Cancer Immunol Immunother (2001) 49:671–678 

ORIGINAL ARTICLE

**Susanne Heinzel · Delphine Rea**
**Rienk Offringa · Graham Pawelec**

# The self peptide annexin II (208–223) presented by dendritic cells sensitizes autologous CD4+ T lymphocytes to recognize melanoma cells

Received: 3 August 2000 / Accepted: 19 October 2000

**Abstract** Annexin II is known to be over-expressed in different types of tumours. We show here that annexin II protein is expressed by melanoma cell lines in various amounts, consistent with previous findings that an annexin II (208–223) peptide could be eluted from isolated HLA-DR molecules of a constitutively MHC class II-positive melanoma line. T cells sensitized to annexin II (208–223) in vitro using peptide-pulsed autologous dendritic cells responded only to the lines which over-expressed annexin II, in a peptide-specific, HLA-DR-restricted fashion. These CD4+ T cells proliferated strongly and secreted large amounts of type 1 cytokines in response to annexin II (208–223) peptide or annexin II protein-positive melanoma cell lines. These results demonstrate that the annexin II (208–223) peptide, corresponding to a non-mutated sequence of a normal protein, induces antigen-specific T cells which can respond to melanoma cells over-expressing the annexin II molecule. This peptide may therefore be useful in immunotherapy for recruiting CD4+ type 1 helper cells active locally in the tumour environment.

**Key words** Cancer immunotherapy · MHC class II-binding peptides · Annexin II (208–223) · Tumour immunology · CD4+ T cells

S. Heinzel[1] · G. Pawelec (✉)
University of Tübingen, Section for Transplantation Immunology and Immunohaematology,
Second Department of Internal Medicine,
Zentrum für Medizinische Forschung ZMF,
Waldhörnlestrasse 22, 72072 Tübingen, Germany
e-mail: graham.pawelec@uni-tuebingen.de
Tel.: +49-7071-2982805; Fax: +49-7071-295567

D. Rea · R. Offringa
Department of Immunohematology and Blood Bank,
Leiden University Medical Center, Albinusdreef 2,
Postbus 9600, 2300 RC Leiden, The Netherlands

*Present address*:
[1]Hanson Centre for Cancer Research,
Institute of Medical and Veterinary Science,
Frome Road, Adelaide, SA 5000, Australia

## Introduction

The importance of tumour-reactive CD4+ T lymphocytes in tumour rejection is increasingly appreciated. Diverse immunotherapy models show benefit or requirement for CD4+ as well as CD8 + cells [8, 13, 14] either directly by secreting cytokines which may act on CD8+ cytotoxic T lymphocytes (CTLs), endothelial cells and/or the tumour cells themselves. In addition, CD4+ cells are required for so-called "cross-priming" in the draining lymph nodes by activating dendritic cells (DC) via CD40-CD40L interactions [2, 20]. Although tumour-non-specific CD4 activation together with tumour-specific CD8 activation represents an improvement over CD8 alone, only specific co-activation of both cell types is likely to result in optimal tumour rejection [12]. However, compared to known class I epitopes, only a limited number of MHC class II-restricted tumour antigens have been described [3, 9, 15, 22, 24, 25, 27]. It is therefore important to search for further novel antigens of this type.

Several different approaches to seeking novel tumour antigens can be taken. These may be indirect, involving, for example, searching for MHC-binding motifs within the amino acid sequences of known candidate molecules, or transfecting their genes into antigen-presenting cells (APC). An alternative, direct approach is to isolate MHC molecules from tumour cells, elute and sequence bound peptides, and study these. We have taken this latter approach, isolating HLA-DR-bound peptides from melanoma cells [7] and then aiming to establish whether these peptides act as tumour antigens. In addition to some other potentially interesting candidates, a peptide sequence from the protein annexin II was identified in this way. Unlike the majority of eluted peptides, it was found in relatively large amounts that might reflect the high expression of protein in the cell. Annexin II (lipocortin II, p36) is a calcium- and phospholipid-binding protein and a substrate for protein kinases which exists in membrane-bound form both intra- and

extracellularly as well as in soluble form in serum. Annexin II is over-expressed in different tumours, such as glioma cell lines and brain tumour biopsies [11, 18], and in human pancreatic carcinoma cells [23]. Interestingly there is some evidence that annexin II is involved in tumour metastasis due to its tenascin-C-binding capacity [21], and the level of annexin II surface expression in tumour cells has been correlated with their metastatic potential [26]. It may be an especially good target for immunotherapy because it is also expressed by endothelial cells, so that downregulation of this protein might lead to a double disadvantage for the tumour cell. Thus, locally stimulated CD4+ T cells might contribute to inhibition of angiogenesis as well as having direct effects on the tumour or on CD8+ cells.

Previous studies had indicated that the annexin II (208–223) peptide might well be immunogenic, despite representing a normal non-mutated sequence. However, we demonstrated this by using xenogeneic CHO cells as APC, which by themselves had a very strong stimulating (adjuvant) effect [10]. It was thus not clear whether "normal" APC would present this self peptide. We therefore tested whether the annexin II (208–223) peptide was indeed immunogenic when presented to autologous T cells by peptide-pulsed DC, the type of pre-clinical system appropriate to screening immunotherapy procedures. The results clearly show that this peptide, despite its derivation from a normal self protein, is highly immunogenic, and, moreover, that annexin II peptide-stimulated CD4 type 1 T helper cells are able to recognize melanoma cells over-expressing annexin II. Thus, this peptide may be a candidate to join that group of class II-restricted antigens which may be useful in immunotherapy as a result of their over-expression by tumour cells [5].

