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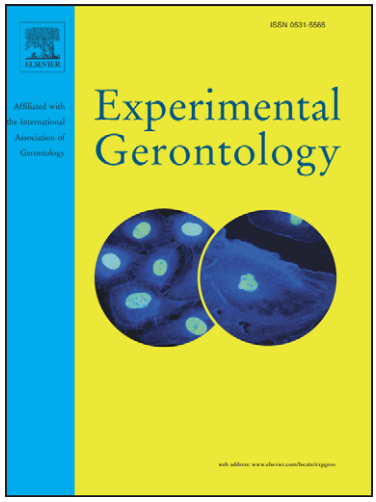

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T-cells from advanced atherosclerotic lesions recognize hHSP60 and have a restricted T-Cell Receptor-repertoire

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Abstract

Atherosclerosis is a multifactorial, chronic inflammatory disease for which the underlying cause remains unknown. It is also well documented that T-cells are among the first cells to migrate into the arterial intimal vessel layer, but their function there is still unexplained. Clinical and experimental data have provided evidence that atherosclerosis starts as an autoimmune reaction based on humoral and cellular immunity against a phylogenetically highly conserved stress protein, heat shock protein 60 (HSP60). In the present study, we phenotypically characterized T-cells from endarterectomised specimens of the carotid artery, and tested their reactivity to human HSP60. In addition, the T-cell receptor repertoire of the T-cell lines was defined by immunoscope analysis. We found a mixed population of $CD4^+$ and $CD8^+$ intralesional T-cells, with a slight predominance of $CD8^+$ cells. IFN-γ production prevailed over IL-4 production. The T-cell reaction against human HSP60 was significantly increased in intralesional cells compared to peripheral T-cells. The lesion-derived T-cells showed an oligoclonally restricted repertoire, in contrast to the polyclonal pattern of PBMC. These results clearly show that HSP60 is a major antigenic candidate, and that an oligoclonal T-cell expansion takes place in advanced human atherosclerotic lesions.

Introduction

In the last decade, it became apparent that atherosclerosis is a multifactorial chronic inflammatory disease (Hansson, 2005), the consequences of which (myocardial infarction or stroke) are the most common causes of death in developed countries. Along with classical risk factors such as hypertension, hyperlipidemia, diabetes mellitus and smoking, immunological-inflammatory processes are now recognized to play a crucial role in the development of the disease (Hansson, 1999; Libby, 2002; Wick et al., 2004). It has been shown that the immune system, especially T-cells, plays an important role in the initiation of arterial wall thickening (Xu et al., 1990). The exact role of the intralesional T-cells has not yet been defined (Wick et al., 2004; Xu et al., 1990). Animal experiments and human studies from our laboratory on advanced atherosclerotic lesions have provided evidence that cellular and humoral immunity against heat shock protein 60 (HSP60) may lead to the development of atherosclerosis. HSP60 is an evolutionarily highly conserved protein with an amino acid sequence homology of more than 97% among microbes. Due to the high homology between microbial and human HSP60, antibodies or T-cells directed against microbial HSP60 may also be capable of cross-reacting with human HSP60 epitopes present on the surface of stressed endothelial cells (EC) (Perschinka et al., 2003). HSP60 expression can be induced by various atherosclerosis risk factors, such as mechanical stress (hypertension), oxygen radicals (H_2O_2), and proinflammatory cytokines (TNF-α; Amberger et al., 1999). Experiments performed on rabbits have revealed that immunization with mycobacterial HSP65 (mHSP65) induced atherosclerosis, and lesion-derived T-cells showed a stronger reaction against HSP60 compared to peripheral T-cells from the same animals (reviewed in Wick

et al., 2004). T-cells isolated from human atherosclerotic lesions also showed a significant reaction against human HSP60 (hHSP60), and a response against chlamydial and mycobacterial HSP was also detected (Benagiano et al., 2003; Curry et al., 2000). Several studies have shown a mixed $CD4^+/CD8^+$ phenotype in these cells with a predominant type-1 cytokine profile, with high Interferon-γ (IFN-γ) production and low Interleukin-4 (IL-4) levels (Benagiano et al., 2003; de Boer et al., 1999).

All these findings support the importance of the immune system, and especially T-cells, in the development of atherosclerosis. The present study was designed to scrutinize the hypothesis that T-cell cross-reactivity against microbial HSP60 or a *bona fide* immunity against altered autologous HSP60 plays a role in the development of atherosclerosis. We have phenotypically characterized T-cells generated from advanced atherosclerotic lesions, tested their antigen specificity, determined their cytokine profile, and analyzed the T-cell receptor repertoire by spectratyping the CDR3- region of the T-cell receptor (TCR). To exclude a cell culture induced clonal expansion of T-cells, we additionally analysed randomly selected atherosclerotic carotid plaques by isolating RNA directly from the lesion.

