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The Role of Proteases in Processing of Tumour Associated Antigen(NY-ESO-1)

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Abstract The activation of T cell responses is reliant upon several steps: the uptake and processing/transforming of antigens into small peptides within specialist antigen presenting cells (APC); the binding of peptides to a class of major histocompatibility molecules (MHCs); the presentation of this complex on the surface of the APC; and the interaction of the T cell with the MHC-peptide complex. The melanoma-associated antigen (NY-ESO-1) is one of cancer testis antigens (CTAs). Because of the restricted expression of CTAs to germ cells, it has been suggested that the antigens belongs to this family could be a promising target for cancer vaccines and/or cancer immunotherapy. Therefore, their presentation to the T cells need to be elucidated for design a successful cancer vaccines. This study aims to highlight the processing of NY-ESO-1 antigen inside APC and the enzymes that are engaged in this process. The mechanisms of antigen processing of the immunogenic epitope from NY-ESO-1 antigen were studied using antigen presentation assays followed by ELISA and ELISpot, The results demonstrated that the treatment of the Dendritic cells (DCs) with the protease inhibitors affected the processing of the NY-ESO-1 protein. This indicates that the NY-ESO-1 protein requires proteolysis using proteases which are involved in the major processing (i.e., cysteine and aspartic proteases) for effective presentation to CD4 T cells. It also requires a low pH environment. In conclusion, the findings of the present study may provide further indications that, potentially, a NY-ESO1 antigen could be used in a cancer vaccine. This kind of study is at high importance for tumour immunity in terms of design tumour vaccines and modulation of tumour cells to function as antigen presenting cells and present their own antigens to the T cells.

Keywords: NY-ES0-1, Cancer testis antigen, MHC molecules, Proteases, Antigen processing.

دور الانزيمات المحللة للبروتينات فى تكسير البروتين السرطاني (نييسو –1)

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الملخص يعتمد تحفيز الاستجابة المناعية للخلايا التائية على عدة عمليات وتشمل : عملية أخذ الانتيجين بواسطة خلايا عرض الانتيجين ، ثم عملية تكسير الانتيجين داخل خلايا عرض الانتيجين ، ثم عملية اتحاد القطع الانتيجينية بمركبات التوافق النسيجي وعرض المركب المتكون على سطح خلايا عرض الانتيجين لبتم تمييزه من قبل الخلايا التائية وتكوين الاستجابة المناعية اللازمة. يعتبر الانتيجين السرطانى (نييسو –1) والمرتبط بسرطان الجلد أحد انتيجينات سرطان الخصية ، وبسبب اقتصار وجود هذه الانتيجينات فى خلايا الخصية السرطانية وعدم وجودها فى الخلايا الطبيعية ، وجد أن هذه الانتيجينات يمكن استخدامها كلقاحات او علاج مناعى ضد السرطان. وبناء عليه ، فان عملية عرض هذه الانتيجينات للخلايا التائية تحتاج الى دراسة وتوضيح بهدف تصميم لقاحات سرطانية ناجحة. وتهدف هذه الدراسة الى توضيح الية تكسير الانتيجين المذكور أعلاه ، ومعرفة الأنزيمات الداخلة فى عملية التكسير. وقد تمت دراسة هذه الالية باستخدام اختبارات عرض الانتيجين ، وتلى ذلك تطبيق تفنية الاليزا والاليزسبوت لقياس معدل الاستجابة المناعية. أظهرت النتائج أن معالجد خلايا عرض الانتيجين ، والتى كانت الخلايا الغصنية ، بالمثبطات للانزيمات المسئولة على عملية التكسير البروتيني كان له تأثير على تكسير هذا الانتيجين. وهذا يوضح أن هذا الانتيجين يتطلب انزيمات التحليل البروتينى لتعمل على تكسيره وارتباطه بمركب التوافق النسيجي وعرضه على الخلايا التائية للجهاز المناعي ، وقد وجد ايضا ان الانريمات الداخلة فى عملية تكسير هذا الانتيجين هى الداخلة فى عملية التكسير الرئيسية ، وهى انزيمات السيستين والاسبارتيك ، وانها تتطلب بيئة حامضية لتقوم بعملية التحليل البروتيني. وخلصت هذه الدراسة الى أن الانتيجين المدروس يمكن استخدامه كلقاح سرطاني ، وذلك لتحفيزه على تكوين استجابة مناعية ذات دلالة معنوية. وبالتالى فان هذا النوع من الدراسات يعتبر على درجة عالية من الأهمية من ناحية تصميم اللقاحات السرطانية ، ومن ناحية تحوير الخلايا السرطانية لتعمل كخلايا عرض للأنتيجين وتقوم بعرض انتيجيناتها السرطانية الى الخلايا التائية وتكوين استجابة مناعية ضدها.

الكلمات المفتاحية : نييسو–1، انتيجينات سرطان الخصية ، مركبات التوافق النسيجى ، انزيمات تحليل البروتينات ، تكسير الأنتيجين.

Introduction

The activation of T cell responses is reliant upon several steps: the uptake and processing of antigens into small peptides within specialist antigen presenting cells (APC); the binding of peptides to a class of major histocompatibility molecules (MHCs); the presentation of this complex on the surface of the APC; and the interaction of the T cell with the MHC-peptide complex via a T cell antigen receptor (TCR). Upon activation, T cells recognise small regions (epitopes) of the antigenic peptides bound to MHC proteins; these, as has been said, are present on the surface of other cells known as antigen-presenting cells (APCs). The epitopes are generated by the modification of the native protein, usually following their (the protein's) enzymatic degradation inside the APCs – referred to as 'antigen processing'. The resulting peptides bind to the MHC molecules which are then transported to the APCs surface for presentation to T cells, a process termed 'antigen presentation' [1]. On the surface of the APC, interactions between T cells and the MHC-peptide complex occur via TCRs specific to the antigenic epitope.