## Materials and methods

### Tumour cell lines

FM3 was established from a 78-year-old woman and was a kind gift of J. Zeuthen, Copenhagen, Denmark. This tumour cell line is DRB1*0401 and DRB1*1501 positive. The other cell lines were collected from different laboratories as part of an effort to establish a cell bank of MHC class II-typed and antigenically well-characterized melanoma lines (supported by the EU project EUCAPS, see www.medizin.uni-tuebingen.de/eucaps/ for details): MZ3 (DRB1*11011, DRB1*1501, DRB3*02x, DRB5*0101, DQB1*0301, DQB1*0602) and MZ13 (DRB1*0401, DRB1*11041, DRB3*02x, DRB4*01x, DQB1*0301, DQB1*0301) were kind gifts of E. Jäger, Frankfurt, Germany; Mel 624, Mel 1359, Mel 1102, Mel 1479 (all DRB1*0401) were kind gifts of J. Wunderlich, Bethesda, USA; SkMel 28 (DRB1*0401) is an ATCC cell line; M18 (DRB1*04x, DQB1*0301, DQB1*0302) and M18/B7 (transfected with a gene encoding CD80) were kind gifts of F. Jotereau, Nantes, France; and UKRV Mel 17 (DRB1*03011, DRB1*0401, DRB3*0101, DRB4*01x, DQB1*02x, DQB1*0302) was a kind gift of D. Schadendorf, Mannheim, Germany. Melanoma cell lines were cultured in 10% FCS in RPMI 1640 (or 10% FCS in DMEM for cell lines MZ3 and MZ13) at 5% $CO_2$ and 37 °C. All cell lines were checked by FACS analysis for expression of MHC class II. In M18, M18/B7 and in Mel 624, MHC class II had to be induced by incubation with IFN-$\gamma$ (100 U/ml) for 2 days; the other lines expressed MHC class II constitutively in culture.

### Synthesis of annexin II

Peptide synthesis was performed by automated solid-phase techniques using Fmoc chemistry by Dr. Eschner, Department of Physiological Chemistry, University of Tübingen. Purity exceeded 98%.

### B-cell lines

The EBV-transformed B-cell line FB3 was established from the same patient as FM3 and was also a kind gift of J. Zeuthen, Copenhagen, Denmark. MZ3-EBV and MZ13-EBV were established from the same patients as MZ3 and MZ13 and were from E. Jäger, Frankfurt, Germany. B-cell lines were cultured in 10% FCS in RPMI 1640 at 5% $CO_2$ and 37 °C.

### Peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) from healthy HLA-DRB1*0401-homozygous donors were isolated from fresh buffy coat obtained from the blood bank. Briefly, buffy coat was diluted 1:2 with RPMI 1640 and then centrifuged for 25 min at 1320 *g* on a Ficoll-Hypaque gradient. The interface layer was collected and washed three times in RPMI 1640 (5 min, 680 *g*; 5 min, 300 *g*; 5 min, 540 *g*). Cells were counted and either frozen or immediately used.

### Generation of DC

Monocyte-derived DC were generated from PBMC from healthy donors using a slightly modified standard protocol [19]. Briefly, PBMC were plated in X-Vivo 15 (BioWhittaker) in a six-well plate and incubated for 2 h at 37 °C. The non-adherent cells were washed off, and the adherent cells were cultured in X-Vivo 15 with addition of 500 U/ml IL4 and 800 U/ml GM-CSF. On day 6 cells showed an immature DC phenotype [CD83–, CD14–, CD80–, CD86 low or – and MHC II low (data not shown)]. To generate mature DC, cells were cultured for another 2 days with 500 U/ml IL4 and 800 U/ml GM-CSF and 300 µl MCM (monocyte conditioned medium, previously titrated to determine optimal concentration). Mature DC were CD83+ , CD14–, CD80+ , CD86+ and MHC class II-positive (data not shown). Peptide [10 µg/ml annexin II (208–223)] was added together with this maturation agent.

### Production of MCM

Monocyte conditioned medium has been described as a very effective factor for DC maturation [16, 17]. Briefly, PBMC from five healthy donors were cultured for 2 h, non-adherent cells were removed and fresh medium containing SACS (Pansorbin cells, Calbiochem) (1:10000) was added to the adherent monocytes. Cells were incubated for 24 h at 37 °C, the supernatant harvested, pooled, filtered and frozen in appropriate aliquots at −20 °C.

### Induction of peptide-specific T cells

$1 \times 10^5$ Annexin II-pulsed DC and $1 \times 10^6$ autologous PBMC per well were cultured in 2 ml X-Vivo 15 with an additional 10 µg/ml peptide in a 24-well plate at 37 °C for 14 days. On day 7 and from then on every 2 days, 1 ml medium was replaced by 1 ml fresh medium, containing 20% TCGF (Lymphocult-T, Biotest, Germany). On day 14, the T cells were counted, plated at a density of $2 \times 10^5$ cells per well and restimulated with $4 \times 10^5$ autologous

irradiated PBMC plus 10 μg/ml peptide in 2 ml X-Vivo 15 in a 24-well-plate. TCGF (10%) or IL 2 (20 U/ml) was added on day 2 or 3 and from then on every 2 days. After 14 days of culture (day 28), some of the T cells were tested for specific proliferation in a [$^3$H]thymidine incorporation assay, and the remainder kept in culture (restimulated as above) and/or frozen and stored in liquid nitrogen.