Material and Methods

Patients: Carotid plaques were obtained from the Department of Vascular Surgery, Innsbruck Medical University, after endarterectomy from 10 patients, 9 males and 1 female, ranging from 51-67 years of age (mean age 61). Five had a symptomatic and 5 asymptomatic stenosis of more than 90%. Their risk profiles are summarized in **Table 1**: 3 had diabetes mellitus, 4 were smokers, 4 former smokers and 2 had never smoked. All had hypercholesterolemia/ -lipidemia, and 7 suffered from hypertension. All were taking medication for hypertension and elevated cholesterol levels. The study was approved by the local Ethics Committee, and all participants gave their written consent.

Isolation and expansion of plaque-derived T-cells: The atherosclerotic plaques were cut into small pieces (ca. 2x2mm) and incubated (37°C, 5% CO2) in a Petri dish in 1640 RPMI (Cambrex, Baltimore, USA) supplemented with IL-2 (20U/ml, kindly provided by Dr. E. Liehl, Novartis Research Institute, Vienna, Austria) on days 1 and 3. After 7 days, the supernatant was filtered trough a cell strainer (100µm, Becton Dickenson, Bedford, MA, USA) to clear the suspension of fragments. The resulting cell suspension was transferred into flat-bottomed 24 well-plates (Greiner, Kremsmünster, Austria). The T-cells were stimulated weekly with OKT3 in a concentration of 20ng/ml (Orthoclone OKT3, Janssen-Cilag, Saunderton, UK) and autologous, irradiated (30Gy) peripheral blood mononuclear cells (PBMC). IL-2 (10U/ml) was added twice a week. Half of the 1640 RPMI was exchanged every third day. After 3 weeks of cultivation, the T-cells were harvested and analyzed.

Generation of peripheral T-cell-lines: Peripheral blood from patients was obtained twice, on the date of endarterectomy and 2 weeks after surgery. PBMC were isolated by density centrifugation over Ficoll Paque (Amersham, Buckinghamshire, UK). T-cell-lines were grown the same way as plaque-derived T-cells, i.e. stimulation with IL-2 (10U/ml) and OKT3 (20ng/ml). The peripheral T-cell-lines were harvested after a culture period of 3 weeks and analyzed as the lesion-derived T-cells were.

Phenotypic analysis: The phenotype of the T-cell lines was determined by FACS analysis. Briefly, $3x10^5$ T-cells were incubated at 4°C for 1h with appropriate dilutions of directly labeled monoclonal antibodies (CD3FITC, CD4PE, CD16PE, CD25FITC, CD56PE, Exbio, Prague, Czech Republic; CD8Pe-Cy7, CD28APC, Becton Dickinson, Franklin Lakes, NJ, USA). After 2 washing steps with 0.02% BSA/PBS (bovine serum albumin, phosphate buffered saline, pH 7.3), fluorescence was analyzed on a FACS Calibur (Becton Dickinson).

Intracellular cytokine staining: T cell cytokine production was determined after 4h (37°C, 5%CO_2) of stimulation with Brefeldin A (10µg/ml), Ionomycin (500ng/ml) and Phorbol-Myristate-Acetate (PMA, 30ng/ml; all reagents from Sigma Aldrich, Vienna, Austria). After surface staining with directly labeled monoclonal antibodies (CD4APC, CD8FITC, Becton Dickinson), the cells were fixed with 2% paraformaldehyde in PBS. After permeabilization of the cell membrane with saponin buffer, the cells were incubated with an appropriate dilution of directly labeled monoclonal antibodies to IL-4 and IFN-γ (both PE,

Becton Dickinson). Fluorescence was measured in a FACS Calibur (Becton Dickinson).

Proliferation assays: The T-cell-lines derived from endarterectomy specimens and peripheral blood were tested for their reactivity against recombinant LPS-free human HSP60 (hHSP60, 20µg/ml, batch 04-1, Lionex, Braunschweig, Germany) and recombinant LPS-free mycobacterial HSP65 (mHSP65, 10µg/ml, batch 99-1, Lionex). As positive controls, Concanavalin A (ConA, 10µg/ml, Sigma Aldrich) and OKT3 (20ng/ml) were used, and as negative controls, T-cells and PBMC without antigen. Proliferation assays were performed as described (31). Results are expressed as stimulation indices (SI): [(counts per minute in presence of antigen)–(counts per minute in absence of antigen)] / (counts per minute in absence of antigen) (Knoflach et al, 2003).

