Incomplete tolerance to the tumour-associated antigen MDM2

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Abstract

MDM2 is a tumour-associated antigen widely expressed by normal tissues and over-expressed by many tumours of different origin. We wanted to define the level of immunological tolerance against MDM2 and explore its potential in tumour immunotherapy. Two murine MDM2 epitopes, pMDM100 and pMDM441, differ in their affinity for MHC class I molecules. Previous observations made in vitro suggested that pMDM100, due to its high affinity for Kb, induces a high level of tolerance, whereas tolerance to pMDM441, which binds poorly to Db, is incomplete. In the present article we test the immunogenicity of these two peptides in vivo. Surprisingly, mice immunized with pMDM100 generated cytotoxic T lymphocytes (CTL) that killed tumour cell lines expressing MDM2 endogenously, indicating the existence of high-avidity CTL specific for a widely expressed protein. However, the response was limited as effector CTL disappeared after continued in vitro stimulation. While immunization with the individual MDM2 peptides did not protect against tumour challenge, mice immunized with both pMDM100 and pMDM441 were partially protected. These observations suggest that targeting of multiple epitopes may be required to vaccinate against tumours expressing elevated levels of CTL-recognized self-proteins.

Introduction

The MDM2 protein is a tumour-associated antigen over-expressed in tumours of different origin (1,2). MDM2 is an antagonist of p53 (3), hence its function is essential for most cells of the body. Although it is expected that the ubiquitous expression of MDM2 induces immunological tolerance, there is evidence that the normal T cell repertoire contains autoreactive T cells. For example, autoreactive T cells can be found in transgenic mice expressing defined TCR and their specific antigens in the periphery (4,5). In addition, most human cytotoxic T lymphocyte (CTL) specific for tumour antigens recognize unaltered self-proteins (6–8). In some of these cases the level of expression of self-antigens in normal cells may be below the threshold of recognition by T cells. Over-expression of such self-antigens in tumour cells will make these cells the potential target of autoreactive CTL and there is evidence to support this view (9–11).

In a previous report from our group, two mouse MDM2 peptides able to induce CTL in vitro were characterized (12). Peptides pMDM100 and pMDM441 differ in their binding affinity to mouse MHC class I molecules. Both peptides bind to different MHC molecules, but with different affinity—pMDM100 binds with high affinity to Kb and pMDM441 binds with low affinity to Db. It was possible to raise high-avidity pMDM441-specific CTL in vitro and these cells were able to kill tumour cell lines expressing MDM2. However, several cycles of peptide stimulation were required to generate these CTL, suggesting a low precursor frequency. In contrast, stimulation with the high-affinity pMDM100 peptide generated low-avidity CTL that did not recognize cells expressing MDM2 endogenously. The proposed hypothesis was that peptide affinity for MHC class I determines the level of tolerance, since the high affinity of pMDM100 may result in a high level of presentation in thymus and normal tissues, thus purging high-avidity pMDM100-specific CTL from the repertoire. Interestingly, it is possible to obtain high-avidity allo-MHC-restricted CTL specific for pMDM100 (13). These CTL kill tumour cell lines expressing MDM2, demonstrating that the pMDM100 epitope is naturally presented. These observations support the notion that, contrary to allo-MHC-restricted CTL, self-MHC-restricted high-avidity pMDM100-specific CTL are tolerized and then undetectable in vitro.

To date, only in vitro studies have been performed to analyse the immune response against MDM2 (12,13). In the present study we extend these observations and analyse the
level of tolerance against the two MDM2 peptides by using in vivo immunization. Peptide immunization with incomplete Freund’s adjuvant (IFA) elicited a weak pMDM441-specific CTL response, in agreement with the low affinity of the peptide to MHC class I. In contrast, it was possible to raise a strong pMDM100-specific CTL, although the response was variable between individual mice. Some mice generated high-avidity pMDM100-specific CTL capable of killing tumour cell lines expressing MDM2 endogenously. Labelling with pMDM100-Kb tetramer confirmed the presence of pMDM100-specific CD8+ T cells, although the staining level was not able to discriminate between high- and low-avidity CTL. Attempts were made to obtain high avidity pMDM100-specific CTL lines by re-stimulation in vitro. However, after the second round of stimulation low-avidity CTL dominated the culture and high-avidity CTL were undetectable. Although this could be a consequence of the culture conditions, it is more likely that a physiological mechanism limits the expansion and/or effector function of autoreactive MDM2-specific CTL.

