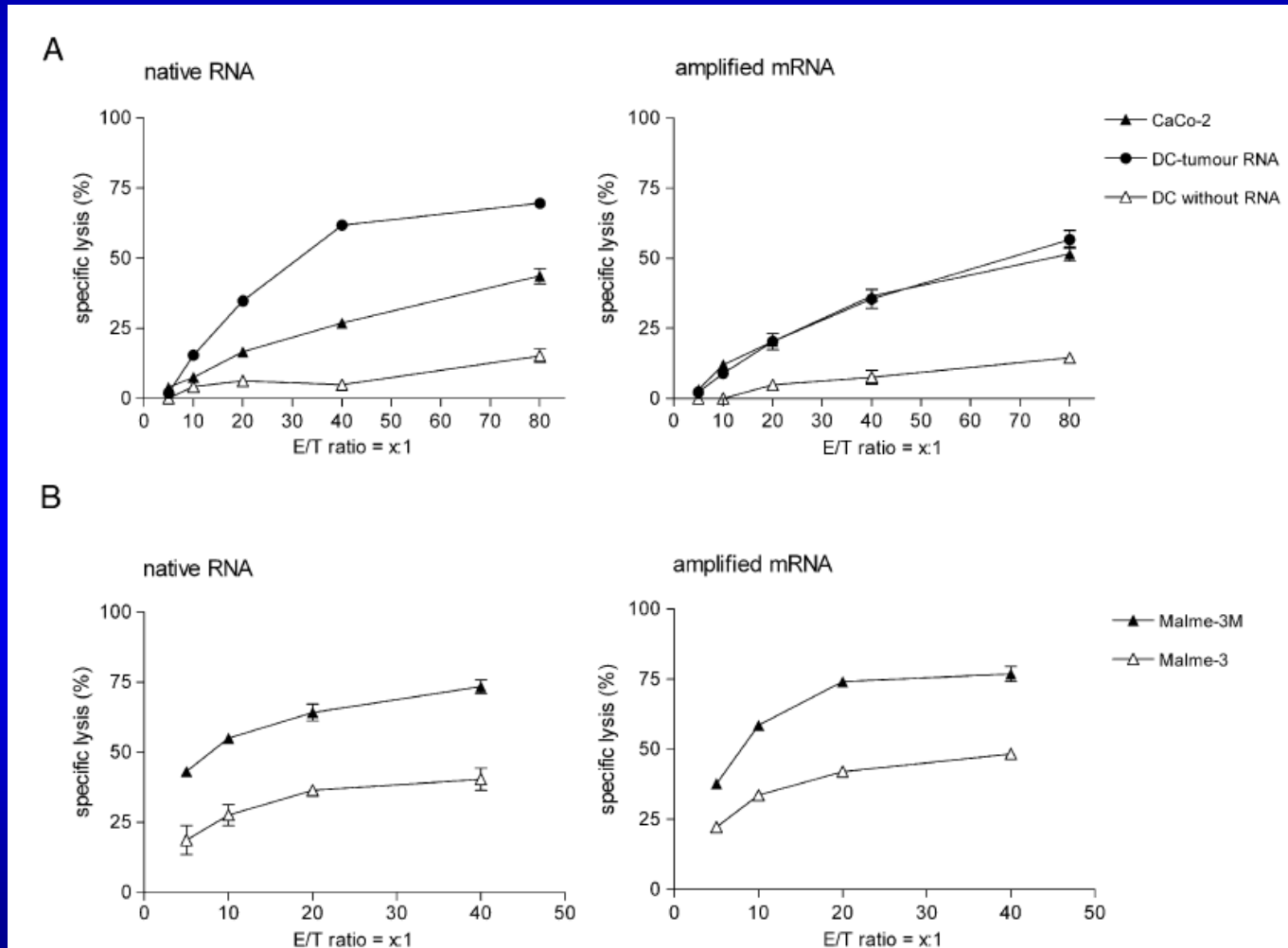


# CEA-specific CTL activity induced by tumour RNA transfected DCs



Target cells:  
HLA A\*0201 positive T2 cells  
loaded with A\*0201 restricted  
CEA-specific peptide YLSGANLNL

# Strong *in vitro* anti-tumour CTL activity



# Detection and quantification of immunohybridomas

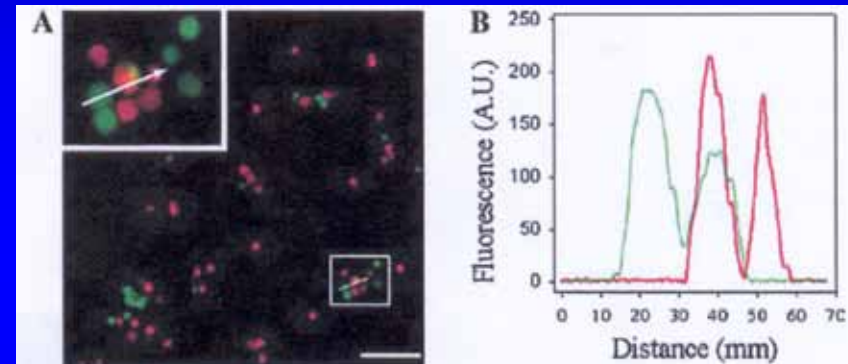
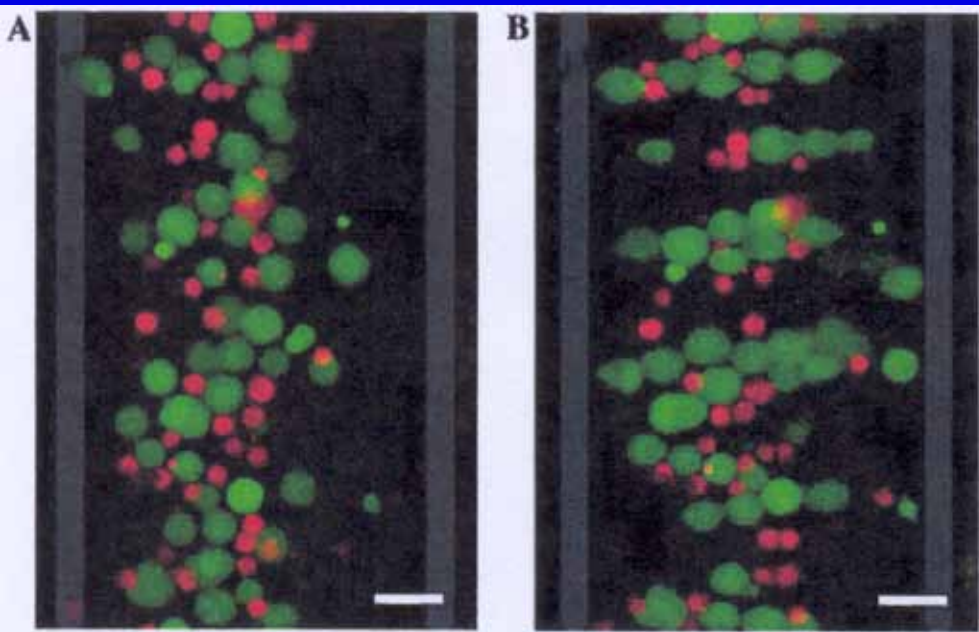


Fig. 2. Identification of yellow hybrid cell. Fused hybrid cells appear yellow, since their cytoplasm contains a mixture of the green (CMFDA) and the red (CMTMR) fluorescent dyes (A). Bar: 100  $\mu$ m. (B) It shows a line intensity profile (see arrow in A), used to confirm that a cell is a hybrid. A cell was considered to be a hybrid when the intensity of the red and the green pixels was at least 16% of the maximum intensity level.

BBRC 2004, 314: 717-723

# Alloantigens and alloreactivity

- o many alloepitopes (HLA class I and II alleles + peptides);
- o the most potent immune response – involves 1 to 10 % of all lymphocytes;
- o cellular (T cells, CD4<sup>+</sup>, CD8<sup>+</sup>) as well as humoral (allosensibilisation, B cells);
- o abundant pro-inflammatory cytokines;
- o but unfortunately – life threatening GvHD effects in transplantation;
- o MHC compatibility prevents alloreactivity;
- o MIS – potency of alloreactivity preserved without the risk of aGvHD – a possible way to uncouple GvHD and GvM/GvT/GvL effects?