### Proliferation assay

Proliferation of T cells was determined by a standard [$^3$H]thymidine incorporation assay. Briefly, T cells were plated in triplicate at $2 \times 10^4$ cells/well in a 96-well plate (round bottom). Stimulators were either irradiated (30 Gy) autologous PBMC ($5 \times 10^4$/well) with or without peptide, fixed melanoma cells ($2.5 \times 10^4$/well) or fixed EBV-B cells ($2.5 \times 10^4$/well). Fixation of stimulator cells was done for 2 min with 0.025% glutaraldehyde. Blocking of HLA-DR/TCR interactions was achieved by addition of HLA-DR mAb L243 (ATCC) (hybridoma cell culture supernatant; one fourth of total volume) to the culture. Control antibodies were against HLA-DP (B7/21; kind gift of Dr. I. Trowbridge, San Diego) and -DQ (Tü 22; Tübingen local mAb). After incubation for 24, 48 or 72 h, 37 kBq [$^3$H]thymidine was added to each well and incubation continued for another 18 h. Cells were harvested and measured in a Betaplate. In all assays, proliferation of fixed or irradiated stimulator cells alone was determined and no thymidine incorporation could be detected. Results are shown as mean of triplicates with standard deviation.

### Enzyme-linked immunosorbent assay

Cytokines produced by the T cells were assayed by sandwich enzyme-linked immunosorbent assays (ELISAs). Supernatants from a 48 h coculture of T cells with different stimulators were collected and stored at −20 °C (short term) or −70 °C (long term). Antibody pairs used were mouse anti-human GM-CSF, TNF-α, IFN-γ, IL4 and IL10 as capture antibodies and biotinylated rat anti-human GM-CSF, TNF-α, IFN-γ, IL4 and IL10 as detection antibodies (all from Pharmingen, Hamburg, Germany), and ELISA was carried out according to the manufacturer's instructions.

### Immunoblot (western blot)

Annexin II protein levels in different cell lines were assessed by immunoblotting. Cell lines were cultured as usual, harvested, washed and lysed at a concentration of $1 \times 10^6$/ml in electrophoresis sample buffer. Samples of 25 μl of these cell lysates were analyzed. The samples were separated by SDS-PAGE and blotted on nitrocellulose membranes electrophoretically. The membranes were washed, blocked and incubated with anti-annexin II mouse IgG1 Abs (Transduction Laboratories, Pharmingen, Hamburg, Germany) and AP-conjugated secondary antibody according to the manufacturer's protocol. Sigma Fast BCIP/NBT tablets were used as substrate according to the manufacturer's instructions, and bands (36 kDa) could be seen after 5–10 min. Cell lysate from the MCDK cell, delivered together with the annexin II antibody from Transduction Laboratories, served as the manufacturer's pre-screened positive control. For the quantification of the bands, the immunoblot membrane was scanned with a Sharp JX-325 scanner and the intensity of bands was calculated with the Image Master software (Amersham/Pharmacia Biotech) and shown as ODI (optical density integrated).

## Results

### Annexin II (208–223) presented by DC can sensitize autologous T cells for peptide-specific autocrine proliferation

Naive T cells were cocultured with peptide-pulsed autologous DC. After 2 weeks (with addition of TCGF after 7 days), restimulation was effected using PBMC and peptide, resulting in the generation of a T-cell population designated T13A. T13A cells were first screened for peptide reactivity in a test for specific autocrine proliferation at 66 h in the presence of annexin II (208–223) peptide at two different concentrations, using PBMC as the APC (Fig. 1). Although the level of thymidine incorporation in this experiment was rather low overall, T13A cells did show autocrine proliferation in the presence of the annexin II peptide. In contrast, there was no proliferative response when a control peptide gp100 (44–59), also eluted from FM3 and known to be immunogenic when presented on DRB1*0401 [7], was added. As measured by FACS analysis, the T cell line T13A was 100% CD4+; no CD8+ or CD56+ cells could be detected (data not shown).

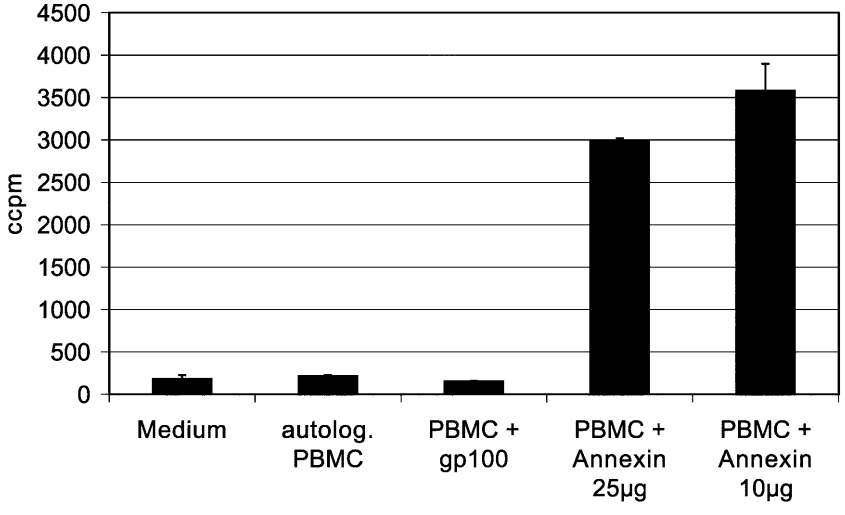


**Fig. 1** [$^3$H]Thymidine incorporation of T-cell line T13A after stimulation with autologous irradiated PBMC alone or with addition of 10 μg/ml gp100 (44–59) or annexin II (208–223) at different concentrations (25 and 10 μg/ml). Shown are mean values of triplicates ± standard deviation, measured after 66 h

DC-sensitized annexin II-specific T cells show autocrine proliferation in the presence of the native tumour cell line FM3, but not in the presence of B-cell line FB3 from the same patient

Because the peptide annexin II (208–223) was originally eluted from the melanoma cell line FM3, it was of interest to establish whether the annexin II peptide-specific T-cell line T13A was able to recognize the native tumour itself. To this end, the ability of fixed FM3 cells to stimulate T13A cells was compared with that of an EBV-transformed B-cell line from the same patient (FB3). Figure 2 shows that FM3 but not FB3 cells strongly stimulated T13A cells. The figure also shows that preincubating FM3 cells in a saturating concentration of the HLA-DR-specific mAb L243 completely blocked the response of T13A cells to FM3 cells (shown as FM3/L243), whereas anti HLA-DP and -DQ antibodies failed to do so (latter data shown in Fig. 5).

