UNIVERSITÀ DEGLI STUDI DI BOLOGNA

ALMA MATER STUDIORUM

DOTTORATO DI RICERCA IN

"SCIENZE PNEUMO-CARDIO-TORACICHE DI INTERESSE MEDICO E CHIRURGICO"

CICLO XIX

CHARACTERIZING THE INTERACTION BETWEEN SUSCEPTIBILITY GENES TO BERYLLIOSIS AND BERYLLIUM.

DETERMINING THE ANTIGEN BERYLLIUM AND MOLECULAR MECHANISMS TO PREVENT THE BE/HLA BINDING AS A IMMUNO-MODULATORY THERAPY AGAINST BERYLLIOSIS.

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OMISSIS

Dott.ssa **Berretta Floriana**
curriculum seguito: Indirizzo internistico – Malattie dell'Apparato Respiratorio.
titolo tesi di Dottorato: Caratterizzazione della interazione tra i geni di suscettibilità alla berilliosi e berillio. Determinazione del "antigene berillio" e meccanismi molecolari di prevenzione del legame Be/HLA quale terapia immuno-modulatoria della berilliosi.

"presentazione" La Dott.ssa Berretta ha prevalentemente sviluppato studi sulla: Caratterizzazione della interazione tra i geni di suscettibilità alla berilliosi e berillio. Determinazione del "antigene berillio" e meccanismi molecolari di prevenzione del legame Be/HLA quale terapia immuno-modulatoria della berilliosi.

Il lavoro è stato eseguito presso l'Università di Roma "Tor Vergata", Dipartimento di Medicina Interna, dove ha sviluppato una linea di ricerca sotto la supervisione del Prof. Cesare Saltini.

Una prima parte del lavoro ha permesso una dettagliata analisi strutturale del sito di legame della molecola HLA-DPGlu69 e degli effetti del polimorfismo Glu/Lys sulla specificità di legame peptidico (Tissue Antigens 2003; 62: 459-71).
E' stato poi identificato un marker di suscettibilità all'ipersensibilità al berillio HLA-DR Phenylalanin β47 in soggetti che non presentano il marker associato alla berilliosi HLA-DPGlu 69 (Respir Res. 2005; 6: 94).
Successivamente è stata analizzata l'interazione berillio/HLA-DP attraverso la capacità del berillio di competere con CLIP e con i peptidi CLIP-derivati per il legame con molecole solubili HLA-DPGlu69.
I risultati preliminari suggeriscono che:
- i peptidi CLIP-derivati ad alta affinità: CLIP-YY, CLIP-QY, CLIP-RF venivano solo marginalmente influenzati dalla presenza del berillio nei saggi di competizione (le affinità erano superiori a quelle di CLIP da 11,843 a >21,429 volte).

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L'effetto di questi peptidi ad alta affinità sulla presentazione del berillio era determinato dalla misura di IFN-γ rilasciato dai PBMC stimolati con il berillio prelevati dai soggetti ipersensibilizzati al berillio.
CLIP-YY inibiva la presentazione del berillio e l'attivazione delle cellule T, mentre CLIP-QY e CLIP-RF stimolavano marcatamente il rilascio di IFN-γ in risposta al berillio. Anticorpi monoclonali anti HLA-DP bloccavano il rilascio di IFN-γ stimolato dal berillio in presenza di CLIP-QY (88%) e di CLIP-RF (76%). Effetti simili venivano osservati per CLIP-YY in grado di bloccare il rilascio di IFN-γ indotto dal berillio in presenza di CLIP-QY (79%) e di CLIP-RF (76%).
Questi risultati hanno potenziale implicazione per la diagnosi e il trattamento della berilliosi attraverso il disegno di (i) peptidi stimolanti la presentazione in grado di incrementare i segnali di attivazione delle cellule T e di (ii) peptidi bloccanti che potrebbero essere usati come base per una terapia competitiva per bloccare o anergizzare la reazione T-cellulare *in vivo*.

La Dott.ssa Berretta ha svolto la sua attività di ricerca con dedizione e profitto degni di nota.

Il suo lavoro, documentato dalle pubblicazioni prodotte, è stato apprezzato ed è valutato come eccellente dal Collegio dei Docenti del Dottorato .

Il Coordinatore
Prof. Sandro Mattioli

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Abbreviation used in this thesis

BAL	**Bronchoalveolar Lavage**
BH	**Beryllium hypersensitivity**
CLIP	**Class II associated Invariant Chain Peptide**
HLA	**Human Leukocyte Antigen**
ELISA	**Enzyme-linked Immunosorbent Assay**
IFN-γ	**Inteferon-γ**
IL-2 (4/10)	**Interleukin 2 (4/10)**
MHC	**Major Histocompatibility Complex**
MoAb	**Monoclonal antibody**
PBMC	**Peripheral Blood Mononuclear Cells**
TCR	**T-cell receptor**
TNF-α	**Tumor Necrosis Factor–α**

CHAPTER 1 – BERYLLIOSIS

INTRODUCTION

The lung is constantly burdened by an extraordinary number of airborne particles contained in the seven to ten thousands liters of inhaled air daily. Particles, chemical agents and antigens are cleared by the mucociliary escalator or destroyed by the cells of the lung immune system; they do cause though a constant state of activation of the lung-compartmentalized immune-effector cells (1) and in a fraction of the exposed individuals, exaggerated reactions leading to tissue damage and lung dysfunction.

With the expanding use of metals and chemicals in high-technology industries, immunotoxic compounds are now found in the industrial environment as well as in manufactured consumer products. Exposure to occupational and/or environmental metal compounds capable of interacting with the immune system is responsible for a variety of adverse immune reactions including granulomatous inflammations and allergic/atopic IgE-mediated type I immune responses (1, 2). In fact, they may either overpower the clearance systems of the lung, as in non complicated silicosis, or trigger an exaggerated immune and inflammatory reaction as in complicated silicosis, occupational asthma and berylliosis. A number of inorganic compounds such as cadmium, beryllium (Be), cobalt, mercury, manganese, titanium, antimony or zinc are known to cause acute chemical pneumonitis and bronchitis (1, 2). Zinc, cadmium, copper, manganese and aluminum oxide fumes are the cause of a non-fibrosing acute alveolitis (3, 4), crystalline silica, fibrous and non fibrous silicates cause fibrogenic pneumoconioses, while non fibrogenic pneumoconioses are caused by exposure to iron, barium, tin (5). Exposure to tungsten carbide and cobalt complexes is associated with a giant cell or a desquamative interstitial pneumonitis and lung fibrosis, also known as hard metal lung disease (1). Chronic granulomatous

lung disorders are caused by Be (6), zirconium (7-9), titanium (10) and aluminum (11).

The discovery of immune response genes in the 1970s has started a widespread search for genes modulating individual susceptibility to exaggerated lung immune reactions to silica, asbestos, isocyanates, tungsten carbide, cobalt, nickel and Be as well as for genes of susceptibility to chronic immune disorders of the lung such as sarcoidosis and idiopathic pulmonary fibrosis which are pathologically indistinguishable from berylliosis and asbestosis and for which an environmental cause has been postulated.

THE BERYLLIOSIS MODEL

Due to its unique chemical and physical properties, Be continues to be utilized in a variety of high-technology industries, including aerospace, electronics, and nuclear defense (12). As a consequence, berylliosis, or chronic beryllium disease (CBD) for US authors, is one of the most thoroughly investigated environmental chronic granulomatous disorder of the lung: the causative agent is known, both a specific immunologic test and a mineralogical test are available to confirm the pathological diagnosis and a disease-associated immune response gene has been identified in the human leukocyte antigen (HLA) locus.

Thus, berylliosis lends itself as a very promising model to analyze gene-environment interactions in an attempt to understand the pathogenesis of more common and less understood chronic inflammatory disorders of the lung. Further, unlike the majority of immune-mediated disorders in which the antigenic T cells are sequestered within an inaccessible target organ, berylliosis allows the study of an immune-mediated disease with a known pathogenic antigen and accessible target organ.

Epidemiology

Exposure to Be is the cause of a spectrum of reactions. According to the type and level of Be exposure, these vary from acute tracheobronchitis, chemical pneumonitis and metal fume fever to a chronic granulomatous lung disorder. Be toxicity has been reported since the 1930's. Marradi-Fabroni in 1935, first attributed pulmonary toxicity of Be compounds to Be metal and, at about the same time, cases of acute chemical pneumonia (13), of dermatitis (14) and of granulomatous lung reactions, initially diagnosed as sarcoidosis, (15) were reported. Notwithstanding the histopathology characterized by non caseating granulomas, it was hotly debated at the time whether the disease was caused by Be toxicity (16, pages 1-2) or by the immunologic reaction to the metal (17).

The acute type of disease was typically due to exposure to concentrations of soluble Be ranging from 25 to 600 mg/m3 (18) while the granulomatous could occur after exposure to much lower concentrations of insoluble Be, with a variably long latency period. Of the 649 cases described by Tepper an co-workers in 1961, the majority of the acute cases (85.9%) came from the extraction industry, while the majority of the chronic cases from the fluorescent lamp industry (56.2%), metallurgical plants (17.6%), ceramics (6%) and research (0.7%). Strikingly, 11% of the chronic cases were associated with bystander or environmental exposures (16, page 24). Berylliosis is still observed in association of a number of Be productions. The larger numbers of cases have been reported from the extraction, ceramics, metallurgic and nuclear industries; some cases have also been observed in a variety of other exposure situations including precious metal recovery operations (19, 20), dental shops (21) and subjects wearing dental prostheses containing Be alloys (22).

Consistent with the observations that machinists in metallurgical plants had higher disease prevalence (23), Kreiss and co-workers, using job history analysis and historic exposure

assessment have shown that disease rates vary in fact with the exposure type and intensity, from less than 1% in less exposed workers and clerical workers to more than 10% in more exposed machinists (24-27). These findings, demonstrating a dose-response effect to the levels of inhaled Be, raise again the question of the role of hypersensitivity in the pathogenesis and the prevalence of disease.

Berylliosis diagnosis.

The histopathologic hallmark of the chronic form of berylliosis is the non necrotizing non-caseating granuloma. However, the granulomas of berylliosis are indistinguishable from those of sarcoidosis (28), non-caseating tuberculosis, hypersensitivity pneumonitis, Crohn's disease (29, 30) and those induced by aluminum (31) and titanium (32). The lung tissue shows a characteristic profusion of sarcoid-like non-caseating granulomas, occurring together with interstitial mononuclear-cell pneumonitis, which may be the dominant feature in a minority of cases, and fibrosis. Granulomatous lesions can also be found in the skin, the liver, the spleen, lymph nodes, the myocardium, skeletal muscles, the kidney, bone and salivary glands (33). The granulomas are comprised of epithelioid cells and Langhans giant-type cells surrounded by lymphocytes interspersed with hyaline material and, rarely, areas of focal necrosis. Some types of inclusions, such as Schaumann bodies and asteroid bodies, are typical of granulomatous diseases but none can be considered specific or diagnostic of berylliosis (16, 30, 34, 35). As end stage fibrosis overtakes inflammation, persistent Shauman bodies in the interstitium may be the only clue to previous granulomas formation.

The classic diagnostic criteria for berylliosis included: a positive history of exposure to Be or demonstration of Be in tissue specimens, a chest X-radiograph showing diffuse reticulo-nodular infiltrates and or hilar adenopathy; abnormal pulmonary function tests and a lung biopsy

showing non-caseating granulomas (36). Since then the introduction of lymphocyte testing has added a specific etiologic test to this list.

Current immunologic testing for Be sensitization use *in vitro* radiometric Be-stimulated proliferation tests of blood and lung T cells (blood Be-LPT and lung Be-LPT). Several reports claiming test sensitivities in the range of 80 to 100% for the blood Be-LPT (24, 37-39) have not been confirmed by the studies of Stokes and Rossman, who determined 38% test sensitivity and 97% test specificity (40) and by Markhan who describes test sensitivities ranging from 40 to 70% with an even lower inter-laboratory reproducibility (41). The test still remains the standard diagnostic test for Be hypersensitivity (42) since it adds an etiologic component of the classic criteria. The lung Be-LPT, that is performed on mononuclear cells obtained by bronchoalveolar lavage (BAL), is thought to be more sensitive and specific (43).

Berylliosis immunopathology.

In a normal nonsmoking individual, 10-15% of BAL cells are lymphocytes (44). On the other hand, dramatic increases in the percentage and number of both BAL macrophages and lymphocytes occur in berylliosis (45, 46). Most of the BAL lymphocytes are $CD4^+$ T cells, and the majority of Be-specific $CD4^+$ T cells in blood and lung express markers consistent with an effector-memory T-cell phenotype (e.g., expression of CD45RO and lost of both CD62L and CCR7 expression) (46-48).

$CD4^+$ T cells play a critical role in the immunopathogenesis of berylliosis (44, 45, 49-51). The Be-LPT, the diagnostic test for berylliosis, detects Be-induced proliferation of blood and BAL T cells in culture. The development of granulomatous inflammation in the lung of both berylliosis and sarcoidosis patients is temporally associated with the accumulation of $CD4^+$ T cells in the BAL (46, 52, 53). In addition, T-cell receptor (TCR) expression of these lung cells

frequently shows a pattern specific for berylliosis patients and indicative of conventional antigen stimulation (54, 55). The importance of T cells is further supported by studies of berylliosis patients who have been patch tested with $BeSO_4$. Patch testing resulted in $CD4^+$ T cell infiltration of the dermis and subsequent granuloma formation in patients with berylliosis but not control subjects (52, 56). Comparing TCR usage by $CD4^+$ T cells infiltrating skin to those clones found in BAL, identical oligoclonal T-cell populations were seen, confirming the importance of these Be-specific $CD4^+$ T cells in the initiation of the granulomatous response (52).

Once $CD4^+$ T cells are activated in the lungs of berylliosis patients, these cells clonally proliferate and secrete Th1-type cytokines such as interleukine-2 (IL-2), interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α) (47, 57). Th2-type cytokines are not detectable in the lungs of berylliosis patients (47). This polarized Th1-type response results in macrophage activation, accumulation, and aggregation and the development of granulomatous inflammation (48, 58). The ensuing immune response occurs in a self-perpetuating manner, which appears to be compartmentalized to the lung (47, 54, 55). Interleukine-10 (IL-10) is also produced by BAL cells after Be stimulation and has minimal inhibitory effects on the ongoing immune response occurring in the lungs of berylliosis patients, possibly due to the persistence of the Be antigen (59). Thus, in the absence of down-regulation of the immune response by endogenous IL-10 and/or interleukine-4 (IL-4), the Be-stimulated immune response occurs unabated with the eventual development of fibrosis and pulmonary dysfunction.

Unlike most immune-mediated diseases in which the antigen-specific T cells in the target organ are inaccessible, Be-induced disease serves as an ideal system in which to investigate the role of different human memory $CD4^+$ T-cell subsets in disease. Using intracellular cytokine staining in a subset of berylliosis patients, it has been found that a large frequency of Be-specific $CD4^+$ T cells (mean 18%) in the BAL (47). For example, a similar percentage of BAL $CD4^+$ cells

expressed IFN-γ (mean ± SEM, 15.3 ± 2.7%) and TNF-α (17.8 ± 3.6%) after Be exposure in culture. A lower frequency of Be-specific BAL-$CD4^+$ T cells expressed IL-2 (8.8 ± 1.9%) after Be exposure, and these cells represented a subset of the IFN-γ and TNF-α secreting cells. These findings reveal a remarkably large number of antigen-specific $CD4^+$ T cells in the target organ of berylliosis patients. Be-specific $CD8^+$ T cells were not identified in the BAL.

Conversely, the frequency of Be-responsive $CD4^+$ T cells in the blood of berylliosis patients ranged from undetectable to ~1.0% and directly correlated with the extent of alveolar inflammation, as measured by both BAL WBC and lymphocyte counts (48). Greater numbers of IFN-γ- and IL-2-producing Be-specific $CD4^+$ T cells were seen in the blood of berylliosis patients compared to Be-sensitized subjects (48).

Interestingly, a lack of correlation between the frequency of Be-specific, cytokine-producing-$CD4^+$ T cells in blood and/or BAL and Be-induced proliferation has been observed (47, 48). These data suggest that antigen-specific T cells may retain their ability to secrete Th1-type cytokines but lose their ability to proliferate. Despite the fact the majority of Be specific $CD4^+$ T cells express an effector-memory T-cell phenotype, a recent study showed that the ability of Be-specific $CD4^+$ T cells to proliferate in the presence of Be salts in culture was strongly correlated with the fraction expressing a central-memory T-cell phenotype (48). Thus, the functional properties of the Be-specific $CD4^+$ T cell in blood are determined by the relative proportion of memory T-cell subsets, which is influenced by the degree of target organ inflammation.

Activation of Be-specific $CD4^+$ T cells requires the engagement of the TCR-surface with an unknown antigen bound by major histocompatibility complex (MHC) class II molecules on the surface of antigen-presenting cells in presence of Be (45). Analysis of TCR expression on BAL $CD4^+$ T cells from berylliosis patients has shown the presence of oligoclonal expansions

characteristic of T-cell responding to a conventional T-cell antigen (54, 55). These TCRs were specific for Be, compartmentalized to the lung and persisted in the lung at high frequency in patients with active disease. In certain berylliosis patients, a *TCRBV3* motif was identified with a related complementarity-determining region 3, expressing an invariant aspartic acid (Asp) at position 96 of the β-chain, as well as certain other conserved residues (55-60). These studies suggest that T cells bearing this TCR motif were selected and expanded in the lung in response to Be (49, 55, 60).

Berylliosis immunogenetics.

Early epidemiological studies of residents living within 0.25 miles of the Lorain (Ohio) extraction plant showed an incidence of 1%, not dissimilar to that of 1.3% of the extraction workers in the same plant; this first observation suggested that hypersensitivity played a major role in disease incidence. On the contrary, the machinists showed an incidence of 4.9% (61).

Further, animal studies have shown that the response to Be is genetically determined. Inhalation of BeO induced marked macrophage and lung T-lymphocytes activation in strain 2 but not in strain 13 of guinea pigs (62), and Be-induced lung granulomatous reactions are associated with certain but not all H2 genes of mice (63). The finding that lung T cells from individuals with berylliosis recognize Be as a specific HLA class II restricted hapten/antigen prompted the search for immune response genes associated with Be hypersensitivity.

In 1993, Richeldi and co-workers (64) in a retrospective study of 33 cases of berylliosis, found the HLA-DPB1*0201 allele positively associated with disease i.e., with "disease risk" and the allele HLA-DPB1*0401 was negatively associated with disease i.e., with "disease protection". Furthermore, sequence analysis identified the polymorphism coding for a lysine to glutamic acid (Glu) change at position 69 of the β–chain of the HLA-DP molecule (HLA-

DPGlu69) as the disease risk locus (64) (see table 1 for data details).

This finding was confirmed by 8 independent studies where HLA-DPGlu69 was found associated with the disease, with a prevalence of 73 to 95% in 270 disease cases versus 30 to 48% in 988 exposed controls (table 1) (65-72). The Glu69 marker was also associated with Be-sensitization without disease in 3 studies (table 1) (68-70). In one study HLA-DPGlu69 was associated with disease but not with sensitization (table 1) (67), and in one both with disease and sensitization, with a significantly higher Glu69 prevalence in the 33 diseased (phenotype frequency 82%, allele frequency 52%) than in the 68 sensitized (phenotype 68%, allele 32%) (71).

Table 1. Summary results of the previous reported studies on the association between HLA-DPGlu69 and berylliosis.

Original study[1]	N of BEC[2] evaluated	N of BHwG[3] evaluated	N of BHnoG[4] evaluated	HLA-DPGlu69 association in the different studies			
				Phenotype frequency			
				BEC	BHwG	BhnoG	P
Richeldi et al. 1993 (ref.64)	44	33	Not tested	27%	97%	/	0.0001
Richeldi et al. 1997 (ref. 65)	119	6	2	83%	30%	0%	0.01 BEC vs BHwG
Wang et al. 1999 (ref. 66)	75 evaluated for Glu69 frequency; 34 HLA-typed	20 evaluated for Glu69 frequency 19 HLA-typed	2 (included in BEC)	45%	95%	/	0.0006
Saltini et al. 2001 (ref. 67)	93	22	23	40%	73%	39%	0.02 BEC vs BHwG; 0.05 BHwG vs BhnoG
Wang et al. 2001 (ref. 68)	163	20	25	37%	95%	88%	0.001 respect to BEC
Rossman et al 2002 (ref. 69)[5]	82	25	30	48%	84%	87%	0.002 BEC vs BHwG; 0.003 BhnoG vs BEC
Maier et al. 2003 (ref. 70)	94 out of 104 typed for HLA-DP	48 out of 50 typed for HLA-DP	115 out of 125 typed for HLA-DP	38%	86%	85%	<0.0001 BHwG vs BEC, <0.0001 BhnoG vs BEC
McCanlies et al. 2004 (ref. 71)[6]	730	64	90	33%	82%	68%	0.01 BEC vs BHwG;and BhnoG vs BEC
Amicosante et al. 2005m (ref. 72)	86	36	38	48%	86%	55%	<0.0001 BEC vs BHwG; 0.008 BHwG vs BhnoG

Notes:

1. All studies are cited in references section.

2. BEC: Be-exposed controls.

3. BHwG: Be-hypersensitive with granuloma proven berylliosis.

4. BHnoG: Be-hypersensitive without granulomas.

5. Alleles presenting a Post hoc cell contribution > $\pm$1.96 compared with controls at which is associated a high probability to be significant.

6. The study analyzes only the HLA-DPGlu69 marker.

Further, a role for HLA-DPGlu69 homozygosity was also hypothesized in the determination of disease susceptibility. In seven studies DPGlu69 homozygosity frequencies were higher in berylliosis cases, (0 to 30%, CTR 1.3 to 10.9%) but lower than the frequencies expected from the Hardy-Weinberg equation (17.6 to 39.1; CTR 3.3 to 8.6%) (see table 2 for details). Although in three studies the difference between affected and control populations was statistically significant (66, 68, 70), the results of the one study large enough for precise statistical analysis (71) indicated that increased homozygosity could be attributed to excess heterozygosity i.e., to increased prevalence of the HLA-DPGlu69 gene variant driven by environmental pressure. The observation that 3 to 27% of berylliosis-affected subjects do not carry HLA-DPGlu69, prompted the search for additional susceptibility markers including other HLA genes and cytokines polymorphisms. These questions have represented some of the topics on which the work reported in this thesis focused. Thus, these aspects are largely debated in chapter 2 of the thesis in which specific data are presented.

Table 2. Analysis of the Hardy-Weinberg (HW) law in the immunogenetic studies on berylliosis in which HW equilibrium was not taken into consideration.

	Glu69 homozygosity in berylliosis		**Glu69 homozygosity in Be-sensitised**		**Glu69 homozygosity in Be-exposed CTRs**		
	Observed	**HW expected**	**Observed**	**HW expected**	**Observed**	**HW expected**	
Richeldi 1993 (ref. 64)	8/33 (24%)	36.7%	NA	NA	4/44 (9%)	3.3%	Glu69 homozygosity 1.5-fold LOWER than expected in CBD Glu69 homozygosity 2.7-fold HIGHER than expected in controls
Wang 1999 (ref. 66)	6/20 (30%)	39.1%	NA	NA	1/75 (1.3%)	5.4%	Glu69 homozygosity 1.3-fold LOWER than expected in CBD Glu69 homozygosity 4.1-fold LOWER than expected in controls
Wang 2001 (ref. 67)	6/20 (30%)	39.1%	6/25 (24%)	31.4%	5/163 (3.1%)	4.2%	Glu69 homozygosity 1.3-fold LOWER than expected in CBD Glu69 homozygosity 1.3-fold LOWER than expected in BeS Glu69 homozygosity 1.3-fold LOWER than expected in controls
Saltini 2001 (ref. 68)	5/21 (23.8%)	22.8%	1/23 (4.3%)	4.7%	5/93 (5.4%)	5.1%	Observed Glu69 homozygosity almost EQUAL (between 0.9-1.1) than expected in all study groups
Rossman 2002 (ref. 69)	0/25 (0%)	17.6%	5/30 (17%)	28.4%	9/82 (10.9%)	8.6%	Glu69 homozygosity NOT observed in CBD Glu69 homozygosity 1.7-fold LOWER than expected in BeS Glu69 homozygosity 1.3-fold HIGHER than expected in controls
Maier 2003 (ref. 70)	24/94 (25.5%)	31.1%	7/48 (14.6%)	25%	2/115 (1.7%)	4.2%	Glu69 homozygosity 1.5-fold LOWER than expected in CBD Glu69 homozygosity 1.7-fold LOWER than expected in BeS Glu69 homozygosity 2.5-fold LOWER than expected in controls

HLA-DP structure and function in susceptibility to berylliosis

The HLA-DPGlu69 could be a DNA marker associated with berylliosis due to linkage disequilibrium with other nearby immune response genes or it might play a direct role in the disease as the antigen-Be receptors for the presentation of Be to lung T cells. The data generated in several laboratories strongly suggest that the latter is the likely case.

Ex vivo and *in vitro* studies have shown that Be is presented to $CD4^+$ T cells in the context of the MHC class II molecules (45, 60, 73). Using T cells from berylliosis patients or Be-specific T cell lines and clones derived from blood and lung of different berylliosis patients, both Be-induced proliferation and cytokine secretion were nearly completely and selectively inhibited by the addition of a monoclonal antibody (MoAb) directed against HLA-DP. As a result, HLA-DP was the primary immune response gene involved in Be presentation (47, 60, 73-75). In these studies, only certain HLA-DP molecules, all carrying HLA-DPGlu69, were capable of Be presentation, and the restricting MHC class II molecules closely matched those implicated in disease susceptibility (Table 3).