# MIS and MIT effectors and their targets

- o MIS effectors: *in vitro* pre-treated (patent protected) allogeneic PBMC, BM/SC, strictly Th1 programmed (T help and cytotoxicity), targeting the tumour protecting Treg or Ts, Th2, M2 and DC2 that persist after the precedent leukodepletion step carried out by using chemotherapy, irradiation and monoclonal or polyclonal Abs (OKT-3, anti-CD25, ATGAM,...). Due to their controlled (limited) life span they can not induce aGvHD.
- o MIT effectors: *in vitro* pre-treated (patent protected) allogeneic PBMC, BM/SC, strictly Th1 programmed to produce and secrete specific cytokines (IL-2, IFN-g, IL-12), deprived of cytotoxicity and targeting Mφ as well as DC to turn them into M1, DC1 state and to maintain it. They are highly immunomodulatory. Due to their controlled (limited) life span they can not induce aGvHD.

# MIS effectors and their targets

- o allogeneic MIS GvM/I (donor specific) effectors are able to deblock patient's own GvM/II immune cells by purging tumour protecting Treg or Ts and by switching APC M2 and DC2 subsets into M1 and DC1, respectively. Thereby they provide proinflammatory (Th1) conditions that enhance proper and persisting priming of naive T cells and prevent negative immunoregulation (suppression).
- o these effects can be further on potentiated by applying selected single and combined substances (out of approx. 400 tested), a patent protected procedure, that helps to remove the nocive effects of hyperactivated APCs and dysregulated T-cell clones persisting in chronic inflammation processes typical for cancer, atherosclerosis, etc.
- o MIS effectors can also be primed (pre-activated) by patient's own cells (MLR) to potentiate their activities.
- o when tumours lack MHC class I expression, MIS-NK/LAK effectors could be safely used as well.
- o MIS effectors could therefore strongly enhance and potentiate immunotherapy: cellular vaccines, adoptive transfer of specific T cells, use of different monoclonal antibodies, etc.

# Leukodepletion enhances T-cell mediated tumour immunotherapy

- o leukodepletion per se eliminates the cellular cytokine "sinks" (competitors) for homeostatic  $\gamma\gamma$  cytokines (for example: IL-7, IL-15 and possibly IL-21) as well as regulatory CD4<sup>+</sup>CD25<sup>+</sup> T-cells (Treg) & CD8<sup>+</sup>CD28<sup>-</sup> (Ts) that suppress tumour reactive T-cells; it additionally induces tumour apoptosis and necrosis in conjunction with APC activation (M1, DC1). [*IL-7 promotes naive CD4<sup>+</sup> and CD8<sup>+</sup> homeostatic proliferation and has little impact on memory T-cell expansion and survival in a lymphopenic environment; IL-15 in contrast has a pivotal role on memory CD8<sup>+</sup> T-cell proliferation and durability; IL-21 shares homology with IL-2 and IL-15 and binds to their common  $\gamma\gamma$  receptor*]  
Trends in Immunology 2005; 26(2): 111-117 & Trends in Immunology 2005; 26(6): 298.
- o leukodepletion can be additionally supported by allo-MIS effectors (PBMC, BM-SC, NK/LAK), leading to the fine tuning and resetting of the immune system (deblocking of active tumour protection); the final result being the reappearance and amplification of patient's own anti-tumour immune responses; these could further on be enhanced by using autologous or even haploidentical DC-based cellular vaccines (combination of alloreactivity and Ag/MHC specific immune responses) or adoptive transfer of autologous anti-tumour-reactive T cells.

# Impressive results of extensive pre-clinical studies

- o syngeneic, semi-allogeneic and allogeneic B16 melanoma mouse experimental model with over 200 different protocols tested on more than 7000 mice; certain combinations resulting in 100 % long-term survival of animals with established B16 melanoma.
- o basic protocol: induction of tumour (inoculation of  $2 \times 10^6$  B16 cells/mouse), 9 days later cyclophosphamide (Cy = 120 mg/kg) or irradiation (800 cGy); 10 days after B16 inoculation tumour excision (1-3 hours before, 50 mg of mouse anti-Th 1.2-MoAbs given), splenocyte or/and M1S application (syngeneic, semi-allogeneic or allogeneic donors);
- o numerous additional protocols (variations) tested as well;
- o monitoring of survival rate – selection of the most successful protocols (complete, 100 % long-term remission of tumours);
- o the most successful ones selected for re-challenge with high dose of B16 cells (up to  $10^7$ ) - assessment of the survival rate.

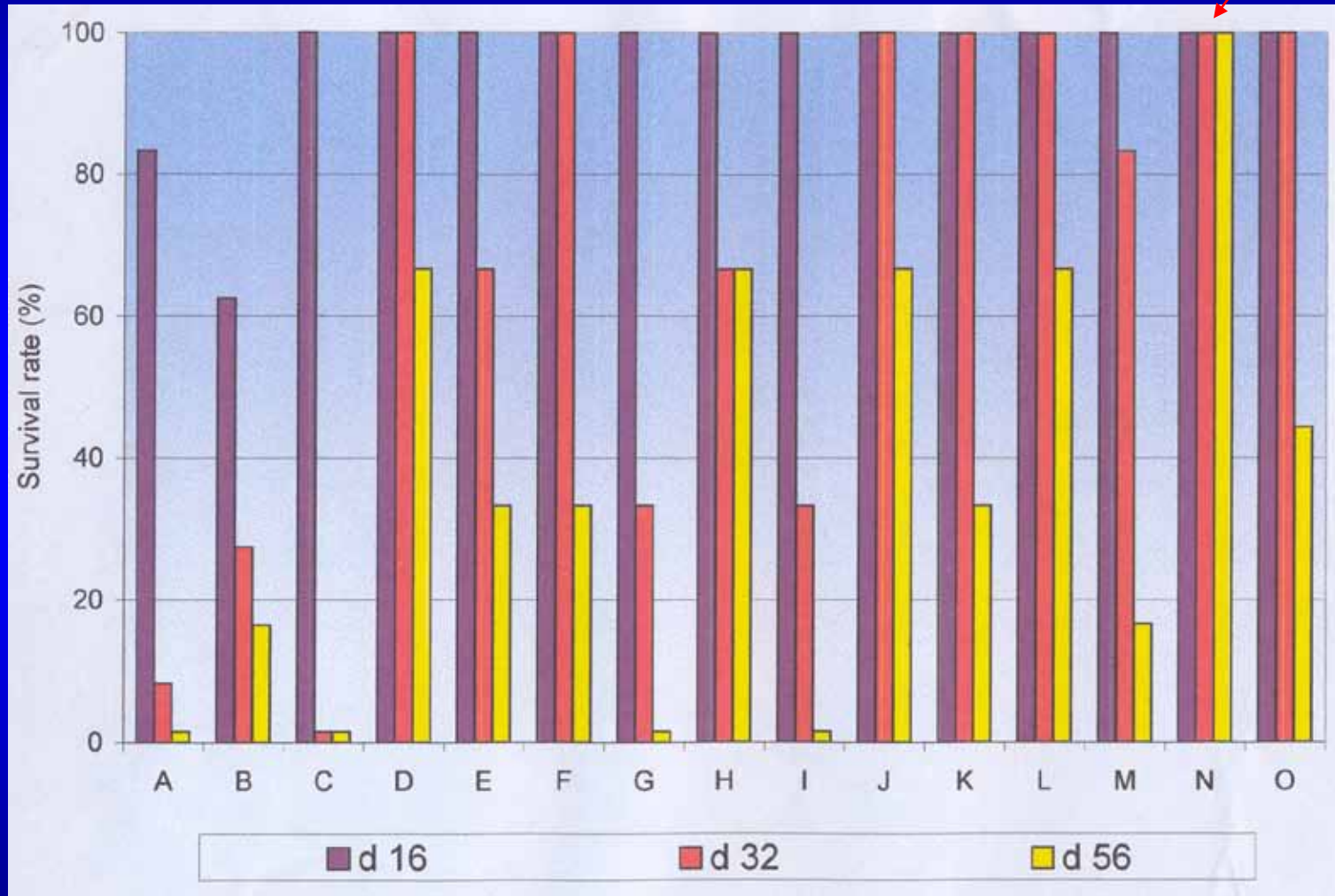


# Impressive results of extensive pre-clinical studies

- o tumour excision combined with a radical *in vivo* depletion of tumour-protecting T cells, followed by the application of donor PBLs, pre-manipulated *in vitro* in a multistep procedure, consisting of: (a) programming of their lifespan, (b) pre-programming of their cytotoxic capacity and (c) pharmacological pre-programming of their resistance to hyperactivation-induced anergy (activation of  $K_{Ca}$ -channels and blocking of L-type-voltage-dependent  $Ca^{++}$ -channels) - "anergy-resistant effectors".
- o the high efficiency of the procedure due to: (1) thorough Treg and Ts depletion through the use of mAbs and alloreactive donor effectors (2) functional re-programming of patient's APCs from type 2 (M2, DC2) to type 1 (M1, DC1) through IL-2 and IFN-g signals of allo-stimulated donor Th1 cells (3) Th1-commitment of naive tumour-specific T cells on the surface of the type-1 re-programmed patient's APCs, replacing the pre-depleted set of patient's misregulated (hyperactivated) mature T cells.
- o the antitumour effect of MIS effectors was therefore based on: (a) a strong GvHD-free GvT/GvM-effect, (b) depletion of tumour-protecting Treg and Ts cells, and (c) type1 (M1, DC1) re-programming of patient's APCs; a repeated MIS treatment resulted in a 100% long-term survival rate; re-inoculation of  $1 \times 10^7$  tumour (B16) cells into long-term survivors had only minimal impact on the excellent overall survival rate.