RNA isolation and TCR CDR3 spectratyping: Total RNA was extracted from PBMC (blood donation for immunoscope analysis was approximately 6 months after surgery), plaque-derived T-cells using TRI-reagent, according to the manufacturer's instructions (Sigma Aldrich). In addition, to examine if there was any effect of 3 weeks tissue culture on the T-cell repertoire of the original donors A-J, total RNA isolated directly from PBMC and atherosclerotic plaques without in vitro expansion of T-cells from a further six subjects (denoted 1-6, age range 60-71 years) was also investigated. Total RNA (1µg)was used for first-strand cDNA synthesis using a reverse transcription system (Promega, Mannheim, Germany). As previously described by Herndler-Brandstetter et al. (2005), TCR Vβ transcripts were amplified by PCR using a HotStart Taq Master Mix kit

(Qiagen, Vienna, Austria) and primers (MWG Biotech, Ebersberg, Germany) specific for each of the human Vβ families and a specific primer for the constant region of the β-chain (labeled with the fluorescent dye marker 6-FAM). A Gaussian-like profile emerged in the case of normal polyclonal distribution of individual clones within a given Vβ family. A distortion of the Gaussian distribution and the appearance of a dominant peak suggested the presence of an oligoclonal or monoclonal T-cell populations, respectively. The distribution of clonality was calculated by a standard calculation of percentage.

Statistics: The TCR repertoire of the donors was compared with the two-sided Mann-Whitney test for independent samples, while in all other cases, the Student T-test was applied. Statistical analyses were performed using Microsoft Excel and SPSS 11.0 software.

Results

Plaque derived T-cells have a mixed, but predominantly $CD8^+$, phenotype

Fragments of carotid plaques from 10 patients were cultivated with IL-2 and OKT3, allowing for an *in vitro* expansion of T-cells previously activated *in vivo*. For analysis, all T-cells were gated for CD3. Most of the donors had a mixed phenotype of $CD4^+$ (mean 39%) and $CD8^+$ T-cells (mean 53%). The CD4/CD8 ratio was significantly decreased compared to peripheral T-cells (**Fig. 1A**). An average of 97% of the $CD4^+$ and 79% of the $CD8^+$ T-cell population expressed CD28, meaning that most of the cells had reached their full proliferative potential. The α-chain of the IL-2 receptor (CD25) was also found on virtually all $CD4^+$ (mean 90%) and $CD8^+$ T-cells (mean 98%). A subgroup of $CD8^+$ T-cells was CD16- (mean 21%) and CD56-positive (mean 8%), possibly affording a non MHC-restricted cell lysing potential (**Tab. 2**).

Plaque derived T-cells produce IFN-γ

Overall, $CD8^+$ T-cells were more capable of producing cytokines than the $CD4^+$ T-cells (**Fig. 1B and C**). Over a third of the $CD8^+$ (38%), and 22% of the $CD4^+$ T-cell population derived from the vessel wall produced IFN-γ, whereas IL-4 secretion ranged from 29% ($CD8^+$) to 9% ($CD4^+$). A smaller percentage of peripheral T-cells produced IFN-γ (mean of $CD4^+$ 5.3%; $CD8^+$ 9.3%) and IL-4 (mean of $CD4^+$ 3.4%, $CD8^+$ 2.9%; (**Fig. 1C**).

T-cell response against hHSP60 is stronger in plaque-derived T-cells

Plaque- and peripheral blood-derived T-cell-lines expanded from donors A to J were tested for their response against hHSP60 (**Fig. 2A**). In addition, the T-cell reactivity of donors E to J was also tested against mHSP65. In all cases, the positive control with OKT3/IL-2 and ConA showed good responses (SI- ranges from 17 to 160; **Fig. 2B**). The proliferative response of peripheral- and plaque-derived T-cells against mHSP65 was very low (SI<1). As shown in **Fig. 2A**, the reactivity of plaque-derived T-cells against hHSP60 (SI mean 2.3) was significantly increased compared to peripheral T-cell lines (SI mean 0.7; $p<0.0001$).

T-cells from advanced lesions have a restricted T-cell repertoire

To analyze the clonal composition of plaque- and peripheral blood-derived T-cells, we used the immunoscope technique (Pannetier et al., 1995). This analysis could only be performed on 8 of 10 donors, due to insufficient donor material. As displayed in **Fig. 3A**, 86.3% of plaque-derived T-cells showed an oligoclonally-restricted repertoire compared to 52.4% of peripheral cells. No differences were found in monoclonal T-cell expansion, i.e. 9% in both periphery and lesion. In the periphery, a statistically significant (10-fold) increase (38% vs. 4% in plaque-derived T-cells, $p<0.0001$) of polyclonal TCR Vβ transcripts with a Gaussian-like distribution was detected. The results obtained in isolated, cultivated T-cells from donors A-J were confirmed by Immunoscope analysis on RNA isolated directly from atherosclerotic lesions and PBMC (donors 1-6; n = 6). Also under these conditions, plaque T-cells showed a significantly increased oligoclonal restriction (66% of TCRs) compared to autologous peripheral T-cells (36%, $p<0.003$). In the

periphery, a predominantly polyclonal T-cell-pattern (67%) was found, while almost no polyclonality was present in the plaque T-cells (1%, $p<0.00006$; **Fig. 3B**). This pattern is shown in both directly isolated (numbered donors 1-6) and in vitro expanded (lettered donors A-F) T-cell populations (**Fig.3C**). Interestingly, the monoclonally-restricted TCR of peripheral T-cells was in some cases also the dominant clone of plaque derived T-cells.

