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Journal of Immunology & Clinical Research

Research Article

Identification of two Immunogenic T cell Epitopes of ApoB-100 and their Autoimmune Implications

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Submitted: 12 May 2014

Accepted: 21 May 2014

Published: 02 June 2014

ISSN: 2333-6714

Keywords
- Heart disease
- Atherosclerosis
- ApoE-/- mice
- Ldlr-/- mice
- ApoB-100
- LDL
- T cell epitopes
- T cell proliferative responses

Abstract

While it is generally accepted that Th1 cells play a major role in the development of atherosclerosis, the mechanisms of how these T cells are activated during the initial stages of disease development is far from clear. The lack of this information has hitherto greatly hindered the development of specific therapies targeting these T cells. Since T cells are stimulated by specific antigens, we reason that identifying the atherosclerotic epitopes that bind to these T cells will lead to isolation of atherosclerotic T cells for in-depth studies. To this end, we used the described MHC binding motifs for IA^b to identify 18 potential immunologic sequences of the autoantigen, ApoB-100. We selected 6 sequences for functional analysis. Two peptides, $apoB_{3501-3515}$ and $apoB_{978-992}$, designated P3 and P6 respectively, were found to stimulate strong T cell proliferative responses from immunized $ApoE^{-/-}$, $Ldlr^{-/-}$ and C57BL/6 mice. These T cells also produce inflammatory IFNγ and IL-17A cytokines. These results indicate that autoreactive T cells capable of recognizing peptides of apoB-100 exist in normal and atherosclerosis-prone mice. The identification and isolation of autoreactive atherosclerotic peptide-specific T cells is a significant achievement that will contribute towards better defining the role of T cells in atherosclerosis and the development of peptide-specific vaccines.

ABBREVIATIONS

LDL: Low Density Lipoprotein; oxLDL: Oxidized Low Density Lipoprotein; HSP: Heat Shock Protein; APC: Antigen Presenting Cells; MHC: Major Histocompatibility Complex; ApoE : Apolipoprotein E; Ldlr: Low Density Lipoprotein Receptor

INTRODUCTION

Atherosclerosis is a chronic disease of the arterial wall, with the resulting cardiovascular diseases affecting more than 81 million people in America, accounting for 1 in every 3 deaths [1]. Clinically, inflamed atherosclerotic plaques can cause coronary artery disease due to luminal narrowing of the arteries or acute coronary syndrome, myocardial infarction or stroke due to plaque instability, rupture and thrombosis. Atherosclerosis is traditionally believed to initiate from the dysfunction of Endothelial Cells (EC) lining the inner surfaces of the vessel wall [2], and the diffusion of unabsorbed Low-Density Lipoproteins (LDL) into the intima of large arteries where they form fatty streaks [3]. In the intima, activated macrophages up-regulate their endocytic pattern-recognition receptors [4] and mediate lipoprotein internalization resulting in their foam-cell appearance [5]. These cells release inflammatory cytokines recruiting inflammatory immune cells into the lesion site. Continuous influx of mononuclear cells to the site and inefficient clearance of dead cells (efferocytosis) eventually gives rise to the formation of atherosclerotic plaques with a central necrotic lipid core and a fibrous cap.

The potential involvement of lymphoid cells in the disease was made in 1960 when Anitschkov observed that several cell types were present in the atherosclerotic plaques [6]. Among

these were smooth muscle cells, macrophages and lymphocytes. Following up on this observation, the pioneering work of Hansson and coworkers [7,8] in the past two decades has established that immunity and inflammation also play important roles in atherosclerotic development. It was first shown that ApoE$^{-/-}$/*scid*/*scid* mice fed standard mouse chow and sacrificed at 18 weeks of age had a 73% reduction in lesion size when compared to control apoE$^{-/-}$ mice [9]. *Scid* mice lack the development of both T and B cells. In addition, CD4^{+} T cells isolated from apoE$^{-/-}$ mice and injected into 6-week-old female apoE$^{-/-}$/*scid*/*scid* recipients led to 164% larger fatty streak lesions and an 8-fold increase in serum interferon-γ level as compared to recipients not transferred the CD4^{+} T cells [9]. Furthermore, auto-antibodies against oxidized LDL were found in the lesions of atherosclerotic humans and animal models, providing further evidence of the involvement of adaptive immunity in the disease process [3]. This paradigm-shifting concept ushered in a period of intensive investigation into the contribution of the adaptive immune system, especially T cells in atherosclerosis. From these studies, it is now generally accepted that atherosclerotic inflammation is mediated by Th1 cells which, with their signature cytokine, IFNγ, are found in abundance in human and murine atherosclerotic plaques [10,11]. Support for this conclusion comes from experiments showing that ApoE$^{-/-}$/IFNγR$^{-/-}$ mice fed a high fat diet for 3 months exhibited significant reduction in atherosclerotic lesion size [12] while injection of exogenous IFNγ to ApoE$^{-/-}$ mice fed a high fat diet significantly increased lesion size by two-fold [13]. In addition, deficiency of signal molecules such as T-bet [14] and cytokines such as IL-12 [15] and IL-18 [16] that promote expression of the IFNγ gene were shown to reduce atherosclerosis in ApoE$^{-/-}$ double knockout mice fed a normal chow.