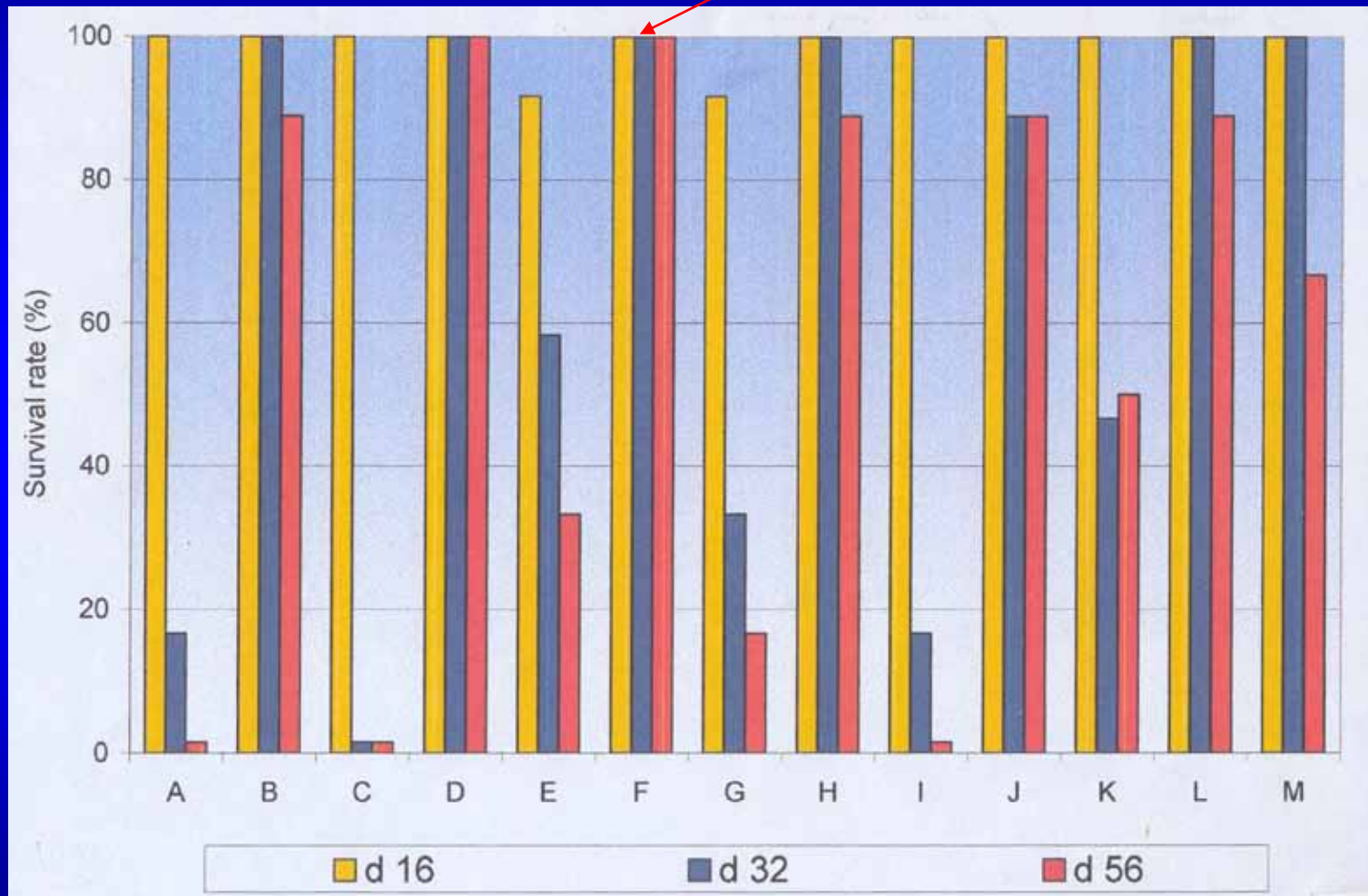
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Semi-allogeneic system  
(R = C57B16xDBA2); MIS eff.



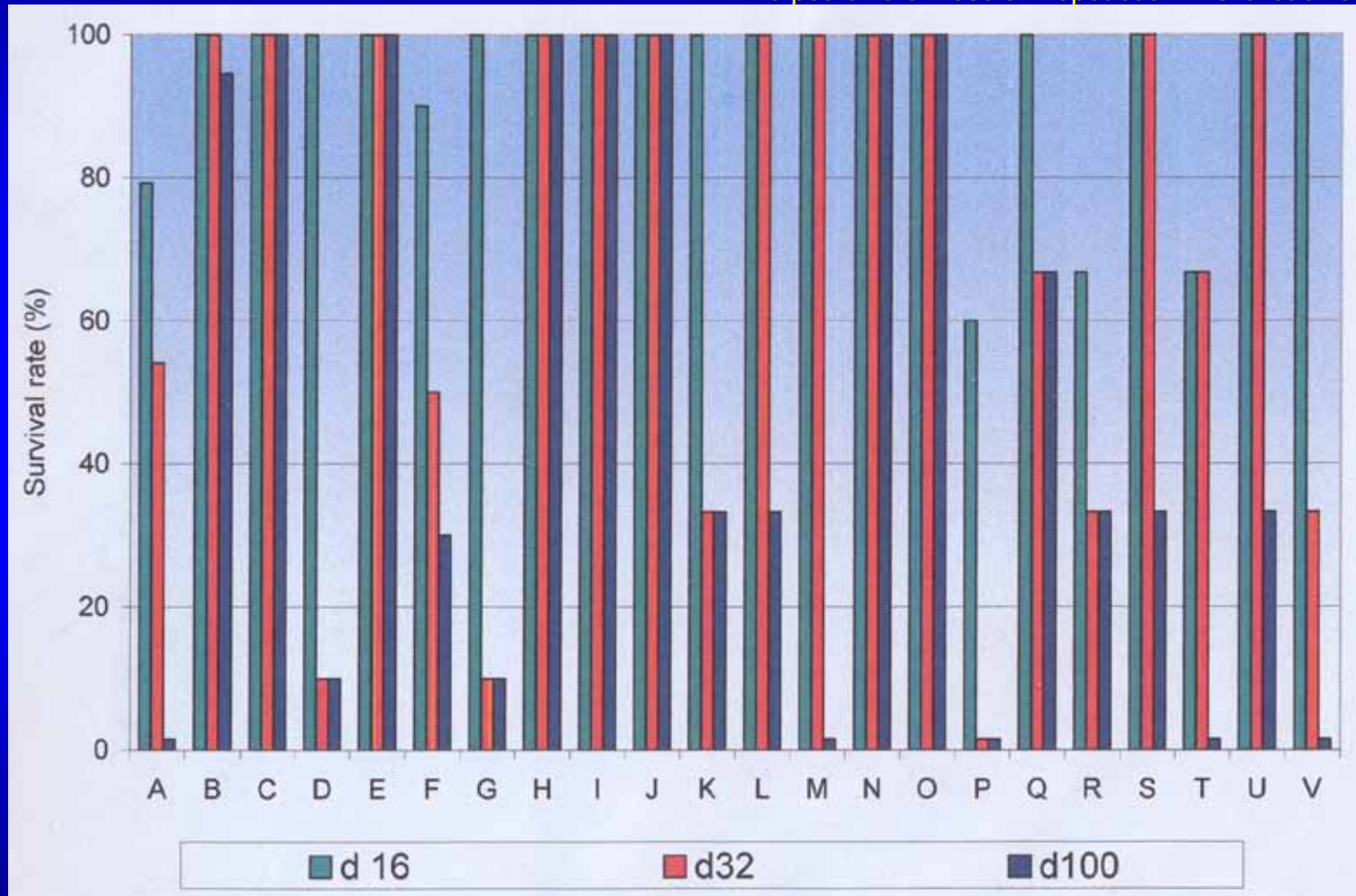
# Impressive results of extensive pre-clinical studies