Table 3. Amino acid residues of *HLA-DPB1* alleles involved in berylliosis susceptibility[1].

			Amino acid position												
***HLA-DPB1* allele**	**Genetic susceptibility**	**Ability to present Be**	**35**	**36**	**55**	**56**	**57**	**67**	**68**	**69**	**76**	**84**	**85**	**86**	**87**
*DPB1*0101*	No	-	Y	A	A	A	E	E	E	K	V	D	E	A	V
*DPB1*0401*	No	-	F	-	-	-	-	-	-	-	M	G	G	P	M
*DPB1*0402*	Yes[2]	-	F	V	D	E	-	-	-	-	M	G	G	P	M
*DPB1*0501*	No	-	F	V	E	-	-	-	-	-	-	-	-	-	-
*DPB1*0201*	Yes	+	F	V	D	E	-	-	-	**E**	M	G	G	P	M
*DPB1*0601*	Yes	+	F	V	D	E	D	-	-	**E**	M	-	-	-	-
*DPB1*1001*	Yes	+	F	V	D	E	-	-	-	**E**	-	-	-	-	-
*DPB1*1301*	Yes	+	-	-	-	-	-	-	-	**E**	M	-	-	-	-
*DPB1*1701*	Yes	+	F	V	D	E	D	-	-	**E**	M	-	-	-	-

1. **Sequences for the different HLA-DP alleles are presented on the basis of their homology to *DPB1*0101*, and a dash indicates the same amino acid at that position. The bolded amino acid residues indicate the only shared amino acids between the presenting *HLA-DPB1* alleles.**

2. **Marginal association only in one immunogenetic study (ref. 69).**

Further, with the limitation of only a small number of functional studies, the data suggest that the critical amino acid residue in the HLA-DP molecules for the Be presentation is the Glu at position 69 of the β-chain (60, 73-76). In fact, using fibroblasts expressing mutated HLA-DP2 molecules, Be recognition dependent solely on the Glu at position 69 of the DP β-chain (38). Mutagenesis of other polymorphic amino acid residues such as the Lys and Glu at positions 55 and 56 of the DPβ-chain had no effects on either Be-induced proliferation or Th1-type cytokine expression (76).

The β69 residue of HLA-DP molecule is the homologue of the residue 71 of the HLA-DRβ–chain molecule located in the pocket 4 of the peptide-binding groove. Our previous study (77) suggests that Be presents an affinity from 40 to 100-fold higher for the HLA-DP molecules expressing a Glu at position β69 compared to HLA-DP molecules expressing its counterpart, the Lys β69, suggesting a direct interaction between HLA-DPGlu69 molecule and Be.

Specific aspects of the structure/function relationship of HLA class II molecules and Be in the presentation to Be-specific T cells are some of the topics reported in this thesis. Chapter 3 and 4 of the thesis discuss these questions in detail, data in hand.

AIM OF THE THESIS

This thesis summarizes two already published papers and one manuscript submitted for publication in the past 3 years, describing the studies in the immunogenetic, functional immunogenetic and *in vitro* model of berylliosis therapy. These studies have represented important advances in the field of berylliosis immunogenetics and functional immunogenetics and also in the contribution of HLA-DP to immune response.

Chapter 2 reports immunogenetic studies on berylliosis, more specifically the identification of the secondary immune response gene associated with susceptibility to develop Be hypersensitivity *(Respir Res. 2005; 6:94).*

Chapter 3 reports the studies on the role of peptide binding and T-cell activation of HLA-DP polymorphisms and in particular the role of the Glu/Lys polymorphism at position β69 *(Tissue Antigen. 2003; 62:459-71).*

Finally, in chapter 4, with the background of functional immunogenetic and HLA-DP peptide-binding studies, an *in vitro* model of specific therapy for Be disease is investigated *(submitted in 2006).*

CHAPTER 2 - IMMUNOGENETIC STUDIES

IDENTIFICATION OF HLA-DR PHENYLALANIN β47 AS THE SUSCEPTIBILITY MARKER OF HYPERSENSITIVITY TO BERYLLIUM IN INDIVIDUALS LACKING THE BERYLLIOSIS-ASSOCIATED SUPRATYPIC MARKER HLA-DPGLUβ69.

The data reported in this paragraph have been already published (Respir Res. 2005; 6: 94) and are fully available at http://respiratory-research.com/content/6/1/94.

Introduction

The observation that Be disease affects only 1 to 16% of Be-exposed individuals led to the hypothesis that genetic susceptibility may play an important role in the pathogenesis of this disease (6). In 1993, the HLA-DP supratypic variant characterized by a Glu at position 69 of the HLA-DP molecule β–chain (DPGlu69) was identified as a genetic marker of susceptibility to Be hypersensitivity (BH), an observation subsequently confirmed by seven independent studies (64-71), as already reported in chapter 1. Two independent studies have also identified the HLA-DPGlu69 marker as the immune response gene responsible for presentation of Be to Be-specific T cells (60, 73); an immunochemical study has also suggested that the structural basis for Be presentation by the HLA-DPGlu69-positive molecules is in its unique ability to bind Be with high affinity possibly in the context of a coordination bond formed by the contribution of other electron-donor groups present in the fourth pocket of the peptide-binding groove of the HLA-DP molecule (77). Further, Ab-inhibition studies have shown that Be-presentation to blood and lung T cells in DPGlu69-positive subjects is inhibited almost exclusively by anti-HLA-DP Abs (73, 74), strongly indicating

HLA-DPGlu69 as the immune response gene used by DPGlu69-positive subjects i.e., about 80% of the BH-affected population (64-71).

In contrast, the HLA gene which might function as the immune response gene in DPGlu69-negative BH-affected subjects i.e., in the remaining 20% of the BH-affected population, has not yet been determined.

Previous studies have identified the HLA-DRB1 alleles belonging to the *01 group (70) as negatively associated with berylliosis, while the HLA-DRB1 variants Ser11 (69), Tyr26 (69), Asn37 (69), Glu71 (69) and Arg74 (67) and the HLA-DQ variant Gly86 (69) were positively associated with BH. Analysis of the role of these markers has, however, been hampered by the small size of the populations examined in most studies. In all studies published so far, the putative susceptibility markers covered only 40 to 50% of the DPGlu69-negative subjects. In this context, our previous study (67) on 45 individuals affected by Be sensitization with or without demonstrable lung granulomas, showed that HLA-DRArg74 and Tyr26 were associated with sensitization without lung granulomas, and HLA-DPGlu69 with sensitization accompanied by lung granulomas, thereby suggesting a different role for Glu69 and these markers (67). However, in the HLA-DPGlu69 negative subjects reported in the Saltini et al. study population, HLA-DRArg74 and Tyr26 were expressed only by 11 out of 19 DPGlu69-negative sensitized subjects 10 of which without and one with demonstrable lung granulomas (67). In another study in the field conducted by Rossman et al. (69) evaluating 56 BH-affected subjects, 4 out of 7 DPGlu69-negative patients carried either DRAsn37, DRGlu71 or DQGly86 (67). Finally, Maier and co-workers (70), in 19 HLA-DP Glu69-negative BH subjects, found that HLA-DRB1*13 alleles were associated with BH susceptibility; however, they were only expressed by 12 of these subjects.

In order to search for this(these) disease associated immune response gene(s), we re-evaluated a previously described population (67) after a follow-up of 7 years that allowed us to

extend the study to other 29 newly identified BH subjects (14 with biopsy proven lung granulomas and 15 with Be-sensitization without lung granulomas) for a total number of 74 BH subjects and 86 Be-exposed controls. This panel included a sufficiently large number of DPGlu69-negative subjects to analyze phenotypic frequencies of all aminoacid variants of the HLA-DPB1, -DQB1, -DRB1, -DRB3, -DRB4 and -DRB5 genes in BH-affected and Be-exposed controls.

Methods

Study population. The study population, already described in part in a previous study (67), includes 86 Be-exposed healthy controls and 74 subjects affected by Be hypersensitivity, all working in the same Be manufacturing plant, 45 of whom (23 Be-sensitized subjects and 22 berylliosis-affected i.e., Be-sensitized subjects with biopsy proven lung granulomas) have been already described in the Saltini et al. report (67). Study subjects are categorized as (i) Be-exposed controls, when having negative blood Be-LPT test, (ii) Be-sensitized, when having 2 blood Be-LPT positive tests and (iii) berylliosis-affected when having 2 blood Be-LPT-positive tests and/or biopsy proven lung granulomas (67). While 4 control subjects were diagnosed with Be sensitization and 3 with berylliosis during the 7-years follow up, none of the subjects in the previous study progressed from sensitization to berylliosis.

Overall the population in this report included 36 berylliosis (age 40 ± 7 years; 33 Caucasians, 2 African-Americans and 1 Asian; 32 males and 4 females; mean duration of Be-exposure 11±7 years) and 38 showed Be-sensitization without lung granulomas detected by trans-bronchial biopsy (age 43±9 years; 37 Caucasians and 1 Afro-American; 31 males and 7 females; mean age of Be-exposure 17±9 years) and 86 Be-exposed controls (age 44 ± 9 years; 81 Caucasians, 2 African-American, 2 Hispanics, 1 Asian; 71 males and 15 females; mean duration of Be-exposure 16 ± 11 years).

High resolution HLA class II typing. High resolution HLA class II typing for the HLA-DPB1, -DQB1, -DRB1, -DRB3, -DRB4, -DRB5 loci were performed by standard protocols as already reported (10).

Be-lymphocyte proliferation test (Be-LPT). The Be-LPT as measures of the T-lymphocytes response against Be in peripheral blood was performed by standard method (79). Briefly, peripheral blood mononuclear cells (PBMC) were tested against three doses Be sulfate ($BeSO_4$*$4H_2O$) at 1, 10, and 100 μM at 3 and 5 days. T-cell proliferation were evaluated by tritium [^{3}H] labeled thymidine (TdR) incorporation and a stimulation index (SI) calculated as the ratio of the radioactivity (counts per minute) of Be-exposed cell cultures to the count rate of unstimulated cultures. A test was defined as abnormal when two or more stimulation index values of six possible values exceeded the normal standard ratio of 3.0 (stimulated to unstimulated) as the cut-off.

Lymphocyte proliferation to Be salt and MoAb inhibition of lymphocyte activation. T-cell proliferation in response to $BeSO_4$ and inhibition by anti-HLA class II MoAbs were performed as previously described (74). Briefly, PBMCs obtained from patients with Be hypersensitivity were isolated from heparinized whole blood by density centrifugation on Ficoll Hypaque gradient. PBMCs (2×10^5 cells/well) were then cultured in 96-well flat-bottomed microtiter plates in RPMI 1640 tissue culture medium supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in the presence of Be sulfate ($BeSO_4$*$4H_2O$) at 10-50-100 μM (all reagents form Sigma Co., St. Louise, MO). Phytohemoagglutinin (PHA, 5 μg/ml, Sigma) and *Candida albicans* (10 μg/ml) were used as positive controls. T-lymphocyte proliferation was measured by [^{3}H]TdR incorporation. Cells were pulsed with 1μCi of [^{3}H]TdR (Amersham International, Amersham, UK) after 5 days of culture and harvested onto glass fiber filters 18 hours later. Proliferation was measured as [^{3}H]TdR incorporation by liquid scintillation spectroscopy and

the test was scored as positive in the presence of a greater than twofold proliferation index. Protein A-Sepharose purified MoAbs directed against HLA-DR (L243), HLA-DP (B7/21), HLA-DQ (L2), HLA class I (W6/32) (60) were used at increasing concentrations (10, 20 and 50 μg/ml) to inhibit antigen presentation and lymphocyte proliferation as previously described (45, 60, 74). The 19 kDa *Mycobacterium tuberculosis* (MTB19) protein MoAb (HYT6) (80) was used as control.

Statistical analysis. Statistical analysis was carried out as previously described (67, 81, 82). Phenotypic frequency data are expressed as percentages with Odds Ratio (OR) with respect to the Be-exposed control group when appropriate. Comparisons between phenotypic frequencies in the study groups were done by χ^2 test with the Yates correction where necessary. Linkage disequilibrium analysis was carried out as previously described (80). Forward and stepwise multiple logistic regression multivariate analysis were applied for identifying independent parameter(s) in multiple comparisons. Be-stimulated lymphocyte proliferation data are expressed as mean $\pm$ standard deviation of the mean (SD). Comparisons between groups in Be-lymphocyte proliferation data were done using the Student's t test with Welch's correction when appropriate. All the statistical analysis were carried out with the SPSS (SPSS inc., Chicago, IL) and GraphPad Prism (GraphPad Software Inc., San Diego, CA) packages.

Results

The allelic frequencies for HLA-DPB1, DQB1 and DRB1, 3, 4 and 5 in general population are reported in the tables 4-7.

Similarly to the previous study on this population (10) the HLA-DPGlu69 marker was carried with higher frequency by berylliosis-affected (31 out of 36, 86%) than subjects with Be-sensitization without granuloma (21 out of 38, 55%, p=0.008 vs berylliosis-affected) and Be-

exposed controls (41 out of 86, 48%, $p<0.0001$ vs berylliosis-affected, $p=0.55$ vs Be-sensitized).

The HLA-DPGlu69 was previously proposed as a marker of progression from the sensitization state to the lung granulomatous reaction of berylliosis (67, 70). In this study population there were no cases of progression from systemic sensitization to lung disease notwithstanding the substantial follow-up period of 7.0 ± 3.7 years from the first positive Be-LPT test, while 4 control subjects were diagnosed with Be sensitization and 3 with berylliosis during the 7-years follow-up. Hence, we could not directly look at the HLA-DPGlu69 association with disease progression. In addition, the frequency of the HLA-DPGlu69 homozygosity, another marker associated with disease progression (70), was higher in the disease-affected population compared to the sensitized and the Be-exposed control population [5 out of 86 healthy exposed controls (5.8%), 3 out of 38 sensitized without disease (7.9%; $p=0.97$ compared to controls) and 8 out of 36 disease-affected (22.2%; $p=0.06$ compared to controls; $p=0.10$ compared to the sensitized)] although the difference was not statistically significant.

Table 4. Frequencies of the HLA-DPB1 alleles in the study populations.

	Be-exposed controls		Be-hypersensitives (berylliosis + Be-sensitized without disease)		Berylliosis		Be-sensitized without disease	
Allele	**N alleles (172)**	**%**	**N alleles (148)**	**%**	**N alleles (72)**	**%**	**N alleles (76)**	**%**
0101	6	3.5%	6	4.1%	2	2.8%	4	5.3%
0201	36	20.9%	40	27.0%	25*	34.7%	15	19.7%
0202	1	0.6%	0	0.0%	0	0.0%	0	0.0%
0301	15	8.7%	8	5.4%	4	5.6%	4	5.3%
0401	65	37.8%	41	27.7%	13*	18.1%	28	36.8%
0402	26	15.1%	13	8.8%	8	11.1%	5	6.6%
0501	3	1.7%	6	4.1%	1	1.4%	5	6.6%
0601	1	0.6%	4	2.7%	1	1.4%	3	3.9%
0901	0	0.0%	2	1.4%	2	2.8%	0	0.0%
1001	5	2.9%	6	4.1%	4	5.6%	2	2.6%
1101	3	1.7%	3	2.0%	1	1.4%	2	2.6%
1301	1	0.6%	4	2.7%	2	2.8%	2	2.6%
1401	2	1.2%	3	2.0%	3	4.2%	0	0.0%
1501	2	1.2%	1	0.7%	0	0.0%	1	1.3%
1601	1	0.6%	3	2.0%	1	1.4%	2	2.6%
1701	0	0.0%	3	2.0%	3	4.2%	0	0.0%
1901	1	0.6%	2	1.4%	2	2.8%	0	0.0%
2001	0	0.0%	2	1.4%	0	0.0%	2	2.6%
2301	1	0.6%	1	0.7%	0	0.0%	1	1.3%
3501	1	0.6%	0	0.0%	0	0.0%	0	0.0%
7701	1	0.6%	0	0.0%	0	0.0%	0	0.0%
7801	1	0.6%	0	0.0%	0	0.0%	0	0.0%

Notes
***: Uncorrected $p<0.03$ and Bonferroni's corrected $p>0.05$ vs Be-exposed controls.**

Table 5. Frequencies of the HLA-DQB1 alleles in the study populations.

	Be-exposed controls		Be-hypersensitives (berylliosis + Be-sensitized without disease)		Berylliosis		Be-sensitized without disease	
Allele	**N alleles (172)**	**%**	**N alleles (148)**	**%**	**N alleles (72)**	**%**	**N alleles (76)**	**%**
0201	24	14.0%	33	22.3%	15	20.8%	18	23.7%
0202	0	0.0%	2	1.4%	0	0.0%	2	2.6%
0301	37	21.5%	23	15.5%	11	15.3%	12	15.8%
0302	19	11.0%	10	6.8%	4	5.6%	6	7.9%
0303	8	4.7%	8	5.4%	4	5.6%	4	5.3%
0304	1	0.6%	0	0.0%	0	0.0%	0	0.0%
0402	4	2.3%	2	1.4%	0	0.0%	2	2.6%
0501	14	8.1%	17	11.5%	7	9.7%	10	13.2%
0502	4	2.3%	1	0.7%	0	0.0%	1	1.3%
0503	5	2.9%	3	2.0%	1	1.4%	2	2.6%
0601	2	1.2%	0	0.0%	0	0.0%	0	0.0%
0602	28	16.3%	26	17.6%	13	18.1%	13	17.1%
0603	14	8.1%	15	10.1%	12	16.7%	3	3.9%
0604	9	5.2%	6	4.1%	4	5.6%	2	2.6%
0609	3	1.7%	2	1.4%	1	1.4%	1	1.3%

Table 6. Frequencies of the HLA-DRB1 alleles in the study populations.

	Be-exposed controls		Be-hypersensitives (berylliosis + Be-sensitized without disease)		Berylliosis		Be-sensitized without disease	
Allele	**N alleles (172)**	**%**	**N alleles (148)**	**%**	**N alleles (72)**	**%**	**N alleles (76)**	**%**
0101	10	5.8%	15	10.1%	6	8.3%	9	11.8%
0102	2	1.2%	1	0.7%	0	0.0%	1	1.3%
0103	1	0.6%	0	0.0%	0	0.0%	0	0.0%
0301	13	7.6%	24*	16.2%	9	12.5%	15*	19.7%
0401	18	10.5%	5*	3.4%	3	4.2%	2	2.6%
0402	1	0.6%	2	1.4%	0	0.0%	2	2.6%
0404	4	2.3%	4	2.7%	1	1.4%	3	3.9%
0405	3	1.7%	1	0.7%	0	0.0%	1	1.3%
0407	1	0.6%	0	0.0%	0	0.0%	0	0.0%
0408	2	1.2%	0	0.0%	0	0.0%	0	0.0%
0410	1	0.6%	0	0.0%	0	0.0%	0	0.0%
0701	17	9.9%	18	12.2%	10	13.9%	8	10.5%
0801	3	1.7%	2	1.4%	0	0.0%	2	2.6%
0806	0	0.0%	2	1.4%	2	2.8%	0	0.0%
0810	0	0.0%	1	0.7%	0	0.0%	1	1.3%
0901	0	0.0%	1	0.7%	0	0.0%	1	1.3%
1001	1	0.6%	1	0.7%	1	1.4%	0	0.0%
1101	14	8.1%	9	6.1%	5	6.9%	4	5.3%
1102	2	1.2%	2	1.4%	1	1.4%	1	1.3%
1103	5	2.9%	1	0.7%	1	1.4%	0	0.0%
1104	3	1.7%	4	2.7%	3	4.2%	1	1.3%
1201	1	0.6%	2	1.4%	1	1.4%	1	1.3%
1301	15	8.7%	18	12.2%	14*#	19.4%	4	5.3%
1302	12	7.0%	8	5.4%	5	6.9%	3	3.9%
1303	4	2.3%	2	1.4%	0	0.0%	2	2.6%
1401	5	2.9%	3	2.0%	1	1.4%	2	2.6%
1406	1	0.6%	0	0.0%	0	0.0%	0	0.0%
1501	26	15.1%	21	14.2%	9	12.5%	12	15.8%
1502	3	1.7%	0	0.0%	0	0.0%	0	0.0%
1601	4	2.3%	1	0.7%	0	0.0%	1	1.3%

Notes
***: Uncorrected p<0.03 and Bonferroni's corrected p>0.05 vs Be-exposed controls.**
#: Uncorrected p<0.02 and Bonferroni's corrected p>0.05 vs Be-sensitized without disease.

Table 7. Frequencies of the HLA-DRB3, -DRB4 and -DRB5 alleles in the study populations.

	Be-exposed controls		Be-hypersensitives (berylliosis + Be-sensitized without disease)		Berylliosis		Be-sensitized without disease	
Allele	**N DRB3 alleles (77)**	**%**	**N DRB3 alleles (77)**	**%**	**N DRB3 alleles (40)**	**%**	**N DRB3 alleles (33)**	**%**
3*0101	21	27.3%	29	37.7%	9	22.5%	20*#	60.6%
3*0201	2	2.6%	0	0.0%	0	0.0%	0	0.0%
3*0202	42	54.5%	36	46.8%	26	65.0%	10*#	30.3%
3*0301	12	15.6%	8	10.4%	5	12.5%	3	9.1%
	N DRB4 alleles (48)		**N DRB4 alleles (31)**		**N DRB4 alleles (14)**		**N DRB4 alleles (17)**	
4*0101	10	20.8%	7	22.6%	3	21.4%	4	23.5%
4*0103	38	79.2%	24	77.4%	11	78.6%	13	76.5%
	N DRB5 alleles (34)		**N DRB5 alleles (22)**		**N DRB5 alleles (9)**		**N DRB5 alleles (13)**	
5* 0101	28	82.4%	21	95.5%	9	100.0%	12	92.3%
5* 0102	2	5.9%	0	0.0%	0	0.0%	0	0.0%
5* 0202	4	11.8%	1	4.5%	0	0.0%	1	7.7%

Notes
***: Uncorrected p<0.02 and Bonferroni's corrected p>0.05 vs Be-exposed controls.**
#: Uncorrected p<0.02 and Bonferroni's corrected p>0.05 vs berylliosis.

A total number of 22 BH subjects (17 Be-sensitized and 5 berylliosis-affected) and 45 Be-exposed controls were HLA-DPGlu69 negative. They did not differ from the DPGlu69-positive subjects (neither the BH-affected nor the Be-exposed controls) in terms of gender, ethnicity, age or length of Be-exposure ($p>0.05$, all comparisons). The allelic frequencies for HLA-DPB1, -DQB1 and -DRB1, 3, 4 and 5 in HLA-DPGlu69-negative subjects are reported in the tables 8-11.

This subgroup of HLA-DPGlu69-negative subjects was analyzed for the distribution of all HLA class II polymorphic aminoacid residues, with the exception of HLA-DPGlu69, by univariate analysis. No associations were found between any of the HLA-DP polymorphic residues and BH. Strikingly, among the polymorphic residues of the HLA-DRβ-chain coded for by the HLA-DRB1 locus, residues Ser13, Tyr26, His32, Asn37, Phe47 and Arg74 were associated with Be hypersensitivity (Table 12). Similarly, the HLA-DRβ-chain HLA-DRB3 locus polymorphic residues Arg11, Tyr26, Asp28, Leu38, Ser60 and Arg74 were found associated to Be hypersensitivity (Table 12). No polymorphisms associated with Be hypersensitivity were found in the HLA-DRB4 and -DRB5 loci. Finally, a statistically significant association with Be hypersensitivity in HLA-DPGlu69 negatives was found for the HLA-DQB1 gene polymorphic residue Leu26 (Table 12).

However, as a linkage disequilibrium could exist between the HLA-DRB1 gene coded residues and other HLA-DRB1, -DRB3 and -DQB1 loci, in order to identify the independently associated residue(s) in the HLA-DPGlu69-negative BH subjects, multiple logistic regression models were carried out on all the HLA variants above. As a result, only HLA-DRPhe47 (OR 2.956, $p<0.05$) was identified as independently associated with Be hypersensitivity in the HLA-DPGlu69-negative subgroup, hence suggesting that the other HLA-DRB1, -DRB3 and –DQB1 loci shown in table 1 could be associated with Be hypersensitivity due to linkage disequilibrium with HLA-DRPhe47. Of the 22 HLA-DPGlu69-negative subjects with BH, 21 were HLA-DRPhe47 (16 Be-

sensitized and 5 berylliosis-affected) and only one, a Be-sensitized individual, was HLA-DPGlu69 and HLA-DRPhe47-negative.

Further, in order to identify which of the HLA isotypic molecules associated with Be hypersensitivity could function as the restriction elements of Be-stimulated T-cell proliferation in HLA-DPGlu69-negative subjects, we analyzed PBMC proliferation in response to $BeSO_4$ in a subgroup of 15 BH-affected subjects, using Abs directed against HLA-DR, HLA-DQ and HLA-DP as probes.

In 4 HLA-DPGlu69-negatives subjects, Be-stimulated T-cell proliferation was inhibited by anti-HLA-DR MoAbs (range 70-92% inhibition) significantly more than by anti-HLA-DP MoAbs (range: 6-29%; $p<0.02$ compared to anti-HLA-DR) while it was not affected at all by the anti-HLA-DQ, anti-HLA class I or the anti-MTB19 control MoAbs (Figure 1), suggesting a role for HLA-DR molecules in Be presentation in HLA-DPGlu69-negative subjects. All the 4 HLA-DPGlu69-negative subjects carried the HLA-DRPhe47 polymorphism.