Proliferation of T13A cells in the presence of other melanoma cell lines and B-cell lines

To investigate whether the annexin II peptide might represent a common antigen shared between different melanoma cell lines, the proliferation of T13A cells in the presence of various melanoma cell lines and B-cell lines as controls was tested. Melanoma lines matched for at least one DRB1*0401 allele, or mismatched for DR, as well as the DRB1*0401-positive B-cell line FB3 were used as stimulators (Fig. 3). Three out of 5 melanoma cell lines sharing the DRB1*0401 allele were able to stimulate T13A. The other two DR-matched melanoma cell lines (Mel 1479 and SkMel 28) were not recognized. Additionally, the two melanoma cell lines that did not share an HLA-DR type (M18 and MZ3) also could not induce autocrine proliferation of T13A. These data suggest that in vitro peptide-sensitized T cells are able to recognize allogeneic melanoma cells only when they express the relevant DR allele and are annexin II-

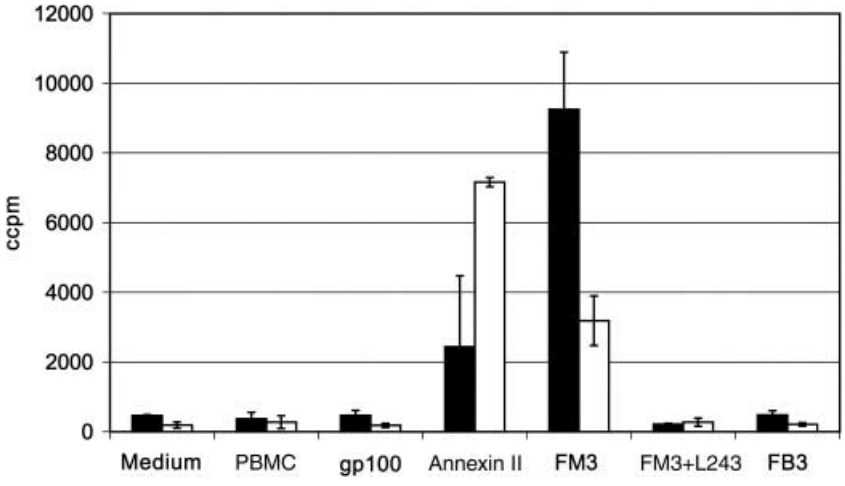


**Fig. 2** [$^3$H]Thymidine incorporation of T-cell line T13A after stimulation with autologous irradiated PBMC alone or with addition of 10 μg/ml gp100 (44–59) or 5 μg/ml annexin II (208–223); melanoma cell line FM3, with and without anti-HLA-DR mAb L243; and B-cell line FB3. Shown are mean values of triplicates ± standard deviation, measured after 42 h (*filled bars*) and 66 h (*open bars*)

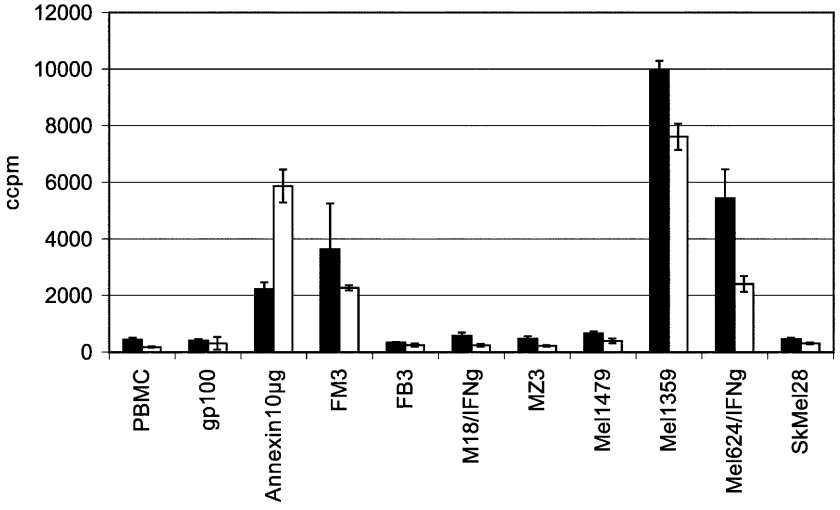


**Fig. 3** [$^3$H]Thymidine incorporation of T-cell line T13A after stimulation with autologous irradiated PBMC alone or with addition of 10 μg/ml gp100 (44–59) or 10 μg/ml annexin II (208–223), with different melanoma cell lines either sharing the HLA-DRB1*0401 allele (FM3, Mel 1479, Mel 1359, Mel 624 and SkMel 28) or not DR-matched (MZ3, M18). The B-cell lines FB3 M18 and Mel 624 were preincubated with IFN-$\gamma$ to induce expression of MHC class II. Shown are mean values of triplicates ± standard deviation, measured after 42 h

positive, and further show that annexin II is commonly processed and expressed as the relevant peptide by the majority of melanoma cells. However, not all DR-matched melanomas were capable of stimulating these T cells.