Methods

Animals

C57BL/6 (B6) mice (KbDb) from Harlan (Bicester, UK) were used. Female mice, at 8–16 weeks of age, were used for immunization and as a source of accessory cells for in vitro CTL stimulation.

Peptides

Peptides pMDM100 (YAMIYRNL) and pMDM441 (GRPKNGCIV) were synthesized by the Peptide Synthesis Laboratory of Imperial College, Faculty of Medicine. The quality of the peptide was assessed by HPLC analysis and the mol. wt confirmed by mass spectrometry. All peptides were dissolved in PBS at 2 mM concentration and kept at -20°C. DTT (Fisher Scientific, Loughborough, UK) was added when required at 2 mM final concentration.

Cell lines

RMA cells (KbDb) and their derivative RMA-S cells (14) were cultured in RPMI/10% FCS (Labtech, Ringmer, UK).

Immunization protocols

Mice were immunized s.c. in the base of the tail with 0.2 ml of peptide emulsified 1:1 with incomplete Freund’s adjuvant (IFA) (Sigma-Aldrich, Poole, UK). In most experiments, 25 mg/mL peptide was injected per mouse; in some experiments, a range of peptide quantities was employed.

In vitro CTL stimulation and generation of CTL lines

Spleen cells from immunized mice were stimulated in vitro at 3 x 10^6 cells/ml with syngeneic spleen cell blasts loaded with the specific peptide. Spleen cell blasts were prepared by stimulation of B6 spleen cells (1.25 x 10^6 cells/ml) with 25 mg/ml lipopolysaccharide (Sigma) and 7 mg/ml dextran sulphate (Sigma) (15). Three days after activation cells were irradiated (3000 rad) and incubated with peptide before mixing with spleen cells from immunized mice. In most experiments spleen cell blasts were loaded with 100 mM peptide; lower peptide concentrations were also used in some experiments. RMA cells were irradiated with 8000 rad when used as stimulator cells.

CTL lines were generated by culturing responder cells from immunized mice 7–14 days after the first in vitro stimulation (2.5 x 10^5/ml) in the first re-stimulation and 1 x 10^5/ml subsequently) with 5 x 10^4/ml irradiated RMA-S cells pulsed with 10 mM peptide. Irradiated naive spleen cells at 1 x 10^5/ml and 10 U/ml of mouse rIL-2 were also added.

CTL assay

CTL cultures were analysed in standard 51Cr-release assays 5 or 6 days after in vitro stimulation (12). When RMA-S cells were
used as target, they were temperature induced overnight before the assay. Assays were performed by mixing $5 \times 10^3$ $^{51}$Cr-labelled target cells with varying numbers of effector T cells and incubating at 37°C for 4 h. At the end of the incubation, 100 µl of the supernatant was analysed on a γ-counter. CTL were always tested against the specific peptide as well as an irrelevant control peptide. All assays were performed at least in duplicates. Specific cytotoxicity was calculated using the formula: 

\[
\text{Specific Cytotoxicity} = \frac{(\text{Experimental Release} - \text{Spontaneous Release})}{(\text{Maximum Release} - \text{Spontaneous Release})} \times 100%.
\]

Tetramer staining

Cells were labelled with anti-mouse CD8±allophycocyanin (BD Biosciences, Cowley, UK), anti-mouse CD3±FITC (BD Biosciences) and tetramer pMDM100-Kb±phycoerythrin (PE) (ProImmune Oxford, UK) in PBS containing 0.02% BSA and 10 mM azide at 4°C. Propidium iodide was added prior to analysis to discard dead cells. Labelled cells were analysed on a FACSCalibur (BD Biosciences).

Tumour challenge

Mice were immunized with 25 μg of pMDM441, pMDM100, a mixture of both pMDM441 and pMDM100 peptides or with the pSV9 control peptide. Between 10 and 14 days after immunization mice were injected i.p. with $10^3$ RMA cells. The weight of mice was recorded to monitor tumour growth. Mice that surpassed 25% weight increase were killed. The statistical significance of the results was analysed by the log-rank test.

Results

*pMDM100 peptide induces CTL after immunization with IFA*

B6 mice were immunized with pMDM441 or pMDM100 peptides emulsified in IFA. A highly immunogenic peptide from Sendai virus (pSV9 peptide, FAPGNYPAL) (16) was used as a positive control. Between 10 and 14 days after immunization, spleen cells were removed and activated with irradiated blast splenocytes loaded with the specific peptide. At 5 or 6 days after in vitro stimulation, cytotoxicity was determined in a standard $^{51}$C-release assay using peptide-loaded RMA-S cells as target. Figure 1 shows that immunization with pSV9 induced a high level of CTL activity (Fig. 1A), whereas the pMDM441-specific CTL response was very low (Fig. 1B). The pMDM100 peptide induced a specific response, but smaller than the pSV9 response (Fig. 1C).