From the above account, macrophages and T cells were shown to be two key players in the inflammatory aspects of atherosclerosis. A majority of the T cell studies in the literature utilize ApoE$^{-/-}$ or Ldlr$^{-/-}$ mice fed a high fat diet to induce disease and to measure the *consequential* changes in immune parameters and signal pathways. As such, these studies investigate the end stages and aggregate effects of immune activities of mixed populations of cells and do not investigate what triggers the T cell immune responses in the first place. The lack of this knowledge about the nature of the atherogenic T cells themselves has greatly hindered designs of new therapeutics to halt the harmful effects of these specific T cells.

Typically, T cells are triggered by binding to specific antigens. In this respect, two major autoantigens, oxidized low density lipoprotein (oxLDL) and the human heat shock protein 60/65 (hsp60/65), have been implicated in the pathogenesis of atherosclerosis [17-20]. T cell clones derived from atherosclerotic plaques were shown to be cytotoxic to human hsp60-pulsed autologous APC [20]. It has not been determined whether the atherosclerotic responses were due to the human form or the microbial form of hsp60 as these proteins are highly conserved phylogenetically. For LDL, the oxidized form of this antigen (oxLDL) is rapidly taken up by the scavenger receptors of macrophages and antibodies to oxLDL are abundant in plasma of patients and experimental animals [3]. oxLDL consists of a mixture of heterogeneously modified particles, including its apolipoprotein component, apoB-100. ApoB-100 is immunogenic as serum antibodies specific for this protein have been detected [21]. The epitopes of apoB-100 that bind to T cell receptors to trigger the T cells, however, have not been elucidated.

To undertake this challenge, we reasoned that by identifying the atherogenic peptide of apoB-100, we would be able to identify and isolate the atherogenic T cells these peptides bind to. We made use of a published strategy [22] to identify IA^{b} binding motifs of ApoB-100. We scanned the entire 4505 amino acid sequence of murine ApoB-100 and identified 18 sequences that fit these binding motifs. We selected and synthesized 6 of these sequences, designated peptides 1 to 6. In this report, we show that two of the peptides, P3 (apoB$_{3501-3515}$) and P6 (apoB$_{978-992}$), were able to stimulate T cell proliferation and cytokine production in antigen recall assays.

MATERIALS AND METHODS

Mice

Male and female C57BL/6, Ldlr$^{-/-}$ and *ApoE*$^{-/-}$ mice on C57BL/6 background were bred in our colonies. Mice were fed a regular chow diet until use. Mice were used between 10 to 12 weeks of age. All experimental procedures involving live animals received prior approval from IACUC.

Identification of Apob-100 H-2^{b} Binding Motifs

Based on analysis of the alignment of sixty-four peptides that bound to IA^{b}, Liu et al. [22] delineated a set of possible residues that most frequently serve as anchor residues for IA^{b} binding. In this analysis, four MHC contact positions are defined (peptide positions 1, 4, 6 and 9) and the three most common amino acids used at each site were listed (position 1, A, F and Y; position 4, S, A and P; position 6, V, P and A; position 9, S, V and A). This analysis also provided potential T cell interacting residues in positions 2, 3, 5 and 8. We arbitrarily deemed any 9-mer peptide with three MHC contact residues to be potential I-A^{b} binding peptides. We developed a strategy to scan for such binding motifs along the 4505 amino acid sequence of ApoB-100. We first searched for any of the three amino acids found in MHC contact position 1 (A, F, Y). We designated this amino acid position 1 of our potential 9-mer. We then determined the amino acids corresponding to positions 4, 6 and 9 downstream of the first position. If any two of the remaining three positions also had an amino acid used frequently in I-A^{b} binding peptides, we designated the 9-mer as a candidate for further characterization. Similarly, we searched the ApoB-100 sequence for amino acids corresponding to those used frequently in position 4. We then searched up and downstream from this residue for amino acids corresponding to positions 1, 6 and 9. This strategy defined another subset of potential 9-mers. We repeated this search starting with amino acids often used in positions 6 and 9 of the I-A^{b} binding motif. Two more subsets of potential 9-mers were thus defined. In all, 18 potential I-A^{b} binding 9-mers were designated. Given their primary sequence, 6 of these were determined to have adequate water solubility to be used in experiments.