Tumour antigens are proteins which degrade into peptides that, in the context of MHC molecules, can be presented in order to induce T lymphocyte-mediated immune responses [2]. Tumour epitopes are normally presented by MHC class I molecules, but can also be loaded into MHC class II grooves after escaping from the vascular network into the cytoplasm. Tumours have several mechanisms for escaping detection by the immune system. A previous study discussed the process whereby tumour cells escape surveillance by shedding certain membrane antigens [3]. A recent study found that tumour antigens shedding into the circulation may suppress antitumor $CD8^+$ T-cell function [4].

Most tumour antigens have been identified as being recognized by $CD8^+$ T lymphocytes; cytotoxic T lymphocytes (CTLs) in the context of MHC class I molecules [5-6]. An example is the melanoma-associated antigen (MAGE) – the first human leukocyte antigen (HLA) class I-restricted tumour antigen described [7]. However, some tumour antigens have been shown to be recognized by $CD4^+$ T helper lymphocytes. One such antigen is tyrosinase; this expresses in melanoma cells. It is HLA-DR4 restricted as it binds to this allele with high affinity and so induces a T cell response [6]. Tyrosinase was the first tumour antigen identified as being recognized by $CD4^+$ T cells [8].

Due to the restricted expression of cancer testis antigens (CTAs) in cancer cells, these antigens have been targeted by researchers for potential use in tumour vaccines. Several studies have focussed on the identification of T cell epitopes of CTAs ; this is for the purpose of creating potent cellular tumour immunity. The emergence of successful immune responses to tumours demands the recognition of epitopes in tumour cells; this is in order to create effective T-cell responses targeted at the tumour-associated antigens (TAAs). Previous studies have revealed that potent immune responses against TAAs such as HER2, CEA, MUC1 and p53 may be limited due to immune tolerance resulting from the fact that these TAAs are self-antigens. However, CTAs can be considered to be non-self-antigens because of their germ-restricted expression and their immunogenicity, which may induce strong anti-cancer immunity [9]. As a result, their potential role in tumour immunology has been highlighted in recent studies. The potential for an immune tolerance against CTAs has not yet been reported, and several studies have shown that some of these antigens are immunogenic. Among the CTAs, NY-ESO-1 or New York esophageal squamous cell carcinoma 1, is the main focus, here, as it is considered the most immunogenic CTAs known to date. Therefore, more attention has been paid to NY-ESO-1 – due to its cellular and humoral immunogenicity. The fact that it is widespread in many tumours also argues for its use as a potential candidate for a cancer vaccine [10]. A recent review has focused on NY-ESO-1 and discussed the past and current NY-ESO-1 targeted immunotherapeutic strategies [11]. It highlighted that several CTAs have been found to induce a spontaneous immune response, NY-ESO-1 being the most immunogenic among the family members. Currently, there are 12 clinical trials registered using a NY-ESO-1 cancer vaccine, 23 using modified T cells, and 13 using combinatorial immunotherapy. Exploring such new strategies have resulted in several novel treatments that are currently in clinical trial [11].Therefore, a deeper understanding of the mechanisms whereby NY-ESO-1 is processed is essential in order to develop a successful cancer vaccine of this type. The aim of this study is to reveal the role of the proteases in the processing of NY-ESO-1 antigen.

Materials and methods

1. $CD4^+$ T cell clone

NY-ESO-1 (the melanoma-derived protein) was used as a candidate tumour antigen in this project. The $CD4^+$ T cell clone specific to the NY-ESO antigen was obtained via collaboration with The Fred Hutchinson Cancer Research Centre in the USA. The clone was provided by Prof. Cassian Yee at that centre. The clone was generated by isolating PMBC from a melanoma patient who had high levels of NY-ESO-1. This procedure involved many steps, starting with the isolation of the monocytes layer from PBMCs [12].

2. Expansion of $CD4^+$ T cell clone

T cell clones were cultured in small flasks at $5*10^6$ cells /ml per flask. Human Interlukin-2 (IL2) was used at a concentration of 50 IU/ml to propagate the cells. They needed 14 days to reach optimal growth, and then they were re-stimulated again with IL-2.

3. HLA-DPB1-PBMC

The commercial human PBMCs were cryopreserved. They were a PBMC characterised for the MHC class II allele, HLA-DPB1. They were

thawed, cultured in AIM-V medium and harvested by using a cold Hanks' buffer.

4. Antigens

An immunogenic peptide, $ESO_{157-170}$ (SLLMWITQCFLPVF), was kindly provided by Dr Weisan Chen of the Laboratory of Viral Diseases, the National Institute of Allergy and Infectious Diseases, Australia [13].This peptide contains a cysteine, and its sequence is hydrophobic. Therefore, it had to be dissolved in 100% DMSO at a concentration of 3 mg/ml, and stored in small aliquots at 20 ^{0}C until use. Exposing the peptide to oxygen would result in the formation of dimers which would reduce its capacity. Additionally, it was found that the immunogenicity of the synthetic peptides containing cysteine was enhanced markedly by treatment with reducing agents [13]. The experimental dose of the peptide was optimized. Recombinant human NY-ESO-1 protein (Cat. no. RP-39227; Thermo Scientific) was used for the study of the antigen processing of the immunogenic peptide derived from the whole protein. It was at 0.1 mg/ml and was then diluted to 10 µg/ml for all the assays. The experimental dose of the protein was optimized. A pool of lyophilized peptides extracted from the NY-ESO-1 protein (PepTivator NY-ESO-1 human, Cat. no. 130-095-380, Miltenyi biotec) was used as another source of peptide and/or protein. This pool consists of 15-mer sequences with 11aa overlap, covering the complete sequence of the human NY-ESO-1 protein. The concentration of each peptide in that pool was 6nmol (about 10 µg) per peptide. The pool was reconstituted in 200 µl of sterile water to make a stock solution at 30nmol (about 50 µg) of each peptide per ml. The doses for each experiment were diluted from the stock solution at the time of the experiment. The experimental dose of the peptide pool was optimized.