In contrast, in the 3 subjects who were HLA-DPGlu69-positive and HLA-DRPhe47-negative, proliferation was completely inhibited by anti-HLA-DP MoAbs (range 68-100%) but not by anti-HLA-DR (range 7-14%, $p<0.05$ compared to anti-HLA-DP), nor by anti-HLA-DQ, anti-HLA class I or the anti-MTB19 control MoAbs (Figure 1). Finally, in the 8 subjects carrying both HLA-DPGlu69 and HLA-DRPhe47, the proliferative response to $BeSO_4$ was always inhibited by anti-HLA-DP MoAbs (range: 63-100%) and variable inhibited by anti-HLA-DR MoAbs (range: 0-94%), with the inhibition by anti-HLA-DP MoAbs being significantly stronger than anti-HLA-DR MoAbs (paired t-test, $p<0.01$).

Table 8. Frequencies of the HLA-DPB1 alleles in the HLA-DPGlu69-negative population.

	Be-exposed controls		Be-hypersensitives (berylliosis + Be-sensitized without disease)	
Allele	**N alleles (90)**	**%**	**N alleles (44)**	**%**
0101	3	3.3%	2	4.5%
0301	14	15.6%	5	11.4%
0401	43	47.8%	19	43.2%
0402	18	20.0%	7	15.9%
0501	3	3.3%	4	9.1%
1101	2	2.2%	2	4.5%
1401	2	2.2%	1	2.3%
1501	1	1.1%	1	2.3%
2001	0	0.0%	2	4.5%
2301	1	1.1%	1	2.3%
3501	1	1.1%	0	0.0%
7701	1	1.1%	0	0.0%
7801	1	1.1%	0	0.0%

Table 9. Frequencies of the HLA-DQB1 alleles in the HLA-DPGlu69-negative population.

	Be-exposed controls		Be-hypersensitives (berylliosis +Be-sensitized without disease)	
Allele	**N alleles (90)**	**%**	**N alleles (44)**	**%**
0201	13	14.4%	14	31.8%*
0202	0	0.0%	1	2.3%
0301	17	18.9%	4	9.1%
0302	10	11.1%	1	2.3%
0303	5	5.6%	2	4.5%
0402	1	1.1%	1	2.3%
0501	9	10.0%	4	9.1%
0502	3	3.3%	0	0.0%
0503	4	4.4%	2	4.5%
0602	16	17.8%	9	20.5%
0603	6	6.7%	3	6.8%
0604	6	6.7%	3	6.8%

Notes
***: Uncorrected $p<0.05$ and Bonferroni's corrected $p>0.05$ vs Be-exposed controls.**

Table 10. Frequencies of the HLA-DRB1 alleles in the HLA-DPGlu69-negative population.

	Be-exposed Controls		Be-hypersensitives (berylliosis + Be-sensitized without disease)	
Allele	**N alleles (90)**	**%**	**N alleles (44)**	**%**
0101	8	8.9%	4	9.1%
0103	1	1.1%	0	0.0%
0301	7	7.8%	12	27.3%*
0401	11	12.2%	2	4.5%
0402	0	0.0%	1	2.3%
0404	4	4.4%	0	0.0%
0405	1	1.1%	0	0.0%
0408	1	1.1%	0	0.0%
0410	1	1.1%	0	0.0%
0701	11	12.2%	5	11.4%
0801	0	0.0%	1	2.3%
1101	5	5.6%	1	2.3%
1102	1	1.1%	1	2.3%
1103	2	2.2%	0	0.0%
1104	1	1.1%	0	0.0%
1301	6	6.7%	5	11.4%
1302	6	6.7%	2	4.5%
1401	4	4.4%	2	4.5%
1406	1	1.1%	0	0.0%
1501	15	16.7%	8	18.2%
1502	1	1.1%	0	0.0%
1601	3	3.3%	0	0.0%

Notes
***: Uncorrected $p<0.01$ and Bonferroni's corrected $p>0.05$ vs Be-exposed controls.**

Table 11. Frequencies of the HLA-DRB3, -DRB4 and -DRB5 alleles in the HLA-DPGlu69-negative population.

	Be-exposed controls		Be-hypersensitives (berylliosis + Be-sensitized without disease)	
Allele	**N DRB3 alleles (33)**	**%**	**N DRB3 alleles (22)**	**%**
3* 0101	9	27.3%	14	63.6%*
3* 0201	2	6.1%	0	0.0%
3* 0202	16	48.5%	6	27.3%
3* 0301	6	18.2%	2	9.1%
	33	100.0%	22	100.0%
	N DRB4 alleles (29)		**N DRB4 alleles (8)**	
4* 0101	6	20.7%	3	37.5%
4* 0103	23	79.3%	5	62.5%
	29	100.0%	8	100.0%
	N DRB5 alleles (19)		**N DRB5 alleles (8)**	
5* 0101	16	84.2%	8	100.0%
5* 0202	3	15.8%	0	0.0%

Notes
***: Uncorrected p<0.02 and Bonferroni's corrected p>0.05 vs Be-exposed controls**

Table 12. Phenotypic frequencies of the polymorphisms found associated with Be-hypersensitivity in HLA-DPGlu69-negative subjects.

	Be-exposed controls (n=45)	**Be-hypersensitives (n=22)**		
HLA-DRB1 polymorphisms[1]	**N positive subjects (%)**	**N positive subjects (%)**	**OR[2]**	**p[3]**
Ser13	28 (62.2%)	20 (90.9)	6.07	0.015
Tyr26	7 (15.6%)	10 (45.5%)	4.52	0.009
His32	21 (46.7%)	17 (77.3%)	3.89	0.017
Asn37	17 (37.8%)	16 (72.7%)	4.39	0.007
Phe47	30 (66.7%)	21 (95.5%)	10.50	0.011
Arg74	7 (15.6%)	10 (45.5%)	4.52	0.009
HLA-DQB1 polymorphism[4]				
Leu26	32 (71.1%)	21 (95.5%)	8.53	0.021
HLA-DRB3 polymorphisms[5]	**N=30**	**N=17**		
Arg11	8 (26.7%)	12 (70.6%)	6.60	0.008
Tyr26	8 (26.7%)	12 (70.6%)	6.60	0.008
Asp28	8 (26.7%)	12 (70.6%)	6.60	0.008
Leu38	8 (26.7%)	12 (70.6%)	6.60	0.008
Ser60	8 (26.7%)	12 (70.6%)	6.60	0.008
Arg74	8 (26.7%)	12 (70.6%)	6.60	0.008

Notes:

1. **HLA-DRB1 polymorphisms found associated with Be-hypersensitivity in HLA-DPGlu69-negative subjects among the overall HLA-DRB1 polymorphic variants analyzed at positions: 9, 10, 11, 12, 13, 16, 26, 28, 30, 32, 37, 38, 47, 57, 58, 60, 67, 70, 71, 73, 74, 77, 85, 86.**
2. **Odds ratio with respect to Be-exposed controls.**
3. **p value (χ^2 analysis) with respect to Be-exposed controls.**
4. **HLA-DQB1 polymorphisms found associated with Be-hypersensitivity in HLA-DPGlu69-negative subjects among the overall HLA-DQB1 polymorphic variants analyzed at positions: 9, 13, 14, 23, 26, 28, 30, 37, 38, 45, 46, 47, 52, 53, 55, 56, 57, 66, 67, 70, 71, 74, 75, 77, 84, 85, 86, 87, 89, 90.**
5. **HLA-DRB3 polymorphisms found associated with Be-hypersensitivity in HLA-DPGlu69-negative subjects among the overall HLA-DRB3 polymorphic variants analyzed at positions: 8, 11, 26, 28, 30, 37, 38, 39, 51, 57, 58, 60, 67, 74, 77, 86. No polymorphisms were found associated to Be hypersensitivity in HLA-DPGlu69-negatives in the HLA-DRB4 and -DRB5 loci and HLA-DP locus.**

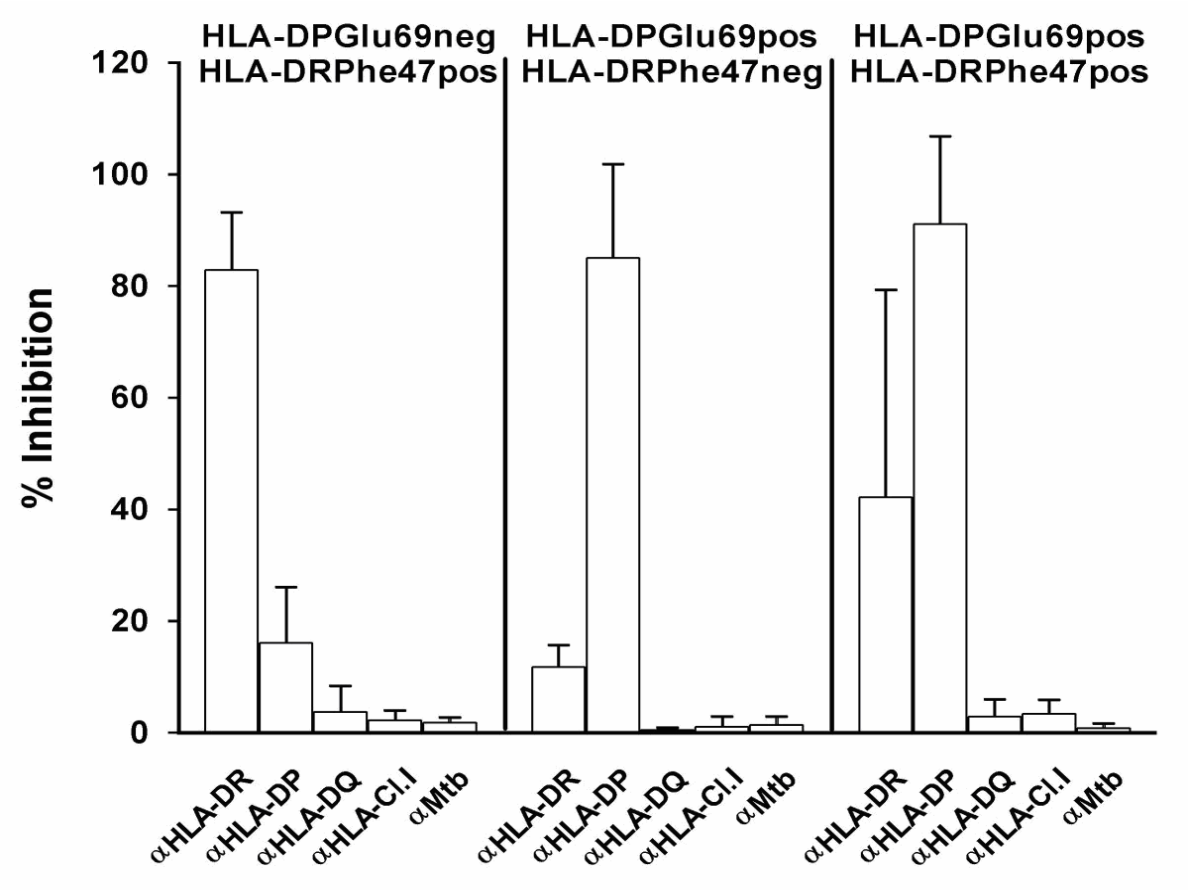


Figure 1. Inhibition of Be ($BeSO_4$)-induced proliferation, by MoAbs directed against HLA-DR, HLA-DP, HLA-DQ, HLA class I and the 19 kDa *M. tuberculosis* protein in PBMCs from BH subjects carrying or not the HLA-DPGlu69 and the HLA-DRPhe47 markers. They were 4 HLA-DPGlu69-negative/HLA-DRPhe47-positive (3 sensitized and 1 berylliosis-affected), 3 HLA-DPGlu69-positive/HLA-DRPhe47-negative (0 sensitized and 3 berylliosis-affected) and 8 HLA-DPGlu69-positive/HLA-DRPhe47-positive (3 sensitized and 5 berylliosis-affected). On the ordinate is shown the percentage of inhibition (with respect to the MoAb-untreated cells) of T-cell proliferation obtained by co-culturing the PBMCs from berylliosis patients with $BeSO_4$ in the presence of each MoAb reported on the abscissa (anti-HLA-DR: DR, anti-HLA-DP: DP, anti-HLA-DQ: DQ, anti-HLA class I: C.I, anti-19 kDa *M. tuberculosis:* Mtb).

Interim discussion

Similarly to our previous report (67) and consistently with the more recent study by McCanlies et al. (71), the re-evaluation of this patient population shows a higher prevalence of HLA-DPGlu69 among the subjects with lung granulomas compared to the Be-sensitized without lung involvement (86% vs 55%, p=0.008). However, not having identified any case of disease progression from Be sensitization to lung disease during the 7-years follow-up, we could not formally assess the association of the HLA-DPGlu69 marker with progression from sensitization to disease. Although the knowledge that HLA-DPGlu69 is the primary immune response gene of Be hypersensitivity (60, 73, 77) makes it attractive to hypothesize that the gene might induce a stronger immune reaction hence inducing granuloma formation. This hypothesis is suggested by Maier et al. (70) in which 11 out of 12 subjects progressed from sensitization to disease status were HLA-DPGlu69-positives (70), as well as in more recent publication in which Be-sensitized subjects progress to berylliosis at a rate of 6-8% per year (83). However, it could be also considered that all these data could be inferred by the possibility of misdiagnosis in the identification of the Be-induced granuloma.

The finding that a sizeable fraction of BH-affected subjects, varying from 3 to 27% in published reports (64-71), do not carry HLA-DPGlu69 has indicated that other HLA molecules may provide the restriction element of Be-stimulated T-cell proliferation and may be implicated in the pathogenesis of susceptibility to BH.

In this regard, with all the limitations imposed by the need of performing specific antigen-presentation studies using specific reagents such as HLA-DRPhe47 restricted antigen-presenting cells and/or HLA-DRPhe47-engineered transfectants as already made for HLA-DPGlu69 (60), the T-cell studies using isotype-specific inhibition of $BeSO_4$-stimulated T-cell proliferation with anti-HLA isotype specific Abs in HLA-typed subjects support the notion that HLA-DR genes are

implicated in Be presentation in HLA-DPGlu69-negative subjects. A role for HLA-DRPhe47 in Be presentation is conceivable, and the data presented suggest it.

In fact, multiple analysis has indicated that the HLA-DR aminoacid variants Ser13, Tyr26, His32, Asn37, Arg74 are indirectly associated with Be hypersensitivity due to linkage disequilibrium with Phe47. It is worth noticing that this analysis accounts for the association of HLA-DRArg74 and HLA-DRTyr26 with Be sensitization found in a previous study (67). These markers were identified for their positive and negative association of HLA-DR alleles with disease or sensitization using univariate analysis in the overall population analyzed (67), while in this re-evaluation of the same study population after 7-years of follow-up including more subjects with Be-sensitization and disease we could use multiple logistic regression multivariate analysis on HLA-DPGlu69-negative subjects only. The fact that the alleles of the HLA-DRB1*03 group (alleles found associated with Be sensitization and not disease in the previous evaluation of this study population) (67) carrying almost exclusively the HLA-DRArg74 and HLA-DRTyr26 are also carrying HLA-DRPhe47 support the notion of the linkage disequilibrium between them. Further, only a fraction of HLA-DPGlu69-negative carry only HLA-DRArg74 and –DRTyr26 (10 out of 22; 45.5%) while all except one (21 out of 22, 95.5%) carry HLA-DRPhe47 suggesting that more than an allele or set of them are involved in the Be-presentation to T cells determining Be-susceptibility.

Consistent with the T-cell Ab-inhibition study, multiple regression analysis also indicates that the association between the HLA-DQ marker Leu26 and Be hypersensitivity is attributable to linkage disequilibrium between HLA-DR and HLA-DQ loci (84). It is well known that the HLA-DQLeu26 residue is expressed by all the HLA-DQB1*02 alleles and most of *03 and *06 alleles that are in linkage with the HLA-DRB1*03, 11, 12, 13 or 15 alleles which, in turn, express HLA-DRPhe47. Similar to our data, Maier and co-workers (70) obtained evidence for an association of HLA-DQB1*06, a Leu26 expressing group of alleles, with Be hypersensitivity in HLA-DPGlu69-

negative subjects. They too attributed the increased frequency of this HLA-DQ marker to linkage disequilibrium with HLA-DR and in particular to HLA-DR*13 alleles, a group of alleles expressing Phe47 (70).

The data of this study take the above observations (67, 70) a step further by suggesting that the HLA-DR gene, possibly the HLA-DRPhe47 supratypic variant, ought to play a functional role in Be presentation, as this (Tyr/Phe 47) polymorphism is known to be important for peptide binding and presentation to T cells (85, 86). Interestingly, HLA-DRPhe47 has been also implicated in susceptibility to the histopathological alike of berylliosis, sarcoidosis, in a very large case control population study (81).

In conclusion, both the HLA typing and the *in vitro* T-cell data in this study indicate a role for HLA-DR genes in determining susceptibility to Be hypersensitivity among individuals not expressing the HLA-DPGlu69 variant. The typing data point to the HLA-DRPhe47 supratypic variant as the susceptibility gene in this sub-population, and analysis of the molecule's structure suggests that HLA-DRPhe47 could bind Be and present it to T cells, using a different mechanism from what used by HLA-DPGlu69. Together, HLA-DPGlu69 and HLA-DRPhe47 could account for susceptibility in almost 100% of the affected population.

CHAPTER 3

HLA-DP POLYMORPHISMS AND PEPTIDE BINDING

DETAILED ANALYSIS OF THE EFFECTS OF GLU/LYS β69 HLA-DP POLYMORPHISM ON PEPTIDE-BINDING SPECIFICITY.

The data presented in this paragraph have been already published (Tissue Antigens 2003; 62: 459-71) and are fully available at http://www.blackwell-synergy.com/doi/full/10.1046/j.1399-0039.2003.00131.x

Introduction

HLA-DPB1 mismatches at position β69 were shown to play a critical role in helper T-cell recognition (87). Specifically, the change of Glu (E) to Lys (K) at residue β69 prevents the recognition of several HLA-DPw2 alloreactive $CD4^+$ cytotoxic T lymphocytes, demonstrating the requirement for a negatively charged residue at this position for allostimulation of these T-cell clones (88) and a similar substitution alters the characteristics of the Ab-binding epitope in the peptide-binding groove (88, 89). Importantly, the HLA-DP2 (HLA-DPA1*0301/HLA-DPB1*02012) molecule, carrying the βGlu69 residue, can bind metals such as Be (77) and cobalt (90) and is able to present Be to Be-specific T cells (60). All these data suggest that the HLA-DPβ69 residue plays a crucial role in HLA-DP-restricted immune responses.

To gain further insight into the functional role of this residue, and, in particular, to evaluate the importance of HLA-DPβ69 in peptide binding, we tested a panel of invariant chain (Ii) derived

peptide (CLIP) variants in competition-binding assays on HLA-DP molecules carrying the two most frequent aminoacids (E and K) found at position β69. In particular, peptide-binding and competition tests were performed on soluble HLA-DP2 (as prototype of Glu69-positive allele) and HLA-DP2K69 molecules generated in *Drosophila melanogaster* (*D. melanogaster*) and also on cell-surface-expressed molecules using B-lymphocyte cell lines (B-LCLs) expressing the HLA-DPB1*02012 and HLA-DPB1*02012-K69 alleles.

In the first instance, we assessed the binding frame of the CLIP peptide to the soluble HLA-DP molecules. Then, we used molecular modeling to predict a detailed structural model of HLA-DP2 complexed with CLIP. As K is the aminoacid most frequently found in position β69, the model was then compared with a mutated HLA-DP2K69 molecule carrying K instead of E. This mutation generates a sequence equivalent to that of the naturally occurring allele HLA-DPB1*0402. Secondly, we analyzed the peptide selection rules of HLA-DP2 and –DP2K69 by using mutated CLIP peptides at the relative positions interacting with pocket 4 and 6 (the two pockets mostly affected by the conformational changes induced by the E to K mutation at position β69) in competition assays. The experimental results were then used for assessing the best molecular modeling approach for predicting the aminoacid selection rules of HLA-DP pocket 4 and 6.

Methods

Antibodies and peptides. The anti HLA-DP monomorphic MoAb B7/21 (91), that is able to recognize only the assembled HLA-DP molecules (91), was purified from culture supernatants as described (92). Tetanus toxoid 947-967 derived peptide (FNNFTVSFWLRVPKVSASHLE) (93), CLIP 89-101 (SKMRMATPLLMQA) (94) and all its mutants at position P4 (A 94) and P6 (P 96) were purchased with free aminoacid termini from Advanced Biotech (Bergamo, Italy). Full-length

CLIP 86-114 (KPVSKMRMATPLLMQALPMGALPQGPMQN), CLIP 85-101 PKPVSKMRMATPLLMQA) and truncated CLIP peptides biotinylated at the amino or C terminus were purchased from Sigma-Genosys (Cambridge, UK). All synthetic peptides were purified by reverse-phase chromatography (RPC) to >90% purity. Sequence and purity were confirmed by mass spectrometry and analytical RPC.

Lymphoblastoid cell lines. Lymphoblastoid cell line 45.EM1 was derived from 45.1, a mutant haploid for HLA (remaining haplotype *A2, B5, DR1, DQw1, DPw2 (DPA1*0301, DPB1*02012*), by ICR191 irradiation and subsequently selected by resistance to lysis by a HLA-DPw2-allospecific CTL clone. This cell line is HLA-DPw2 negative, expressing normal levels of DPA mRNA but is not able to transcribe DPB1. 45.EM1 transfectants expressing wild-type DPB1*02012 allele and the site-directed mutant DPB1*02012-K69 allele were obtained as previously described (88).

Production of soluble HLA-DP molecules. HLA-DP2 and HLA-DP2K69 molecules were produced in soluble form in *D. melanogaster* Schneider-2 (S2) cells as previously described with minor modifications (77). Briefly, constructs containing the soluble form of the HLA-DPA1*0103, HLA-DPB1*02012 and its mutated form (Lys for Glu) at position 69 of the β-chain (HLA-DPB1*02012K69) were used as source for inserting the cDNA of the HLA-DP molecules in front of the Fos (alpha chain) and Jun (beta chains) zipper domains fused (95) by overlap extension PCR as described (96), yielding hybrid cDNAs. Hybrid HLA-DPβ chain cDNAs were digested with appropriate restriction enzymes and reinserted into pRmHA-3 insect cell expression vector; while HLA-DPα–chain cDNA after digestion with appropriate restriction enzymes was reinserted into pRmHA-3 insect cell expression vector modified with an oligonucleotide containing a 5' in frame Bgl II site, followed by the coding sequence for six histidine residues, a stop codon, and a Sal I

cloning site (77, 97). *D. melanogaster* (S2) cells ($2x10^6$ cells/ml in 100 mm Petri dish) were transfected with 9.5 μg of cDNA constructs for HLA-DP α– and β–chains in pRMHa-3 (DPA1*0103/DPB1*02012 or DPA1*0103/DPB1*02012-K69 with leucine zipper tails) together with a plasmid containing a Hygromycin resistance gene, pCoHygro (Invitrogen, Carlsbad, CA), using the calcium phosphate technique (77, 97). Resistant cells were selected using 300 μg/ml hygromicin-B in Schneider's medium containing 10% FCS, 2 mM l-glutamine and antibiotics. For large scale protein purification from culture supernatants, stable S2 transfectants were expanded up to 3-4 liters in serum free Insect X-Press medium (BioWhittaker, Walkersville, MD). Protein synthesis was induced with 1 mM copper sulphate. Soluble HLA-DP2 and HLA-DP2K69 molecules were purified to homogeneity with two affinity chromatography steps using a Ni-NTA (Qiagen, Hilden, Germany) column and an anti-HLA-DP (B7/21) affinity column followed by neutralization in the presence of an excess of tetanus toxoid peptide (tt 947-967) specific for both the HLA-DP molecules in order to stabilize the HLA class II αβ heterodimer. Purified soluble HLA-DP complexes were stored at -80°C in the presence of a mixture of protease inhibitors. Prior to use in binding tests, purified HLA-DP molecules were characterized by SDS-PAGE, western blotting with specific MoAbs, size exclusion chromatography and ELISA, as already described (77).

Peptide-binding and competition assays using HLA-DP soluble molecules. Prior to peptide-binding assays, 5 μg of HLA-DP molecules were incubated twice for 30 min at 37°C with 100 volumes excess of 20 mM sodium acetate/100 mM NaCl buffer pH 5.0 and concentrated to the original volume by ultrafiltration using a Microcon-10 concentrator (Millipore, Milano, Italy). This treatment was able to remove the tetanus toxoid 947-967 stabilizing peptide from the HLA-DP groove used during the purification of HLA-DP molecules, as assessed by the lost of the stability of the HLA-DP dimer after incubation at room temperature in SDS-PAGE buffer (data not shown).

Direct peptide-binding assays were performed as already described with minor modifications (98) by incubating soluble HLA-DP molecules (0.3 μg), with increasing concentrations of biotinylated peptide (0.15-150 μM) at pH 5.0 in 50 μl of 20 mM sodium acetate/100 mM NaCl buffer, PMSF and NaN_3, O/N at 37°C. Similarly, competition assays were performed by using 0.3 μg of soluble HLA-DP molecules incubated O/N at 37°C with biotinylated-CLIP peptide (10 μM) at pH 5.0 in 50 μl of 20 mM sodium acetate/100 mM NaCl buffer in the presence of non-biotinylated competitor peptide (0.3 μM-300 μM), PMSF and NaN_3. To block binding and competition assays, 50 μl of 2X PBS containing 3% BSA was added to each reaction tube.