## Annexin II protein expression in different cell lines and correlation of high-level expression with ability to stimulate T13A cells

To investigate whether the response of T13A to different stimulator cells was associated with their annexin II expression levels, the latter were tested in immunoblotting. Because we used a fixed number of stimulator cells per well in the proliferation assay, the amount of annexin II in lysates from the same number of cells was compared, rather than in the same amount of whole cell protein. Annexin II was detected in all of the various melanoma cell lines (Fig. 4). However, the level of expression differed between the lines, and was strongest in FM3, MZ3, MZ13, Mel 1102, Mel 1359 and UKRV Mel 17. In the remaining melanoma cell lines, a weaker but still clear expression of annexin II was detected.

**Fig. 4** Expression of annexin II protein in different cell lines as assessed by Western blot analysis with anti-annexin II mAb. FM3 (*1*); FM3.13 (*2*); MZ3 (*3*); MZ13 (*4*); M18 (*5*); M18/B7 (*7*); Mel 624 (*8*); Mel 1102 (*9*); Mel 1359 (*11*); Mel 1479 (*12*); SkMel 28 (*13*); UKRV Mel 17 (*15*); FB3 (*16*); MZ3-EBV (*17*); MZ13-EBV (*18*); PHA-stimulated T-cell lymphoblasts (*19*). Molecular weight markers are in lanes *6* and *14*, positive controls in lanes *10* and *20*

In proliferation assays, T13A cells were stimulated by all melanoma lines which were both HLA-DRB1*0401 positive and showed strong expression of annexin II (Figs. 3 and 5, and summarized in Table 1). In contrast, the HLA-DRB1*0401-positive melanoma lines which expressed lower levels of annexin II in Western blotting (Mel 1479 and SkMel 28), failed to stimulate T13A cells (Fig. 3). The two lines not expressing HLA-DRB1*0401, MZ3 and M18, also failed to stimulate, despite high annexin II expression in one of them (Fig. 3). B cells, which completely lacked annexin II, also failed to stimulate T13A cells, whether or not they were HLA-DRB1*0401 positive. Stimulation by B cells was blocked by anti-DR antibody at the same level as FM3 stimulation, indicating essentially complete restriction of T13A responses by HLA-DR rather than -DQ or-DP (Fig. 5). Consistent with this, anti-DQ and -DP antibodies almost completely failed to block FM3-stimulation (Fig. 5).

## GM-CSF release by T13A cells in the presence of different stimulators

Release of GM-CSF by T13A cells in the presence of different stimulators was measured at the same time as the proliferation assay in certain experiments. For this purpose, supernatant was collected from the plates in which the thymidine incorporation was carried out, just before the thymidine was added. This was done after 48 h of coculture. Comparable to the results of the proliferation assay, GM-CSF release could only be

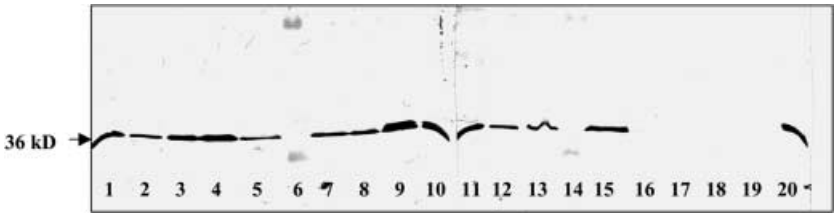


**Fig. 5** [$^3$H]Thymidine incorporation of T-cell line T13A after stimulation with HLA-DRB1*0401-positive melanoma cell lines (FM3 alone or with antibodies anti-DR, anti-DP and anti-DQ), MZ13, Mel 1102, UKRV Mel 17 or B-cell lines FB3 and MZ13-EBV. Mean values of triplicates ± standard deviation, measured after 42 h, are shown

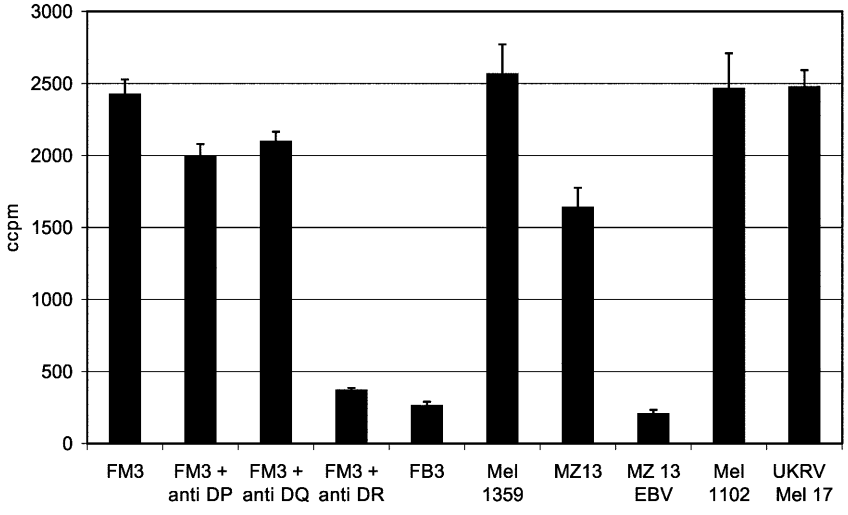

**Table 1** Expression of annexin II in and proliferative response of T13A to various melanoma cell lines

| | FM3 | MZ13 | Mel 1102 | Mel 1359 | UKRV Mel 17 | Mel 624 | Mel 1479 | M18 | SkMel 28 | MZ3 |
|---|---|---|---|---|---|---|---|---|---|---|
| ODI (×1000) | 223 | 462 | 343 | 433 | 265 | 184 | 120 | 78 | 115 | 349 |
| Proliferative response | + | + | + | + | + | + | – | – | – | – |