5. Inhibitors

Key inhibitors were used to study the mechanisms of antigen processing and presentation. After the treatment of the APC with these inhibitors, antigen presentation assays were applied straightaway. Table 1 lists the inhibitors used for this purpose.

Table 1. A list of the inhibitors used for the treatment of the DCs

Inhibitor	Solvent	Dose	Cat. no.	Company
NH_4Cl	H_2O	100 mM	A0171	Sigma
Pepstatin A	DMSO	1µg/ml	P4265	Sigma

Additionally, a protease inhibitor cocktail (Cat. no. 11 836 170 001; Roche) in tablet form was used in the investigation of the patterns of antigen processing. This protease inhibitor cocktail was designed to inhibit a broad spectrum of serine, cysteine, and metallo proteases. Each tablet was dissolved in 10 ml of water or PBS (pH 7.0) in order to make the working solution. For treatment of the DCs, 100 µl/well was added.

6. T Cell proliferation assay

This assay aims to measure the T cell response to the NY-ESO-1 protein (the latter in the form of peptide or of whole protein). T cells were plated into a round-bottomed 96 well plate ($5*10^4$ cells /ml and 100 µl/well). The antigen was added, to triplicates, as follows: 3 doses of NY-ESO-1 peptide ($ESO_{157-170}$) at 20, 40 and 60 µg/ml. On the other hand, the NY-ESO-1 peptide pool and the protein were added at the doses 5, 10 and 20 µg/ml. The cultured plate was then incubated for 60 hrs. at 37 °C in a humidified 5 % CO2 incubator. A triplicate treated with a mitogenic agent; Concanavalin A (ConA: Cat no. L7647; Sigma) at a concentration of 1µg/ml was used as a positive control. Other triplicates had the following: medium only; cells treated with cell death reagent, camptothecin (CPT), (Cat no. C9911 from Sigma) at a concentration of 4 mM/ml; and cells without peptide. These were used as negative controls. After the set incubation time, the viability of the cells was confirmed by light microscopy. Then, 20 µl of Cell Titer 96 AQ_{ueous} One Solution (Cat no: G3582; Promega) was added to the wells and these were re-incubated for a further 1-4 hours. The principle of the above test is explained in the datasheet of the reagent. Afterwards, a cell proliferation assay was carried out using an ELISA microplate reader (VersaMax) to record the absorbance at 490 nm.

7. The role of proteases in antigen processing

The cocktail of enzyme inhibitors, along with pepstatin A, were used to study the processing pattern related to the $ESO_{157-170}$ - derived peptide from the NY-ESO-1 protein. Briefly, the protocol of these experiments was as follows: a treatment of the DCs with the appropriate inhibitor for 30 min took place before the addition of the antigen. The antigen was then added in duplicates of one dose (40 µg/ml) of NY-ESO-1 peptide ($ESO_{157-170}$), one dose of NY-ESO-1 protein (10 µg/ml), and one dose of NY-ESO-1 peptide pool (20 µg/ml). Afterwards, all were washed three times with HANKS, and then T cell clones were added. Negative and positive controls were used. Cells without inhibitor were used as a negative control.

8. The role of endosomal alkalization in antigen presentation

The Role of endosomal alkalization on antigen presentation was examined by using three concentrations of ammonium chloride (25 mM, 50 mM and 100 mM). The same protocol of treatment with inhibitors was applied.

9. A study of the kinetics of antigen presentation

A determination of the kinetics of antigen presentation was performed by the incubation of the antigen with the DCs for three different durations (75 min, 150 min and 300 min) before the fixation of the DCs. Then, the same procedure of antigen presentation assay was carried out.

10. ELISA

The Enzyme-Linked Immunosorbent Assay (ELISA) was used to measure the IL2 level secreted by T cells. The ELISA for human IL2 kit (Cat. no. 3440-1A-6; Mabteck) was used, and the procedure provided with the kit was applied. The optical density (OD) was measured at 405 nm, using an ELISA microplate reader (VersaMax).

11. ELISpot

The NY-ESO-1-specific CD4+ T cell response to NY-ESO-1-derived peptide and protein was also assessed via an enzyme-linked immunosorbent spot (ELISpot) assay specific to human IFN-γ – using the IFN-γ ELISpot kit (Cat. no. EL285, R & D Systems). The ELISpot assay specific to human IFN-γ was used to ascertain the effects of the metabolic inhibitors on the processing and presentation of NY-ESO-1-derived peptide. The procedure of this assay was applied according to the manufacturer's recommendations. The spots of the duplicate wells of each sample were counted manually using a stereomicroscope (Leica Zoom 2000), and the results were presented as a mean value. A response was considered positive when the mean of the spot count in the duplicate wells was significantly higher than that observed in the control wells.