Discussion

Our present study established a method for the isolation of T-cells from atherosclerotic lesions, characterized them phenotypically, and determined their cytokine profile. In contrast to previously published studies, the T-cell receptor repertoire was also determined by immunoscope analysis. All donors had greater than 90% stenosis of the *arteria carotis*, with a mean age of 61 years. Since the immune response generally decreases with age, it is not surprising that the immune reactions against HSP and various other antigens are lower than that seen in healthy young people. In the Atherosclerosis Risk factors in Male Youngsters-Study (ARMY-Study), a cross-sectional analysis carried out on healthy male adolescents showed a correlation between Intima Media Thickness (IMT) and peripheral T-cell reactivity against human HSP60 (Knoflach et al., 2003). No such correlation was found in the BRUNECK-Study, a prospective atherosclerosis prevention study examining people from 50 to 69 years of age. In the present investigation, we show a clear increase of the T-cell response against human HSP60 in lesion derived compared to peripheral T-cells, confirming observations by others (Benagiano et al., 2005; Curry et al., 2000). This is further strong evidence supporting the role of an immune reaction directed against HSP60 in atherogenesis, supporting our original hypothesis.

As known from other autoimmune diseases, e.g. Hashimoto's thyroiditis, circulating antibodies or T-cells against self antigens decrease in the peripheral blood at advanced disease stages (Wick et al, 1987). Considering atherosclerosis in analogy to Hashimoto's disease, one would expect lower levels of HSP60-reactive T-cells in the periphery compared to the target lesion in the arterial wall, probably due to migration of hHSP60-reactive T-cells from the blood to the

affected tissue. Further experiments are needed to address this hypothesis in the context of atherosclerosis.

As reported by others and confirmed in this study, the majority of the T-cells isolated from the lesion produced high amounts of IFN-γ, while IL-4 secretion, although present, was very low (Benagiano et al., 2003; de Boer et al., 1999). Interestingly, an even higher proportion of CD8+ T-cells showed IFN- response than the CD4+ cells, which reverses the traditional roles of the cells where CD4+ T-cells are pro-inflammatory. It has been previously shown, however, that CD8+ T-cells show a shift towards a pro-inflammatory status and IFN- production in old age (Sauerwein-Thiessl et al. 2002). The role cytokines actually play in the development of atherosclerosis in humans remains to be clarified. It is known, however, that IFN-γ activates macrophages and T-cells, induces MHC class II molecules on various cell types including SMC and EC (Jonasson et al., 1986), and instigates apoptosis of vascular SMC (Geng et al,. 1996). Another important consequence of IFN-γ production in atherosclerotic plaques is an increased expression of leukocyte adhesion molecules on EC, which play a role in the recruitment of immunocompetent cells (De Caterina et al., 2001). Mice lacking an IFN-γ receptor showed >60% reduction in atherosclerosis, probably as a result of reduced adhesion molecule expression (Gupta et al., 1997).

The question remains as to how cytokine profiles of T-cell lines *in vitro* correspond to the cytokines produced *in vivo*. *In vivo*, a balance is maintained between the production of IFN-γ, which induces a type-1 pro-inflammatory environment, and IL-4, which promotes a type-2 anti-inflammatory response (Abbas et al,. 1996). In our study, the balance was shifted towards a type-1

response, although IL-4 was also produced in small amounts by the plaque-derived T-cells.

We found a mixture of $CD4^+$ and $CD8^+$ T-cells, with a slight predominance of the $CD8^+$ phenotype. Current literature offers inconsistent results regarding the phenotype of intralesional T-cell lines (Curry et al., 2000; Mosorin et al., 2000). Virtually all of the T-cells expressed the costimulatory CD28 molecule, which interacts with the B7 complex on macrophages and dendritic cells. Binding of B7 to CD28 leads to the augmentation of several T-cell functions, such as proliferation, cytokine production, adhesion and cytotoxicity. Considered together with the expression of IL-2 receptor (CD25) on lesion derived T-cells, it can be concluded that all of these cells were in an activated state and able to reach their full stimulatory potential after interaction with APC.

Most of the lesion-derived T-cells display an oligoclonally-restricted T-cell receptor repertoire compared to the peripheral blood of the same donor. This is strong evidence for antigen driven T-cell proliferation within the atherosclerotic plaque itself. Only 3 of 192 TCR Vβ chains examined showed a PBMC pattern more restricted than in the plaque T-cells. Due to the age of the donors, it was to be expected that some restriction would also be seen in the periphery (Saurwein-Thiesl et al. 2002), and was a useful "inbuilt" control for the immunoscope method. In addition, the long term (3 weeks) culturing of plaque T-cells, even though unspecifically stimulated, does not lead to a restriction in clonality, as confirmed by results obtained from directly isolated T-cell RNA. The predominantly polyclonal TCR and the fact that most of the monoclonally-restricted TCR detected in the plaques were also found in the PBMC of donors

suggests that the repertoire remained unchanged during the time period between surgery and blood collection for immunoscope.