Peptides

ApoB-100 peptides were synthesized by Genemed Synthesis (San Antonio, TX).

Although the minimal sequence to bind to the MHC Class II peptide groove is a 9-mer, all peptides were custom synthesized as 15-mers because additional flanking residues are required for TCR interactions. Peptides were dissolved in Phosphate Buffered Saline (PBS).

Immunization and T Cell Proliferative Assay

Individual peptides were emulsified in Complete Freund's Adjuvant (CFA) and mice were immunized with 100 µg total in four sites in the flanks. Ten days later, draining lymph nodes were harvested and teased into single cell suspension. 3 x 10^5 viable cells per microtiter well were cultured with 50 to 100µg/ml of the peptides for 4 days. In some cases, PPD (purified protein derivative) served as a positive control and a peptide of MOG (myelin oligodendrocyte glycoprotein) or ovalbumin (OVA) served as negative control. Sixteen hours before harvesting, 1 µCi of tritiated thymidine was added to each well. Cells were harvested and incorporation of ^3H was determined in a scintillation counter. Results are expressed as stimulation indices using the following formula (cpm = counts per minute); (S.I =Stimulation index)

$$\frac{\text{cpm experimental} - \text{cpm medium control}}{\text{cpm medium control}}$$

Detection of Cytokines

Primed lymph node cells were cultured in 96-well round-bottom plates (5 x10^4 cells/well) with 2 x 10^5 irradiated spleen cells and with or without the priming peptides for 24h. The supernatants were collected and stored at -80°C until analyzed. Culture supernatants were analyzed using SABiosciences (Federick, MD) Cytokine Multi-Analyte ELISArray kit according to the manufacturer's instructions.

RESULTS AND DISCUSSION

IA^b binding motifs of murine ApoB-100

T cells are activated by recognition of specific antigens. However, it is important to realize that the T cell receptor (TCR) only recognizes a short peptide of the antigen in association with Major Histocompatibility Complex (MHC) gene products [23,24]. As such, the antigen molecule is first broken down by Antigen Presenting Cells (APC) into short peptides of 8 to 30 amino acids. These peptides are expressed on the cell surface of APC in association with MHC class I or Class II proteins. It is this complex composed of peptide/MHC that binds to the TCR for T cell activation [23]. To be able to isolate and directly study the disease-causing T cells in atherosclerosis, our focus is directed towards elucidating the peptides of apoB100 that form the peptide/MHC ligand for activation of atherogenic T cells. Because different MHC haplotypes have different binding motifs for peptide sequences and that most atherosclerosis experiments are carried out in the *ApoE*$^{-/-}$ mice which is of the IA^b haplotype, we searched for H-2^b binding motifs along the whole 4505 amino acid sequence of murine apoB-100 according to the prediction model of Liu et al. [22]. We identified eighteen such peptides, all with at least two MHC anchor residues matched. We selected six of the sequences based on solubility analysis, and 15-mers of the 6 sequences, designed P1 to P6, were synthesized. The sequences are shown in Table 1. 15-mers of the peptides were chosen to assure inclusion of the minimal epitopes necessary for conferring immunogenicity. The numbers of MHC anchor residues contained in each peptide are also indicated in Table 1.