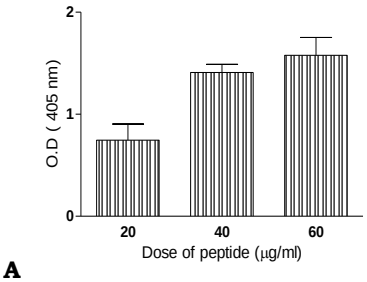
12. Statistical analysis

Microsoft GraphPad Prism 4.0 was used for statistical analysis. For calculating the mean, the standard error of the mean (SEM) and the standard deviation (S.D) of the data in terms of duplicates and/or wells as required. The results represent the mean of duplicate wells ± S.D. The results of the ELISpot assays were expressed as a mean spot count. The P values were calculated, as required, by applying a Student's t-test – where only two sets of conditions must be examined. Where more than two sets of data had to be analysed, a one-way ANOVA was used. When different conditions across more than two groups of data needed to be analysed, a two-way ANOVA was used. Finally, Bonferroni's Multiple Comparison Test was applied for multiple comparisons. Overall, the differences between the examined data sets were regarded as statistically significant when the P values < 0.05.

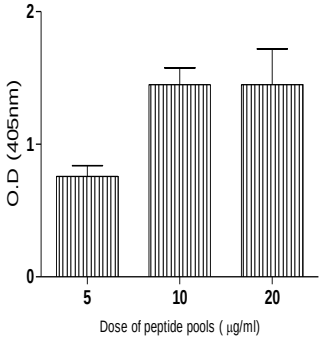
Results

1. Specificity of CD4⁺ T cell clone

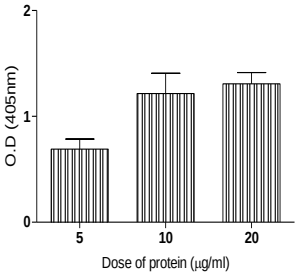
T cell responses to the immunogenic antigen were measured by using a cell proliferation assay. The results of this assay are presented in Fig.1; this demonstrates the immune responses of CD4⁺ T cell clones to ESO-1 antigen as sourced by peptide (ESO $_{157\text{-}170}$), peptide pool, and whole protein, and these results reflect the responses' specificity to the antigen.



A



B



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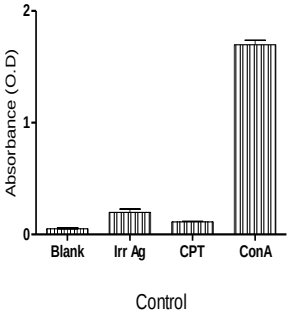

D

Fig.1: Specificity of CD4⁺ T cell clone to NY-ESO-1 antigen.

T cell responses to the immunogenic peptide of NY-ESO-1 (ESO$_{157\text{-}170}$; SLLMWITQCFLPVF), to peptide pool and to whole NY-ESO-1 protein were tested in a dose-dependent procedure in order to find the optimal dose for inducing T cell responses. A T cell proliferation assay was applied after adding three doses of A: peptide (20, 40 and 60 μg/ml), B: peptide pool (5, 10 and 20 μg/ml), and C: protein (5, 10 and 20 μg/ml. All the doses used gave rise to significant T cell responses (P < 0.001), as compared to the controls. These results confirm the specificity of the CD4⁺ T cell clone to the antigen under study. Two negative controls were used (cells with irrelevant antigen and cells

with CPT), ConA was used as a positive control, and medium was used as a background (D).

2. Effect of the protease inhibitor cocktail

To determine whether NY-ESO-1 requires processing or can bind to MHC class II as a native protein, a protease inhibitor cocktail was used for the inhibition of the serine, cysteine, and metallo proteases. The ELISA assay was applied after that. The IL-2 level obtained by ELISA, as compared to those yielded by the experimental controls, demonstrated that there had been no T cell response to the NY-ESO-1 (in the form of peptide pool and protein) in the presence of the protease inhibitor cocktail. However, the T cell response to the antigen was not affected in the absence of the protease inhibitor cocktail. Fig.2 presents the results of ELISA for the IL-2 secreted by T cells both in the presence and in the absence of the protease inhibitor cocktail – as compared to the controls (Fig.8)

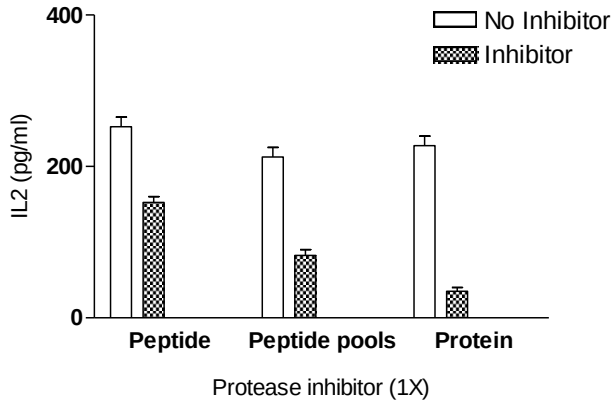

Fig.2: The effect of the protease inhibitor cocktail on the processing of $ESO_{157-170}$ peptide as indicated by an ELIZA.

DCs were treated with 100 µl/well of the working solution of the cocktail for 30 min. Afterwards, one dose (40 µg/ml) of the peptide ($ESO_{157-170}$), one dose (10 µg/ml) of the protein (NY-ESO-1), and one dose of the peptide pool (20 µg/ml) were pulsed with the treated DCs. After the incubation time (5 hrs.), T cell clones were added to the wells and incubated for 60 hrs. The plate of the assay was stored at -20 ^{o}C overnight**,** and the supernatants were collected after this period of freezing. Afterwards, ELISA was employed to measure the secreted IL-2. The results represent the mean ± S.D. of duplicate wells.