The amount of biotinylated-CLIP peptide bound on the HLA-DP molecules at the end of direct binding or competition assays was determined as already described (98) by incubating for 1 hr the reaction mixture in duplicate on 96-well plates (Nunc) coated overnight at 4°C with 50 μl of 50 μg/ml of the HLA-DP specific MoAb B7/21 in PBS, pH 7.4 and blocked with 360 μl of 3% nonfat dry milk in PBS followed by 360 μl of 3% BSA in PBS for 30 min each. The plates were then washed five times with 10 mM Tris/l40 mM NaCI/0.05% NaN,/0.05% Tween-20, pH 8 (TBST) followed by Streptavidin-alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN) incubation for 60 min at room temperature, followed by an additional series of five TBST washes. Alkaline phosphatase substrate (100 μl of p-nitrophenylphosphate, Sigma) was added, and the absorbance was read at 405 nm after incubation for 100 min. In each experiment, the background given by the same amount of biotinylated peptide in the absence of soluble HLA-DP molecules was subtracted from the binding and competition data.

The concentrations of competitor peptide required to compete the binding of biotinylated-CLIP peptide to 50% of maximum (IC50) were calculated by using the least squares fit methods of the titration data using GraphPadPrism software (GraphPad Software Inc, San Diego, CA) and

normalized to 1 μM of biotinylated-CLIP peptide.

Peptide-binding assays using B-LCL. $5x10^5$ B-LCLs were incubated at 37ºC for 4h with biotinylated-CLIP (20 μM) and different amounts (1-100 μM) of non-biotinylated peptides, in RPMI 1% STF, at pH 5-6. Then, 0.5 μg/100μl of Streptavidina-R-phycoerythrin (PE) (Caltag, Burlingame, CA) was added and incubated for 30 min at 4ºC; the cells were washed off using PBS 1% STF after each incubation. Stained cells were analyzed by flow cytometry on a FACScan analyzer (Becton Dickison, San Jose, CA), excluding dead cells from the analysis by staining with propidium iodide.

Since transfectant B-LCLs used in these experiments express HLA-DQ and -DR molecules other than HLA-DP and CLIP peptide binds not only to HLA-DP but also to HLA-DR and -DQ, the parental cell line 45.EM1 expressing the same HLA-DR and -DQ alleles, but not HLA-DP, was always used as negative control and the fluorescence intensity subtracted from the total fluorescence obtained in each experiment. Moreover, control cells incubated in the absence of peptide under the same conditions were taken as background.

Relative binding of biotinylated CLIP to HLA-DP molecules was calculated by the following formula: 100x (MCF from LCLs incubated with biotinylated CLIP- MCF from LCLs incubated with both biotinylated and non-biotinylated CLIP / MCF from LCLs incubated with biotinylated CLIP), where MCF is mean channel fluorescence.

Molecular modeling of HLA-DP and prediction of HLA-DP binding rules for pocket 4 and 6. Sequence and domain structure information of HLA-DP2 molecule were obtained from the Swiss-Prot database. Sequences corresponding to the extracellular parts of α and β chain, respectively, were individually submitted to the Swiss-Model Protein Modeling Server (http://www.expasy.ch/swissmod/) via the "first approach mode". Both resulting models were

subsequently re-submitted via the "combine mode" allowing the Swiss-Model Protein Modelling Server to choose the best crystal structure template available for modeling the HLA-DP2 molecule. The resulting three-dimensional model of the extracellular domain of HLA-DP2 was modeled on the crystal structure of the HLA-DR4 molecule (99). Further processing was carried out using InsightII/Discover (Accelrys, San Diego, CA). The coordinates of the CLIP peptide in the HLA-DR3 crystal structure (100) were superimposed onto the HLA-DP2 model. Optimization of the model was performed using the conjugate gradients method and the consistent-valence (cvff) forcefield. The effects of solvent were simulated using a distance-dependent dielectric constant 4.0 x r. Position β69 (E) was then changed to K generating the HLA-DP2K69 model after similar optimization.

The CLIP residues in pocket P4 and P6, Ala (A) and Pro (P) respectively, were modified with different aminoacid residues and the corresponding models again optimized for all protein residues within 10Å of the CLIP peptide and the peptide itself. Each of these models was subjected to a molecular dynamics simulation at 600°C for 5000 iterations (plus 200 iterations for temperature equilibration). Conformations selected as possible low-energy configurations were again optimized. Intermolecular coulombic and van der Waals energies were calculated between the protein and the CLIP peptide using the Docking module of Insight II with a cutoff of 100A.

Results

Characterization of the CLIP-binding frame of HLA-DP2 and DP2K69 molecules. The Ii protein was shown to bind to all MHC class II molecules through the portion named CLIP as a standard peptide with a medium/low affinity (101, 102, 100). No detailed data are available, however, about the binding frame of CLIP to HLA-DP molecules although the fragment 89-101 had been already

used in competition tests (98, 77).

In order to precisely determine the binding frame used by Ii in the binding of CLIP to HLA-DP2 and -DP2K69 molecules we used a panel of truncated CLIP peptides spanning the entire CLIP sequence (aa 86-114 of human Ii).

Figure 2 shows direct binding data for the different truncated biotinylated-CLIP peptides to HLA-DP2 and HLA-DP2K69 variant as a percentage of the maximum binding observed for full length CLIP 86-114. In particular, only the biotinylated peptides that included the complete sequence of aa 91-99 of Ii demonstrated a significant dose-dependent binding to both HLA-DP molecules, suggesting that the minimum peptide-binding core is composed of 9aa. Interestingly, as it has been reported for other MHC class II molecules, peptide aminoacids at relative positions P-1 and P-2, and, in less extent, at positions P10 and P11 determined an increment of the peptide affinity to the HLA-DP molecule, thus suggesting a stabilizing role for these portions of the peptide extending outside the HLA-DP2 binding groove.

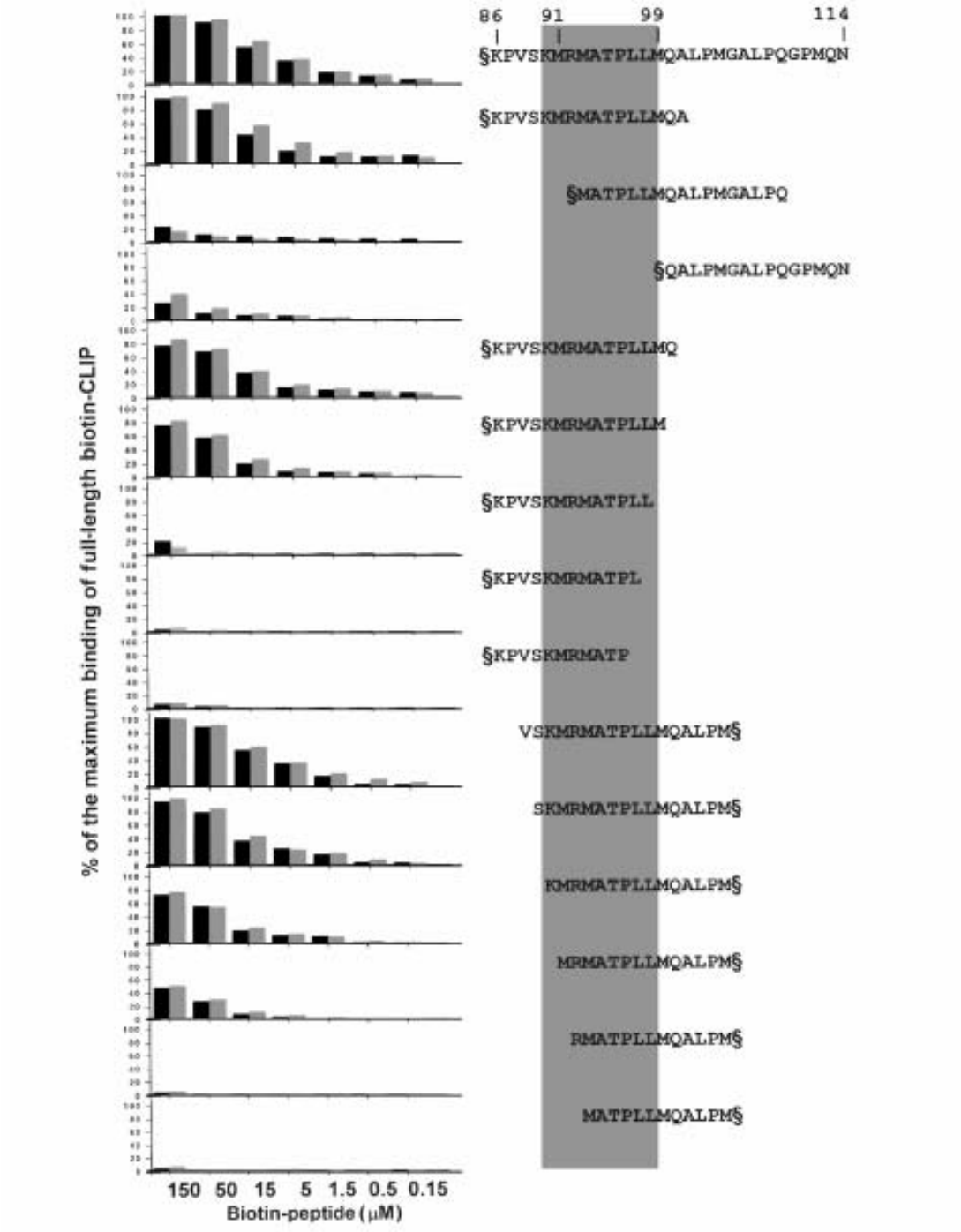


Figure 2. CLIP-binding frame of the HLA-DP2 and HLA-DP2K69 molecules. Increasing concentrations (0.15-150 μM) of truncated biotinylated-CLIP peptides (aminoacid sequence shown on the right, §: biotin) were tested for direct binding to HLA-DP2 (black bars) and HLA-DP2K69 (grey bars) molecules and reported on the abscissa. The data are expressed as a percentage of the maximum binding obtained with 150μM of full-length CLIP peptide. The grey area on the aligned CLIP sequences indicates the principal binding frame of CLIP to HLA-DP2 in agreement with the plotted data on the left.

HLA-DP2 and HLA–DP2K69 molecular models. In order to predict the possible structural interaction between CLIP and HLA-DP molecules, HLA-DP2 and HLA–DP2K69 molecular models carrying the CLIP peptide in the binding groove were obtained by homology modeling using the crystal structure of HLA-DR4 (99) for the αβ HLA-DP dimer coordinates and HLA-DR3 (100) for CLIP coordinates inside the groove, as for HLA-DR3 (100) present the same binding core for CLIP as that assessed for the HLA-DP2 and DP2K69 molecules. Figure 3 panel A shows the surface analysis of the binding regions (top view) of the HLA-DP2 molecule interacting with the CLIP peptide (shown in stick). As expected from the homology with HLA-DR4, the binding sites presented four major contact sites with the peptide accommodated in the groove. These four pockets interacted with the peptide aminoacid residues P1 (M91 in CLIP), P4 (A94 in CLIP), P6 (P96 in CLIP) and P9 (M99 in CLIP).

Due to the deletion of two aminoacids in the region comprised between the aminoacids 22-27 of HLA-DR β chain, position HLA-DPβ69 is homologous to HLA-DRβ71. As already described for DRβ71, DPβ69 is located in the HLA-DP models in the α–helix of the β-chain and the aminoacid residue is directly involved in the formation of pocket 4. Interestingly, by mutating Glu (in HLA-DP2) to Lys (in HLA-DP2K69) in the position β69 important changes were observed in the shape and charge distribution not only of pocket 4 but also of the nearby pocket 6 (figure 3 panel B, surface analysis of the binding regions, top view, of the two molecules after removing the CLIP peptide). In a deeper and more negatively charged pocket 4 and a different charge distribution in pocket 6 with particular, HLA-DP2 displayed respect to HLA-DP2K69. Moreover, some structural differences between the two molecules could also be observed in α1β1 domains outside the binding groove (figure 3 panel C, superimposition of HLA-DP2 and HLA-DP2K69 molecules, surface analysis; top view).

The structural changes observed between the HLA-DP2 and –DP2K69 models did not appear to be due to a different conformation of the CLIP peptide inside the groove of the two models, since this showed an essentially similar structure in both models and Ala94 at position P4 did not even penetrate into the pocket (figure 3 panel D, superimposition of CLIP peptide from HLA-DP2 and HLA-DP2K69 molecular models; front view). Therefore, the differences in pocket 4 structure are probably directly due to the HLA-DP aminoacid residues involved in the pocket itself and not to a different interaction of these aminoacids with the Ala residue present in CLIP at this position.

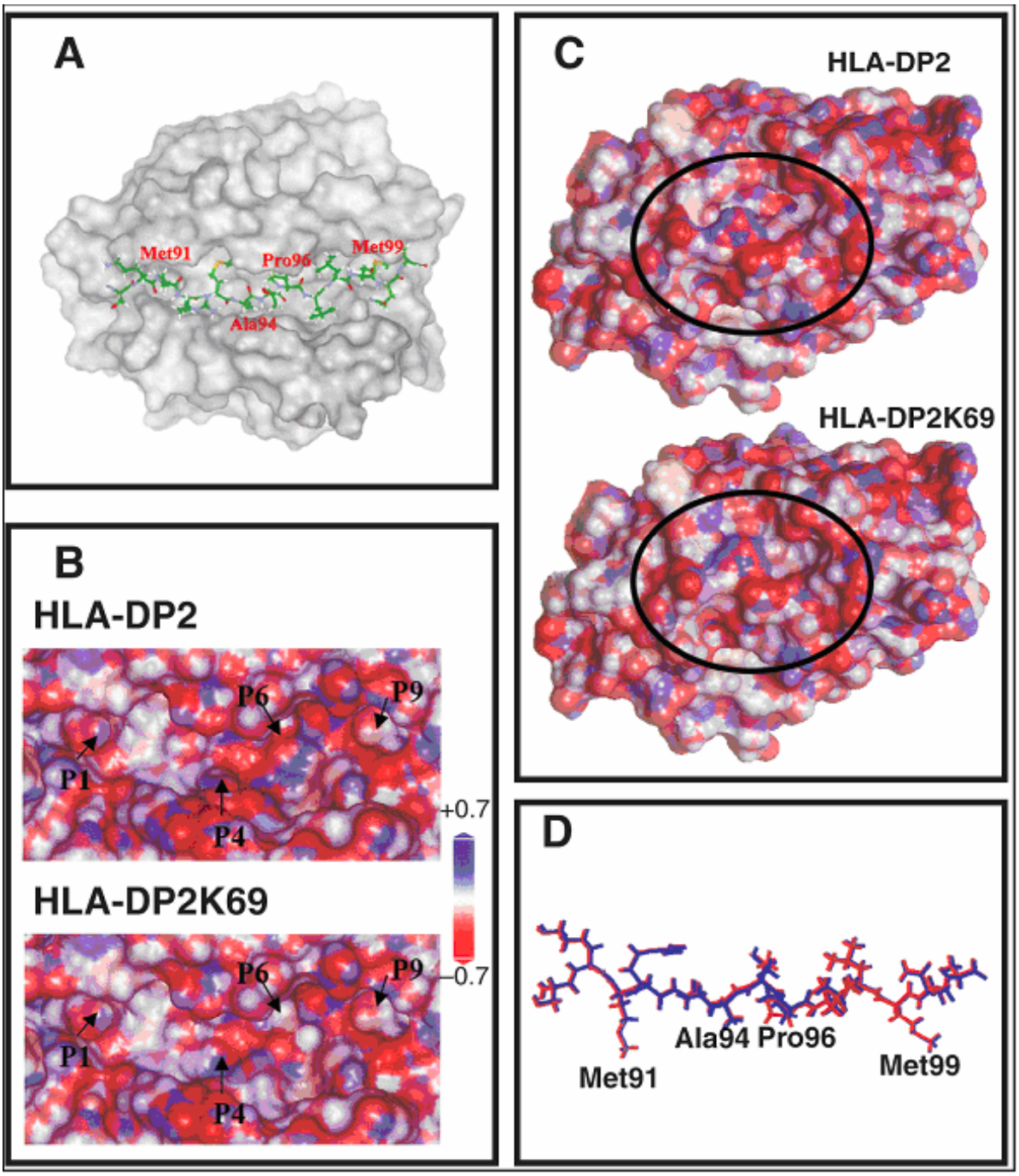


Figure 3. Molecular model analysis of the HLA-DP2 and DP2K69 molecules.
Panel A: top view of the surface analysis of the peptide-binding region of the HLA-DP2 molecule (in light blue) interacting with the CLIP peptide (shown in CPK color in stick stile). Aminoacid residues interacting with the main HLA-DP2 pockets are indicated. Panel B: surface analysis of the HLA-DP2 and -DP2K69 binding grooves after removing the CLIP peptide from the models. The main peptide-interacting pockets are indicated with arrows. The partial surface charge distribution is shown color-scaled from red (negatively charged) to blue (positively charged) as indicated on the right. Panel C: top view of the surface analysis of HLA-DP2 molecule (red transparent surface) with super-imposed the HLA-DP2K69 surface analysis (blue filled surface). Panel D: frontal view of the superimposed CLIP peptides from HLA-DP2 (red) and HLA-DP2K69 (bleu) molecular models. Aminoacid residues interacting with the main HLA-DP pockets are indicated.

Competition tests between CLIP and CLIP variants. In order to define the role of the polymorphism E/K at position β69 of the HLA-DP in determining peptide-binding rules we characterized the binding affinities of CLIP peptides modified at relative positions P4 and P6 in competition tests on soluble HLA-DP2 and –DP2K69 molecules using a fixed amount of biotinylated-CLIP (able to give 90% of the maximum binding at pH 5.0 and 60% of the maximum binding at pH 7.5; data not shown).

Figure 4 (panel A) shows the IC50 values obtained in the competition tests at pH 5.0 using HLA-DP2 soluble molecules for the mutated CLIP peptide at relative peptide position P4. In particular, with respect to the wild type residue (Ala –A-) present in CLIP at this position, HLA-DP2 had an increased affinity for CLIPs carrying polar (Gln –Q- and Asn –N-) or positive-charged (Arg –R- and K) residues, non-polar aromatic residues (Trp –W-, Phe –F-, Tyr –Y-, and His –H-) or residues carrying S–groups (Cys –C- and Met –M-). Non-polar aliphatic residues such as Val (V) and Leu (L), and to a lesser extent Ile (I), Pro (P) and the polar residues Thr (T) and Ser (S), were disfavored with respect to A at position P4. Other residues (Asp –D-, E and Gly –G-) showed no significant advantage or disadvantage.

Similarly, as shown in figure 4 (panel B), the IC50 values for the mutated CLIP peptides and the soluble HLA-DP2K69 molecules indicated that pocket 4 of HLA-DP2K69 presented a less general aminoacid selectivity than HLA-DP2. In particular, an increased affinity with respect to A was observed for CLIP variants presenting aromatic residues (F, W, Y, P and H) and charged or polar residues independently of the charge (Q, R, N, D, K and possibly M and E). Interestingly, in contrast to HLA-DP2, non-polar aliphatic residues like L and I were favored with respect to A at position P4. Other residues (V, S, C, T and G) were slightly disfavored.

Figure 4 panels C and D show similar IC50 values obtained in the competition tests using

HLA-DP2 and HLA-DP2K69 soluble molecules for the CLIP peptides mutated at relative peptide position P6. With respect to the wild type residue (P) present in CLIP at this position, HLA-DP2 showed an increased affinity for mutated CLIPs with aromatic residues (Y, W, F and H). Positively charged residues (K and R), the polar residue Q and, among the non-polar aliphatic residues, we also demonstrated an increase of affinity, although less than that for non-polar aromatic residues. Negatively charged residues (E and possibly D), polar residues like T and S, residues carrying S–groups (M and C) and non-polar aliphatic residues (L, V and A), G and perhaps N are disfavored with respect to P at position P6 of HLA-DP2. Pocket 6 of HLA-DP2K69 also had an increased affinity with respect to P for mutated CLIPs presenting non-polar aromatic residues (F, Y, W and H), but the affinity for charged residues was partially independent of the charge (R, K, E, but not D) although positively charged residues seemed to be favored. Polar residues like N, Q (and D) did not present any advantage in binding affinity with respect to the wild type aminoacid P. Smaller polar and non–polar aliphatic residues like A (and G), L, T, I, S, V and residues carrying an S–group (C and M) were disfavored, with the smaller side-chains apparently most affected.

The HLA-DP2 and –DP2K69 peptide-binding data were further confirmed by the analysis of a subset of the tested peptides in peptide-binding studies using cell bound HLA-DP molecules expressed by EBV transformed B-cell lines 45.EM1 transfected with wild-type (45.EM1/DPB1*02012) or site-directed mutant (45.EM1/DPB1*02012-K69) alleles. CLIP peptide mutants carrying K, V, I, C or F at P4 and A, R, W, E or G at P6 were used. A general correlation was observed between the binding data using soluble HLA-DP molecules and HLA-DP molecules expressed at the cell surface of B-LCLs (overall correlation coefficient between IC50 with soluble molecules and EC50 determined with B-cell lines 0.71, $p<0.03$).

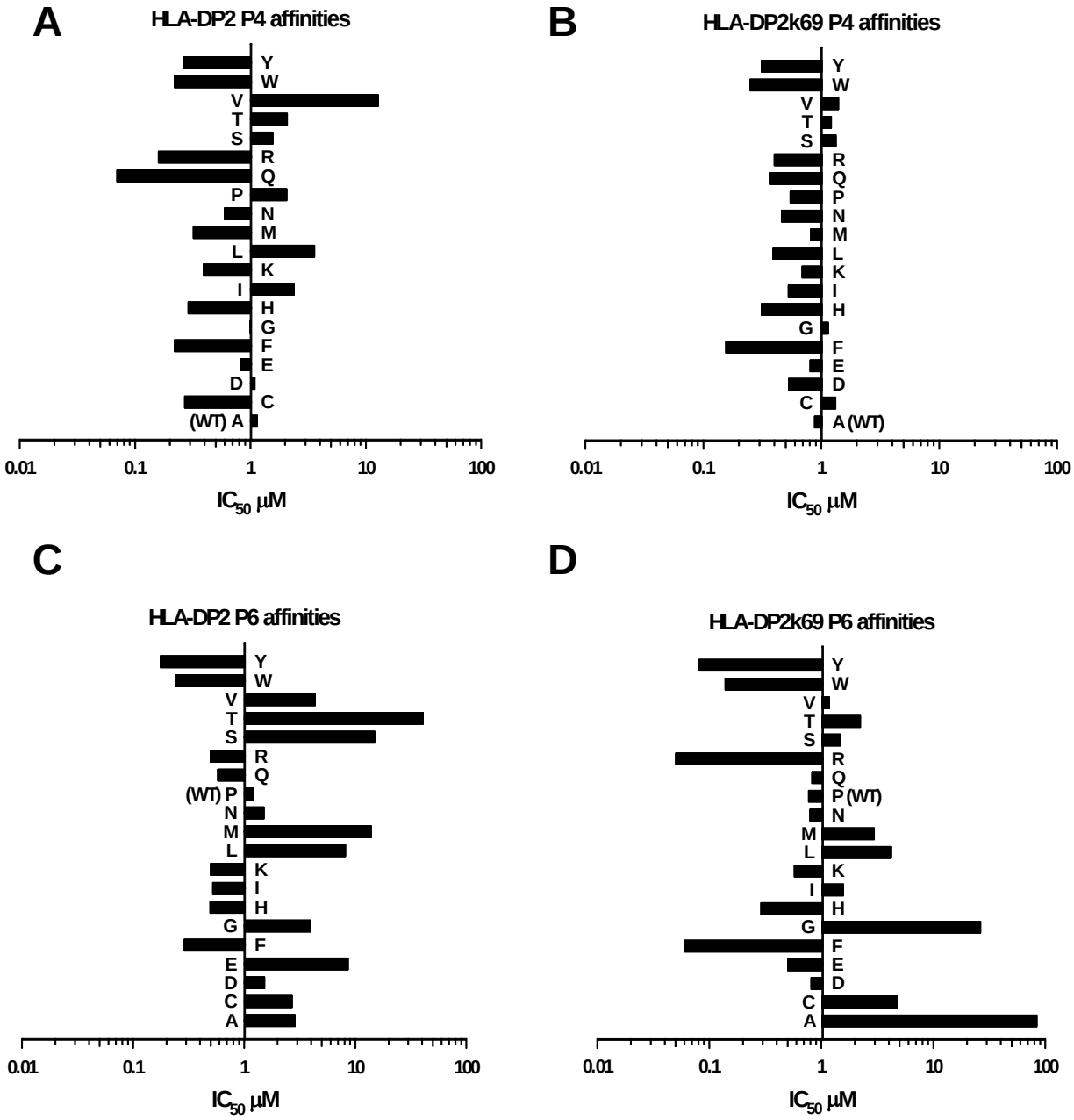


Figure 4. Competition tests between CLIP and mutated CLIPs at P4 and P6 on HLA-DP soluble molecules. The IC50 results (as µM values on the abscissa of each panel) for all the peptides evaluated are reported by molecule and position. Panel A, HLA-DP2 P4 affinities; panel B, HLA-DP2K69 P4 affinities; panel C, HLA-DP2 P6 affinities; panel D, HLA-DP2K69 P6 affinities. The ordinate is drawn through the point of equimolar competition with the biotinylated-CLIP peptide (1 µM). Thus, bars extending to the left of the vertical axis represent CLIP mutated peptides that bind with higher affinity than biotinylated CLIP; bars extending to the right represent CLIP mutated peptides that bind with lower affinity than biotinylated CLIP. Each aminoacid mutation of CLIP peptide tested is reported at basis of the bar. wt: wild type peptide (i.e. non-mutated CLIP). The data shown are the mean value obtained in three independent experiments.