Several experiments, with similar results to the one shown in Fig. 1, have confirmed that pMDM441 induces a low response. The response was always above the cytotoxicity against RMA-S loaded with an irrelevant peptide, suggesting the presence of a small number of pMDM441-specific CTL. This is supported by the observation that repeated in vitro stimulation of naive lymphocytes with pMDM441 peptide generated specific CTL (12). In contrast, the response of cells from mice immunized with pMDM100 was consistently stronger than the pMDM441 response, although generally smaller in intensity than the pSV9 response. It was also apparent, in the course of these experiments, that the pMDM100 response was highly variable among mice. Figure 2 shows the specific response of spleen cells from individual mice immunized with 25 μg of pMDM100 or pSV9 peptides and analysed in parallel. The specific killing was calculated as the cytotoxicity against target cells loaded with the specific peptide minus the cytotoxicity against an irrelevant peptide. All mice immunized with pSV9 generated a strong CTL response (six out of six). In contrast, although all mice immunized with pMDM100 peptide generated specific CTL, four out of seven mice were low responders (<25% specific killing), while the response from three of the animals was comparable to the response of mice immunized with pSV9 peptide.
pMDM100-specific CTL kill tumour cell lines

The pMDM100-specific CTL were tested against tumour cell lines expressing MDM2 endogenously. Spleen cells from B6 mice immunized with pMDM100 or pSV9 were stimulated in vitro once with blast splenocytes loaded with the specific peptide. Five days after in vitro stimulation, cultures were assayed for cytotoxicity versus peptide-loaded RMA-S cells (A) and versus RMA cells (B). Varying numbers of effector cells were used to give a final E:T ratio ranging from 100:1 to 0.8:1. Results are depicted as the percentage of specific cytotoxicity and are representative of three independent experiments.

Fig. 3. pMDM100-specific CTL kill tumour cell lines expressing MDM2 endogenously. Spleen cells from B6 mice immunized with pMDM100 or pSV9 were stimulated in vitro once with blast splenocytes loaded with the specific peptide. Five days after in vitro stimulation, cultures were assayed for cytotoxicity versus peptide-loaded RMA-S cells (A) and versus RMA cells (B). Varying numbers of effector cells were used to give a final E:T ratio ranging from 100:1 to 0.8:1. Results are depicted as the percentage of specific cytotoxicity and are representative of three independent experiments.

pMDM100-specific CTL were more efficient at killing pMDM100-loaded RMA-S cells than RMA. Cytotoxicity against RMA cells was more apparent in cell cultures from mice that were high responders to pMDM100-loaded RMA-S cells.

Low-avidity pMDM100-specific CTL predominate after in vitro re-stimulation

We tried to generate high-avidity pMDM100-specific T cell lines. We followed a standard procedure which is effective in generating and maintaining high-avidity pMDM100-specific...
allo-MHC-restricted CTL (13). Cultures were stimulated with pMDM100-loaded RMA-S cells and syngeneic B6 spleen cells as accessory cells. Figure 4 shows that it is possible to obtain pMDM100-specific CTL lines displaying a strong cytotoxic response against pMDM100-loaded RMA-S cells. However, cells from these cultures were not able to kill RMA cells, suggesting that low-avidity CTL predominated in these cultures. We have employed different stimulation protocols trying to select high-avidity CTL, including stimulation with low epitope density, as described by others (17,18). RMA cells or RMA-S loaded with a range of peptide concentrations (from 1 nM to 0.1 mM) were used to stimulate CTL. None of these modifications were effective at maintaining high-avidity pMDM100-specific CTL in vitro (data not shown). Two different outcomes were obtained in these experiments: a large peptide-specific response, but not reactivity against RMA tumour cells (Fig. 4), or a loss of the peptide-specific response. These results indicate that high-avidity CTL disappear or fail to display cytotoxicity after repeated in vitro stimulation.