An ELISpot assay specific to human IFN-γ was employed to ascertain the effects of the metabolic inhibitors on the processing and presentation of the NY-ESO-1-derived peptide ($ESO_{157-170}$). The effects of the inhibitors were studied by pre-treating the DCs with the inhibitor at the indicated dose. The antigen was added in duplicates of one dose (40 µg/ml) of NY-ESO-1 peptide ($ESO_{157-170}$). The ELISpot results relating to the effects of the proteases inhibitor, as compared to the controls, demonstrated that the T cell response to the NY-ESO-1 peptide and protein was affected by the presence of the protease inhibitor cocktail. In contrast, there was a T cell response to the antigen in the absence of the protease inhibitor cocktail. Fig.3 shows the results of ELISpot for the secreted IFN-γ by T cells in the presence and absence of the protease inhibitor cocktail – compared to the controls (Fig.9).

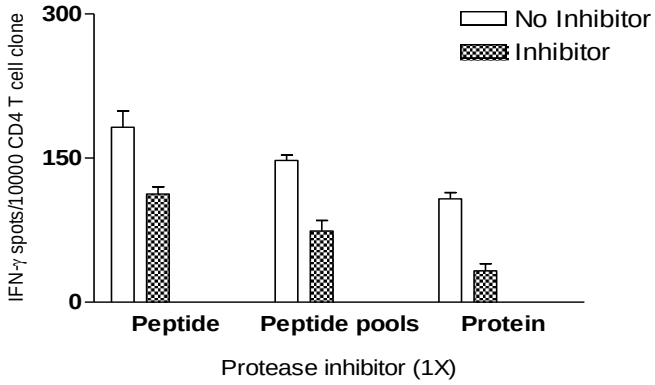

Fig.3: The effect of the protease inhibitor cocktail on the processing of $ESO_{157-170}$ peptide as indicated by an ELISpot assay.

Following the treatment of the DCs with the inhibitor, they were pulsed with the antigen as described in the methods, and then T cell clones were added. The experimental plate was incubated for 40 hrs. at 37 ^{0}C in an incubator. Afterwards, an ELISpot assay was employed which involved counting the IFN-γ spots using a stereomicroscope. The result represents the mean ± the S.D. of duplicate wells.

3. Effect of pepstatin A

Pepstatin A is an inhibitor of aspartic protease. The result of the ELISA assay showed that pepstatin A had a slight effect on the processing of the $ESO_{157-170}$ epitope. Fig.4 illustrates the result of the ELISA assay both in the presence and in the absence of pepstatin A – compared to the controls.

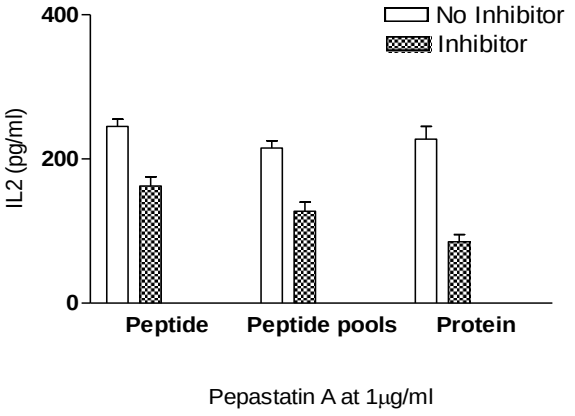

Fig.4: The effects of pepstatin A on the processing of $ESO_{157-170}$ peptide by ELIZA.

DCs were treated with 1 µg/ml of pepstatin A for 30 min. Afterwards; one dose (40 µg/ml) of the peptide ($ESO_{157-170}$), one dose (10 µg/ml) of the

protein (NY-ESO-1), and one dose of the peptide pool (20 µg/ml) were pulsed with the treated DCs. After the incubation time (5 hrs.), T cell clones were added to the wells and incubated for 60 hrs. The plate was stored at -20 °C overnight, and the supernatants were collected after this period of freezing. Afterwards, ELISA was employed to measure the secreted IL-2. The results represent the mean ± S.D. of duplicate wells.

The results concerning the effect of pepstatin A as elicited by an ELISpot assay, compared to controls, was not in agreement with the results obtained via ELISA (refer to figure 8). The EliSpot result demonstrated that there was not a significant difference between the number of spots which emerged in the treated (with inhibitor) and in the untreated wells (no inhibitor) – and in the control wells. Therefore, the results of the ELISpot assay did not give any clear evidence regarding the effects of pepstatin A on the processing of the candidate NY-ESO-1 peptide. Fig.5 illustrates the results of the ELISpot assay on the experimental samples, compared to the controls (Fig.9).

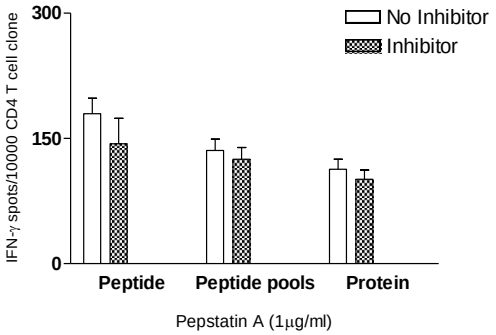

Fig.5: The Effects of pepstatin A on the presentation of $ESO_{157-170}$ peptide, as elicited by an ELISpot assay.