P3 and P6 stimulate antigen-specific T cell proliferative responses

To determine if the synthesized peptides were immunogenic, we immunized *ApoE*$^{-/-}$, *Ldlr*$^{-/-}$ and C57BL/6 mice and tested for T cell proliferative responses. Briefly, mice were immunized with 100 µg of individual peptide emulsified in CFA in four sites in the flanks. Ten days after immunization, draining lymph node cells were cultured with the priming peptide or with PPD (purified protein derivative) as a positive control and a peptide emulsified in CFA of MOG (myelin oligodendrocyte glycoprotein) as a negative control. Proliferative responses were measured by incorporation of tritiated thymidine added to each well in the last 16 hours of the cultures. Results are expressed as Stimulation Indices (SI). ApoE$^{-/-}$ mice which lack the ability to remove circulating LDL are typically used in atherosclerosis studies. Immunization of ApoE$^{-/-}$ mice with the peptides showed that P1, P2, P4 and P5 did not induce proliferative responses (Table 2). Mice immunized with P3 ($apoB_{3501-3515}$) or P6 ($apoB_{978-992}$), on the other hand, mounted strong T cell proliferative responses to these two peptides. Positive control PPD and negative control MOG_{35-55} all responded as expected. These results unequivocally indicate that autoreactive P3- and P6-specific T cells exist naturally in ApoE$^{-/-}$ mice and immunization expands these cells to detectable level.

To test if autoreactive P3- and P6-specific T cells are also present in normal mice, we immunized C57BL/6 (B6) mice with P3 and P6 peptides. In these experiments, B6 mice were immunized with 100 µg of either P3 or P6 in four sites in the flanks. In the in vitro cultures, draining lymph node cells were cultured with titrating concentrations of the peptides ranging from 25 to 400µg/ml. T cell proliferative responses were measured in the same manner as before. As shown in Figure 1, autoreactive P3 and P6-specific T cells proliferated in a dose-dependent manner, with the response reaching a plateau at about 100µg/ml. Similar to ApoE mice, B6 mice did not respond to P1, P2, P4 and P5 (data not shown). It was concluded that autoreactive P3- and P6-specific T cells are present not only in atherosclerosis-prone ApoE$^{-/-}$ mice, but also in normal C57BL/6 mice.

To show that P3 and P6 are immunogenic in another atherosclerosis-prone mouse strain, we immunized Ldlr$^{-/-}$ mice with the peptides. In this series of experiments, PPD served as positive control and OVA (chicken ovalbumin) as negative control in in vitro proliferative cultures. Table 3 shows that P3 and P6 induced strong T cell proliferative responses in these mice. Furthermore, there is no cross-reaction between P3 and P6 and other non-immunogenic peptides. Mice immunized with P3 do not show responses to P6 or other peptides and vice versa.

The observed T cell response to ApoB100 reported here is the first delineation of the immunogenic epitopes of this autoantigen. Recently, Wick and co-workers synthesized 113 15-mer peptides of the 573 amino acid sequence of the human HSP60 protein and divided these peptides into 12 peptide pools for stimulation of

Table 1: Synthetic ApoB-100 peptide sequences matching IA^b binding motifs.

Peptides	Peptide sequences	ApoB100 aa	MHC Anchor*	TCR sites#
Peptide1 (P1)	N'-NNYAL**F**L**S**P**R**AQQA**S**-C'	3066 - 3080	4	1
Peptide2 (P2)	N'-SRATL**Y**AL**S**HA**V**N**S**Y-C'	438 - 452	4	0
Peptide3 (P3)	N'-SQE**Y**S**G**S**V**A**N**EANVY-C'	3501 - 3515	4	4
Peptide4 (P4)	N'-YEN**F**AA**S**NKLD**V**TFS-C'	1578 - 1592	3	1
Peptide5 (P5)	N'-HLE**Y**V**S**S**E**LRK**S**LQV-C'	4054 - 4068	3	2
Peptide6 (P6)	N'-TGA**Y**S**N**A**S**STE**S**ASY-C'	978 - 992	3	2

*Number of MHC anchor residues in the predicted binding motifs. Predicted binding motifs are underlined in the peptide sequences and the MHC anchor residues are highlighted in bold letters.
#according to the predictive model of Lie et al. [22] for positions 2, 3, 5 and 8.

Table 2: T cell proliferative responses of $ApoE^{-/-}$ mice to six ApoB-100 peptides.

	T Cell Proliferative Responses in vitro (Stimulation Index)		
Immunization w/	**Antigen for in vitro stimulation (μg/ml)**		
ApoB-100 Peptides	PPD (20)	MOG35-55 (50)	Priming Peptide (50)
P1	**29.33**	1.67	-0.16
P2	**19.76**	0.64	-0.51
P3	**19.76**	0.35	**11.15**
P4	**13.57**	-0.08	-0.44
P5	**23.43**	-0.08	-0.68
P6	**23.31**	1.82	**7.75**

T Cell Proliferative Responses in vitro (Stimulation Index).
Three $ApoE^{-/-}$ mice for each peptide were immunized in four sites in the flanks with 100 μg/mouse of peptides. Immunization and analysis procedures were the same as described in Figure 1.