R = F1 hybrid + M1S effectors (C57B16 strain)



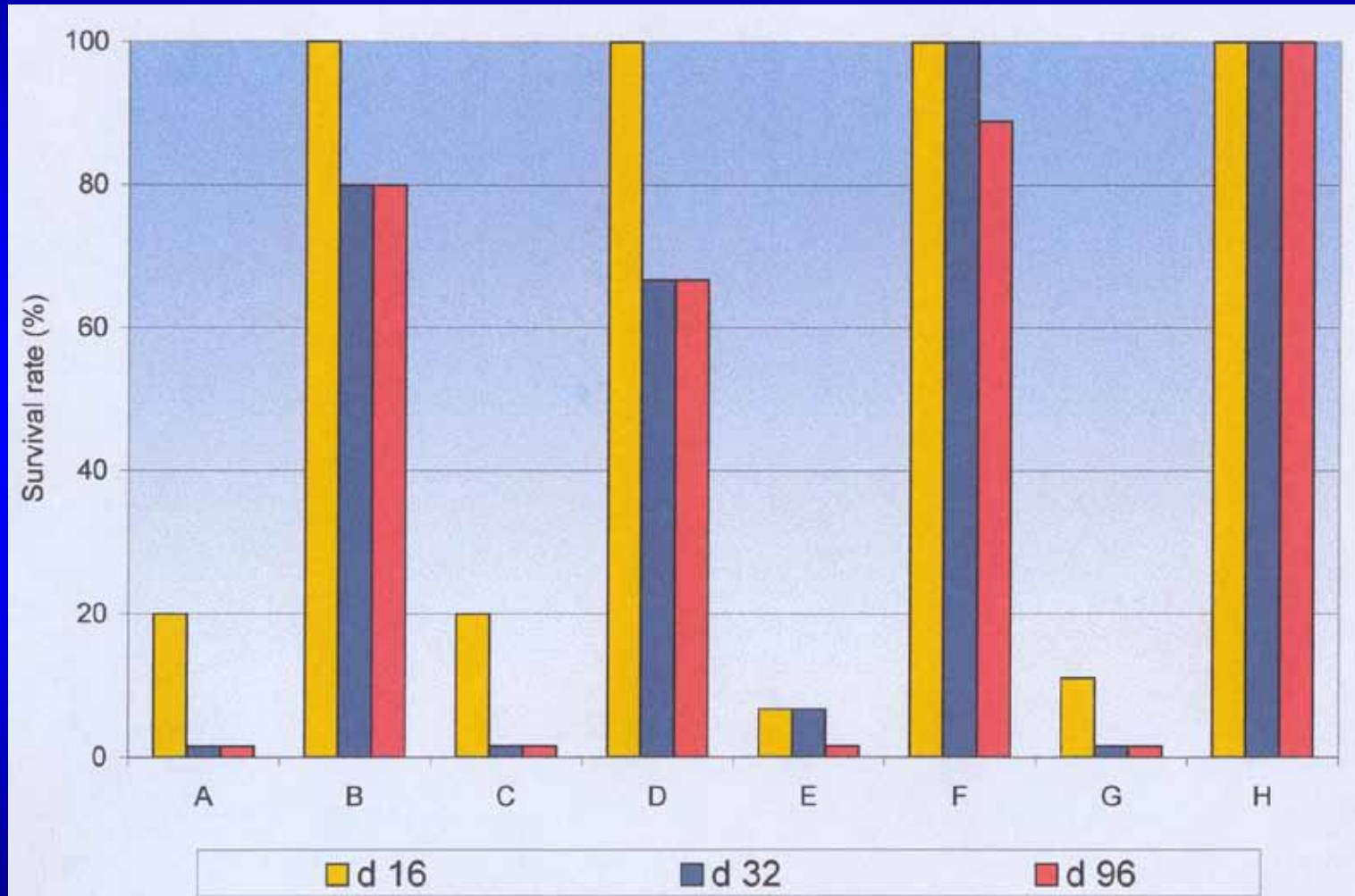
# Impressive results of extensive pre-clinical studies

The positive effect of repeated MIS treatment (2x)