The procedure of this assay was applied as specified in the methods. The experimental plate was incubated for 40 hrs. at 37 0C in an incubator. Afterwards, an ELISpot assay was undertaken by counting the IFN-γ spots using a stereomicroscope. The results represent the mean ± S.D. of duplicate wells.

4. Effect of ammonium chloride

The results gave a clear indication that a T cell epitope requires low pH (endosomal acidification) for optimal antigen processing in relation to the NY-ESO-1 protein. This was in agreement with the results obtained by using the above protease inhibitor since the candidate epitope requires processing at low endosomal acidification. Cells without NH_4Cl (0 mM) were considered as a control for the assay. Fig.6 presents the result of this assay by ELISA, compared to the controls (Fig.8).

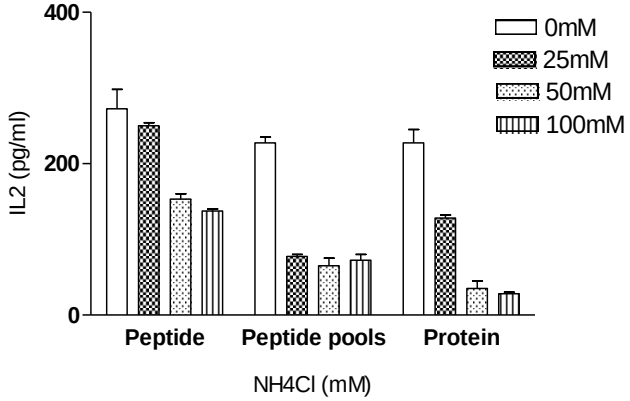

Fig.6: The effect of NH_4Cl on the processing of the $ESO_{157-170}$ peptide. DCs were treated with three concentrations of ammonium chloride (25 mM, 50 mM and 100 mM) for 30 min. Afterwards, one dose (40 µg/ml) of the peptide ($ESO_{157-170}$), one dose (10 µg/ml) of the protein (NY-ESO-1), and one dose of the peptide pool (20 µg/ml) were pulsed with the treated DCs. After the incubation time (5hrs), T cell clones were added to the wells and incubated for 60 hrs. The plate for the assay was stored at -20 °C overnight, and the supernatants were collected after this period of freezing. Afterwards, ELISA was employed to measure the secreted IL-2. The result represents the mean ± S.D. of duplicate wells.

5. A Study of the kinetics of antigen presentation

Fig.7 shows that the ESO-1 peptide needs a long time for processing and presentation as 300 min was the optimal time for its effective presentation – compared to the controls (Fig.8).

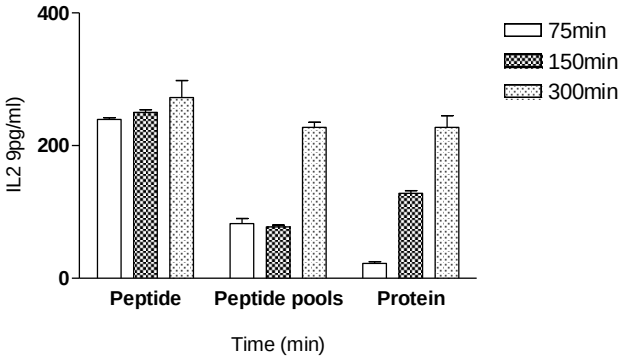

Fig.7: The kinetics of the presentation of the $ESO_{157-170}$ peptide.

The DCs were incubated for 75 min, 50 min and 300 min with $ESO_{157-170}$ peptide (40 µg/ml), NY-ESO-1 protein (10 µg/ml) and peptide pool (20 µg/ml). After these incubation periods, the DCs were fixed with 1% paraformaldehyde, washed to remove unbound antigens, and then, the T cell clones were added and incubated for 60 hrs. in the incubator. Afterwards, the experimental plate was frozen at -20 °C overnight. Subsequently it was subjected to an ELISA assay in order to measure the IL2 level. The result represents the mean ± S.D. of duplicate wells.

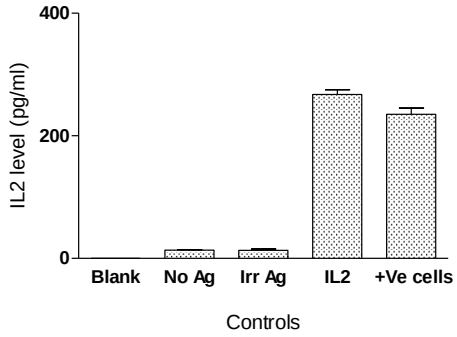

Fig.8: The results for the control samples in relation to the antigen processing and presentation assays (ELISA).

Wells with just medium, no antigen and irrelevant antigen were used as negative controls, while wells with cytokine secreted cells (+Ve cells), and wells with IL-2 present were used as positive controls.

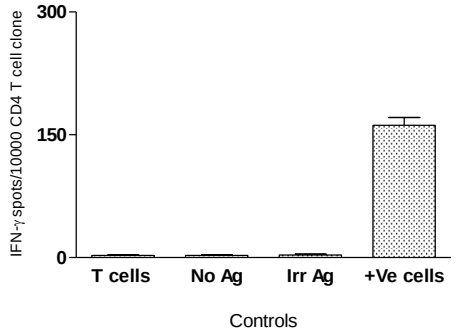

Fig. 9: The results from the control samples for the antigen processing and presentation assays (ELISpot).

T cells alone, un-stimulated cells (no Ag), and cells stimulated with an irrelevant antigen (Irr Ag) were used as negative controls. Wells with cytokine secreted cells (+Ve cells) were used as positive controls.