Molecular modeling of the interaction between HLA-DP molecules and mutated CLIPs: prediction of peptide selection rules. The modeling of HLA-DP2 and -DP2K69 molecules had enable to identify differences in the peptide-binding groove of the two molecules. On the basis of the data from peptide-binding experiments, we tried to define if molecular modeling could be successfully used for predicting the peptide selection rules of HLA-DP. In order to achieve this, we introduced all the possible aminoacid mutations at P4 (A) and P6 (P) of the CLIP peptide in the two HLA-DP/CLIP models generated and determined the energies (in terms of van der Waals and Coulombic energies) of the interaction between the HLA-DP molecules and the peptides.

Figure 5 panel A shows the correlations between the Δ energies of the HLA-DP/mutated CLIP and the HLA-DP/wild-type CLIP complexes and the IC50's experimentally determined for the same mutations. The observed coefficients of correlation varied widely between the different HLA-DP molecules and the pocket evaluated (range: 0.17-0.74). Van der Waals energies seemed to play the most relevant role among total energy results of the interaction (data not shown). In order to minimize the effects of the interaction between the HLA-DP molecule and the CLIP peptide at positions not involved in the analysis, we re-performed the molecular modeling evaluation using the single aminoacids inside the pocket of interest and fixed the coordinates of the peptide backbone and other side-chains and excluded these atoms from the energy calculations. Figure 5 panel B shows the results of the correlations between the Δ energies of the HLA-DP/mutated aminoacid and the HLA-DP/wild-type aminoacid complexes and the IC50s experimentally determined for the peptides carrying the same mutations. Good and homogeneous coefficients of correlation (range: 0.64-0.80) were observed between Δ total energies and IC50 for both molecules and in both the analyzed pockets. Once again, the most relevant role in the total energies was played by the van der Waals energies.

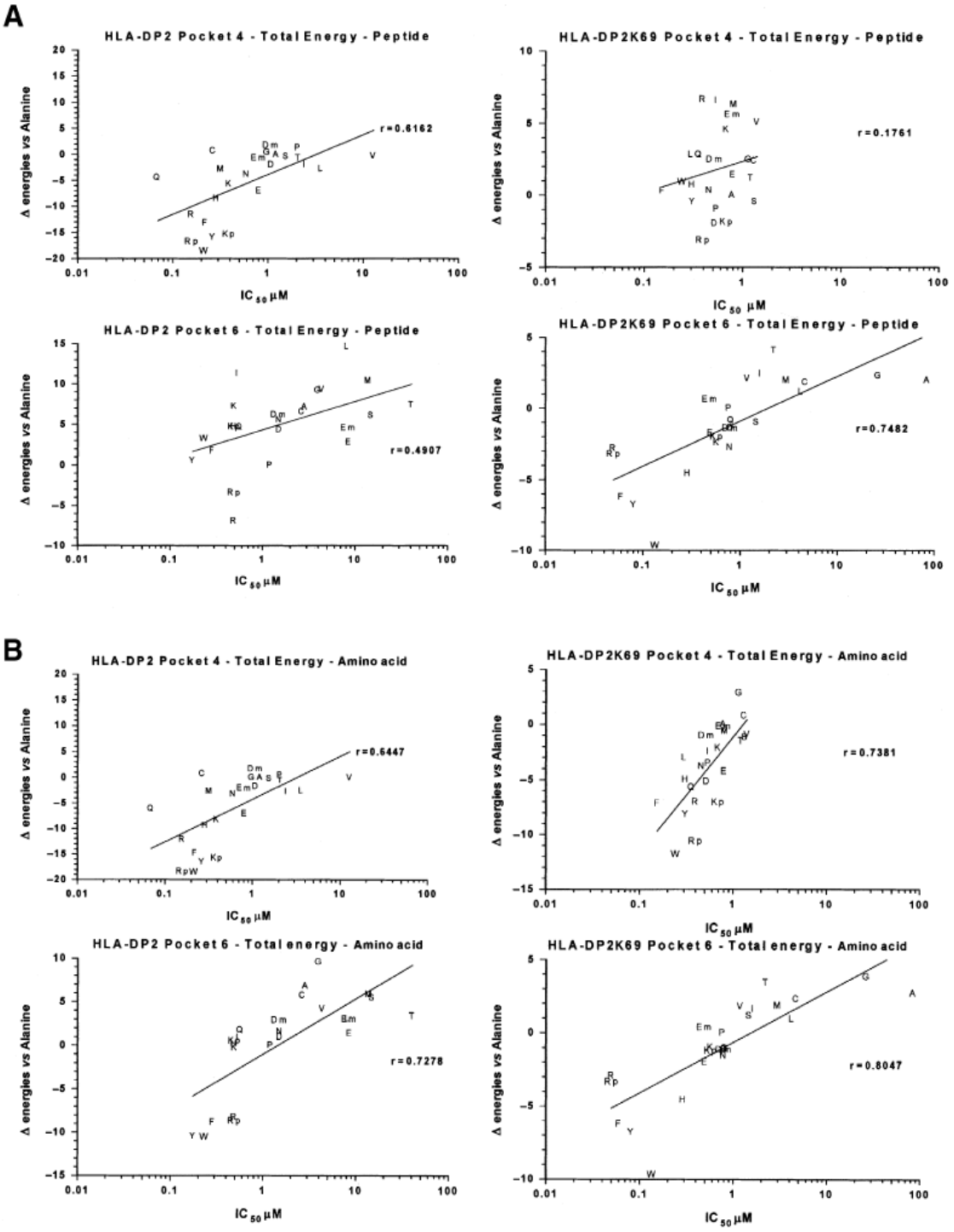


Figure 5. Correlation between predicted binding affinity (Δ energies in KJ/mol) and IC50 (μM) for all the aminoacid residues in P4 and P6 of CLIP with respect to HLA-DP2 and DP2K69. Panel A shows the correlation with the experimental IC50 and the data for total energies obtained by modeling the complete peptide in the groove. Panel B shows the correlation with the experimental IC50 and the data for total energies obtained by modeling only the single aminoacid in the groove. Each aminoacid is represented in a single letter code. The correlation coefficient (r) and the linear regression line are reported for each plot.

Interim discussion

The polymorphism E/K at position 69 of the HLA-DPβ chain plays a critical role in HLA-DP-restricted immune responses (64, 65, 67). In this study we define peptide selection rules of HLA-DP molecules carrying E at position β69, using HLA-DP2 as the prototype, together with the selection rules of its mutated counterpart i.e., HLA-DPK69 corresponding to the naturally occurring allele HLA-DPB1*0402. For this purpose, we used variants of the peptide CLIP in competition peptide-binding assays using soluble or cell membrane-bound HLA-DP molecules. The CLIP peptide is known to bind all HLA class II molecules with low to medium affinity. This peptide carries two M residues at positions 91 and 99 which interact with pockets 1 and 9, respectively, of the HLA class II molecule (100-102). Interestingly, the aminoacid residues carried by the CLIP peptide at positions P4 and P6 (A 94 and P 96), the pockets whose conformation and electrostatic properties are dependent upon residue 69, do not lock into pockets 4 and 6 of the HLA peptide-binding groove. This confers low stability upon the CLIP-MHC complexes thereby allowing CLIP to be released in the endosomal compartment and antigenic peptides to be subsequently loaded (100-104). For its ability of forming low stability complexes, CLIP is particularly suitable for assessing both positive and negative effects of aminoacid substitutions at position P4 and P6 when defining the peptide-binding rules of the HLA-DP2 molecule and of its mutated form HLA–DP2K69.

Molecular modeling has been widely used to predict the structure of MHC class I and class II molecules with unknown crystal structure (105, 106), and frequently the validity of the results obtained with this approach was later confirmed, as with the characterization of the CLIP's structure inside the HLA-DR3 groove (100, 105). Based upon the homology with HLA-DR4 molecule, the peptide-binding site of both HLA-DP alleles used in this study exhibits four major contact sites for bound peptides. The side-chains of the CLIP peptide aminoacid residues P1 (M 91), P4 (A 94), P6

(P 96) and P9 (M 99) fit naturally into these pockets, suggesting that the HLA-DP groove can accommodate nonameric peptide core sequences. This hypothesis has been confirmed by the results of this study where the CLIP fragment 91-99 showed significant binding to HLA DP2 and HLA–DP2K69 molecules, as already suggested by observations with naturally bound peptides eluted from HLA-DP2 (98) and HLA-DP4 (107). In this context, the scanning of all putative CLIP-binding frames for the HLA-DP molecules using molecular modeling suggests that the 91-99 CLIP fragment generates the most stable HLA-DP/CLIP complex with the lowest total free energy (data not shown). Finally, the direct binding study with the truncated CLIP peptides suggested a stabilizing role in the HLA-DP/CLIP complex for the aminoacids of CLIP extending outside the groove at N-terminal and in less extent at the C-terminal. Although further peptide-binding and structural studies are needed to clear assess this point, these data are in agreement with similar observation reported for other peptides and MHC class II molecules (108) suggesting that the N-terminal P -1 and P -2 as well the C-terminal P10 and P11 extended residues of the bound peptide interact with conserved position in the MHC class II molecule increasing the stability of the MHC/peptide complex.

The molecular model of the HLA-DP2 molecule predicts that the β-chain's residue 69 is directly involved in the formation of pocket 4, in agreement with other molecular models already used for describing the properties of HLA-DP molecules (90, 98, 107). Moreover, here we reported that the single E (in HLA-DP2) to K (in HLA-DP2K69) aminoacid substitution at position β69 of the HLA-DP molecule introduces significant changes in the shape and charge distribution of both pocket 4 and the nearby pocket 6. In particular, HLA-DP2 displays a deeper and more negatively charged pocket 4 compared to HLA-DP2K69. Further, HLA-DP2 also shows a different charge distribution in pocket 6. The molecular model predicted changes in charge distribution between the two models of HLA-DP molecules determining a change in peptide selectivity in both pockets as

demonstrated by our peptide-binding results. Finally, consistently with the critical role that the HLA-DP β69 polymorphism plays in allorecognition (87, 88), differences in the α1β1 domains that are outside the binding groove were also predicted by molecular modeling in HLA-DP2 compared to HLA-DP2K69.

In agreement with the predictions of molecular modeling, the competition tests using CLIP mutants demonstrated that pocket 4 of HLA-DP2 has high affinity for positive polar residues like Q, R, K and N or non-polar aromatic residues (F, W, Y and H) and residues carrying the S–groups such as C and M, but reduced affinity for large non-polar aliphatic residues. Similarly, pocket 6 of HLA-DP2 showed high affinity for aromatic residues (Y, W, F and H) but reduced affinity for very small side-chains such as A and G.

Pocket 4 of the HLA-DP2K69 molecule shows lower selectivity than HLA-DP2 in that only aromatic residues present high affinity to it. Interestingly, in addition to altering the binding characteristics of pocket 4, K at position β69 also contributes to aminoacid selectivity of pocket 6. In fact, in addition to select for aromatic residues (F, W, Y and H) as in HLA-DP2, the K69 residue of HLA-DP2K69 favors the binding of large residues endowed with the capacity to form H-bonds (such as R) with residue Qα60. The observation that mutations within pocket 4 of HLA-DP2 affect peptide-binding properties of pocket 6 on the same molecule is not unique. Similar findings were reported for HLA-DR1*0401 and HLA-DR1*0402 (109). In HLA-DRβ*0401, the Kβ71 residue, that's homologous to the Kβ69 residue of HLA-DP2K69, contributes to increased binding affinity for peptides carrying a R in P6 position, while the Qβ71 residue of DRB1*0402 rejects peptides with an R in P6 position (109-111).

Overall, our data are in agreement with previous observations on peptide selectivity for P4 and P6 of HLA-DP2 (98) and of HLA-DP4 (107), and extend them at the quantitative level. In

particular, Chicz and colleagues predicted the general properties of the HLA-DP2 pockets from the analysis of naturally processed peptides eluted from HLA-DP2 which showed that T, S and Q were prevalent in P4 and Y, F and W in P6 (98). Also in agreement with our data, they also found that an S to A change in P4 did not significantly alter the peptide's IC50, while an F or Y to A change in P6 significantly decreased binding affinity (98).

With regard to HLA-DPB1*0402, the natural equivalent of the molecule here reported as HLA-DP2K69, Castelli and co-workers analyzed peptide-binding specificity by using critical aminoacid substitutions on a high affinity peptide (107). Similar to the data reported here, the P4 pocket of HLA-DP0402 showed reduced aminoacid selectivity, with aromatic residues being most favored (107). In agreement with our data, Castelli and colleagues also indicated aromatic residues such as F, W and Y as most favored for selection by pocket 6 of the HLA-DP4 molecule, whereas residues A, E, N, K and T were less tolerated (107). Since they did not evaluate the role of large hydrophobic residues in peptide binding to the P6 pocket (107), a full and critical comparison is not feasible.

In addition to using molecular modeling to make structural predictions, we used it to predict peptide selection rules by HLA-DP molecules. Actual peptide-binding affinities were determined by competition assays. The comparison between the predicted affinity expressed as total energy of interaction between the HLA molecule and the CLIP variants carrying mutations at positions P4 and P6, and the measured affinity, expressed as IC50, showed that van der Waals forces are most critical in defining actual peptide-binding affinity. Although further studies are needed to increase the power of molecular modeling in predicting the binding pocket selectivity, the data reported here suggest that this approach can help with determining the role of aminoacid substitutions in anchor positions 4 and 6 in modifying binding affinity of peptides to HLA allelic variants, therefore indicating it as the technique of choice for *in silico* vaccine design (112).

Together with the available information (113), the results of this study, suggest that aminoacid binding by the HLA-DP groove is controlled by a very limited number of key aminoacid combinations. Thus, the binding results presented in this paper constitute a promising way of selecting peptides for vaccination. Peptide-based vaccination typically uses HLA-DR-restricted peptides selected for their ability to bind multiple HLA-DR allelic variants and to be immunogenic for the largest fraction of the population are used (112) and a number of studies have been carried out to identify such peptides in microbial and tumor antigens and allergens (107, 111, 112, 114). It is worth noticing that HLA-DP-restricted immunodominant peptides have been shown to elicit T-cell response as strong as the typical HLA-DR-restricted T-cell reactions (60, 115-119). In the context of the knowledge that HLA-DP carries a rather limited number of supertypes in the general population, one might expect that peptides known to bind by the two most frequent HLA-DP alleles i.e., HLA-DP2 and HLA-DP4, might have the same impact upon a population as peptide selected for the ability to bind eight or more HLA-DR allelic variants (120). Thus identifying specific HLA-DP restricted peptides might be important for the design of new synthetic vaccines to complement panels of HLA-DR-restricted peptides in multi-epitopic strategies aiming at reducing the risk of pathogen evasion.

CHAPTER 4 *(SUBMITTED)*

IN VITRO MODULATION OF BERYLLIUM STIMULATED T-CELL ACTIVATION USING HLA-DPGLU69 POCKET 4 SPECIFIC CLIP-DERIVED PEPTIDES

Introduction

As pointed out in the previous chapters, in berylliosis the immune response genes, primary the HLA-DPGlu69 and, to a lesser extent, the HLA-DRPhe47, are responsible for antigen presentation of Be to T cells in affected subjects (60, 73) driving higher T-cell stimulation (121).

The granulomatous reaction to Be is thought to be initiated by the exaggerated release in the alveolar milieu of TNF-α by alveolar macrophages caused both by direct Be stimulation (74) and by Be-stimulated HLA-restricted T-cell activation (75). That is maintained by the accumulation of effector-memory $CD4^+$ T cells responding to Be as a specific antigen/hapten (45-47). Previous studies have shown that Be binds to the HLA-DPGlu69 molecule with higher affinity (77) due to the properties of the HLA-DP pocket 4 carrying Glu electron-donor residues at the polymorphic position 69 and Gln, Glu, Arg and Tyr in their invariant and respective positions 13, 14, 27 and 28 on the β–chain. As these residues are in sufficiently close proximity (from 3.1 to 6.4 Å in the HLA-DP2 molecular model) (122), they can directly coordinate the positively charged Be^{2+}. Furthermore, as HLA-DPGlu69 pocket 4 preferentially binds electron-donor aminoacids such as Arg, Asn, Gln, His, Lys, Trp and Tyr (122, 123), it is reasonable to hypothesize that the aminoacid residue of HLA-DPGlu69-pocket 4-bound peptides can also contribute to Be binding.

This study was designed to clarify the role of peptides binding to the HLA-DP groove's pocket 4 in Be binding and to identify peptides with higher affinity for pocket 4 that might either

displace or mask Be, hence making it unavailable for antigen presentation and T-cell stimulation.

Methods

Study population. 13 individuals with Be hypersensitivity were enrolled in the study after informed consent approved by the Cleveland Clinic IRB. They were 11 males and 2 female, all Caucasians, with a mean age 39±6 years and an average time of employment of 9±6 years. 10 out of 13 were HLA-DPGlu69-positive (Table 13) and were used to determine the effect of high affinity peptides upon Be presentation. As control, 5 normal unexposed subjects, 4 male and 1 female, all Caucasians, with a mean age 31 ±3 years were enrolled**.**

HLA typing. HLA class II typing was carried out in all patients and controls as previously described (8, 14).

Reagents. Antibodies and peptides. MoAbs directed against HLA-DR (L243) (15), HLA-DP (B7/21) (74), HLA-DQ (L2) (74), HLA-class I (W6/32) (74) and the 19 kDa *Mycobacterium tuberculosis* protein (HYT6) (74) were purified from culture supernatants. CLIP peptide and its variants used in the study (table 14) either with free aminoacid termini or in biotinylated form were obtained at >90% from Advanced Biotech (Bergamo, Italy). Peptides were designed on the basis of our previous study reported in chapter 3 (122) which aimed to obtain peptides presenting different affinities for pocket 4 and 6 of HLA-DPGlu69 molecules. In particular, one peptide with reduced affinity (CLIP-AA) and three peptides with increased affinity (CLIP-QY, CLIP-RF and CLIP-YY) for HLA-DPGlu69 were used. Soluble HLA-DP2 and -DP2K69 molecules were produced in soluble form in *D. melanogaster* (S2) cells as described (77, 122).

Table 13. Be hypersensitivity status and HLA class II typing of the study population.

#	Patient ID	status	HLA-class II typing			HLA-DPGlu69
			HLA-DR	HLA-DP	HLA-DQ	
1	M.L.	Berylliosis	*01; *11	*0201; *0401	*02; *06	Positive
2	H.P.	Berylliosis	*01 ; *03	*0101 ; *0901	*02; *05	Positive
3	D.W.	Berylliosis	*07	*0401 ; *1701	*02	Positive
4	A.C.	Berylliosis	*01	*0201 ; *0301	*05	Positive
5	M.H.	Berylliosis	*03 ; *04	*0201 ; *0401	*02; *03	Positive
6	W.Q.	Be-sensitized	*01; *13	*0401; *0402	*03; *05	Negative
7	S.E.	Be-sensitized	*04; *11	*0401	*03	Negative
8	J.C.	Be-sensitized	*15; *16	*0401; *1001	*02; *03	Positive
9	M.L.	Be-sensitized	*13	*0402; *1901	*06	Positive
10	R.S.	Berylliosis	*01	*0301; *1001	*05	Positive
11	E.D.	Be-sensitized	*07; *15	*0601; *1401	*02; *03	Positive
12	D.W.	Be-sensitized	*03; *04	*0401	*02; *03	Negative
13	T.C.	Be-sensitized	*04; *13	*0201; *0401	*03; *06	Positive

Table 14. Characteristics of the peptides designed for the study.

Name	Sequence	Details and use in the study
Bt-CLIP	Bt-PKPPKPVSKMRMATPLLMQA	Ii 82-101, reference biotinylated CLIP peptide
Bt-CLIP-AA	Bt-PKPPKPVSKMRMATALLMQA	reference biotinylated-CLIP-AA peptide
Bt-CLIP-RF	Bt-PKPPKPVSKMRMRTFLLMQA	reference biotinylated-CLIP-RF peptide
Bt-CLIP-QY	Bt-PKPPKPVSKMRMQTYLLMQA	reference biotinylated-CLIP-QY peptide
Bt-CLIP-YY	Bt-PKPPKPVSKMRMYTYLLMQA	reference biotinylated-CLIP-YY peptide
CLIP	SKMRMATPLLMQA	CLIP peptide
CLIP-AA	SKMRMATALLMQA	Mutated (P6: Pro->Ala) CLIP peptide with reduced affinity for HLA-DPGlu69 molecules
CLIP-RF	SKMRMRTFLLMQA	Mutated (P4: Ala->Arg; P6 Pro->Phe) CLIP peptide with higher affinity for HLA-DP DPGlu69 molecules
CLIP-QY	SKMRMQTYLLMQA	Mutated (P4: Ala->Gln; P6 Pro->Tyr) CLIP peptide with higher affinity for HLA-DPGlu69 molecules
CLIP-YY	SKMRMYTYLLMQA	Mutated (P4: Ala->Tyr; P6 Pro->Tyr) CLIP peptide with higher affinity for HLA-DPGlu69 molecules
TT30	FNNFTVSFWLRVPKVSASHLE	Tetanus toxoid 947-967; HLA-DP stabilizing peptide

Note: Bt: Biotin

Be-peptide competition tests. Competition assays using HLA-DP soluble molecules were performed as previously described (77, 122). In a nutshell, 0.5 μg of soluble HLA-DP were incubated overnight with biotinylated peptide in the presence of increasing amounts of the appropriate non-biotinylated competitor peptide, of CLIP peptide or of $BeSO_4$ at 0.3 μM-300 μM in 20 mM sodium acetate/150 mM NaCl pH 5.0 or 20 mM phosphate buffer/150 mM NaCl pH 7.5, containing 1 mM PMSF. To terminate the assays, 50 ml of 2X PBS containing 3% BSA were added to each reaction tube.

The amount of biotinylated peptide bound on the HLA-DP molecules at the end of the competition assays was determined as already described (77, 122) by ELISA assay using the HLA-DP-specific MoAb B7/21 as capture agent. IC50 values, i.e., the concentrations of the competitor peptide or of Be required to compete for 50% of maximum binding of the biotinylated peptide were calculated by using the least squares fit methods of the titration data with the GraphPadPrism software (GraphPad Software Inc, San Diego, CA).

Lymphocyte proliferation blocking assays. PBMCs were isolated from heparinized whole blood by density centrifugation on Ficoll Hypaque gradient. PBMCs (0.5×10^5 cells/well) were then plated in 96-well round-bottomed microtiter plates in RPMI 1640 tissue culture medium [2 mM L-glutamine, 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin in the presence of Be sulfate ($BeSO_4*4H_2O$) at 2, 10 and 50 μM (all reagents form Sigma Co., St. Louise, MO)] as previously described (72). Purified MoAbs were used at increasing concentrations (10, 20 and 50 μg/ml) to inhibit lymphocyte proliferation and cytokine production as previously described (72, 45).

Free aminoacid termini peptides designed for competing with Be in antigen presentation to Be-specific T lymphocytes were used at increasing concentrations (0.4, 2, 10, 50, 250 μM).

The levels of IFN-γ released in the culture supernatants of Be-stimulated PBMCs were measured in triplicate on supernatants collected after 5 days and frozen at -80°C with commercially available solid-phase, two-site ELISA kits (Pierce-Endogen, Woburn, MA) and the results were expressed as the means of triplicate cultures.

Statistical analysis. All the data are expressed as mean ± standard deviation of the mean (SD). Comparisons between groups are made by Student's t test.

Results

Competition assays in the HLA-DP2 and -DP2K69 soluble molecule system. The interaction between the HLA-DPGlu69 molecule, Be and competing peptides was evaluated at pH 5.0 in order to mimic the acidic microenvironment in which peptides are loaded onto HLA class II molecules. Be/HLA-DP interactions were analyzed by the ability of Be to compete with the CLIP and CLIP-derived peptides. As previously shown (77), $BeSO_4$ (0.3-300 μM) was capable of competing with biotinylated CLIP (10 μM) for the binding to soluble HLA-DPGlu69 molecules, as $BeSO_4$ displaced biotinylated CLIP from soluble HLA-DPGlu69 (IC50% 0.07 μM of $BeSO_4$; non-biotinylated CLIP IC50% 1.09 μM; Figure 6, panel A) but not from the HLA-DP2Lys69 molecule (not shown). Similarly, the CLIP-derived low affinity peptide CLIP-AA (50 μM), could not compete out Be (IC50% <0.001 μM of $BeSO_4$; non-biotinylated CLIPAA IC50% 0.83 μM; Figure 6, panel B). To the contrary, the binding of CLIP-derived high affinity peptides CLIP-YY (IC50% 829 μM of $BeSO_4$; non-biotinylated CLIP-YY IC50% 0.78 μM; Figure 6, panel C), CLIP-QY (IC50% 1,236 μM of $BeSO_4$; non-biotinylated CLIP-QY IC50% 1.01 μM; Figure 6, panel D), CLIP-RF (IC50% >1,500 μM of $BeSO_4$; non-biotinylated CLIP-RF IC50% 0.97 μM; Figure 6, panel E) were only marginally influenced by the presence of Be in the competition assay for soluble HLA-DPGlu69

molecules with affinities varying from 11,843- to >21,429-fold that of CLIPs. All experiments with soluble HLA-DPGlu69 were replicated with soluble HLA-DP2Lys69 molecules: in no instance Be was capable to bind with affinity higher than that of any of the CLIPs and CLIP-derived peptides.