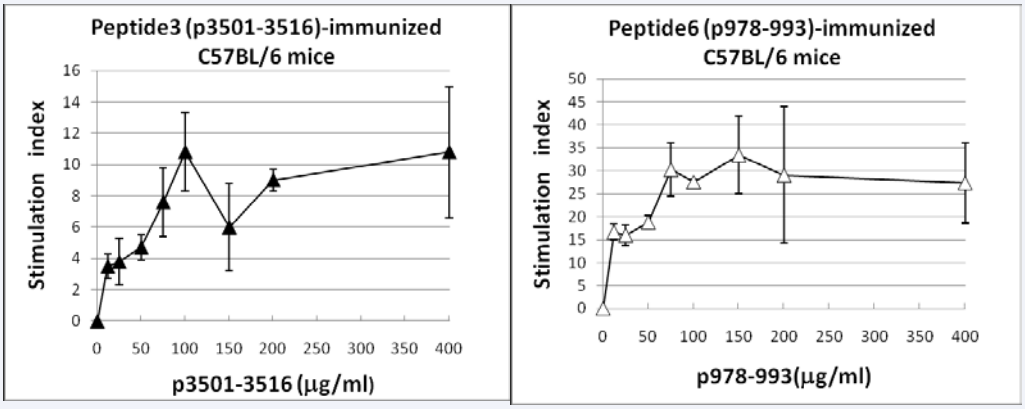

Figure 1 Titration curves of T cell proliferative responses to P3 ($ApoB_{3501-3516}$) and P6 ($ApoB_{978-993}$) from C57BL/6 mice. Synthesized peptides were emulsified in complete Freund's adjuvant (CFA) and 3 C57BL/6 mice in each group were immunized with 100 μg of peptide (total) at four sites in the flanks. Ten days later, 5 x 10^5 viable draining lymph node cells pooled from the 3 mice were cultured with titrating concentrations (ranging from 0 to 400 μg/ml) of P3 or P6 peptides for 4 days. PPD (purified protein derivative) served as a positive control and a peptide of MOG (myelin oligodendrocyte glycoprotein) served as negative control (data not shown). Sixteen hours before harvesting, 1 μCi of tritiated thymidine was added to each well. Cells were harvested and incorporation of ^3H was determined in a scintillation counter. Results are expressed in Stimulation Indices (SI):
(cpm= counts per minute) $\frac{\text{cpm experimental} - \text{cpm medium control}}{\text{cpm medium control}}$

intralesional atherosclerotic T cells isolated from patients with early lesions and late lesions [25]. They identified 8 atherogenic peptides that stimulated T cells isolated from early atherosclerotic lesions and 4 for T cells from late lesions. The identification and isolation of autoreactive atherosclerotic peptide-specific T cells is a significant achievement that will contribute towards better defining the role of T cells in atherosclerosis and development of vaccines [26].

P3 and P6 immunization induce strong inflammatory cytokine responses

We next determined the cytokine profiles of lymph node cells from $ApoE^{-/-}$ mice immunized with P3 and P6. Ten days after immunization, draining lymph node cells were cultured with or

Table 3: T cell proliferative responses of LDLr$^{-/-}$ mice to ApoB-100 peptides.

	T Cell Proliferative Responses in vitro (Stimulation Index)							
Immunization w/	**Antigen for in vitro stimulation (μg/ml)**							
ApoB-100 Peptides	PPD (20)	OVA (50)	P1 (50)	P2 (50)	P3 (50)	P4 (50)	P6 (50)	Con A (1)
(OVA)	**11.74**	**12.48**	-0.49	-0.44	-0.26	-0.15	0.6	**4.97**
P1	**8.48**	1.54	-0.47	-0.29	1.2	0.6	1.11	**6.37**
P2	**13.58**	1.76	-0.63	-0.46	0.3	-0.39	0.16	**10.43**
P3	**6.59**	2.14	-0.55	-0.57	**10.23**	0.72	2.54	**7.06**
P4	**3.34**	2.57	0.56	0.31	0.83	2.38	0.51	**6.31**
P6	**12.16**	1.02	-0.62	-0.41	0.77	0.3	**7.3**	**8.24**