As recently shown by De Palma et al. (2006), T-cells from advanced unstable coronary plaques also show an oligoclonally-restricted TCR repertoire compared to peripheral T-cells of the same donor, implying that a specific T-cell expansion takes place. In addition, our plaque-derived T-cells showed a significantly increased proliferative response against recombinant hHSP60. This data supports our hypothesis where T-cells attach to stressed, HSP60 expressing, endothelial cells and consequently accumulate in the intimal vessel layer, finally leading to the formation of an atherosclerotic lesion. Due to the intralesional expression of HSP60 by SMC and macrophages as well, there is the possibility of continuous *in situ* stimulation of HSP60-specific T-cells (Xu et al., 1993). We not only found a stronger response against hHSP60 comparing lesional and peripheral T-cells, but a more pronounced response than against other antigens such as mHSP65.

The atherosclerotic lesions analyzed were all in the end-stage of the disease, i.e. at the point of surgical intervention. The present study shows an accumulation of antigen specific T-cells in these lesions. Presently, it is not possible to say whether the T-cells isolated from the advanced plaque also reflected the situation in the initial stages. The question remains as to whether the TCR-restriction is present in the early stage of atherosclerosis or is due to antigen-driven selection during disease progression within the arterial wall. The clonal restriction seen is evidence of a specific antigen(s) driving the immune process during atherogenesis. It is not yet known what form this antigen takes, but HSP60 and oxidized lipoproteins are clearly the leading candidates in this respect.

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Figure Legends:

Figure 1: Phenotypic parameters of T-cells isolated from advanced atherosclerotic lesions. A significantly decreased CD4/CD8 ration in plaque-derived T-cells compared to peripheral blood-derived T-cells ($p=0.007$) was found (A). Cytokine profiles of plaque-derived and peripheral $CD4^+$ and $CD8^+$ T-cells were determined as percent of cells producing the respective cytokine. Figure 1B represents the cytokine pattern of $CD4^+$ T-cells, while $CD8^+$ cells are shown in Figure 1C.

Figure 2: Antigenic response of plaque-derived and peripheral T-cells against hHSP60 and control antigens. The response of lesion-derived T-cells was significantly increased compared to peripheral T-cells ($p<0.0001$, Fig 2A). Positive controls for proliferation of plaque derived T-cells were performed against ConA (black bars), and OKT3 (white bars, Fig 2B).

Figure 3: Distribution of TCR Vβ transcripts in plaque- and lesion-derived T-cells. A significant increase in oligoclonal TCR repertoire was detected in cultivated lesion derived T-cells, while in PBMCs a polyclonal pattern was significantly present ($p=0.001$, Fig 3A).

The TCR-repertoire was also determined from T-cells directly isolated from other advanced atherosclerotic lesions (Fig 3B). White boxes representing plaque derived T-cells have a significantly restricted monoclonal and oligoclonal repertoire ($p<0.004$ and $p<0.003$, respectively). In contrast, peripheral T-cells display a statistically highly significant increase in polyclonal character ($p<0.00006$). The TCR-restriction pattern of Vβ-transcripts shows that plaque

derived T-cells are generally more restricted than their peripheral counterparts (Fig 3C). Donors are indicated by letters or numbers in the upper left corner. Vβ-primers are indicated in the upper right corner. The lower line shows the plaque-derived T-cells, while the upper line represents the PBMC. Most cases show TCR restriction in plaque-derived T-cells, whether the plaque derived T-cells were cultivated or used directly. In some cases clonally- expanded T-cells in the plaque were also found to predominate in the PBMC.

Tables

ID	age	sex	stenosis(%)	clinical event	hypertension	diabetes mellitus	hyperlipidemia	smoke
A	52	m	95, r asympt.	none	+	-	+	+
B	65	m	90, r sympt.	TIA	+	+	+	ex
C	54	m	90, l sympt.	stroke	+	-	+	ex
D	65	m	90, r sympt.	TIA	-	-	+	+
E	63	m	90, l asympt.	none	+	+	+	ex
F	64	m	90, l asympt.	none	+	-	+	ex
G	67	m	90, r asympt.	none	+	+	+	+
H	63	m	90, l asympt.	none	-	-	+	-
I	51	m	90, l sympt.	arm paresis	+	-	+	+
J	65	f	90, l sympt.	TIA	-	-	+	-

Table 1: Characteristics of the 10 donors (m, male, f, female; r, right; l, left; a, asymptomatic; s, symptomatic; +, yes; -, no; ex, former smoker; TIA, transient ischemic attack)