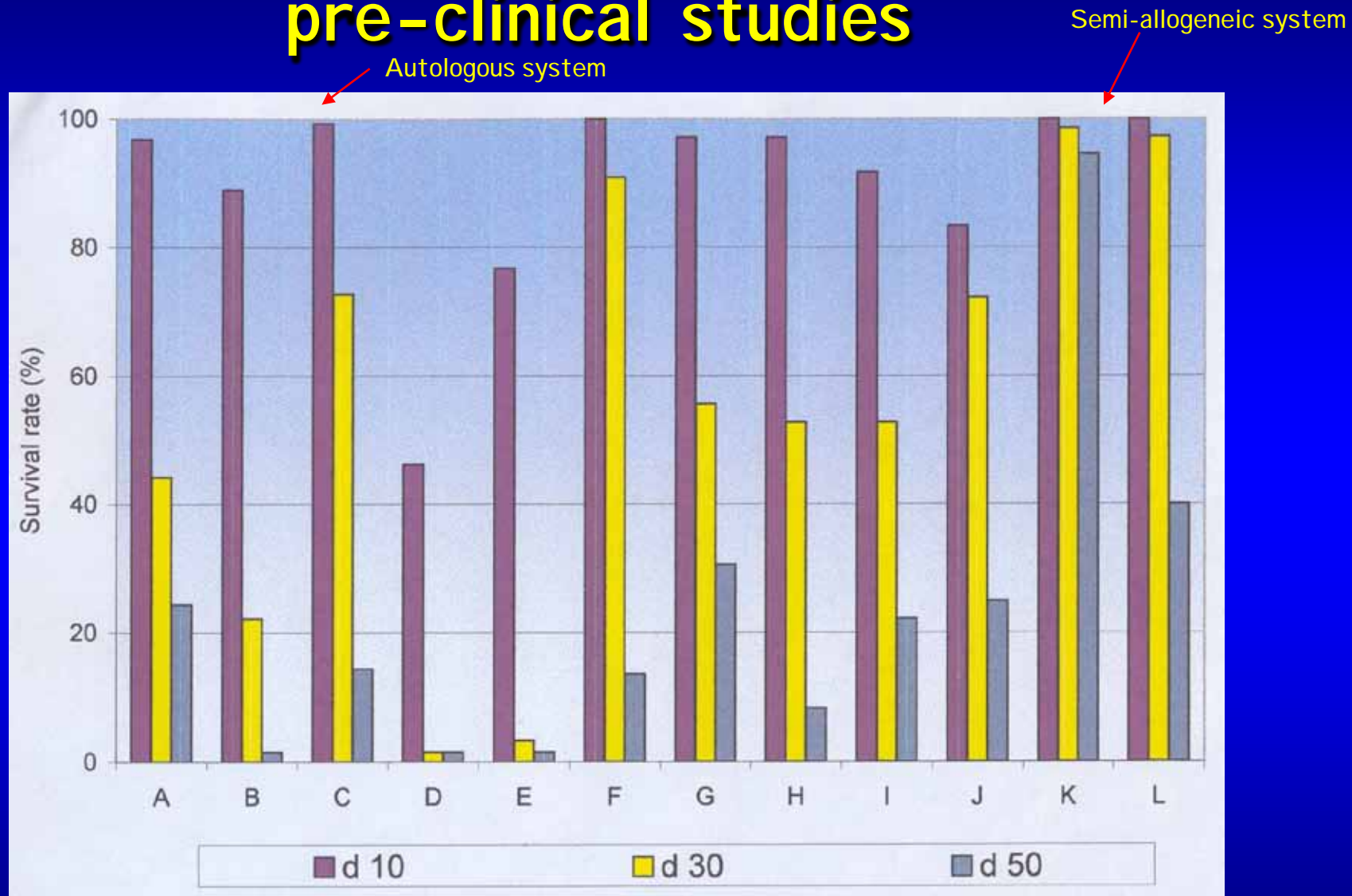


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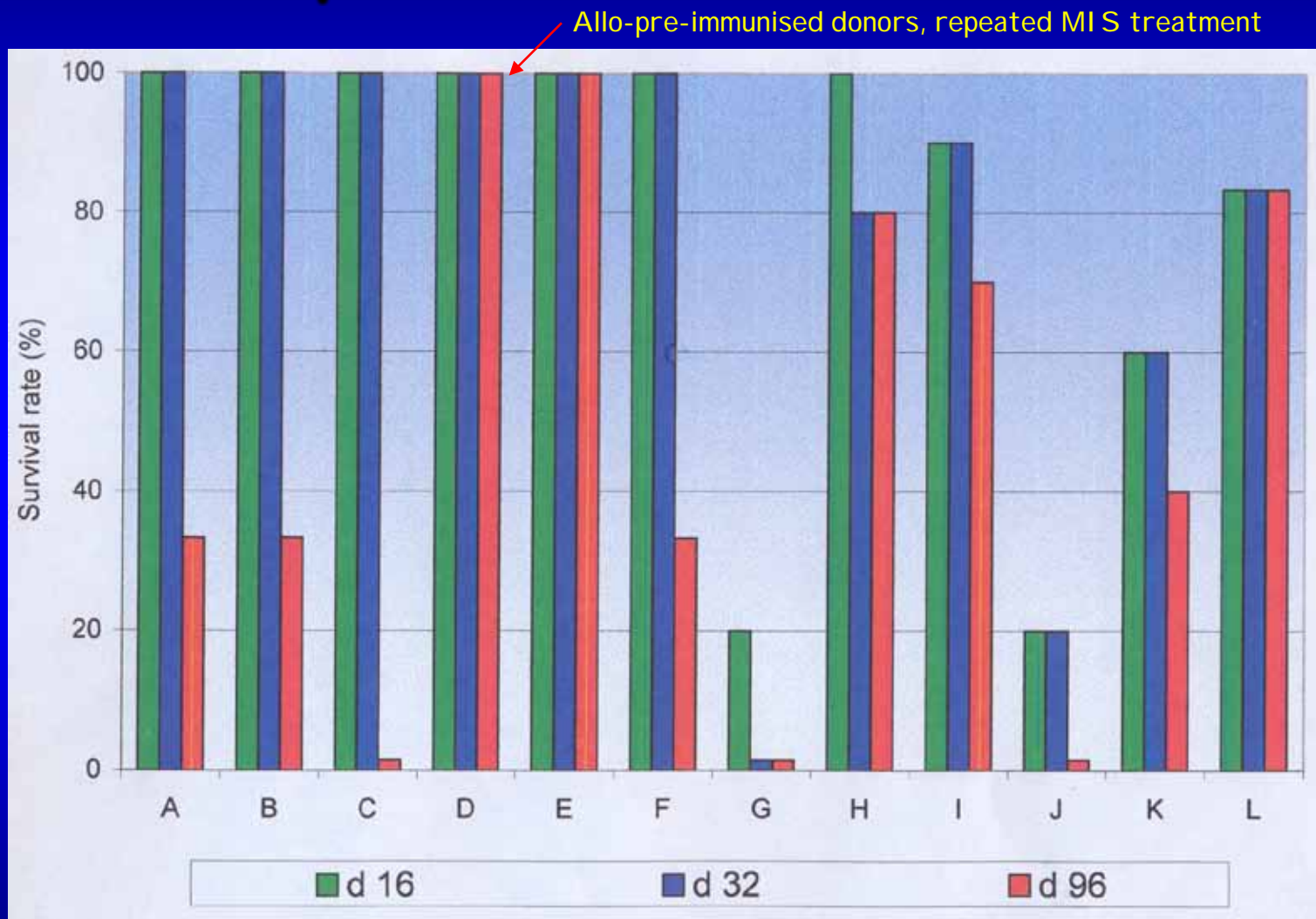
Unmanipulated splenocytes vs MI S effectors



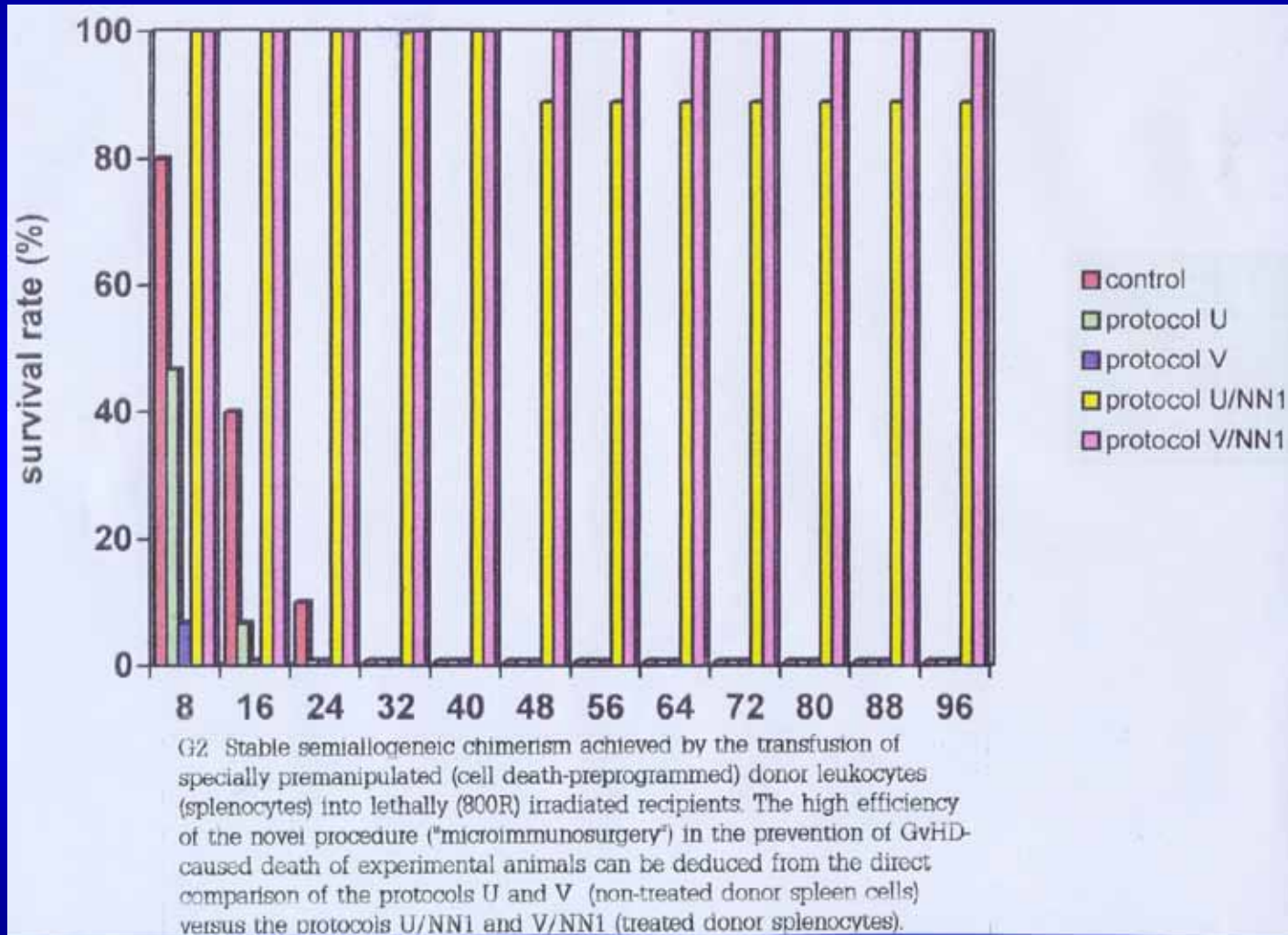
# Impressive results of extensive pre-clinical studies