**pMDM100-K b tetramer binding of pMDM100-specific primary cultures and pMDM100-specific T cell lines**

Cultures were labelled with pMDM100-K b tetramer after one round of in vitro stimulation. Figure 5 shows that spleen cell cultures from mice immunized with pMDM100 and stimulated in vitro with pMDM100 contained a large population of tetramer + cells, ~40% of the CD8 + cells in this particular culture. Figure 3 shows that these cells displayed a high level of cytotoxicity against peptide-loaded RMA-S and RMA cells, so they are identified as high-avidity CTL. Tetramer binding to spleen cells from mice immunized with pSV9 and stimulated in vitro with pSV9 was very low (Fig. 5). The CTL line 3f3b was used as a positive control for tetramer labelling; this is an allo-MHC-restricted CTL line which recognizes pMDM100 with high avidity (13). In this experiment there was a very small CD8 + cell population giving a high signal in the FL2 channel in all cultures (Fig. 5). We believe that these are non-specific events that occurred in this particular experiment, as it was present independently of the CTL specificity and it was absent in other experiments (e.g. Fig. 6).

We also analysed tetramer binding of low-avidity pMDM100-specific T cell lines that killed peptide-loaded RMA-S, but not RMA tumour cells (see CTL assay in Fig. 4). Figure 6 shows the tetramer labelling of a pMDM100 T cell line after three (Fig. 6C) and four (Fig. 6D) rounds of in vitro stimulation. Tetramer staining of 3f3b CTL (Fig. 6A) and a SV9-specific T cell line (Fig. 6B) was used as positive and negative control respectively. The pMDM100-specific T cell line bound the pMDM100-K b tetramer. After the third round of stimulation there were tetramer + and tetramer - cells, whereas after the fourth stimulation the culture became homogeneous regarding tetramer staining. In contrast to the CTL labelled in Fig. 5(F), which killed RMA tumour cells, the CTL line in Fig. 6 showed strong peptide-specific killing, but were not able to kill RMA tumour cells that expressed MDM2 endogenously. These results indicate that tetramer staining did not distinguish between high- and low-avidity CTL. However, tetramer analysis did identify CTL cultures with peptide-specific killing activity, since we found a correlation between the number of tetramer + cells and the killing activity against pMDM100-loaded RMA-S cells in eight independent CTL cultures (data not shown).

**Minor effect of pMDM100 immunization on RMA tumour challenge**

Mice were immunized s.c. with pMDM441, pMDM100 or a mixture of pMDM441 and pMDM100 peptides. The pSV9 peptide was used as negative control and peptide H19 as a positive control for tumour protection. H19 is a T helper peptide from a murine leukaemia virus that confers partial protection against challenge with the tumour cell line RMA (19). Ten days after immunization, mice were injected i.p. with 10^5 RMA cells and tumour growth was monitored by recording the weight of injected mice. Figure 6 shows that the survival of mice injected with RMA cells is not ameliorated by immunization with pMDM100 or pMDM441. However, after immunization with both pMDM100 and pMDM441 peptides together, one mouse was completely protected up to the end of the experiment at day 48 and the survival of another one was substantially increased. The differences between this group and the control were not statistically significant using the log-rank test (P > 0.25). As the challenge with RMA cells provokes 100% mortality in the control group, we believe that the modest protection with the mixture of peptides is biologically relevant. Mice immunized with H19 were partially protected and the differences were statistically significant (P < 0.005).

**Discussion**

In the present article, the immunity of the tumour-associated antigen MDM2 was analysed in order to increase our understanding of immune responses to widely expressed tumour-associated self-antigens. We have found that CTL tolerance to
the pMDM100 peptide that binds to Kb class I molecules with high affinity was incomplete, since peptide immunization induced high-avidity CTL. CTL avidity is here defined by the CTL capacity to kill tumour cell lines expressing MDM2 endogenously. However, the response was variable between mice and faded after a few rounds of in vitro stimulation even when high-avidity CTL were present after in vivo immunization.

In contrast to pMDM100 responses, immunization with pMDM441 emulsified in IFA induced a very small CTL response. The pMDM441 peptide binds MHC class I with low affinity and this predicts poor immunogenicity (15). Nevertheless, co-immunization with pMDM100 and pMDM441 improved survival in a small number of mice challenged with RMA tumor cells, although the protection was not statistically significant. In contrast, immunization with pMDM100 alone had no effect on the survival of tumour-challenged mice, although vaccination stimulated detectable CTL in a substantial number of mice (Figs 2 and 3). This indicates that in vitro CTL responses against pMDM100 did not correlate with in vivo protection. Vaccination with multiple epitopes may be a strategy to improve in vivo protection when targeting tumour-associated self-epitopes that are poorly immunogenic due to tolerance mechanisms.