	CD4+CD28+		CD8+CD28+		CD8+CD56+		CD8+CD16+	
Donor	Plaque	PBMC	Plaque	PBMC	Plaque	PBMC	Plaque	PBMC
A	96	99	71	83	12	7	18	11
B	96	95	85	59	2	20	21	17
C	99	90	76	62	7	8	33	27
D	100	99	78	87	11	10	31	4
E	96	98	54	45	7	7	39	25
F	99	95	91	62	12	25	23	19
G	88	97	77	75	13	12	3	11
H	98	98	87	21	6	61	14	63
I	98	96	78	50	5	28	20	13
J	99	98	93	55	3	25	5	25

Table 2: Distribution of $CD28^+$, $CD56^+$ and $CD16^+$ T-cells among the $CD3^+CD4^+$ and $CD3^+CD8^+$ population.

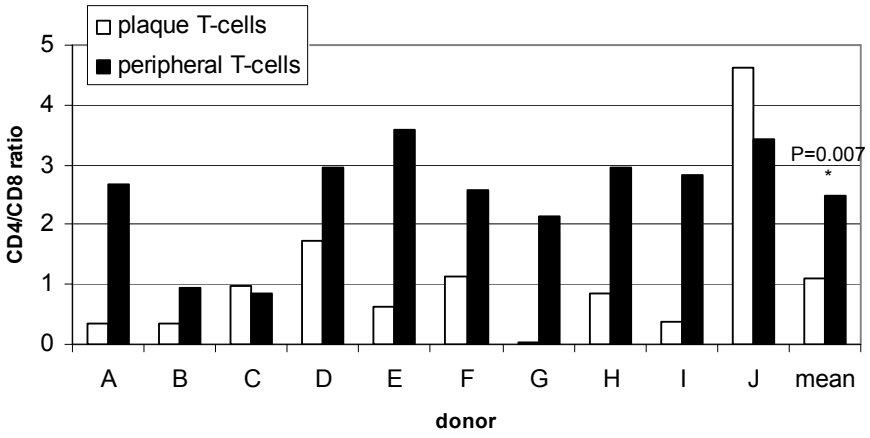

Figure 1a

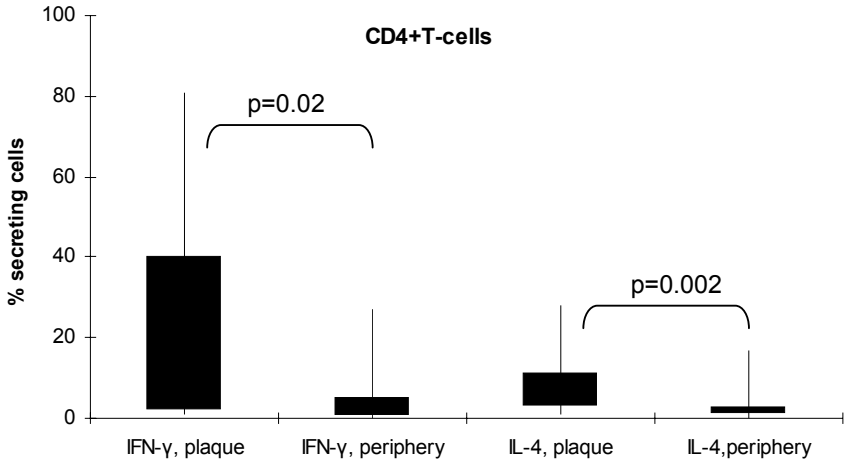

Figure 1b