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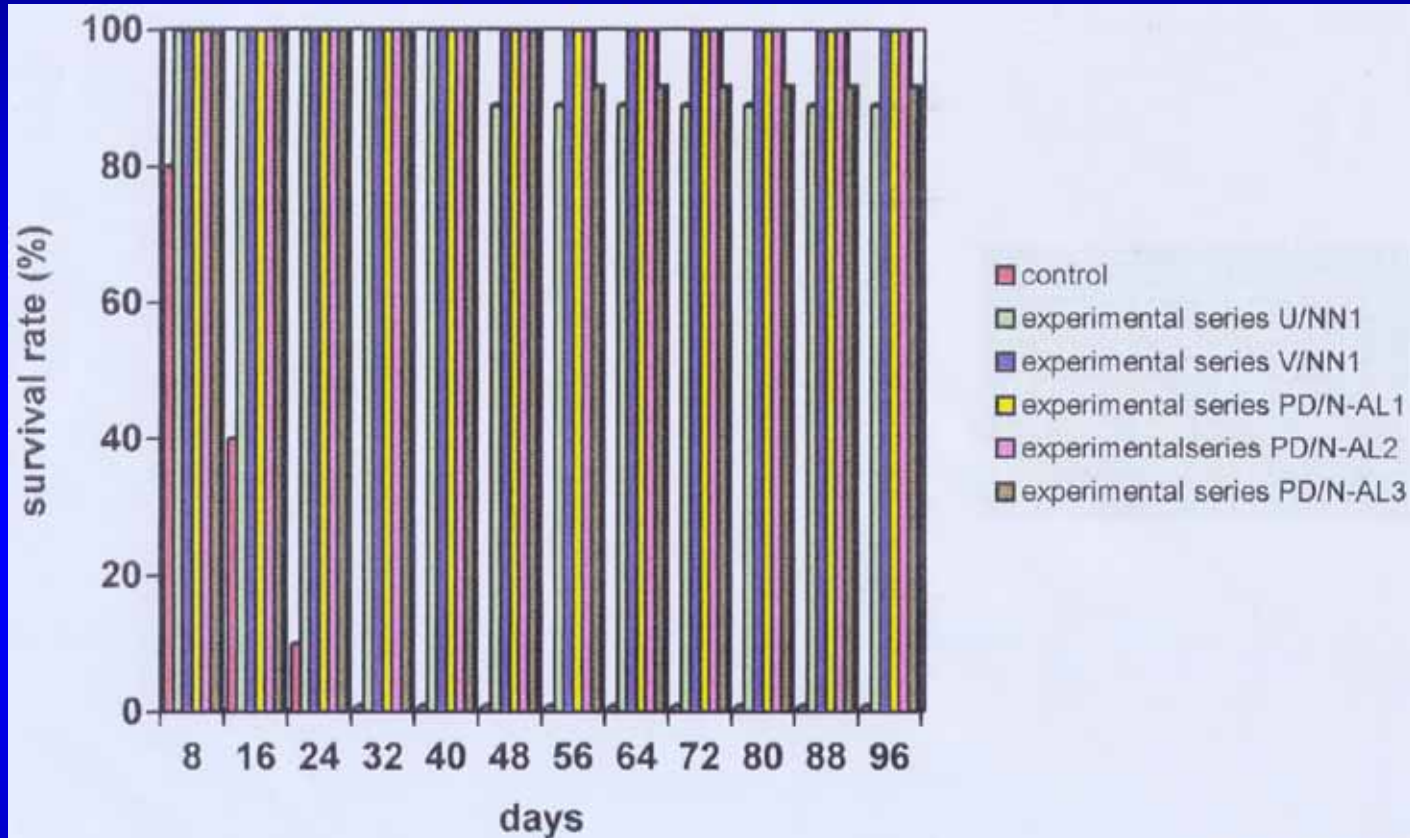


# Impressive results of extensive pre-clinical studies





# Impressive results of extensive pre-clinical studies



G6: Complete prevention of the fatal graft-versus-host-disease (GvHD) by the ex vivo pretreatment of alloreactive donor effector cells (patented multistep procedure) both in lethally (800R) preirradiated (protocols U/NN1 and V/NN1) and sub-lethally preconditioned (120 mg/kg cyclophosphamide plus anti Thy1.2-Mab) mice (protocols PD/N-AL1...3).

# Impressive results of extensive pre-clinical studies



G10 Evidence for the high efficacy of the novel approach ("microimmunotherapy") in the selective elimination of GvHD-establishing donor effector cells, both in the semi-syngeneic (protocols P4b and P8b) and allogeneic (protocols PD/N-AL1...3) system

# Clinical study (1)

- o inclusion criteria: histologically confirmed cutaneous metastatic malignant melanoma patients, resistant to all known conventional therapies; age between 18 and 75 years; ECOG performance <2; life expectancy  $\geq 3$  months (2-4); signed written informed consent with the right to withdraw from the study at any time without prejudice; willingness and capacity to comply with the protocol. [Oncological Institute Ljubljana & Clinical Centre Sarajevo – Department of Oncology]
- o protocol:
  - Day 0: serum and blood samples
  - Days 1 and 2: cyclophosphamide (Endoxan) 400 mg/m<sup>2</sup>
  - Days 8 to 12: ATGAM (15 mg/kg) or OKT3 (5 mg/day)
  - Day 15: infusion of pre-treated allogeneic PBMC (MIS)\* (6-7x10<sup>8</sup>)
    - \*: repeated 3 to 5 times using different healthy blood donors within intervals not exceeding 7 days (5-7 days)
- o monitoring of eventual adverse effects (allergic reactions, anaphylactic shock, dyspnoea, tachicardia, influenza syndrome, ...)
- o evaluation: measurable lesions (tumours), quality of life (ECOG performance), hematology, immunology, biochemistry ....

# Clinical study (1) - results

- o first phase I/II clinical study (1997-2000): 8 patients with stage IV metastatic melanoma (expected survival period 2-4 months) were included and MIS/MIT treated (no other therapy in parallel, except in 1 patient\* - cisplatin + IFN); 6/8 with measurable response, good quality of life, treatment could safely be repeated.

Patient Id.	Sex & age	No. of cycles	Response	Survival period	Adverse effects
1* BJ	M/60	2	CR	18 months	No
2 MK	F/54	1	SD	2 months	No
3 RB	M/60	2	SD	> 3 months	Cytokine release syndrom
4 VP	F/34	1	CR	> 8 months	Allergic to ATGAM
5 DM	M/33	1	PROG	NA at the time of report	No
6 GP	M/39	1	PROG	excluded – brain matastases	ND
7 NS	M/59	1	PR	> 2 months	No
8 SV	M/58	1	SD	> 2 months	No

# Clinical study (2)

- o Hepatobiliary and pancreatic cancer patients: 11 patients with different Dg: pancreatic cancer 7 (radical surgery 5/7), gallbladder cancer 3 (no radical surgery) and biliary duct carcinoma 1 patient (no radical surgery); average age 53 years (range 21-72), 7 male and 4 female patients. MIS/MLT was the only treatment. [Clinical Centre Ljubljana, Department of Gastroenterology]
- o basic protocol:
  - Day 0: serum and blood samples
  - Day 1-3 (5 pat.) or 1-4 (3 pat.) or 1-5 (2 pat.): cyclophosphamide (Endoxan)
  - Day 8-12 OKT3 (5 pat.), 3 pat. 3 days, 1 pat. 2 days
  - Days 16, 23, 37 and 44: MIS pre-treated allogeneic PBMC of different healthy blood donors (1 x pre-alloimmunised with MLR in 5 patients)
- o monitoring of eventual adverse effects (allergic reactions, anaphylactic shock, dyspnoea, tachicardia, influenza syndrome, ...)
- o evaluation: tumour response, quality of life, tumour markers (CEA and CA 19-9), hematology, immunology, biochemistry ....