Competition assays in the T-cell system. The effect of high affinity peptides upon Be binding was biologically determinated by measuring T-cell IFN-γ release upon Be-presentation in the absence/presence of the high affinity peptides CLIP-YY, CLIP-QY and CLIP-RF, using CLIP and CLIP-AA as lower affinity controls. All ten HLA-DPGlu69-expressing berylliosis-affected subjects evaluated responded to $BeSO_4$ with IFN-γ release (no stimulus, 10.8±7.2 IFN-γ pg/ml; $BeSO_4$ 2 μM 59.14±11.32 IFN-γ pg/ml, $BeSO_4$ 10 μM 138.30±13.9 IFN-γ pg/ml, $BeSO_4$ 50 μM 107.24±45.6 IFN-γ pg/ml).

For each of the subjects carrying the HLA-DPGlu69 susceptibility marker, the effect of peptides upon antigen presentation was measured at the optimal $BeSO_4$ concentration in the presence of five different peptide concentrations (0.4, 2, 10, 50 and 250 μM). As expected from the peptide-binding data, CLIP exerted minimal inhibition at very high doses, while CLIP-AA did not exert any inhibitory activity even at the highest doses (Figure 6).

Strikingly, the high affinity peptide CLIP-YY did inhibit antigen presentation and T-cell activation, as measured by IFN-γ release, at doses below the molar concentration of the stimulant (Figure 6).

It is interesting to observe that the high affinity peptides CLIP-QY and CLIP-RF markedly enhanced the IFN-γ response to Be at doses that were equimolar with the stimulant (Figure 6).

To determine whether the potentiating effect of the high affinity peptides CLIP-KF and CLIP-RF upon Be IFN-γ release was caused by enhanced Be binding to antigen-presenting cells or by a non-specific superantigenic or mitogenic effect of the peptides themselves, the same tests were

repeated with PBMCs from 5 Be-unexposed normal donors. In these subjects, IFN-γ release was not induced by $BeSO_4$ alone, neither by $BeSO_4$ with the addition of either CLIP, CLIP-AA, CLIP-YY, CLIP-QY and CLIP-RF, nor by the peptide alone (data not shown).

Finally, to confirm the specificity of CLIP-QY and CLIP-RF augmentation of Be antigen presentation by HLA-DPGlu69, we used HLA-DP-specific MoAb B7/21 inhibition as previously described (72, 74). In addition we tested the ability to the CLIP-YY peptide to block the augmentation of Be presentation by the CLIP-QY and CLIP-RF peptides.

As expected, B7/21 inhibited IFN-γ release, induced by Be in the presence of CLIP-QY (88%) and CLIP-RF (76%). Minimal or no inhibition was observed with anti-HLA-DR (Be/CLIP-QY: 9%; Be/CLIP-RF: 5%, p=0.012 compared to HLA-DP), anti-HLA-DQ (Be/CLIP-QY: 1%; Be/CLIP-RF: 0%, p=0.006 compared to HLA-DP), anti-HLA class I (Be/CLIP-QY: 3%; Be/CLIP-RF: 4%, p=0.009 compared to HLA-DP) or the anti-MTB (Be/CLIP-QY: 0%; Be/CLIP-RF:0%, p=0.005 compared to HLA-DP) MoAbs (Figure 7).

Furthermore, the CLIP-YY peptide inhibited IFN-γ release by Be stimulation in the presence of CLIP-QY and CLIP-RF at almost equimolar concentration (Figure 7).

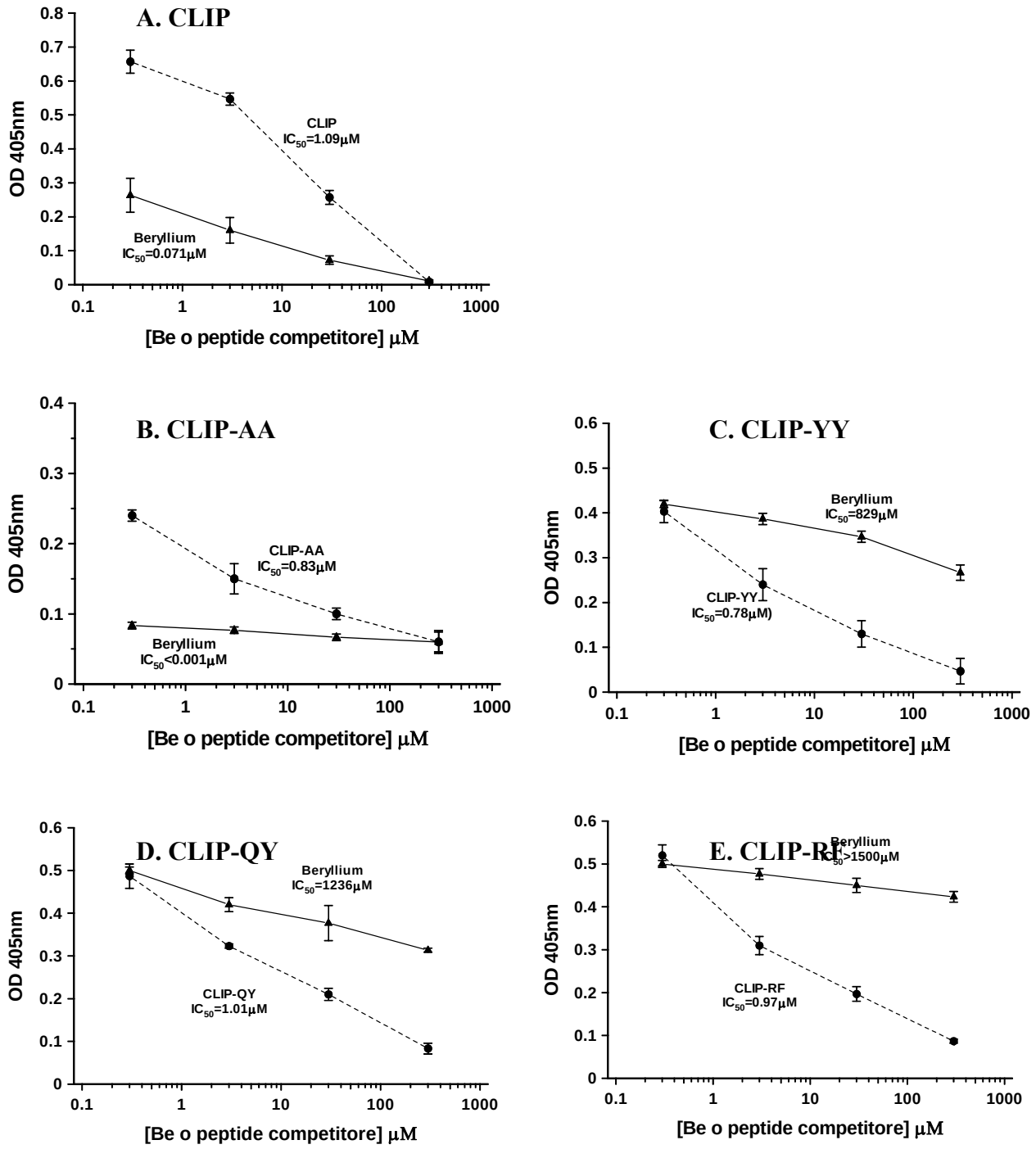


Figure 6. Analysis of the ability of CLIP-derived high affinity peptides to compete for sluble HLA-DPGlu69 molecules with Be *in vitro*. Shown are the peptide/$BeSO_4$ competition curves generated with CLIP, CLIP-AA CLIP-YY, CLIP-QY and CLIP-RF. Peptide affinities for HLA-DPGlu69, relative to CLIP, were 11,843 for CLIP-YY, 17,657 for CLIP-QY and >21, 429 CLIP-RF. Data point represent the mean (±SD) of three separate experiments. In each graph, $BeSO_4$ competitions are represented using triangles with continuous line, while CLIP (or CLIP-derived) peptide competitions are represented using circles with dashed line.

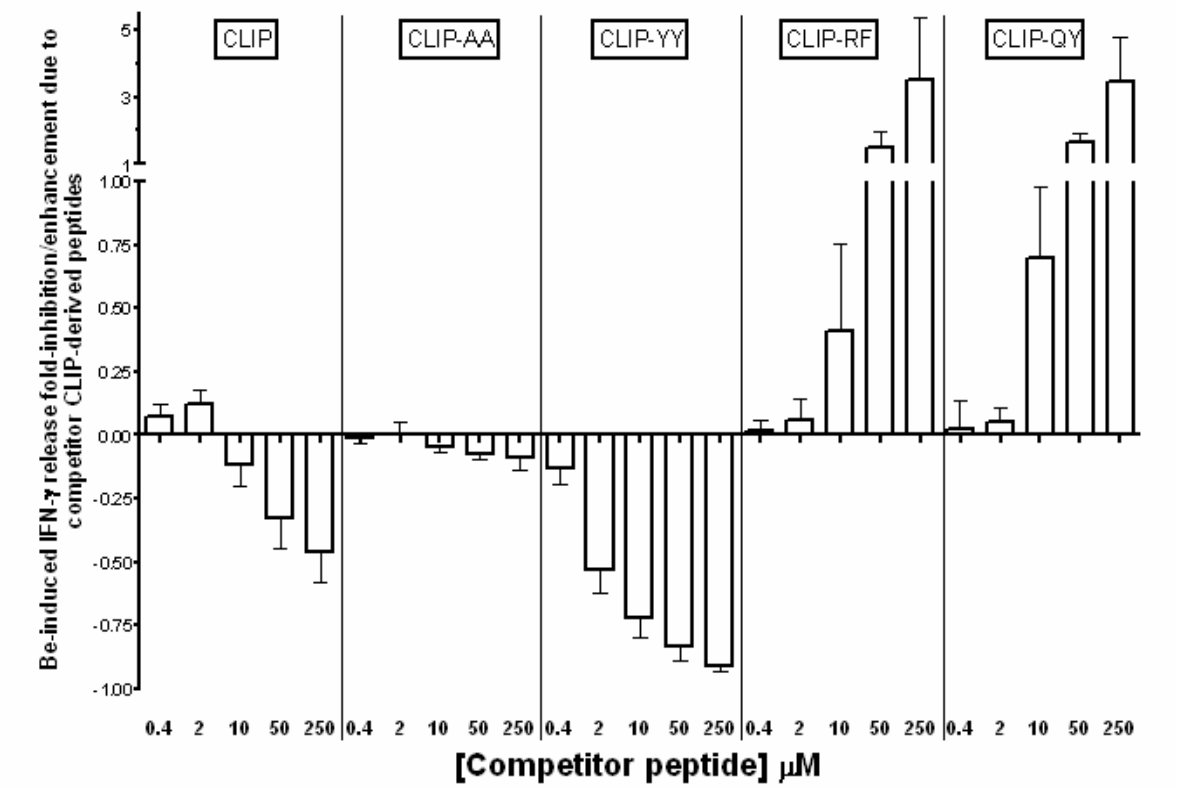


Figure 7. Analysis of the ability of CLIP derived high affinity peptides to interact with T-cell activation by Be antigen presentation in the context of the HLA-DPGlu69 molecule using fresh PBMCs from berylliosis-affected individuals. The inhibition or enhancement curves obtained with the CLIP, CLIP-AA, CLIP-YY, CLIP-QY and CLIP-RF peptides are shown. Each bar represents the mean (±SD) of the triplicates generated from each of five study subjects at the optimal $BeSO_4$ concentration.

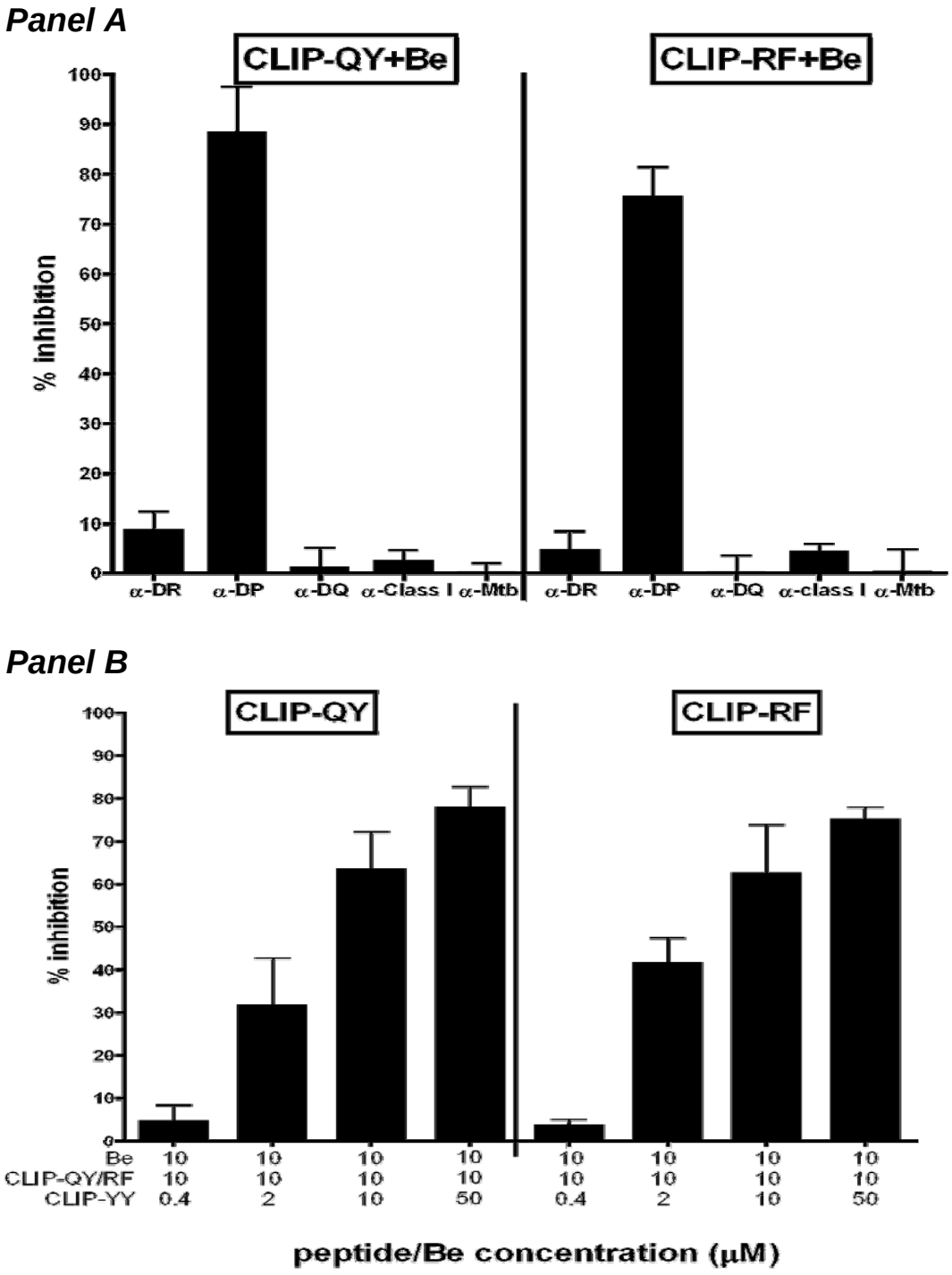


Figure 8 Analysis of the specificity of the enhancement of Be-stimulated T-cell activation in the presence of the high affinity peptides CLIP-QY and CLIP-RF. In <u>panel A</u> are shown the inhibition of Be-stimulated IFN-γ release obtained with the anti-HLA-DR, -DP, -DQ, -class I and *M. tuberculosis* 19kDa protein specific MoAbs in presence of the augmentatory peptides CLIP-QY and CLIP-RF (at optimal concentration of Be and peptide). In <u>panel B</u> are shown the inhibition curves of IFN-γ release obtained with different concentration of CLIP-YY peptide at the optimal or suboptimal concentration of $BeSO_4$ (10μM), CLIP-QY and CLIP-RF (10μM). Each bar represents the mean (<u>+</u>SD) of the triplicates generated from each of study subject at the optimal concentration.

Interim discussion

Structure/function studies have indicated residue Glu69 of the HLA-DP molecule as the key site of Be binding and have suggested that Be might bind to HLA-DPGlu69 molecules by interacting with the Glu residue at position 69 of the β–chain (3, 4, 13, 20). This residue could coordinate the bivalent Be cation together with the electron-donor residues Asnα60, Hisβ9, Glnβ13, Gluβ14, Tyrβ28, Argβ27 and Argβ75 which all line HLA-DP pocket 4. In a molecular model simulation of Be-binding, the most stable Be/HLA-DPGlu69 complex is obtained from coordinating of Be by residues Glnβ13, Gluβ14, Argβ27 and Gluβ69 in pocket 4. However, neither computer modeling or peptide competition assays could precisely determined whether Be binds directly, and exclusively, with the above aminoacid residues in the HLA-DP pocket 4. Thus, the possibility that groove-bound peptides expressing aminoacids carrying electron-donor groups in pocket 4 may participate in the coordination of the Be^{2+} ion by the Glu residue at position 69 within pocket 4, and it has not been ruled out in the stabilization of the Be/HLA-DPGlu69 complex.

In fact, this possibility is suggested by the observation that the CLIP-RF peptide, which carries an Ala to Arg (a stronger electron-donor group) residue substitution in pocket 4, can strongly augment Be presentation and T-cell activation, likely through increased binding affinity of Be for the HLA-DPGlu69 pocket 4 and through increased stability of the complex.

In this context, what was discovered by means of CLIP-YY peptides bring further support to this hypothesis, as CLIP-YY carry an Ala to Tyr substitution in pocket 4 endowing the modified peptide with higher affinity for pocket 4 while expressing lower electron-donor capability. It is important to remark that the CLIP-YY peptide can completely block Be presentation and T-cell activation at doses that are 100-fold lower than those recognized from CLIP even in presence of peptides with similar affinity for pocket 4 (122), but much higher electron-donor ability. This

indicates that the inhibition of Be presentation by CLIP-YY is likely caused by the displacement of a bound peptide participating in the coordination of Be within the pocket.

Current concepts of metal binding to HLA molecules in the determination of metal hypersensitivity are that metal binding may be favored by peptides with high affinity both for the metal and the HLA peptide-binding groove (124, 125). For Be it is reasonable to hypothesize that electron-donor-carrying peptides with high affinity for HLA-DP pocket 4 may cooperate with the existing non polymorphic aminoacid residues pocket 4 in increasing the coordination stability between HLA-DPGlu69 and Be. This concept is in agreement with the recent report that the Be bound to ferritin is able to induce high T-cell proliferation in BH subjects at much lower concentration than Be alone (126).

These findings have potential implication both for the diagnosis and the treatment of berylliosis. With the background of the knowledge that in addition to HLA-DP, HLA-DR variant molecules may determine Be activation of T cells (72, 76) albeit generating weaker and less persistent T-cell responses (121). The extension of these studies to Be binding both to HLA-DP and HLA-DR might allow the design of presentation-enhancing peptides capable of increasing T-cell activation signals in diagnostic tests.

With regard to berylliosis treatment, that today relies upon the use of corticosteroids and, eventually, lung transplantation, the data suggest that blocking peptides could be designed. They could be tried for their potential ability to block or energize T-cell reaction *in vivo*, as the glatiramer acetate (named also copaxone or cop-1) in multiple sclerosis (127), and Fel d1 and PLA on the cat and bee venom allergy (128).

CHAPTER 5 – DISCUSSION

The studies presented in chapters 2-4 represent an advance in the understanding of the functional immunogenetic of berylliosis and, in particular, of the link between MHC class II molecules and pathogenic $CD4^+$ T cells in the initiation and perpetuation of the disease. The understanding of the interaction mechanisms may be of great relevance to understand pathogenesis, epidemiology and prevention strategies of a large number of disorders related to the industrial environment.

In fact, with the expanding use of metals and chemicals in high-technology industries, immunotoxic compounds are now found in the industrial environment as well as in manufactured consumer products. Occupational and/or environmental exposure to metal compounds interacting with the immune system is responsible for a variety of adverse immune reactions including granulomatous inflammations and allergic/atopic IgE-mediated type I immune responses (1, 2).

MODELS FOR METAL PRESENTATION TO $CD4^+$ T CELLS

Since the first observation by Sinigaglia and co-workers in 1985 that nickel was recognized by $CD4^+$ T cells of nickel-allergic individuals as a specific antigen/hapten presented by MHC class II molecules (129), different models of interaction between metals and the immune response genes were proposed (130). Metals might interact with HLA molecules by three different mechanisms: (i) direct binding to the HLA molecule, (ii) binding to the HLA molecule in association with a peptide and (iii) generation of a cryptic peptide capable of binding to the HLA molecule.

1. Direct binding

A model of direct interaction between immune response genes and metals has been proposed for cobalt and gold (Figure 9A). In this model, the metal directly interacts with the HLA molecule and is recognized by the TCR on T-cell surfaces. Potolicchio et al. (90) proposed this interaction model between cobalt and HLA-DP, where the metal directly binds to HLA-DPβ-chain polymorphic residue(s), such as Lys and Glu residues at positions 55-56 and/or 69.

2. Peptide-mediated binding

Another mechanism of interaction between HLA molecules and metals has been proposed for nickel. This metal binds at the same time both to the HLA backbone, by the non-polymorphic His residue at position 81 of the HLA-DRβ-chain, and to an unknown peptide which is bound in the HLA molecule's groove.(Figure 9B) (125).

3. Cryptic peptide

Finally, in addition to the direct or indirect binding to HLA molecules, metals may also generate cryptic peptides which could be presented and recognized as neo-antigens. In fact, divalent cations may change the protease cleavage patterns of proteins (22). As a consequence, changing the conformation of proteins and exposing them to proteolytic enzymes, metals may generate a set of uniquely cleaved peptides recognized as nonself by T cells. A similar mechanism was proposed for mercury salts in generating autoimmunity (Figure 9C) (130).

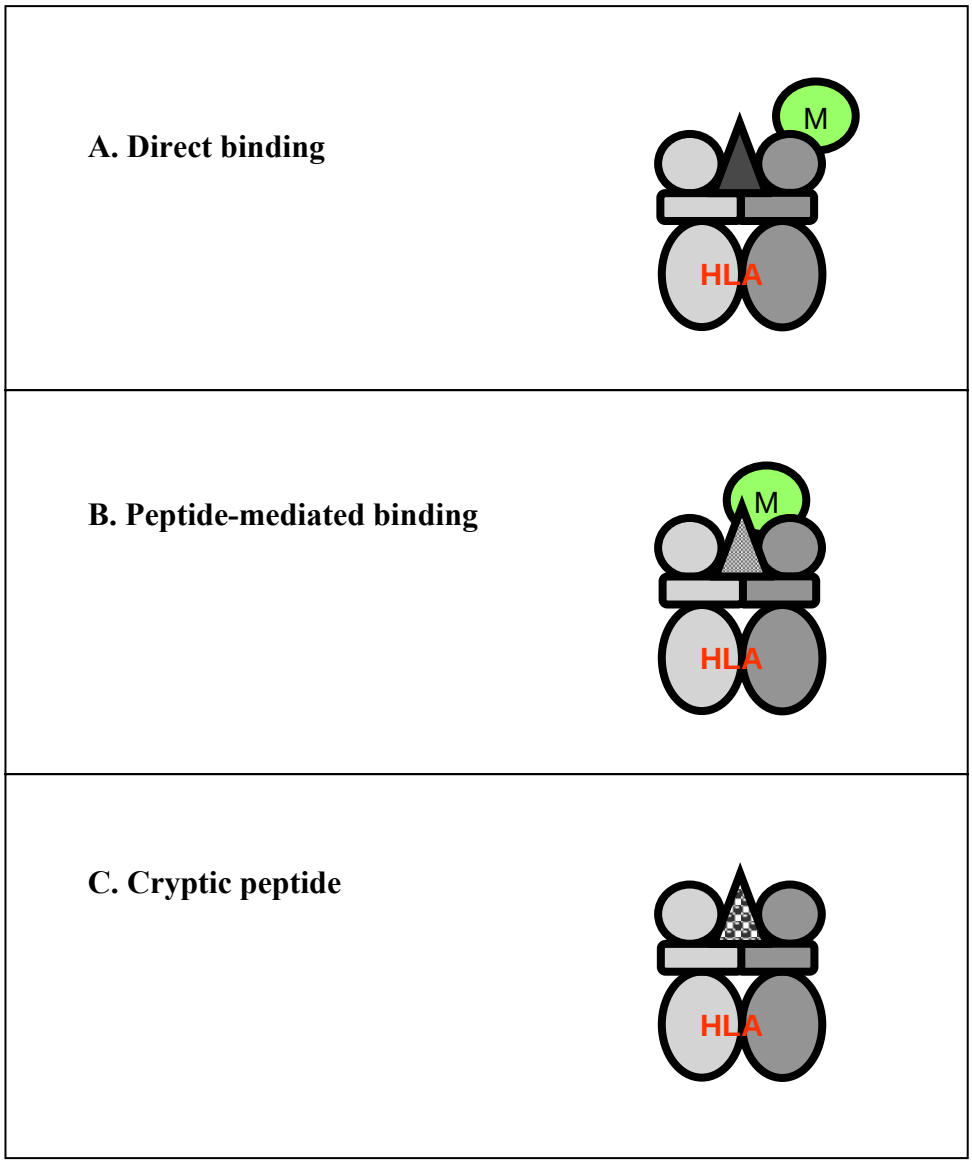


Figure 9. Models of metal presentation to $CD4^+$ T cells in the context of MHC class II molecules. Three models of metal presentation have been hypothesized. Panel A shows the schematic model for the direct binding of a metal (e.g., cobalt and gold) to the MHC class II molecule. Panel B depicts the model of interaction suggested for nickel where the metal binds to the antigenic peptide and the MHC class II molecule. Panel C shows cryptic peptides presented as neoantigens by MHC class II molecule as a result of metal-induced enzymatic cleavage patterns of proteins as suggested for mercury.