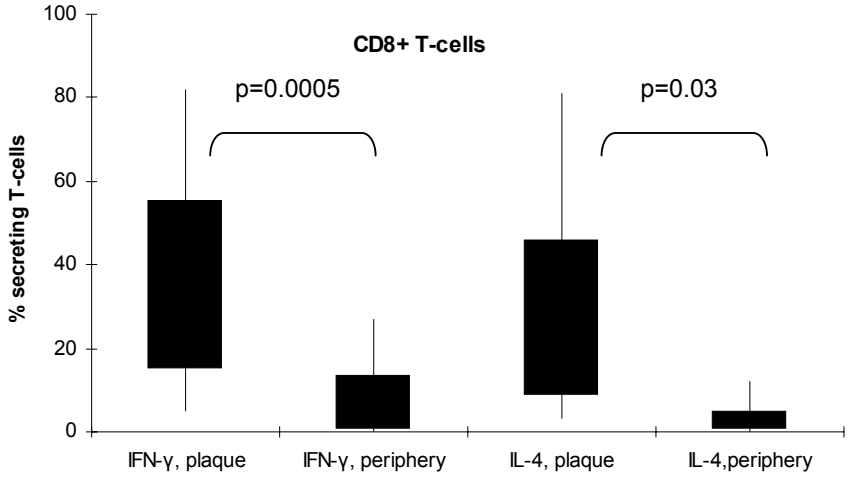

Figure 1c

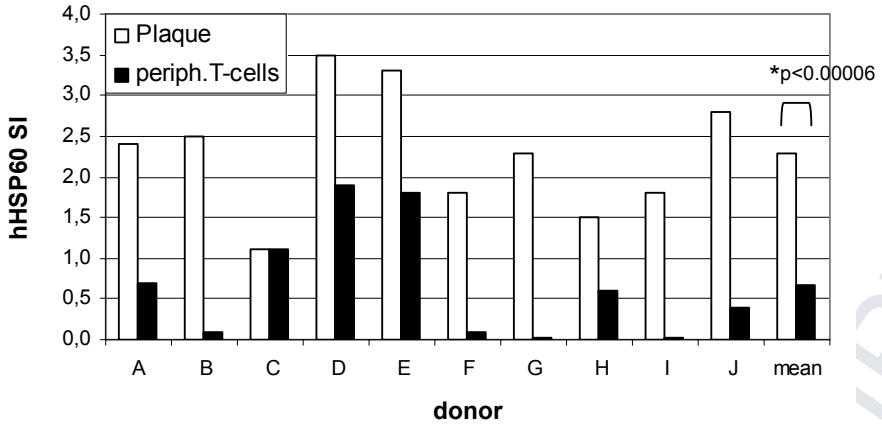

Figure 2a

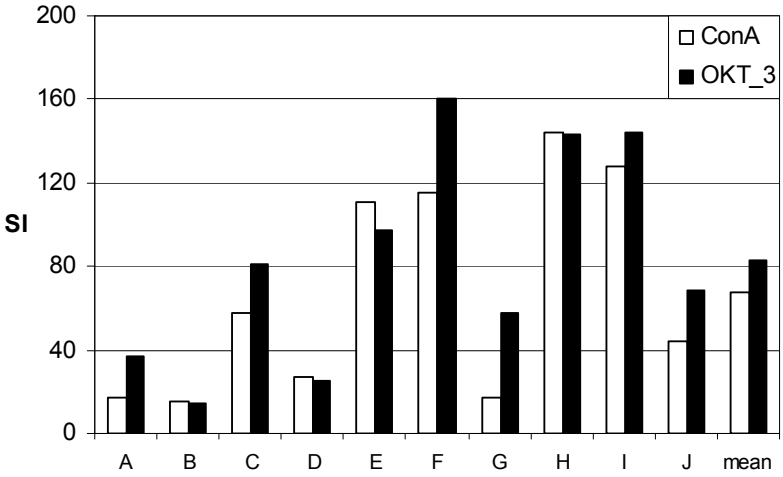

Figure 2b

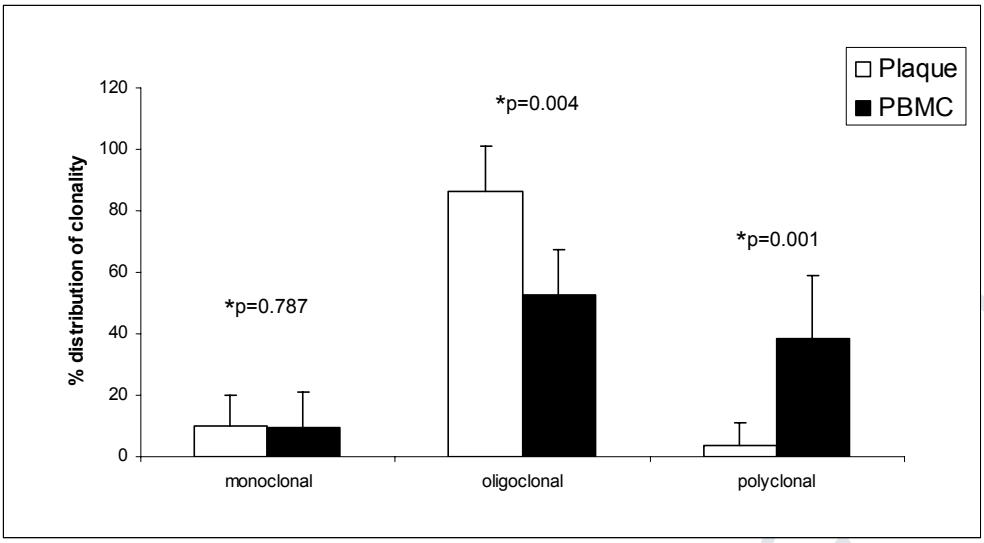

Figure 3a

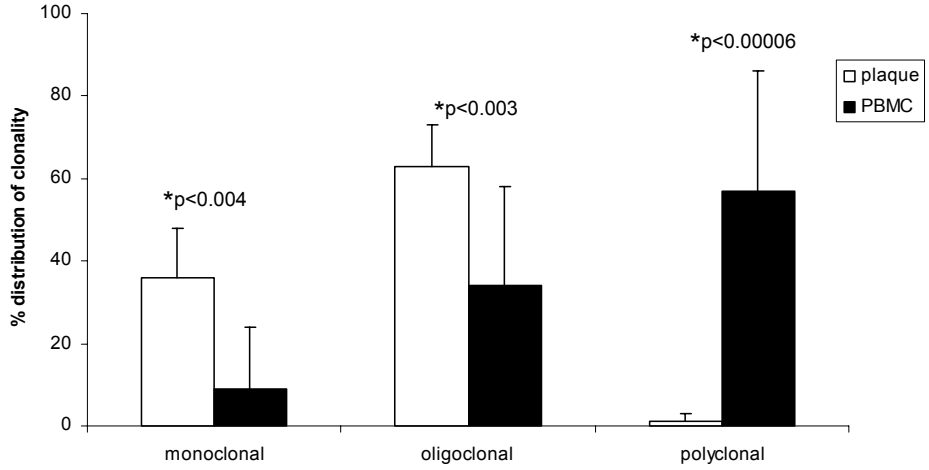

Figure 3b

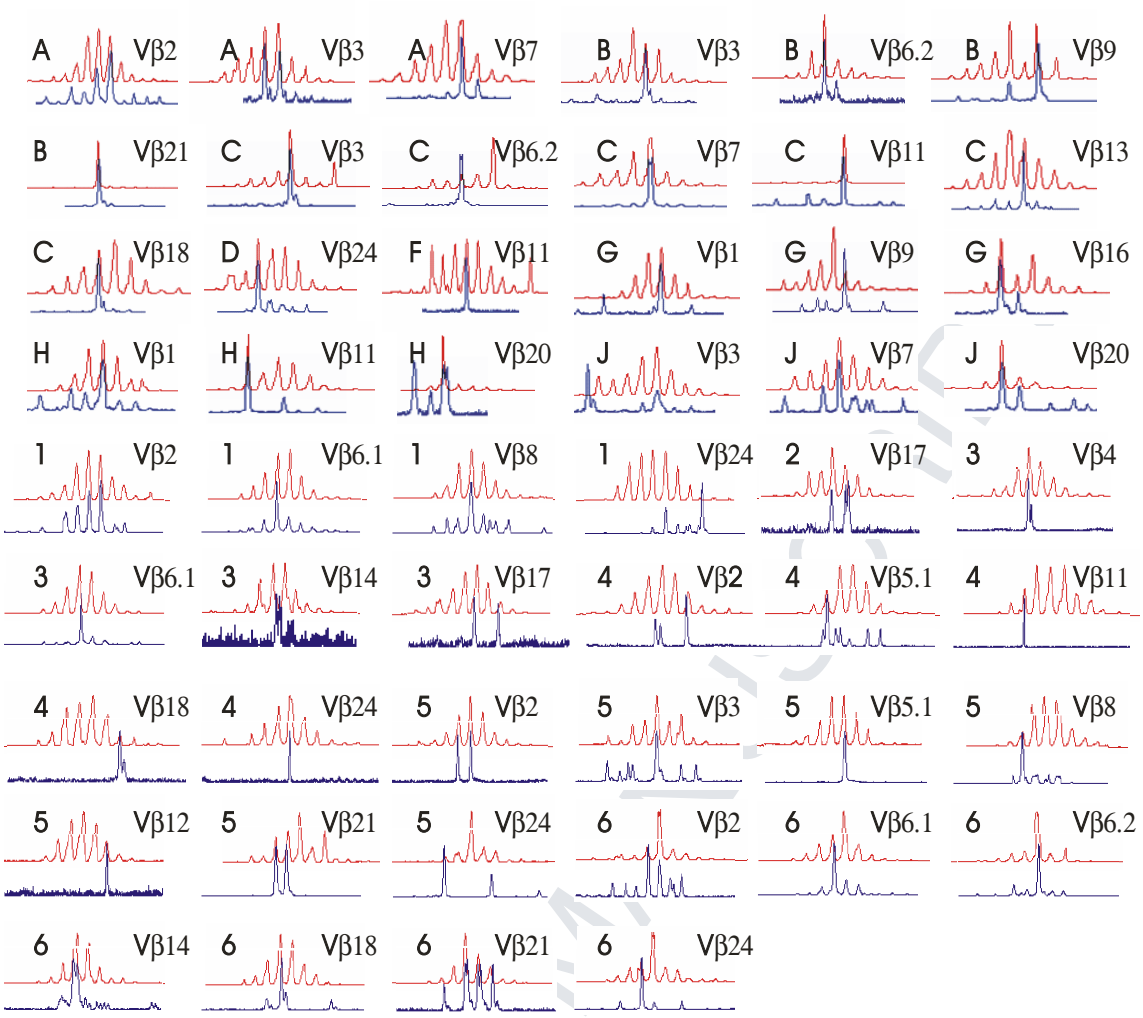

Figure 3c