Summary of level of annexin II expression in and proliferative response of T-cell line T13A to various DRB1*0401-positive melanoma cell lines (except MZ3, which is DR4 negative, and M18, which is DR4 positive but not DRB1*0401 positive). Expression of annexin II is shown as the ODI (optical density integrated) value ×1000, measured from the western blot shown in Fig. 4. This figure depicts a representative experiment performed two to four times on each cell line with essentially identical results, but not including all lines on the same gel (data not shown). For densitometric measurements only bands run at the same time on the same gel were assessed

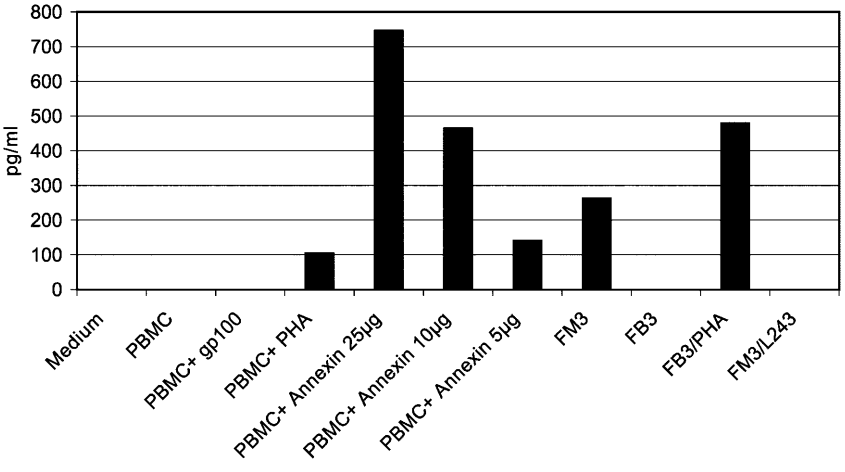


**Fig. 6** GM-CSF release of T-cell line T13A after 48 h stimulation with autologous irradiated PBMC alone or with addition of PHA (1%), 10 µg/ml gp100 (44–59) or different concentrations of annexin II (208–223) (25, 10 and 5 µg/ml); with melanoma cell line FM3 with and without anti-HLA-DR mAb L243; and B-cell line FB3 with and without PHA. Shown are mean values of duplicates

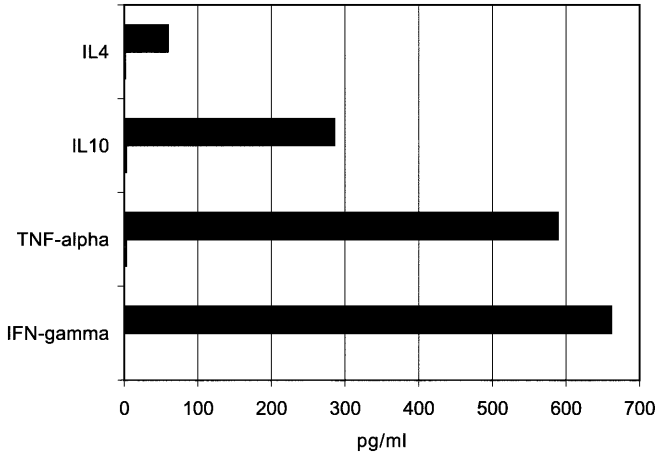


**Fig. 7** Release of IL 4, IL 10, TNF-$\alpha$ and IFN-$\gamma$ of T-cell line T13A after 48 h stimulation with autologous PBMC alone (*open bars*) or with addition of 10 µg/ml annexin II (208–223) (*filled bars*)

detected when annexin II (208–223) was added to the culture, not if gp100 (44–59) was added or with PBMC alone (Fig. 6). Furthermore, GM-CSF release could also be shown in the presence of melanoma cell line FM3, but not with the B-cell line FB3. GM-CSF release by T13A cells in the presence of FM3 could also be blocked with the anti-DR mAb L243. These results suggest a specific GM-CSF release only in the presence of annexin II (208–223), either added to the coculture of PBMC and T13A or naturally presented on FM3, in a DR-restricted manner.

### Cytokine release pattern of T13A cells after specific stimulation

To determine whether T13A shows a $T_H1$, $T_H2$ or other profile, cytokine release of T13A cells in the presence of PBMC alone or with specific peptide was measured. T cells were cocultured with the stimulator cells in 24-well plates and the supernatant was collected at 48 h. The same supernatant was used for all four different cytokines, IL4, IL10, IFN-$\gamma$ and TNF-$\alpha$, tested by ELISA. Large amounts of the $T_H1$ cytokines TNF-$\alpha$ and IFN-$\gamma$ were produced in the presence but not in the absence of specific antigen (Fig. 7). IL10 was also produced, but in smaller amounts, and even smaller amounts of the $T_H2$ cytokine IL4 could be detected. Taking into account the strong autocrine proliferation of these cells (implying IL2 secretion), together with marked production of IFN-$\gamma$, but very little IL4, it is concluded that these annexin II peptide-reactive T cells are predominantly of a $T_H1$ phenotype.