Discussion

Understanding the mechanisms of antigen processing and presentation is of great importance to immunotherapy, and could lead to new contributions to the design of a vaccine. Few studies have focused on investigating the presentation of tumour antigens to the immune system. Tumour antigens are considered endogenous (intracellular) antigens which are usually presented by the MHC class I-antigen presentation pathway. However, targeting tumour antigens towards the MHC class II pathway has been shown to be most effective at inducing anti-tumour T cell responses Therefore, the generation of $CD4^+$ T cell clones specific to a melanoma-associated antigen (NY-ESO-1 protein) by a research group in the USA, using an immunogenic $CD4^+$ T cell epitope ($ESO_{157-170}$ peptide), has been a major contribution to the study presented here [12].

The NY-ESO-1 antigen-specific $CD4^+$ T cell clone was characterized before being subjected to the experiments. CD4 markers and $TCR_{\alpha\beta}$ receptors were detected on the cell surface of the clone, thus indicating that the clone had been generated successfully and that, hence, this clone was the right one for use in antigen presentation assays. The clone was also assessed in terms of its NY-ESO-1 antigen specificity. A T-cell proliferation assay was carried out purely to measure the proliferation of T Cells. Which dose of the NY-ESO-1 protein, the NY-ESO-1 peptide pool, and the NY-ESO-1-derived peptide would be most effective for the pulsing of the DCs was also deduced. It was shown that the best doses, for creating a good response to the NY-ESO-1-derived peptide, were 40 µg/ml for the peptide, 20 µg/ml for the peptide pool, and 10 µg/ml for the NY-ESO-1 protein as these doses yielded the highest optical densities. These doses were used to carry out the antigen presentation assays. A dose of 40 µg/ml of the peptide was also used for pulsing the isolated T cells in order to generate the above $CD4^+$ T cell clones [12]. The results suggest that 40 µg/ml of the peptide or 10 µg/ml of the protein could be used for *in vivo* vaccination.

Since a higher proliferation of cells occurred in the presence of the peptide rather than the protein, it can be inferred that the peptide antigen has a greater specificity for the T cell clone, resulting in a greater immune response. Thus, it can be concluded that the peptide antigen is the most immunogenic epitope, not the whole protein. It is also proposed that a dose of 60 µg/ml be used, although this would require toxicity testing beforehand. Data from the T cell proliferation assays could contribute to a preclinical *in vitro* proof of concept study designed to predict the best starting doses for a dosing schedule in a projected clinical trial on humans. It is important to strike a balance between immunological efficiency and concentration of antigen – striving to achieve maximum immunological response using the lowest dose possible as such is less likely to produce an adverse reaction within the patient. Of course, this is why it is of utmost importance to perform *in vitro* and *in vivo* scientific studies before proposing dosing schedules.

Antigen processing is an essential step in producing T cell epitopes from intact antigens. Four classes of proteases are said to be involved in the degradation of antigens [14-15]. Therefore, the role of proteases in the proteolysis of the NY-ESO-1 antigen was investigated in order to identify whether or not the NY-ESO-1 antigen requires proteolytic processing by DCs. This investigation included looking at the effects of a protease inhibitor cocktail containing metabolic inhibitors for cysteine, serine, and metallo proteases and at the effects of pepstatin A, which is an inhibitor for aspartic protease. The protease inhibitor cocktail was shown to have a significant effect on the processing of the NY-ESO-1 antigen as it blocked the T cell responses when the DCs were treated with it, while pepstatin A had less effect on the processing of the $ESO_{157-170}$ epitope. This suggests that the NY-ESO-1 antigen requires

lysosomal processing by cysteine proteases in order to generate the candidate T cell epitope from the NY-ESO-1 antigen, although it might be relatively independent of the processing by aspartic proteases. Both the ELISA and the ELISpot assays were in agreement in terms of the effectiveness of the protease inhibitor cocktail (compared to the assay controls). However, the ELISA and ELISpot assays produced slightly different results from each other in relation to the effects of pepstatin A – this could be due to experimental error. Additionally, the treatment of the DCs with three doses of ammonium chloride demonstrated that the NY-ESO-1 antigen requires low pH (endosomal alkalization) for optimal antigen processing. This result was in agreement with the results obtained by using the above protease inhibitors: the candidate antigen requires processing at low endosomal alkalization. Also it was confirmed that the T cell epitope ($ESO_{157-170}$ peptide) needs the protease families that are involved in the major processing in order to be elaborated from the NY-ESO-1 antigen for presentation to T cells. Some previous studies on HLA-DR-transgenic mice have used a variety of protease inhibitors in order to determine the protease families which are involved in the processing of some bacterial antigens [16-18].