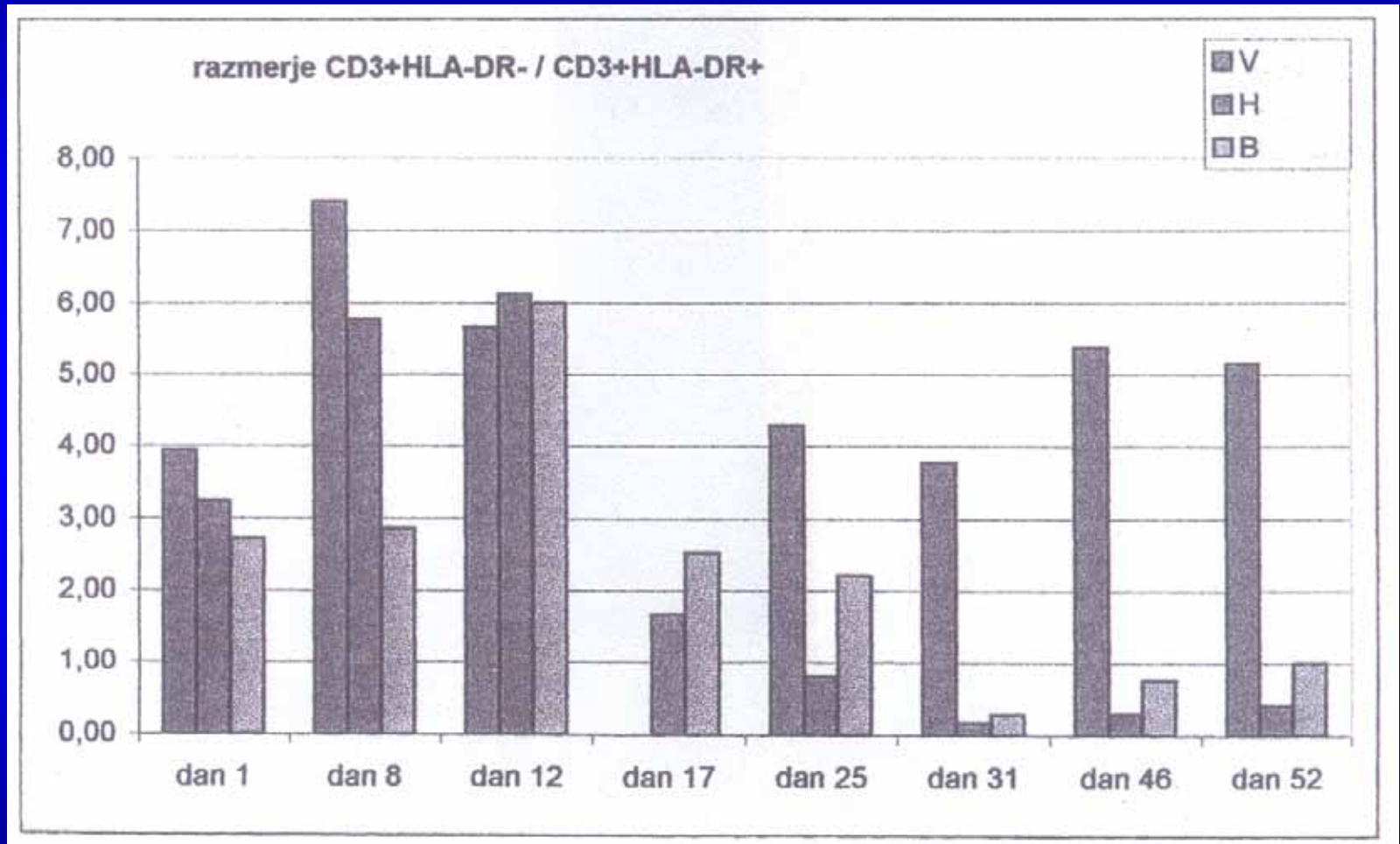
# Clinical study (2) - results

- o first phase I/II clinical study (June 2001- August 2002): 11 patients with hepato-biliary and pancreatic carcinoma (stage III-IV) were included and MIS treated (no other therapy in parallel); 2/11 without signs of disease (DF), 1/11 with stable disease, prolonged expected long-term survival period (by more than 20 %), good quality of life, treatment could safely be repeated as only mild and transient adverse reactions were noticed;
- o follow up time - 6 months (last included pat.) to 14 months;
- o tumour markers CEA and CA 19-9: in most cases fluctuations during the therapy; normal in 3 patients (2 DF pat., No. 3 and 11) and one with the progress of disease (pat. No. 10).
- o loss of weight (> 10%): 7/11 patients

# Clinical study (2) - results

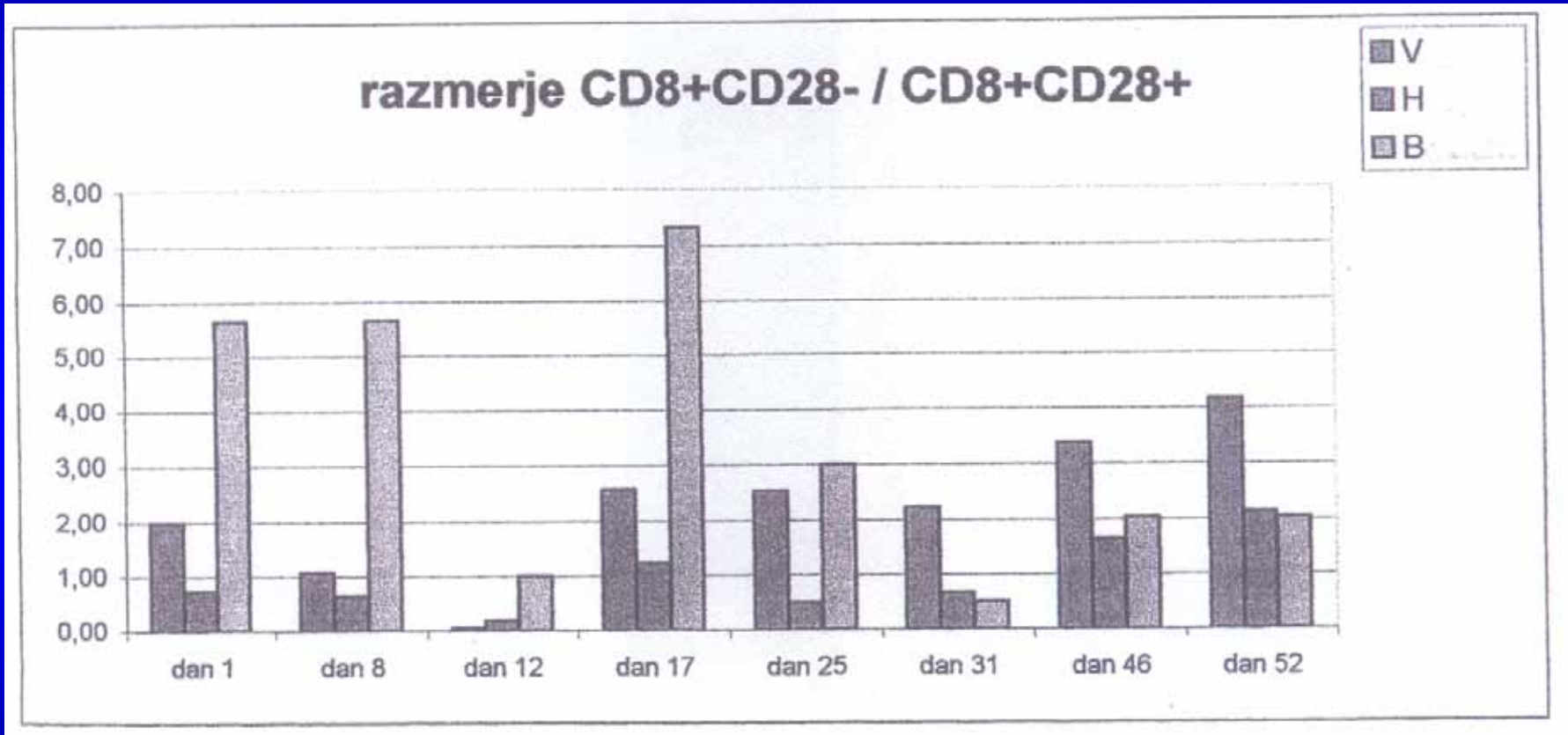
Patient	Sex & age	Dg.	No. of cycles	Response	Survival period	Adverse effects
1 HV	F/66	Ca. pancreatis	1	PROG	10 months*	No
2 VI	M/50	Ca. cholecystae, st.IV	1	SD	10,5 months*	No
3 BA	F/72	Ca. papillae Vateri	1	DF	NA	Epileptic seizures
4 RI	M/21	Ca. pancreatis	Out of the study	PROG	6 months*	-
5 BB	M/42	Cholangiocarcinoma	1	PROG	7 months*	Febrile neutropenia
6 BIG	F/58	Ca. pancreatis, st.III	1 (MLR)	PROG	4,5 months*	No
7 JK	F/57	Ca. cholecystae	1 (MLR)	PROG	8 months*	Raised body T
8 SJ	M/49	Ca. cholecystae	1 (MLR)	PROG	10 months*	Hypotension, sweating
9 LB	M /47	Cy. stadenoca caudae	1 (MLR)	PROG	7,3 months*	NA
10 GF	M/59	Ca. pancreatis	1	PROG	NA	NA
11 BJ	M /56	Ca. papillae Vateri	1 (MLR)	DF	NA	NA