## Discussion

It is now well established that tumours can be rejected by the immune system. HLA class I-restricted CD8+ CTL interact with those tumour cells retaining expression of class I molecules and are directly lytic. In the majority of reported studies, the activity of such CD8 cells is found to be enhanced, or made possible in the first place, by the action of HLA class II-restricted CD4+ T cells,

even when the tumour cells themselves do not express class II [8, 12, 13, 14]. Moreover, in the local tumour environment, CD4 cells may themselves be directly cytotoxic to class II-positive tumours, as well as influencing tumour growth by means of the cytokines that they secrete (either directly or via effects on angiogenesis). Thus far, however, most tumour antigens that have been identified are MHC class I restricted. Additionally, many of these are specific for one kind of tumour, such as the differentiation antigens identified on melanoma. To establish effective tumour biotherapies and tumour vaccines, it may be critical to identify tumour antigens shared by different kinds of tumours, and to extend those which stimulate CD4 cells.

Annexin II is a protein tyrosine kinase substrate that is expressed by normal cells, particularly endothelial cells [6] and over-expressed in various kinds of tumours [11, 18, 22]. The level of cell surface expression in tumour cell lines has been correlated with their metastatic potential [25]. There is also some evidence that annexin II is involved in tumour metastasis due to its tenascin-C-binding capacity [20] and other properties. Moreover, as a protein tyrosine kinase substrate which may be involved in the neoplastic process, annexin II function may be required for tumorigenesis. Thus, specific down-regulation of annexin II can inhibit proliferation of human tumour cells [4]. Therefore, it may represent a target for immunotherapy which the tumour cannot down-regulate to escape immune responses without losing tumorigenicity. In the present communication, to the best of our knowledge, we have shown for the first time that annexin II is variably but mostly strongly expressed in melanoma cell lines and, as expected, is absent in B cells from the same patients. By analogy with tumour antigens such as her2/neu, normal self proteins involved in the tumorigenic process by virtue of their over-expression may represent targets for immunotherapy [5]. Therefore, annexin II (208–223) may be utilizable in this context. We previously obtained some evidence for immunogenicity of this peptide using strongly stimulatory, artificial xenogeneic APC, which generated a very high background response. We now document in a human autologous system that T cells can be sensitized to this peptide presented by autologous DC. In this model, which is appropriate for immunotherapy, the responses of the sensitized T cells are correlated with the level of expression of annexin II by melanoma cells; this provides evidence that only tumour cells over-expressing annexin II are able to stimulate T cells, suggesting that the lower levels expressed by some normal cells would also not be recognized.

Repeated stimulation of T cells with antigen resulted in the generation of a peptide-specific T-cell line, T13A, which proliferated in an autocrine fashion in the presence of annexin II-positive HLA-DRB1*0401-bearing tumour cell lines FM3 and MZ13, but not to annexin II-negative cell lines established from the same patients. This implies peptide specificity of T-cell line T13A. T-cell recognition of tumour was HLA-DR restricted: it could be blocked with anti-DR antibodies, but not anti-DQ or -DP mAb, and HLA-DR-mismatched annexin II-positive melanoma cells failed to stimulate. The recognition of various DR-matched but not mismatched melanoma cell lines also shows that the protein annexin II is processed and presented in an efficient manner not only by the cell line from which it was originally eluted but generally by melanoma lines. Moreover, the peptide 208–223 represents a shared antigen common to these cell lines. There was a good correlation between the levels of annexin II protein assayed in melanoma cell lysates by western blotting and the cells' ability to stimulate the T13A line: only high-level expressors could stimulate. This implies that normal cells, which express annexin II at a lower level [6], will probably not be able to trigger proliferation of such peptide-specific T cells even when the latter have been presensitized by the most potent APC. Taken together, the data so far suggest that the annexin II (208–223) peptide, originally eluted as a dominant endogenous peptide from HLA-DR molecules of a melanoma cell line, is processed and presented by the majority of melanomas, and therefore potentially represents a shared tumour antigen able to stimulate good CD4 T cell responses of a predominantly $T_H1$ type. Because the annexin II (208–223) peptide binds a wide range of different HLA-DR alleles [7] and is known to be over-expressed in several different types of tumours, it may constitute a general tumour antigen which could be applied to many different patients without regard to HLA typing or tumour histology. However, it is clear that further investigations on any potential anti-annexin II-triggered autoimmunity remain to be performed before considering clinical trials with this peptide.

**Acknowledgements** This work was supported by the Deutsche Forschungsgemeinschaft (DFG Pa 361/5-1, 5-2); the Mildred Scheel Foundation (10-1173-Pa3); and the University of Tübingen Medical Faculty (fortüne grant no. 138) under the aegis of the European Commission project "EUCAPS" (Biomed-II-Program, CT98-3058). We thank Dr. Eschner (Tübingen) for peptide synthesis, Dr. Deeg (Tübingen) for peptide analysis, and Drs. Jäger (Frankfurt) and Wunderlich (Bethesda) and Profs. Jotereau (Nantes), Schadendorf (Mannheim) and Zeuthen (Copenhagen), for melanoma cell lines.