The reason for the variability in the pMDM100 response is unknown; at present we cannot correlate the level of the response in individual animals with any biological parameter (i.e. age or sex) or with a particular peptide preparation. The pMDM100-specific CTL response is the final outcome of mechanisms that promote tolerance versus mechanisms that induce effective priming. The variability of the response between individual mice may indicate that the response against pMDM100 is in a delicate balance where small alterations in stimulating conditions tip the balance towards tolerance or priming. Additionally, it was not possible to maintain high-avidity pMDM100 CTL in culture. It has been suggested that the in vitro expansion of high-avidity CTL is dependent on stimulation with a low epitope density (17,18) since high antigen dose may induce apoptosis in high-avidity CTL (20). While trying to expand high-avidity CTL, cells were stimulated with a range of peptide concentrations, from 1 nM to 100 μM, or with tumour cell lines presenting naturally processed pMDM100 peptides. In all instances, only low-avidity pMDM100-specific CTL could be expanded or, when tumour cells were used as stimulators, peptide-specific CTL were lost. While it is possible that pMDM100-specific CTL can be expanded in some specific culture conditions, the simplest explanation is that some tolerance mechanism is in play controlling the response of autoreactive pMDM100-specific CTL. Mechanisms that are capable of limiting CTL responses include the loss of effector function after continued activation (21) or the lack of expansion in response to antigen stimulation (22-24).

In some situations, CD8 T cells proliferate in response to antigen, but do not display any effector function (21). This phenotype has been described in response to certain viral epitopes during chronic infections. It is possible that the loss of effector function may also occur in high-avidity CTL specific for the MDM2 self-antigen, as these cells would be constantly engaged in recognition of this widely expressed antigen. In our experiments, tetramer staining of CTL lines that did not kill RMA tumour cells (Fig. 4) revealed a population of tetramer- cells and a population of tetramer+ cells (Fig. 6C). This raised the possibility that the tetramer+ cells were high-avidity CTL that had lost killing activity, whilst tetramer- cells were low-avidity CTL responsible for the killing of the peptide-loaded targets. This, however, was unlikely, since repeated stimulation resulted in the loss of the tetramer+ population and demonstrated that the tetramer+ population was functionally

![Fig. 7. Immunization with pMDM100 and pMDM441 together induces a small level of protection against tumour challenge. B6 mice were immunized with pMDM100 (open diamonds), pMDM441 (closed diamonds), both pMDM441 and pMDM100 (open triangles), pSV9 (closed triangles) or H19 (closed squares) peptides. Control mice (open squares) were not immunized. Fourteen days after immunization, mice were injected i.p. with 10^3 RMA cells. Mice were weighed to follow the tumour growth. Results are depicted as the percentage of surviving mice along time. Results from four independent experiments are represented. The differences were statistically significant between the control group and mice immunized with H19 (P < 0.005). Other differences were not significant.](image-url)
active against peptide-loaded targets, but not against tumour cells, i.e. they were low-avidity CTL. Therefore, contrary to observations in other studies (25), the tetramer staining profile did not discriminate between high- and low-avidity CTL in our experiments (cf. Figs 5F and 6C). Lack of correlation between tetramer staining and CTL avidity has also been observed by others (26).

Tolerance mechanisms implying lack of proliferation by tolerant CD8 T cells have also been described. It has been reported that, in spite of their inability to proliferate, tolerant CD8 T cells may display effector functions (22-24). These observations may provide a hint to understand the loss of high-avidity pMDM100-specific CTL in vitro. Peptide immunization may induce expansion and differentiation of these CTLs, although they were unable to further expand in response to peptide stimulation. This is consistent with other experimental models of tolerance, where cell proliferation induced by antigen stimulation precedes cell deletion (27,28) or tolerance induction (22).

The presence of high-avidity pMDM100-specific CTL is very relevant from the perspective of designing tumour immunotherapy using tumour-associated antigens as targets. In fact, we observe a small level of protection immunizing with both MDM2 peptides. With the protocol presented here, the pMDM100 response was fragile, but it will be important to explore in the future alternative immunization protocols that may provide extra help for the stimulation of pMDM100-specific CTL. These protocols should involve the participation of CD4 helper T cells and dendritic cells (29,30).

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Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>CFA</td>
<td>complete Freund’s adjuvant</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>IFA</td>
<td>incomplete Freund’s adjuvant</td>
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<tr>
<td>PE</td>
<td>phycoerythrin</td>
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References