FUNCTIONAL IMMUNOGENETICS OF BE HYPERSENSITIVITY

Since the first description of the association of certain HLA-DP molecules and berylliosis in 1993 (64), eight other studies (65-72) confirmed the importance of HLA-DP in the genetic susceptibility of Be-induced diseases. In fact, ~80% of subjects with Be hypersensitivity express an HLA-DP allele with the Glu at position 69 of the β chain (HLA-DPGlu69). Whether homozygosity for Glu69-containing alleles confers a greater risk of disease development in Be-exposed individuals remains a matter of controversy (66, 69, 70). Recent studies have shown that HLA-DPGlu69 is a risk factor for the development of Be sensitization and not simply a marker of progression from sensitization to disease (68-71). Conversely, Saltini et al. (67) identified no association between Glu69-containing DPB1 alleles and Be sensitization. In addition, rare HLA-DP alleles carrying Glu69 (i.e., non HLA-DPB1*0201 alleles), more than the HLA-DPGlu69 itself, were associated with berylliosis in some studies (66, 68, 69).

Approximately 20% of BH subjects do not possess an HLA-DPGlu69-containing allele which suggests the importance of other MHC class II molecules in the genetic susceptibility to Be-induced diseases (67, 70). In this context, the study presented in this thesis suggests that a Phe at position 47 of the HLA-DRβ–chain (HLA-DRPhe47) is associated with Be hypersensitivity in HLA-DPGlu69-negative subjects (72). Similarly, an association with Be hypersensitivity has been noted for HLA-DR*13 alleles in another series of HLA-DPGlu69-negative subjects (70). HLA-DR*13 alleles possess both a Phe at position 47 and a Glu at position 71 of the DRβ-chain (which corresponds to position 69 of HLA-DP). The role of these genetic susceptibility alleles in the immunopathogenesis of Be-induced diseases is believed to occur through the presentation of a critical epitope by the associated MHC class II molecule to disease-relevant $CD4^+$ T cells. In most instances, this mechanism for association in most immune-mediated diseases is poorly defined due

to the unknown initiating autoantigen(s). Conversely, berylliosis provided a unique opportunity to investigate whether the association of MHC class II molecules and disease susceptibility occurs at the level of antigen presentation.

Ex vivo and *in vitro* studies have shown that Be is presented to $CD4^+$ T cells in the context of the MHC class II molecules (45, 60, 73). Using Be-specific T-cell lines and clones derived from bloods and lungs of different berylliosis patients, both Be-induced proliferation and cytokine secretion were nearly completely and selectively inhibited by the addition of a MoAb directed against HLA-DP, which indicates that HLA-DP was the primary MHC class II molecule involved in Be presentation (60, 73-75, 78). In these studies, only certain HLA-DP molecules, all carrying HLA-DPGlu69, were capable of Be presentation, and these molecules closely matched those implicated in disease susceptibility. Together, these data indicate that the HLA contribution to disease susceptibility is related to the ability of certain HLA-DP molecules to bind and present Be to T cells.

In subjects without an HLA-DPGlu69-containing allele, the study here presented, together with other recent studies, have shown that HLA-DR assumes the predominant role in Be presentation (73, 76). Thus, a hierarchy of Be presentation to $CD4^+$ T cells has been established with HLA-DP assuming the predominant role, with a minor role for HLA-DR and no role for HLA-DQ. Functional studies confirming genetic susceptibility studies, have proved that HLA-DP is the primary immune response gene for Be presentation to pathogenic $CD4^+$ T cells.

The HLA-DP gene comprises 107 HLA-DPB1 protein alleles of which 36 alleles carry the HLA-DPGlu69 (IMGT/HLA database Release 2.9.0, 08-Apr-2005). Only a few of these alleles are present in different human populations with an allelic frequency greater than 5%, and most of them being very rare alleles. As discussed above, immunogenetic data have suggested that some other residues and/or different HLA-DP alleles should be preferentially associated with berylliosis more than HLA-DPGlu69 itself. From a functional point of view, these HLA-DP alleles and/or

combination of polymorphic residues should be able to present Be more efficiently than HLA-DPGlu69 alone. However, with the limitation of only a small number of functional studies addressing this problem, the data suggest that the critical aminoacid residue in the HLA-DP molecule is the Glu at position 69 of the β-chain (60, 73, 76). For example, using fibroblasts expressing mutated HLA-DP2 molecules, Be recognition was solely dependent on the Glu at position 69 of the DPβ-chain (76). Mutagenesis of other polymorphic amino acid residues such as the Lys and Glu at positions 55 and 56 of the DPβ-chain had no effects on either Be-induced proliferation or Th1-type cytokine expression (76). In this context, it is worth noting that other polymorphic residues such as Val36, Asp55 and Glu56, of the HLA-DPβ-chain frequently coexist in disease-associated HLA-DP alleles with the exception of a few, very rare alleles. Modeling of the HLA-DP molecule also supports the key role of the HLA-DPGlu69 residue in peptide binding with respect to the other HLA-DP polymorphisms found in association with berylliosis. The β69 residue of HLA-DP molecules is located in the pocket 4 of the peptide-binding groove. The presence of either a Glu or Lys at this position determines critical changes in the shape and charge distribution of the HLA-DP pocket 4 and of the nearby pocket 6. These changes in both shape and charge play a fundamental role in determining peptide-binding selectivity in HLA-DP (107, 122, 123). Conversely, other HLA-DP polymorphisms seem less critical than residue 69 for peptide binding (121).

Whether Glu69 homozygosity plays a role in the genetic susceptibility to berylliosis and/or the progression from Be sensitization to berylliosis remains an unanswered question. Due to the low number of berylliosis cases and Be-exposed controls, this remains a matter of controversy. Only the study by McCanlies et al. (71) tackled the question of HLA-DPGlu69 homozygosity with appropriate population numerosity and appropriate statistical tools. In their study, the increase of the

HLA-DPGlu69 frequency in the berylliosis-patient population was associated with a significant increase in homozygosity. However, this increase was significantly lower than expected in accordance with the Hardy-Weinberg law suggesting an excess of heterozygosity in berylliosis cases. In other words, the inheritance of just one HLA-DPGlu69-containing allele conferred a high risk of berylliosis, as expected when environmental pressures exert a pathologic influence through a genetically determined pathway (121). This concept is supported by information available regarding the HLA-DP molecule surface density and the number of MHC class II/peptides necessary for the induction of an immune response. In fact, Lanzavecchia and Viola showed that the number of TCRs needing to be engaged in order to initiate an immune response varies from 1,500 to 8,000 (131). The number of HLA-DP molecules on naive monocytes has been estimated to be greater than 100,000 per cell, and greater than 400,000 per cell on IFN–γ activated monocytes (89). Thus, it is unlikely that HLA-DPGlu69 homozygosity plays a critical role in driving T-cell responses to a low molecular weight, high affinity hapten such as Be.

MODELS OF BERYLLIUM PRESENTATION IN THE CONTEXT OF HLA-DPGLU69 MOLECULES.

Previous studies have shown that HLA-DPGlu69 is capable of binding Be with an affinity of 40-100-fold higher than that of its polymorphic counterpart, HLA-DPLys69 (77). It is possible that Be binds directly to HLA-DPGlu69 molecules due to the unique properties of the HLA-DP pocket 4 (122). In fact, in addition to the Glu69 residue, other electron-donor groups are present in the HLA-DP pocket 4 or in its proximity (e.g., Asnα60, Hisβ9, Glnβ13, Gluβ14, Tyrβ28, Argβ27 and Argβ75). They could directly bind Be as they are within close proximity (from 3.1 to 6.4 Å in the HLA-DP2 molecular model (122, 113) to coordinate the positively charged Be^{2+} ion. Molecular modeling could help in simulating Be binding and determine the most stable Be^{2+}/HLA-DPGlu69 complex in the pocket 4, which is obtained from the coordination of Be by residues Glnβ13, Gluβ14, Argβ27 and Gluβ69 (Figure 10, panel A). However, computer modeling or peptide competition assays could not precisely determine whether Be binds directly, and exclusively, with the above aminoacid residues in the HLA-DP pocket 4. Thus, it is possible that groove-bound peptides expressing aminoacids which carry electron-donor groups may participate in the coordination of the Be^{2+} ion with the Glu residue at position 69 within the pocket 4. This possibility is suggested by our observation that CLIP-RF or CLIP-QY peptides, which carries an Ala to Arg or a Ala to Gln (both strong electron-donor groups) residue substitution in pocket 4, can strongly augment Be presentation and T-cell activation; likely this is due to increased Be-binding affinity for the HLA-DPGlu69 pocket 4 and to increased stability of the complex (Figure 10 panel B). This is in line with the recent observation that Be bound to ferritin induces greater T-cell proliferation in berylliosis patients at much lower concentrations than $BeSO_4$ alone (42).

Conversely, peptides binding with high affinity a peptide presented by HLA-DPGlu69

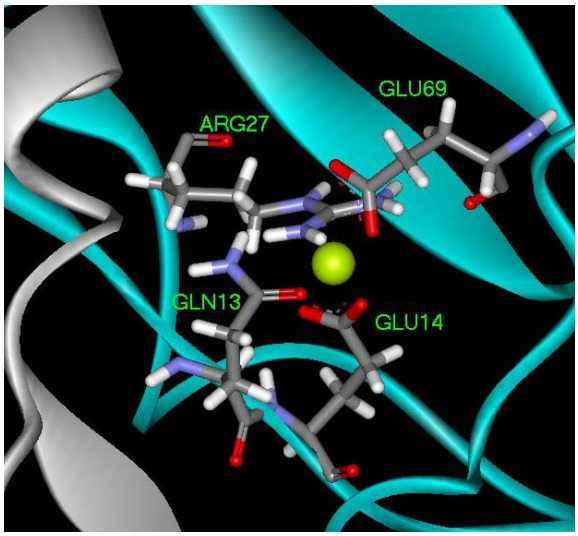
pocket 4 molecule, could block the Be induced T-cell response. In this context, discoveries about the CLIP-YY peptide bring further support to this hypothesis. In fact, CLIP-YY carries an Ala to Tyr substitution in P4 endowing the modified peptide with higher affinity for pocket 4 while expressing lower electron-donor capability. It is worth noting that the CLIP-YY peptide can completely block Be presentation and T-cell activation at doses that are 100-fold lower than those recognized from CLIP even in presence of peptides with similar affinity for pocket 4 (122), but much higher electron-donor ability. This indicates that the inhibition of Be presentation by CLIP-YY is likely caused by the displacement of a bound peptide participating to Be coordination within pocket 4 (figure 10 panel C).

HLA-DR RESTRICTION OF BERYLLIUM PRESENTATION

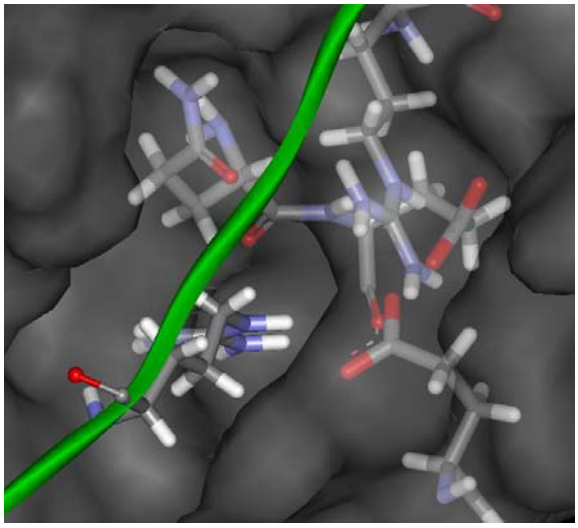
The most recent immunogenetic and functional studies in the field of berylliosis have focused on the fraction of Be-sensitized and berylliosis subjects who do not carry an HLA-DPGlu69-containing allele including the studies presented in this thesis (70, 72, 76). Taking together all of the available information to address the question of which HLA-DR polymorphic residues are critical for Be presentation to pathogenic $CD4^+$ T cells, we might depict several scenarios. First, HLA-DRGlu71 is playing a role in presentation of Be in HLA-DR*13 molecules (76). However, this Glu at position 71 is rare in other HLA-DR alleles and was present in only 7 out of 22 HLA-DPGlu69-negatives subjects in one study (72) and 8 out of 19 in another one (70). Second, HLA-DR-restricted Be-induced T-cell stimulation can occur in subjects who do not express HLA-DR*13 alleles suggesting the importance of other HLA-DR polymorphisms in addition to DRGlu71 (72). Finally, HLA-DRPhe47, present in 21 out of 22 HLA-DPGlu69-negatives BH subjects, could represent only a marker of susceptibility playing no direct role in Be presentation through HLA-DR, although this residue has been shown to be critical in peptide binding (85, 86). In

this context, it is worth noting that residues β71 and β47 are mapping in HLA-DR pocket 4 and the nearby pocket 7 (72). As opposed to HLA-DP where only Glu69 is playing a critical role in the pocket 4 conformation, the pocket 4 of HLA-DR is giving allelic specificity with many different possible conformations (189, 122). By using available HLA-DR crystal structures and molecular models to analyze the pockets 4 and 7 in HLA-DR alleles carrying HLA-DRPhe47 and/or HLA-DRGlu71 with respect to their counterparts (48), it appears that the H-bond network more than the single amino acid residues could play a critical role in Be binding. Thus, it is likely that more than one combination of residues in HLA-DR pockets 4 and 7 area could play a role in Be presentation. The role of HLA-DRPhe47 could be just to leave H-bonds available for interaction, while its counterpart Tyr47 could engage some of them (48). Once HLA-DRPhe47 is present, other residues in the area and/or bound peptide residues could play a direct role in coordinating Be, and changing one of these residues could have the same inhibitory effects on T-cell activation as seen with the mutation of the Glu at position 71 of HLA-DR13 to an Arg (76).

Panel A



Panel B



Panel C

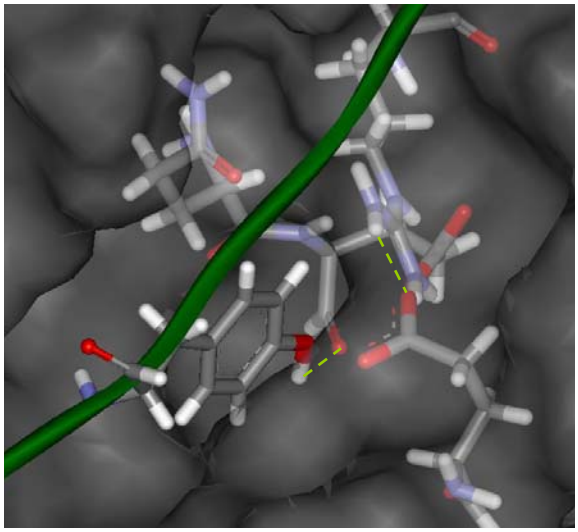


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Figure 10. Panel A. Analysis of the HLA-DPGlu69 molecule and Be interaction in pocket 4 of HLA-DP2 molecule. The figure shown the most stable HLA-DPGlu69/Be complex model among all the possible interactions models evaluated between HLA-DP-groove electron-donor groups and Be as ion. Specifically, the peptide-binding pocket 4 of HLA-DP2 is capable to coordinate Be by using its residues Glnβ13, Gluβ14, Argβ27 and Gluβ69. The HLA-DPα-chain backbone is reported in grey colored ribbon style, while the HLA-DPβ-chain backbone is reported in light blue colored ribbon. Aminoacids reported in stick style and colored by atom (C: grey; O: red; N: blue; H: white). Be is shown as van der Walls radius and colored in green.
Panel B. Analysis of the HLA-DPGlu69 molecule and CLIP-RF interaction in pocket 4 of HLA-DP2 molecule. The figure shown the Arg94 of the CLIP-RF peptide buried in pocket 4 of HLA-DP2. This aminoacid does not form H-bond with the Gluβ14 or Glnβ13 and together with Argβ27 and Gluβ69, that are interacting with H-bond, are representing an electron-donor environment that could determine Be coordination. CLIP-RF backbone is shown in green. The HLA-DP surface is shown in semi-trasparence and electron-donor aminoacids in pocket 4 are shown in stick style colored by atom (C: grey; O: red; N: blue; H: white). H-bonds are shown in green.
Panel C. Analysis of the HLA-DPGlu69 molecule and CLIP-YY interaction in pocket 4 of HLA-DP2 molecule. The figure shown the Tyr94 of the CLIP-YY peptide deeply buried in pocket 4 of HLA-DP2 interacting by H-bond with the Gluβ14. In this way both these electron-donors aminoacids are unavailable for interacting with Be and the Glnβ13 becomes too far from Argβ27 and Gluβ69 for coordinating Be. Argβ27 and Gluβ69 are interacting with H-bond. CLIP-YY backbone is shown in green. The HLA-DP surface is shown in semi-trasparence and electron-donor aminoacids in pocket 4 are shown in stick style colored by atom (C: grey; O: red; N: blue; H: white). H-bonds are shown in green.

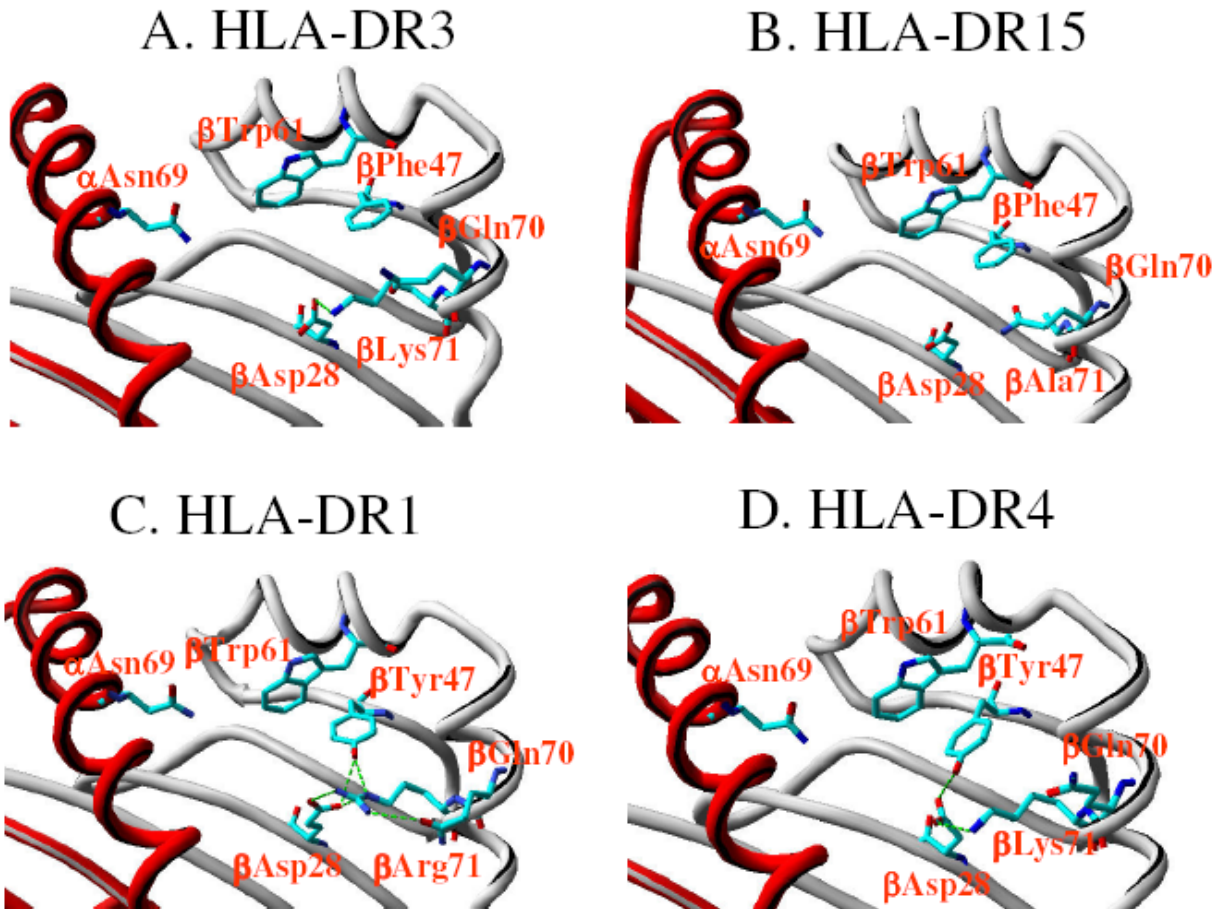

Figure 11. Analysis of the H-bond network in the pocket 7, the peptide binding pocket where the HLA-DR residue β47 is mapping, of HLA-DR molecules carrying HLA-DRPhe47 (panel A: HLA-DR3 and panel B: HLA-DR15) or its counterpart Tyr47 (panel C: HLA-DR1 and panel D: HLA-DR4). Molecular modeling of the PDB entry crystal structures (HLA-DR3: 1A6A; HLA-DR15: 1BX2; HLA-DR1: 1AQD; HLA-DR4: 2SEB) have been evaluated with the SwissPDB viewer v3.7b2 software (free available at http://ca.expasy.org/spdbv/). The HLA-DRα-chain backbone is reported in red colored ribbon style, while the HLA-DRβ-chain backbone is reported in grey colored ribbon. Aminoacids are colored in CPK style (C: light blue; O: red; N: blue) and residue names are reported in red. H-bonds were computed with the SwissPDB viewer (H-bond detection threshold: 1.20–2.76 A when Hydrogen is present and 2.19–3.30 A when hydrogen is absent) and are shown as green dashed lines. All the aminoacids presenting electron-donor groups in the pocket 7, of HLA-DR1, -DR3, -DR4 and -DR15, putatively capable of coordinating Be are shown (residues α69, β28, β61, β70 and β71). In the HLA-DR3 crystal structure (panel A) with the presence of Phe47 only one of the two terminal oxygens of Aspβ28 is engaged in a H-bond network with Lys71, leaving four other contacts **points for co**ordinating Be (specifically residues αAsn69, βAsp28, βTrp61, βGln70). A similar pattern is present in HLA-DR15 (panel B) where, with the presence of Phe47, no H-bonds are present leaving 5 electron-donor groups available for Be coordination (specifically one electron-donor group for each residue αAsn69, βTrp61, βGln70 and two electron-donor groups for βAsp28). When Tyr47, the HLA-DRPhe47 counterpart, is present in HLA-DR molecules as in HLA-DR1 (panel C) and HLADR4 (panel D), the H-bond network of pocket 7 results dramatically modified. Specifically, Tyr47 engages in a H-bond network with residues Asp28 and Arg71 in HLA-DR1 (panel C) or Asp28 and Lys71 in HLA-DR4 (Panel D). As a consequence there is reduced availability of electron donor groups capable to coordinate Be.

IMPLICATIONS FOR THE FUTURE: THE SPECIFIC IMMUNOTHERAPY OF BERYLLIOSIS.

The present treatment of berylliosis relies upon the use of corticosteroids and, eventually, lung transplantation (2, 17). The new advances in the field of berylliosis could figure out some possible more specific alternative treatments based on block of Be processing and/or of HLA class II/Be-complex formation or the block of responding T cells.

Scavengers that could let the rapid elimination of Be from the lung milieu could help in blocking Be-uptake and processing. Humanized MoAbs direct to HLA-DP could be successfully used to block Be-presentation as for *in vitro* models (60, 72-76). Similarly, taking the multiple sclerosis, treated with glatimer acetate, as a model (65), we could hypothesize a potential block of both Be presentation and Be-specific T-cell anergization by using high-affinity peptides for HLA-DP and -DR at the site of the inflammation, as we presented for CLIP-YY peptide in the *in vitro* model of "berylliosis therapy".

Most of the information for all these approaches are now in hand. The new era of the specific immunological treatment of berylliosis is just one step further as much as we believe that studies in Be-induced disease will teach us important lessons for common autoimmune diseases.

REFERENCES

1. Nemery B. Metal toxicity and the respiratory tract. Eur Respir J 1990; 3: 202-219.
2. Saltini C, Amicosante M. Beryllium disease. Am J Med Sci. 2001; 321: 89-98.
3. Fine JM, Gordon T, Chen LC, Kinney P, Falcone G, Beckett WS. Metal fume fever: characterization of clinical and plasma IL-6 responses in controlled human exposures to zinc oxide fume at and below the threshold limit value. J Occup Environ Med 1997; 39: 722-726.
4. Blanc PD, Boushey HA, Wong H, Wintermeyer SF, Bernstein MS. Cytokines in metal fume fever. Am Rev Respir Dis. 1993; 147: 134-138.
5. Robinson DS, Richeldi L, Saltini C, duBois RM. Granulomatous processes. In: The Lung: Scientific Foundations, 2nd edition. Crystal RG, West JB, Weibel ER, Barnes PJ Eds. Raven Press, New York, N.Y. 1997; 2395-2409.
6. Kreiss K, Miller F, Newman LS, Ojo-Amaize EA, Rossman M, Saltini C. Chronic beryllium disease - from the work place to cellular immunology, molecular immunogenetics, and back. Cell Immunol Immunopath 1994; 71: 123-129.
7. Bartter T, Irwin RS, Abraham JL, Dascal A, Nash G, Himmelstein JS et al. Zirconium compound-induced pulmonary fibrosis. Arch Intern Med 1991; 151: 1197-1201.
8. Liippo KK, Anttila SL, Taikina -Aho O, Ruokonen E-L, Toivonen ST, Tuomi T. Hypersensitivity pneumonitis and exposure to zirconium silicate in a young ceramic tile worker. Am Rev Respir Dis 1993; 148: 1089-1092.
9. Romeo L, Cazzadori A, Bontempini L, Martini S. Interstitial lung granulomas as a possible consequence of exposure to zirconium dust. Med Lav 1994; 85: 219-222.