# Clinical study (2) - monitoring

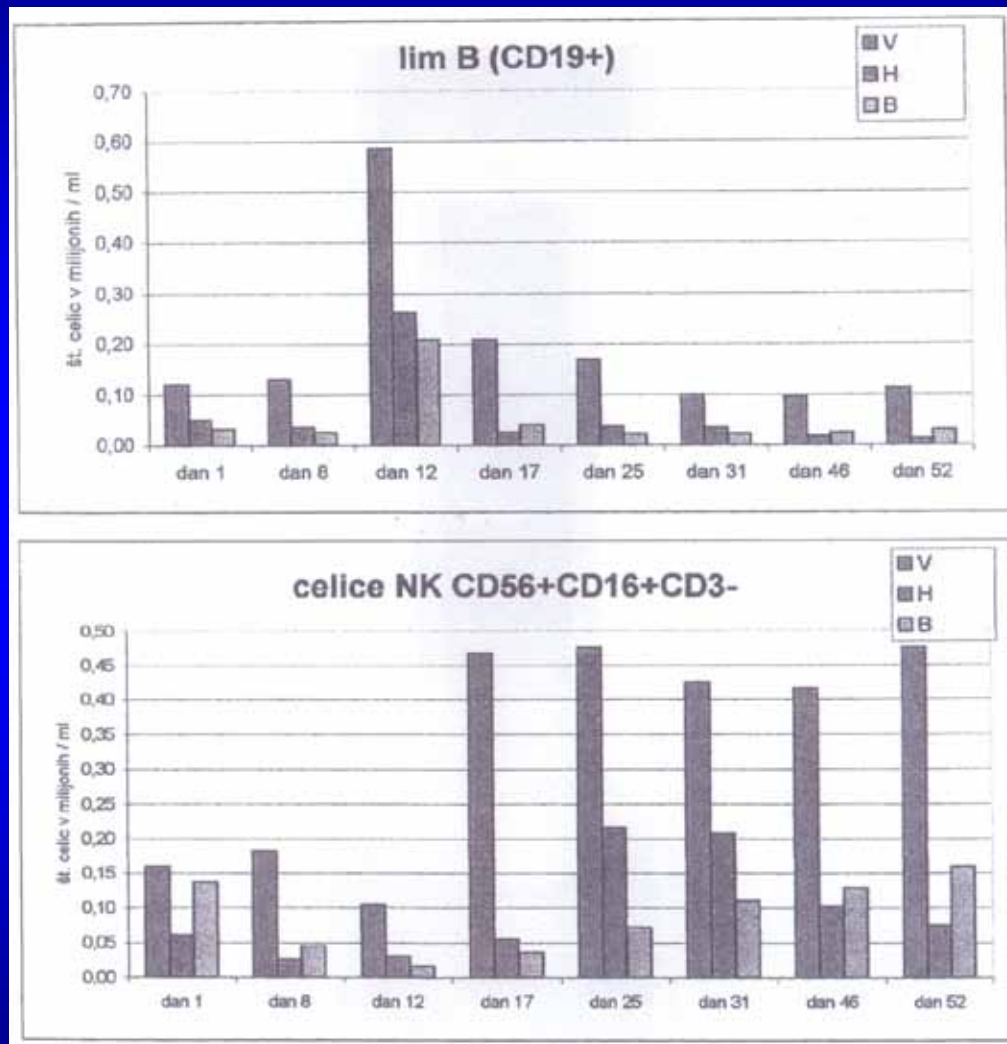




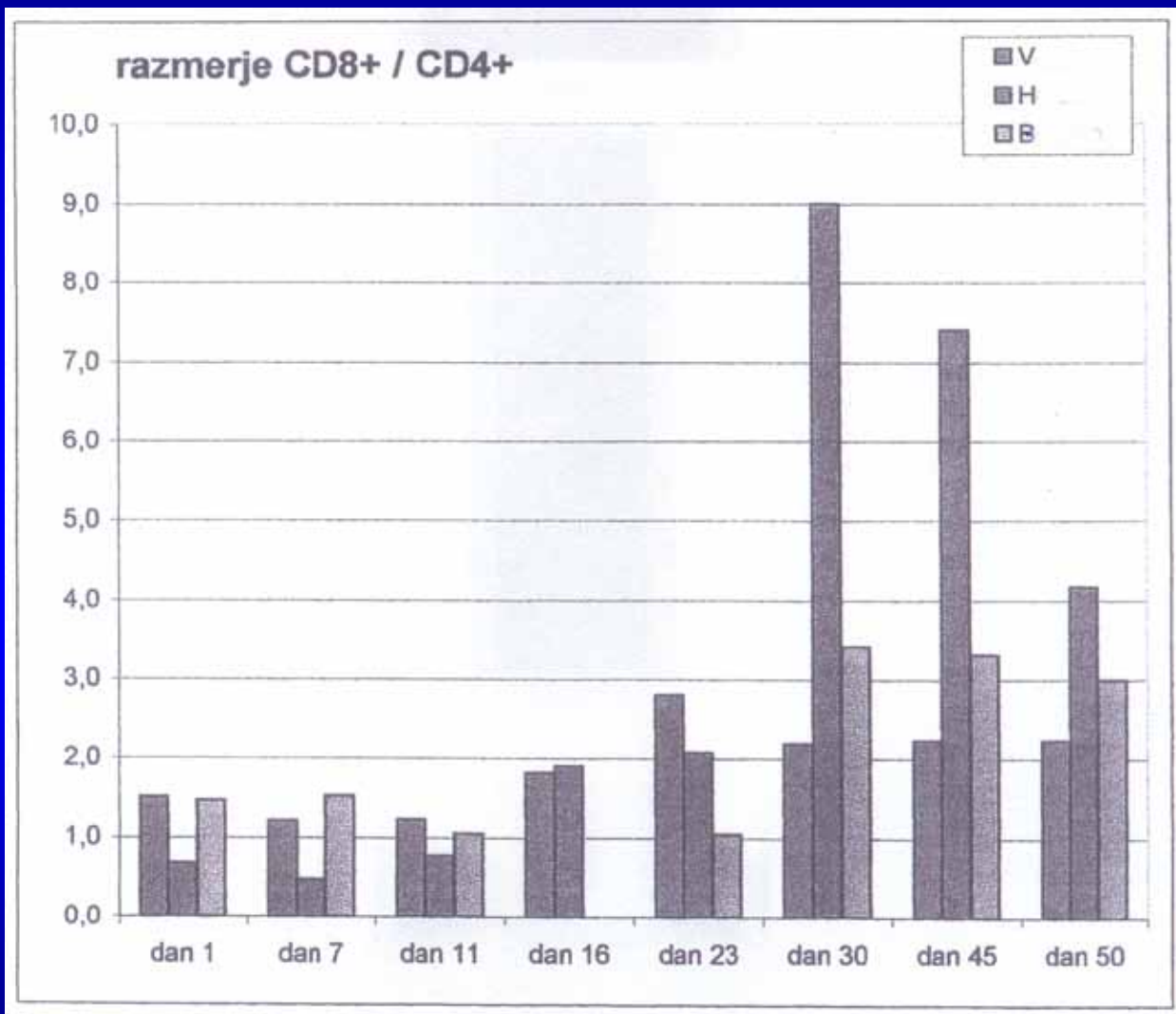
# Clinical study (2) - monitoring



# Clinical study (2) - monitoring



# Clinical study (2) - monitoring



# Potentials of M1S/M1T technology

- o efficient purging of TAA-specific Treg, Ts, Th2 subsets that protect tumour from patient's own immunocompetent cells as well as converting M2/DC2 (tolerogenic, suppressive) APCs into their M1/DC1 counterparts, following the precedent leukodepletion procedure;
- o uncoupling aGvHD and GvM – different implications, for example BM/SC transplantation;
- o enhancement of various types of immunotherapy resulting in better immune responses; (hybridoma/trioma cellular vaccines patent protected already since 1993);
- o possible applications in the treatment of cancer, autoimmune (already tested in animal models) and infectious diseases.