## References

1. Armstrong TD, Clements VK, Ostrand-Rosenberg S (1998) MHC class II-transfected tumor cells directly present antigen to tumor-specific CD4+ T lymphocytes. J Immunol 160: 661
2. Bennett SR, Carbone FR, Karamalis F, Flavell RA, Miller JF, Heath WR (1998) Help for cytotoxic-T-cell responses is mediated by CD40 signalling. Nature 393: 478
3. Chaux P, Vantomme V, Stroobant V, Thielemans K, Corthals J, Luiten R, Eggermont AM, Boon T, van der Bruggen P (1999) Identification of MAGE-3 epitopes presented by HLA-DR molecules to CD4+ T lymphocytes. J Exp Med 189: 767
4. Chiang Y, Rizzino A, Sibenaller ZA, Wold MS, Vishwanatha JK (1999) Specific down-regulation of annexin II expression in human cells interferes with cell proliferation. Mol Cell Biochem 199: 139

5. Disis ML, Cheever MA (1998) HER-2/neu oncogenic protein: issues in vaccine development. Crit Rev Immunol 18: 37
6. Dreier R, Schmid KW, Gerke V, Riehemann K (1998) Differential expression of annexins I, II and IV in human tissues: an immunohistochemical study. Histochem Cell Biol 110: 137
7. Halder T, Pawelec G, Kirkin AF, Zeuthen J, Meyer HE, Li K, Kalbacher H (1997) Isolation of novel HLA-DR restricted potential tumor-associated antigens from the melanoma cell line FM3. Cancer Res 57: 3238
8. Hung K, Hayashi R, Lafond-Walker A, Lowenstein C, Pardoll D, Levitsky H (1998) The central role of CD4+ T cells in the antitumor immune response J Exp Med 188: 2357
9. Kobayashi H, Kokubo T, Sato K, Kimura S, Asano K, Takahashi H, Iizuka H, Miyokawa N, Katagiri M (1998) CD4+ cells from peripheral blood of a melanoma patient recognize peptides derived from nonmutated tyrosinase. Cancer Res 58: 296
10. Li K, Adibzadeh M, Halder T, Kalbacher H, Heinzel S, Muller C, Zeuthen J, Pawelec G (1998) Tumour-specific MHC-class-II-restricted responses after in vitro sensitization to synthetic peptides corresponding to gp100 and annexin II eluted from melanoma cells. Cancer Immunol Immunother, 47: 32
11. Nygaard SJ, Haugland EK, Kristoffersen EK, Lund-Johansen M, Laerum OD, Tysnes OB (1998) Expression of annexin II in glioma cell lines and in brain tumor biopsies. J Neurooncol 38: 11
12. Ossendorp F, Mengede E, Camps M, Filius R, Melief CJ (1998) Specific T helper cell requirements for optimal induction of cytotoxic T lymphocytes against major histocompatibility complex class II negative tumors. J Exp Med 187: 693
13. Overwijk WW, Lee DS, Surman DR, Irvine KR, Touloukian CE, Chan CC, Carroll MW, Moss B, Rosenberg SA, Restifo N (1999) Vaccination with a recombinant vaccinia virus encoding a "self" antigen induces autoimmune vitiligo and tumor cell destruction in mice: requirement for CD4+ T lymphocytes. Proc Natl Acad Sci USA 96: 2982
14. Pardoll DM, Topalian SL (1998) The role of CD4+ T cell responses in antitumor immunity. Curr Opin Immunol 10: 588
15. Pieper R, Christian RE, Gonzales MI, Nishimura MI, Gupta G, Settlage RE, Shabanowitz J, Rosenberg SA, Hunt DF, Topalian SL (1999) Biochemical identification of a mutated human melanoma antigen recognized by CD4+ T cells. J Exp Med 189: 757
16. Reddy A, Sapp M, Feldman M, Subklewe M, Bhardwaj N (1997) A monocyte conditioned medium is more effective than defined cytokines in mediating the terminal maturation of human dendritic cells. Blood 90: 3640
17. Romani N, Reider D, Heuer M, Ebner S, Kampgen E, Eibl B, Niederwieser D, Schuler G (1996) Generation of mature dendritic cells from human blood. An improved method with special regard to clinical applicability. J Immunol Methods 196: 137
18. Roseman BJ, Bollen A, Hsu J, Lamborn K, Israel MA (1994) Annexin II marks astrocytic brain tumors of high histologic grade. Oncol Res 6: 561
19. Sallusto F, Lanzavecchia A (1994) Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. J Exp Med 179: 1109
20. Schoenberger SP, Toes RE, van der Voort EI, Offringa R, Melief CJ (1998) T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. Nature 393: 480
21. Siever DA, Erickson HP (1997) Extracellular annexin II. Int J Biochem Cell Biol 29: 1219
22. Topalian SL, Gonzales MI, Parkhurst M, Li YF, Southwood S, Sette A, Rosenberg SA, Robbins PF (1996) Melanoma-specific CD4+ T cells recognize nonmutated HLA-DR-restricted tyrosinase epitopes. J Exp Med 183: 1965
23. Vishwanatha JK, Chiang Y, Kumble KD, Hollingsworth MA, Pour PM (1993) Enhanced expression of annexin II in human pancreatic carcinoma cells and primary pancreatic cancers. Carcinogenesis 14: 2575
24. Wang RF, Wang X, Rosenberg SA (1999) Identification of a novel major histocompatibility complex class II-restricted tumor antigen resulting from a chromosomal rearrangement recognized by CD4+ T cells. J Exp Med 189: 1659
25. Wang RF, Wang X, Atwood AC, Topalian S, Rosenberg SA (1999) Cloning genes encoding MHC class II-restricted antigens: mutated CDC27 as a tumor antigen. Science 284: 1351
26. Yeatman TJ, Updyke TV, Kaetzel MA, Dedman JR, Nicolson GL (1993) Expression of annexins on the surface of non-metastatic and metastatic human and rodent tumor cells. Clin Exp Metastasis 11: 37
27. Yee C, Gilbert MJ, Riddell SR, Brichard VG, Fefer A, Thompson JA, Boon T, Greenberg PD (1996) Isolation of tyrosinase-specific CD8+ and CD4+ T cell clones from the peripheral blood of melanoma patients following in vitro stimulation with recombinant vaccinia virus. J Immunol 157: 4079