10. Redline S, Barna BP, Tomashefski JF, Abraham JL. Granulomatous disease associated with pulmonary deposition of titanium. Br J Ind Med 1986; 43: 652-656.
11. De Vuyst P, Dumortier P, Schandené L, Estenne M, Verhest A, Yernault JC. Sarcoidlike lung granulomatosis induced by aluminum dusts. Am Rev Respir Dis 1987; 135: 493-497.
12. Newman LS., Maier LA.. Beryllium disease. In *Interstitial Lung Disease.* M. I. Schwarz, and T. E. King, Jr, eds. B.C. Decker Inc., Hamilton 2003; 435-451.
13. Van Ordstrand HS, Hughes R, Carmody MG. Chemical pneumonia in workers extracting beryllium oxide. Report of three cases. Cleve Clin Q Archives 1943; 51:431-439.
14. DeNardi JM, VanOrdstand HS, Carmody MG. Acute dermatitis and pneumonitis in beryllium workers: review of 406 cases in 8-year period with follow-up on recoveries. Ohio State Med J 1949; 45:467.
15. Hardy HL, Tabershaw IR. Delayed chemical pneumonitis in beryllium workers. J Ind Hyg Toxicol 1946;28:197 cited in Tepper, Hardy and Chamberlin.
16. Tepper LB, Hardy HL, Chamberlin RI. Toxicity of beryllium compounds. Elsevier Publishing Company, Amsterdam 1961.
17. Sterner J.H. and Eisenbud M. Epidemiology of beryllium intoxication. Arch Ind Hyg Occup Med 1951; 4:123-151.
18. Eisenbud M. Commentary and update: chemical pneumonia in workers extracting beryllium oxide. Cleve Clin Q 1984; 51:441-447.
19. Sneddon IB. Berylliosis a case report British Med J. 1955;ii:1448-1450.
20. Cullen MR, Kominsky JR, Rossman MD, Cheriack MG, Rankin JA, Balmes JR et al. Chronic beryllium disease in a precious metal refinery. Am Rev Respir Dis 1987; 135: 201-208.

21. Kotloff RM, Richman PS, Greenacre JK, Rossman MD. Chronic beryllium disease in a dental laboratory Am Rev Respir Dis 1993; 147: 205-207.
22. Haberman AL, Pratt M, Storrs FJ. Contact dermatitis from beryllium in dental alloys. Contact dermatitis 1993; 28: 157-162.
23. Kriebel D, Brain JD, Sprince NL, Kazemi H. The pulmonary toxicity of beryllium. Am Rev Respir Dis 1988; 137: 464-73.
24. Kreiss K, Wasserman S, Mroz MM, Newman LS. Beryllium disease screening in the ceramics industry. J Occup Med 1993; 35: 267-274.
25. Kreiss K, Mroz MM, Zhen B, Martyny JW, Newman LS. Epidemiology of beryllium sensitization and disease in nuclear workers Am Rev Respir Dis 1993; 148: 985-991.
26. Kreiss K, Mroz MM, Newman LS, Martyny J, Zhen B. Machining risk of beryllium disease and sensitization with median exposures below 2 mg/m^3. Am J Indust Med 1996; 30: 16-25.
27. Kreiss K, Cox-Ganser J. Metalworking fluid-associated hypersensitivity pneumonitis: a workshop summary. Am J Ind Med 1997; 32: 423-432.
28. Sprince NL, Kazemi H, Hardy HL. Current problem of differentiating between beryllium disease and sarcoidosis. Ann NY Acad Sci 1975; 278: 654-664.
29. Jones Williams W. Beryllium disease, pathology and diagnosis. J Soc Occup Med 1977; 27: 93-96.
30. Jones Williams W. Beryllium disease. Postgrad Med J 1988; 64: 511-516.
31. De Vuyst P, Dumortier P, Schandené L, Estenne M, Verhest A, Yernault JC. Sarcoidlike lung granulomatosis induced by aluminum dusts. Am Rev Respir Dis 1987; 135: 493-497.
32. Redline S, Barna B, Tomashefski JF, Abraham JL. Granulomatous disease associated with pulmonary deposition of titanium. Br J Indust Med 1986; 27: 652-656.

33. Sprince NL, Kazemi H, Hardy HL. Current problem of differentiating between beryllium disease and sarcoidosis. Ann NY Acad Sci 1975; 278: 654-664.

34. Jones Williams W. The nature and origin of Schaumann bodies. J Path Bact 1960; 79:193-203.

35. Freiman DG, Hardy HL. Beryllium disease. Hum Pathol 1970;1:25-44.

36. Hardy HL. Beryllium disease: a continuing medical problem. The Am J Medical Sci 1961; 242:150-155.

37. Williams WR, Jones Williams W. Comparison of lymphocyte transformation and macrophage migration inhibition tests in the detection of beryllium hypersensitivity J Clin Pathol 1982; 35: 684-687.

38. Mroz MM, Kreiss K, Lezotte DC, Campbell PA, Newman LS. Re-examination of the blood lymphocyte transformation test in the diagnosis of chronic beryllium disease. J Allergy Clin Immunol 1991; 88: 54-60.

39. Jones WIlliams W. Diagnostic criteria for chronic beryllium disease (BERYLLIOSIS) based on the U.K. registry 1945-1991. Sarcoidosis 1993;10: 41-27.

40. Stokes RF, Rossman MD. Blood cell proliferation to beryllium: analysis by receive-operating characteristics. JOM 1991; 33: 23-28.

41. Markham TN. Screening for chronic beryllium disease using beryllium specific lymphocyte proliferation testing. Int Arch Occup Environ Health 1996; 68: 405-407.

42. Kreiss K, Miller F, Newman LS, Ojo-Amaize EA, Rossman M, Saltini C. Chronic beryllium disease - from the work place to cellular immunology, molecular immunogenetics, and back. Cell Immunol Immunopath 1994; 71: 123-129.

43. Rossman MD, Kern JA, Elias JA, Cullen MR, Epstein PE, Preuss OP et al. Proliferative response of bronchoalveolar lymphocytes to beryllium. Ann Int Med 1988; 108: 687-693.

44. Fontenot AP, Maier LA. Genetic susceptibility and immune-mediated destruction in beryllium-induced disease, Trends Immunol 2005; 26: 543-549.

45. Saltini C, Winestock K, Kirby M, Pinkston P, Crystal RG. Maintenance of alveolitis in patients with chronic beryllium disease by beryllium-specific helper T cells, N Engl J Med 1989; 320: 1103-1109.

46. Saltini C, Kirby M, Trapnell BC, Tamura N, Crystal RG. Biased accumulation of T lymphocytes with "memory"-type CD45 leukocyte common antigen gene expression on the epithelial surface of the human lung, J Exp Med 1990; 171: 1123-1140.

47. Fontenot AP, Palmer BE, Sullivan AK, Joslin FG, Wilson CC, Maier LA, Newman LS, Kotzin BL. Frequency of beryllium-specific, central memory $CD4^+$ T cells in blood determines proliferative response. J Clin Invest 2005; 115: 2886-2893.

48. Amicosante M, Fontenot AP. T cells recognition in chronic beryllium disease. Clinical Immunology 2006; Nov, 121(2):134-43.

49. Fontenot AP, Newman LS, Kotzin BL. Chronic beryllium disease: T cell recognition of a metal presented by HLA-DP. Clin Immunol 2001; 100: 4-14.

50. Fontenot AP, Kotzin BL. Chronic beryllium disease: immune-mediated destruction with implications for organ-specific autoimmunity. Tissue Antigens 2003; 62: 449-458.

51. Rossman MD, Kern JA, Elias JA, Cullen MR, Epstein PE, Preuss OP, Markham TN, Daniele RP. Proliferative response of bronchoalveolar lymphocytes to beryllium. Ann Intern Med 1988; 108: 687-693.

52. Fontenot AP, Maier LA, Canavera SJ, Hendry-Hofer TB, Boguniewicz M, Barker EA, Newman LS, Kotzin BL. Beryllium skin patch testing to analyze T cell stimulation and granulomatous inflammation in the lung. J Immunol 2002; 168: 3627-3634.

53. Thomas PD, Hunninghake GW. Current concepts of the pathogenesis of sarcoidosis.

American Review of Respiratory Disease 1987; 135: 747-760.

54. Fontenot AP, Kotzin BL, Comment CE, Newman LS. Expansions of T-cell subsets expressing particular T cell receptor variable regions in chronic beryllium disease. Am J Respir Cell Mol Biol 1998; 18: 581-589.
55. Fontenot AP, Falta MT, Freed BM, Newman LS, Kotzin BL. Identification of pathogenic T cells in patients with beryllium-induced lung disease. J Immunol 1999; 163: 1019-1026.
56. Hardy HL, Tabershaw IR. Delayed chemical pneumonitis in workers exposed to beryllium compounds. J Ind Hyg Toxicol 1946; 28: 197-211.
57. Tinkle SS, Kittle LA, Schumacher BA, Newman LS. Beryllium induces IL-2 and IFN-g in berylliosis. J Immunol 1997; 158: 518-526.
58. Saltini C, Amicosante M, Franchi A, Lombardi G, Richeldi L. Immunogenetic basis of environmental lung disease: lessons from the berylliosis model. Eur Respir J 1998; 12: 1463-1475.
59. Tinkle SS, Kittle LA, Newman LS. Partial IL-10 inhibition of the cell-mediated immune response in chronic beryllium disease. J Immunol 1999; 163: 2747-2753.
60. Lombardi G, Germain C, Uren J, Fiorillo MT, du Bois RM, Jones-Williams W, Saltini C, Sorrentino R, Lechler R. HLA-DP allele-specific T cell responses to beryllium account for DP- associated susceptibility to chronic beryllium disease. J Immunol 2001; 166: 3549-3555.
61. Eisenbud M, Lisson J. Epidemiological aspects of beryllium-induced nonmalignant lung disease: a 30 year update. J Occup Med 1983; 25:196-202.
62. Barna BP, Deodhar SD, Chiang T, Gautam S, Edinger M. Experimental beryllium-induced lung disease. I Differences in immunologic responses to beryllium compounds in strain 2 and 13 guinea pigs. Int Arch Allerg Appl Immun 1984; 73: 42-48.

63. Huang H, Meyer KC, Kubai L, Auerbach R. An immune model of beryllium-induced pulmonary granulomata in mice Labor Invest 1992; 67: 138-146.

64. Richeldi L, Sorrentino R, Saltini C. HLA-DPB1 Glutamate 69: a genetic marker of beryllium disease. Science 1993; 262:242-244.

65. Richeldi L, Kreiss K, Mroz MM, Zhen B, Tartoni P, Saltini C. Interaction of genetic and exposure factors in the prevalence of berylliosis. Am J Ind Med 1997; 32: 337-340.

66. Wang Z, White PS, Petrovic M, Tatum OL, Newman LS, Maier LA, Marrone BL. Differential susceptibilities to chronic beryllium disease contributed by different Glu69 HLA-DPB1 and -DPA1 alleles. J Immunol 1999; 163:1647-1653.

67. Saltini C, Richeldi L, Losi M, Amicosante M, Voorter C, van den Berg-Loonen E, Dweik RA, Wiedemann HP, Deubner DC, Tinelli C. Major histocompatibility locus genetic markers of beryllium sensitization and disease. Eur Respir J 2001; 18: 677-684.

68. Wang Z, Farris GM, Newman LS, Shou Y, Maier LA, Smith HN, Marrone BL. Beryllium sensitivity is linked to HLA-DP genotype. Toxicology 2001; 165: 27-38.

69. Rossman MD, Stubbs J, Lee CW, Argyris E, Magira E, Monos D. Human leukocyte antigen Class II amino acid epitopes: susceptibility and progression markers for beryllium hypersensitivity. Am J Respir Crit Care Med 2002; 165: 788-794.

70. Maier LA, McGrath DS, Sato H, Lympany P, Welsh K, Du Bois R, Silveira L, Fontenot AP, Sawyer RT, Wilcox E, Newman LS. Influence of MHC CLASS II in susceptibility to beryllium sensitization and chronic beryllium disease. J Immunol 2003; 171: 6910-6918.

71. McCanlies EC, Ensey JS, Schuler CR, Kreiss K, Weston A. The association between HLA-DPB1Glu69 and chronic beryllium disease and beryllium sensitization. Am J Ind Med 2004; 46: 95-103.

72. Amicosante M, Berretta F, Rossman M, Butler RH, Rogliani P, van den Berg-Loonen E, Saltini C. Identification of HLA-DRPhebeta47 as the susceptibility marker of hypersensitivity to beryllium in individuals lacking the berylliosis-associated supratypic marker HLA-DPGlubeta69. Respir Res 2005; 6: 94.

73. Fontenot AP, Torres M, Marshall WH, Newman LS, Kotzin BL. Beryllium presentation to CD4+ T cells underlies disease-susceptibility HLA-DP alleles in chronic beryllium disease. Proc Natl Acad Sci USA 2000; 97: 12717-12722.

74. Amicosante M, Berretta F, Franchi A, Rogliani P, Dotti C, Losi M, Dweik R, Saltini C. HLA-DP-unrestricted TNF-alpha release in beryllium-stimulated peripheral blood mononuclear cells. Eur Respir J 2002; 20: 1174-1178.

75. Sawyer RT, Parsons CE, Fontenot AP, Maier LA, Gillespie MM, Gottschall EB, Silveira L, Newman LS. Beryllium-induced tumor necrosis factor-alpha production by CD4+ T cells is mediated by HLA-DP. Am J Respir Cell Mol Biol 2004; 31: 122-130.

76. Bill JR, Mack DG, Falta MT, Maier LA, Sullivan AK, Joslin FG, Martin AK, Freed BM, Kotzin BL, Fontenot AP. Beryllium presentation to $CD4^+$ T cells is dependent on a single amino acid residue of the MHC class II b-chain. J Immunol 2005; 175: 7029-7037.

77. Amicosante M, Sanarico N, Berretta F, Arroyo J, Lombardi G, Lechler R, Colizzi V, Saltini C. Beryllium binding to HLA-DP molecule carrying the marker of susceptibility to berylliosis glutamate beta 69. Hum Immunol 2001; 62: 686-693.

78. Fontenot AP, Canavera SJ, Gharavi L, Newman LS, Kotzin BL. Target organ localization of memory CD4(+) T cells in patients with chronic beryllium disease. J Clin Invest 2002; 110: 1473-1482.

79. Stange AW, Furman FJ., Hilmas DE. The beryllium lymphocyte proliferation test: relevant issues in beryllium health surveillance. Am J Ind Med 2004; 46: 453–462.

80. Khanolkar-Young S, Kolk AH, Andersen AB, Bennedsen J, Brennan PJ, Rivoire B, Kuijper S, McAdam KP, Abe C, Batra HV. Results of the third immunology of leprosy/immunology of tuberculosis antimycobacterial monoclonal antibody workshop. Infect Immun 1992; 60: 3925-3927.

81. Rossman MD, Thompson B, Frederick M, Maliarik M, Iannuzzi MC, Rybicki BA, Pandey JP, Newman LS, Magira E, Beznik-Cizman B, Monos D. ACCESS group. HLA-DRB1*1101: a significant factor for sarcoidosis in blacks and whites. Am J Hum Genet 2003; 73: 720-735.

82. Rozas J, Rozas R. DnaSP, DNA sequence polymorphism: an interactive program for estimating population genetics parameters from DNA sequence data. Comput Appl Biosci 1995; 11: 621-625.

83. Newman LS, Mroz MM, Balkissoon R, Maier LA. Beryllium sensitization progresses to chronic beryllium disease: a longitudinal study of disease risk. Am J Respir Crit Care Med 2005; 171: 54-60.

84. Degli-Esposti MA, Leaver AL, Christiansen FT, Witt CS, Abraham LJ, Dawkins RL. Ancestral haplotypes: conserved population MHC haplotypes. Hum Immunol 1992; 34: 242-252.

85. Evavold BD, Sloan-Lancaster J, Allen PM. Tickling the TCR: selective T-cell functions stimulated by altered peptide ligands. Immunol Today 1993; 14: 602-609.

86. Spain LM, Jorgensen JL, Davis MM, Berg LJ. A peptide antigen antagonist prevents the differentiation of T cell receptor transgenic thymocytes. J Immunol 1994; 152: 1709-1717.

87. Potolicchio I, Brookes PA, Madrigal A, Lechler RI, Sorrentino R. HLA-DPB1 mismatch at position 69 is associated with high helper T lymphocyte precursor frequencies in unrelated bone marrow transplant pairs. Transplantation 1996 ; 62: 1347–52.

88. Diaz G, Catalfamo M, Coiras MT et al. HLA-DPß residue 69 plays a crucial role in allorecognition. Tissue Antigens 1998; 52: 27–36.

89. Arroyo J, Alvarez AV, Nombela C, Sanchez-Perez M. The role of HLA-DPß residue 69 in the definition of antibody-binding epitopes. Hum Immunol 1995; 43: 219–26.

90. Potolicchio I, Festucci A, Hausler P, Sorrentino R. HLA-DP molecules bind cobalt. a possible explanation for the genetic association with hard metal disease. Eur J Immunol 1999; 29: 2140–7.

91. Gorga JC, Horejsi V, Johnson DR, Raghupathy R, Strominger JL. Purification and characterization of class II histocompatibility antigens from a homozygous human B cell line. J Biol Chem 1987; 262: 16087–94.

92. Harlow E, Lane D. Antibodies. A laboratory manual. : Cold Spring Harbor Laboratory, 1988.

93. Panina-Bordignon P, Tan A, Termijtelen A, Demotz S, Corradin G, Lanzavecchia A. Universally immunogenic T cell epitopes: promiscuous binding to human MHC class II and promiscuous recognition by T cells. Eur J Immunol 1989; 19: 2237–42.

94. Raddrizzani L, Sturniolo T, Guenot J et al. Different modes of peptide interaction enable HLA-DQ and HLA-DR molecules to bind diverse peptide repertoires. J Immunol 1997; 159: 703–11.

95. Kalandadze A, Galleno M, Foncerrada L, Strominger JL, Wucherpfennig KW. Expression of recombinant HLA-DR2 molecules. Replacement of the hydrophobic transmembrane region by a leucine zipper dimerization motif allows the assembly and secretion of soluble DR alpha beta heterodimers. J Biol Chem 1996; 271: 20156–62.

96. Doebele RC, Busch R, Scott HM, Pashine A, Mellins ED. Determination of the HLA–DM interaction site on HLA-DR molecules. Immunity 2000; 13: 517–27.

97. Aichinger G, Karlsson L, Jackson MR et al. Major histocompatibility complex class II-dependent unfolding, transport, and degradation of endogenous proteins. J Biol Chem 1997; 272: 29127–36.

98. Chicz RM, Graziano DF, Trucco M, Strominger JL, Gorga JC. HLA-DP2. Self peptide sequences and binding properties. J Immunol 1997; 159: 4935–42.

99. Dessen A, Lawrence CM, Cupo S, Zaller DM, Wiley DC. X-ray crystal structure of HLA-DR4 (DRA*0101, DRB1*0401) complexed with a peptide from human collagen II. Immunity 1997; 7: 473–81.

100. Ghosh P, Amaya M, Mellins E, Wiley DC. The structure of an intermediate in class II MHC maturation: CLIP bound to HLA-DR3. Nature 1995; 378: 457–64.

101. Watts C. Capture and processing of exogenous antigens for presentation on MHC molecules. Annu Rev Immunol 1997; 15: 821–50.

102. Germain R. MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation. Cell 1994; 76: 287–99.

103. Cresswell P. Assembly, transport, and function of MHC class II molecules. Annu Rev Immunol 1994; 12: 259–93.

104. Cresswell P. Chemistry and functional role of the invariant chain. Curr Opin Immunol 1992; 4: 87–92.

105. Liang MN, Lee C, Xia L, McConnell HM. Molecular modeling and design of invariant chain peptides with alterated dissociation kinetics from class II MHC. Biochemistry 1996; 35: 14734–42.

106. Sinigaglia F, Hammer J. Predicting major histocompatibility complex-binding sequence within protein antigens. Biochem Soc Trans 1995; 23: 675–6.

107. Castelli FA, Buhot C, Sanson A et al. HLA-DP4, the most frequent HLA II molecule, defines a new supertype of peptide-binding specificity. J Immunol 2002; 169: 6928–34.

108. Arnold PY, La Gruta NL, Miller T et al. The majority of immunogenic epitopes generate CD4+ T cells that are dependent on MHC class II-bound peptide-flanking residues. J Immunol 2002; 169: 739–49.

109. M Rammensee HG. Chemistry of peptides associated with MHC class I and class II molecules. Curr Opin Immunol 1995; 7: 85–96.

110. Hammer J, Gallazzi F, Bono E et al. Peptide binding specificity of HLA-DR4 molecules: correlation with rheumatoid arthritis association. J Exp Med 1995; 181: 1847–55.

111. Sturniolo T, Bono E, Ding J et al. Generation of tissue-specific and promiscuous HLA ligand databases using DNA microarrays and virtual HLA class II matrices. Nat Biotechnol 1999; 17: 555–61.

112. De Groot AS, Bosma A, Chinai N et al. From genome to vaccine: in silico predictions, ex vivo verification. Vaccine 2001; 19: 4385–95.

113. Díaz G, Amicosante M, Jaraquemada D et al. Functional analysis of HLA-DP polymorphisms: crucial role for DPß residues 9, 11, 35, 55, 56, 69 and 84–87 in T cell allorecognition and peptide binding. Int Immunol 2003; 15: 567–76.

114. Sinigaglia F, Hammer J. Defining rules for the peptide–MHC class II interaction. Curr Opin Immunol 1994; 6: 52–6.

115. Celis E, Larson J, Otvos L, Wunner WH. Identification of a rabies virus T cell epitope on the basis of its similarity with a hepatitis B surface antigen peptide presented to T cells by the same MHC molecule (HLA-DPw4). J Immunol 1990; 145: 305–10.

116. Wyss-Coray T, Brander C, Frutig K, Pichler WJ. Discrimination of human CD4 T cell clones based on their reactivity with antigen-presenting T cells. Eur J Immunol 1992; 22: 2295–302.

117. Charron D, Fauchet R, Albert E et al. Genetic diversity of HLA, functional and medical implication. In: Charron D, ed. XIIth International Histocompatibility Workshop and Conference. EDK 1997

118. Schultz ES, Lethe B, Cambiaso CL et al. A MAGE-A3 peptide presented by HLA-DP4 is recognized on tumor cells by CD4+ cytolytic T lymphocytes. Cancer Res 2000; 60: 6272–5.

119. Koelle DM, Reymond SN, Chen H et al. Tegument-specific, virus-reactive CD4 T cells localize to the cornea in herpes simplex virus interstitial keratitis in humans. J Virol 2000; 74: 10930–8.

120. Zeng G, Wang X, Robbins PF, Rosenberg SA, Wang RF. CD4+ T cell recognition of MHC class II-restricted epitopes from NY-ESO-1 presented by a prevalent HLA DP4 allele: Association with NY-ESO-1 antibody production. Proc Natl Acad Sci U S A 2001; March 27; 98(7): 3964–3969.

121. Amicosante M, Deubner D, Saltini C. Role of the berylliosis-associated HLA-DPGlu69 supratypic variant in determining the response to beryllium in a blood T-cells beryllium-stimulated proliferation test. Sarcoidosis Vasc Diffuse Lung Dis 2005; 22: 175-9.

122. Berretta F, Butler RH, Diaz G, Sanarico N, Arroyo J, Fraziano M, Aichinger G, Wucherpfennig KW, Colizzi V, Saltini C, Amicosante M. Detailed analysis of the effects of Glu/Lys beta69 human leukocyte antigen-DP polymorphism on peptide-binding specificity. Tissue Antigens 2003; 62: 459-71.

123. Diaz G, Amicosante M, Jaraquemada D, Butler RH, Guillen MV, Sanchez M, Nombela C, Arroyo J. Functional analysis of HLA-DP polymorphism: a crucial role for DPbeta residues 9, 11, 35, 55, 56, 69 and 84-87 in T cell allorecognition and peptide binding. Int Immunol 2003; 15: 565-76.

124. Lu L, Vollmer J, Moulon C, Weltzien HU, Marrack P, Kappler J. Components of the ligand for a Ni++ reactive human T cell clone. J Exp Med 2003; 197: 567-574.

125. Gamerdinger K, Moulon C, Karp DR, Van Bergen J, Koning F, Wild D, Pflugfelder U, Weltzien HU. A new type of metal recognition by human T cells: contact residues for peptide-independent bridging of T cell receptor and major histocompatibility complex by nickel. J Exp Med 2003; 197: 1345-53.

126. Sawyer RT, Day BJ, Fadok VA, Chiarappa-Zucca M, Maier LA, Fontenot AP, Silveira L, Newman LS. Beryllium-ferritin: lymphocyte proliferation and macrophage apoptosis in chronic beryllium disease. Am J Respir Cell Mol Biol 2004; 31: 470-7.

127. Farina C, Weber MS, Meinl E, Wekerle H, Hohlfeld R. Glatiramer acetate in multiple sclerosis: update on potential mechanisms of action. Lancet Neurol 2005; 4: 567-75.

128. Ali FR, Larche M. Peptide-based immunotherapy: a novel strategy for allergic disease. Expert Rev Vaccines 2005; 4: 881-9.

129. Sinigaglia F, Scheidegger D, Garotta G, Scheper R, Pletscher M, Lanzavecchia A. Isolation and characterization of Ni-specific T cell clones from patients with Ni-contact dermatitis. J Immunol 1985; Dec;135(6):3929-32.

130. Griem P, Gleichmann E. Metal ion induced autoimmunity. Curr Opin Immunol 1995; Dec;7(6):831-8. Review.

131. Viola A, Lanzavecchia A. T cell activation determined by T cell receptor number and tunable thresholds. Science 1996; Jul 5;273(5271):104-6.

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