T Cell Proliferative Responses (Stimulation Index)

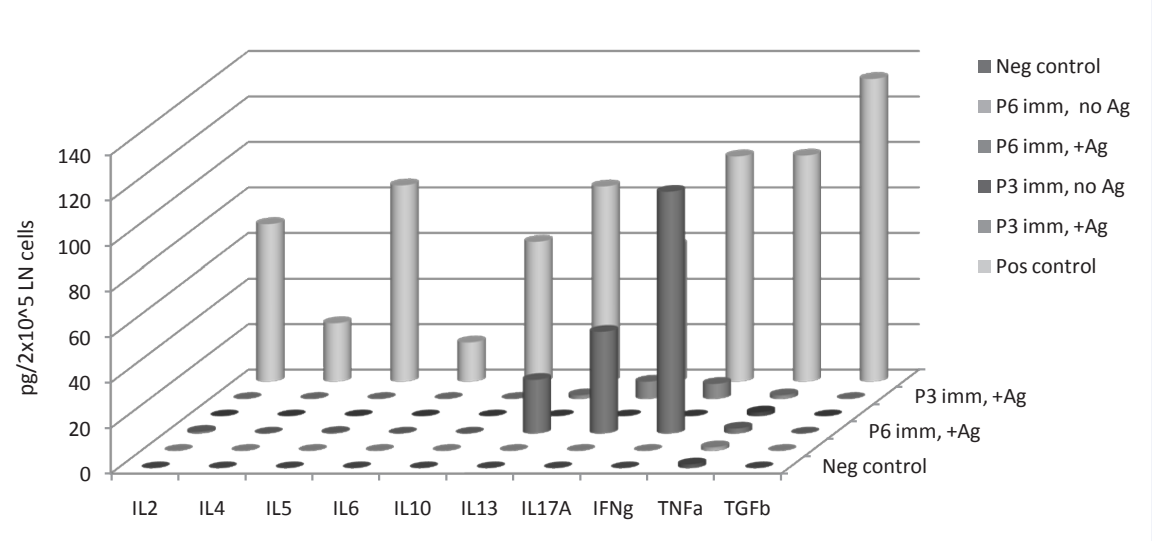

Figure 2 Cytokine profile of lymph node cells from P3- and P6-primed ApoE$^{-/-}$ mice. Three ApoE$^{-/-}$ mice in each group were immunized with P3 or P6. Ten days after immunization, 5 x10^4 draining lymph node cells pooled from the 3 mice were cultured with or without the priming antigen (100 μg/ml) for 24 hours. Culture supernatants were collected and cytokine production analyzed by Cytokine Multi-Analyte ELISArray kit from SABiosciences (Federick, MD).

without the priming antigen for 24 hours. Culture supernatants were collected and cytokine production analyzed by Cytokine Multi-Analyte ELISArray kit from SABiosciences (Federick, MD). As shown in Figure 2, immunization with P6 produced large amounts of IFNγ and moderate amounts of IL-17A. Some IL-13 was also produced. P3 seemed to be less inflammatory than P6. Lymph node cells from mice immunized with P3 produced small amounts of IFNγ and IL17A and negligible amounts of IL-13. Other cytokines such as IL-2, IL-4, IL-10 and TNFα were all at background level. The predominant production of IFNγ by peptide-specific T cells closely correlated with other studies showing Th1 cells as the major T cell subset participating in the development of atherosclerosis. Nevertheless, formal proof that P3- and P6-specific T cells are atherogenic is necessary. Further experiments will clone such T cells and to use them to adoptively transfer disease to naïve recipients.

CONCLUSION

This report is the first to delineate the immunogenic T cell epitopes of the murine autoantigen ApoB-100. Two of the 6 sequences tested, ApoB$_{3501-3515}$ (P3) and ApoB$_{978-992}$ (P6), stimulate strong T cell proliferative responses in immunized ApoE$^{-/-}$, LDLr$^{-/-}$ and C57BL/6 mice and induce production of inflammatory IFNγ and IL-17A cytokines. The development of this experimental system is the first step towards the ultimate development of a peptide vaccine to prevent or reduce atherosclerosis and related diseases.

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Cite this article

Shaw MK, Welch K, Zhao X, Montgomery PC, Thummel R, et al. (2014) Identification of two Immunogenic T cell Epitopes of ApoB-100 and their Autoimmune Implications. J Immunol Clin Res 2(2): 1021.