Some studies have shown that the main locations for antigen processing in the APCs are at the late endosomes and lysosomes – where the formation of the peptide/MHC class II complexes takes place following the unfolding, disulfide bond reduction and denaturation of the antigens [19-23]. These studies demonstrated that lysosomal cysteine and aspartic are implicated in the processing of the antigen and the invariant chain. In the present study, the protease inhibitor cocktail, which includes cysteine, serine, and metallo inhibitors, significantly blocked the presentation of the $ESO_{157-170}$ epitope, but the aspartic inhibitor (pepstatin A) had less effect on the presentation of the $ESO_{157-170}$ epitope to the T cells. This highlights the role of the cysteine protease in the processing of the NY-ESO-1 antigen as the T cell epitope contained a disulphide bond in the cysteine residue. On the other hand, the aspartic inhibitor had no significant role on the processing of the candidate antigen. Serine and metallo proteases are found on cell surfaces and in early endosomes, where they act at neutral pH [24-26]. These latter protease families participate in minor proteolysis of antigens at extracellular sites or in early endosomes, as shown by [17, 27-30]. It has been suggested that the serine and metallo proteases are involved in the pre-processing of the antigens in early endosomes; this is followed by the actions of other protease families in late endosomes or lysosomes [17]. The effect of various inhibitors for antigen processing of another peptide ($NY\text{-}ESO\text{-}1_{95-106}$) from a melanoma cell line (SK-MEL-37) was evaluated by [31] and found that the pretreatment of tumour cells with proteasome inhibitors or protease inhibitors significantly impaired T-cell recognition, indicating that is probably generated by the proteasome and loaded on MHC class II in the endosome and in a protease-dependent manner. In addition to endosomal/lysosomal proteases that are typically involved in MHC class II antigen presentation, several pathways in the MHC class I presentation pathways, such as the proteasomal degradation and transporter-associated with antigen-processing-mediated peptide transport, were also involved in the presentation of intracellular NY-ESO-1 on MHC class II. The presentation was inhibited significantly by primaquine, a small molecular that inhibits endosomal recycling, consistent with findings that pharmacologic inhibition of new protein synthesis enhances antigen presentation [32]. Therefore, it can be concluded that the main protease family that participates in the processing of the NY-ESO-1 antigen is the cysteine protease which is responsible for the major proteolysis of the antigens, and works in low pH environments.

The kinetics of the antigen processing and of the presentation of the $ESO_{157-170}$ T cell epitope from the intact NY-ESO-1 antigen was also studied – in order to confirm the pathway of NY-ESO-1 presentation. Since it is known that the recycling pathway is characterized by faster kinetics of antigen presentation, as compared to the classical pathway, three incubation times were used for incubating the DCs with the NY-ESO-1 antigen (75 min, 150 min and 300 min). The results indicated the use of the classical pathway since the $ESO_{157-170}$ epitope took a long time (300 min) to be processed from the NY-ESO-1 antigen, and then to be presented to the T cell clone. The ELISA controls confirmed this finding. In summary, the classical MHC class II-antigen presentation pathway was found to be involved in the generation of the $ESO_{157-170}$ epitope from the NY-ESO-1 antigen. The recycling pathway, which is characterized by rapid kinetics (within 15 min) in terms of antigen presentation, as compared to the delayed kinetics of the classical pathway (starting at 90 min), has been investigated by others. These studies have shown the rapid kinetics of the following: the T cell epitope from the haemagglutinin antigen ($HA_{307-318}$); [33], the $RNase_{42-56}$ epitope from bovine ribonuclease [21], the T cell epitope from the M5 protein of *Streptococcus pyogeni* M_{17-31} [34-35] and two T cell epitopes from the protective antigen (PA) of *Bacillus anthracis*, $PA_{547-560}$ and $PA_{659-672}$ [17]. These epitopes were all loaded on mature MHC class molecules recycled from early endosomes to the plasma membrane, using the recycling pathway. In contrast, none of the three T cell epitopes studied from *Yersinia pestis* were presented by the recycling pathway [18].

Interestingly, a recent study [36] has discussed a unique interaction which takes place between the NY-ESO-1 protein and the innate immune system via toll like receptor 4 (TLR4). The NY-ESO-1 protein demonstrated its ability to bind to immature DCs, monocytes and macrophages, in vitro. It was found that the strong immunogenicity of the non-mutated NY-ESO-1 protein is attributable to the polymeric structures which it forms through its disulphide bonds. The binding of the NY-ESO-1 protein to the immature DCs is

known to involve TLR4 because it was found that the immunogenicity of NY-ESO-1 is not induced in TLR4-knock-out mice. Also, its immunogenicity was diminished by the substitution of cysteine with serine [36]. This indicates the importance of NY-ESO-1 as a target for immunotherapy and/or a tumour vaccine. More recently, a study has demonstrated that NY-ESO-1 mediates direct recognition of melanoma cells by CD4+ T cells after intracellular antigen transfer [37]. That makes this antigen a promised target for cancer immunotherapy and vaccine.

The key point of all the above was to find a promising CT antigen which could be used as a tumour vaccine and/or for tumour immunotherapy. One issue is that an efficient vaccine for producing an immune response against tumours should have a natural processing [38] or should be delivered into the APC for efficient processing, as was reviewed by [39]. The role of NY-ESO-1 in immunogenicity has been seen in the generation of T and B cell immune responses in some cancer patients [12,38-39,40-51]. Therefore, particular attention has been paid to the NY-ESO-1 antigen as representing a potential immunological approach.

Based on the results achieved by these approaches, vaccine trials have been conducted on some tumours, aimed at inducing long-term anti-tumour immunity. These trials have used different sources of the NY-ESO-1 antigen and different adjuvants [12,43,45-51]. The findings of the present study may provide further indications that, potentially, a NY-ESO1 antigen could be used in a cancer vaccine. In conclusion**,** the treatment of the DCs with the protease inhibitors affected the processing of the NY-ESO-1 protein which results in the generation of the $ESO_{157-170}$ peptide. In conclusion, this indicates that the NY-ESO-1 protein requires proteolysis using proteases which are involved in the major processing (i.e., cysteine and aspartic proteases). It also requires a low pH environment.

Abbreviations and Acronyms

Antigen presenting cells : APC, CTAs : Cancer testis antigens, Cytotoxic T lymphocytes: CTLs, HLA: Human leukocyte antigens , MHC: Major histocompatibility , NY-ESO-1: New York esophageal squamous cell carcinoma 1, T cell receptor : TCR, Toll like receptor 4: TLR4, TAAs: Tumour-associated antigens.

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