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Research Paper

*Ci*8 short, a novel LPS-induced peptide from the ascidian *Ciona intestinalis*, modulates responses of the human immune system

Angela Bonura[a,*,1], Aiti Vizzini[b,1], Sara Vlah[a], Francesco Gervasi[c], Alessandra Longo[a], Mario R. Melis[a], Frank A. Schildberg[d], Paolo Colombo[a]

[a] *Istituto di Biomedicina e di Immunologia Molecolare "Alberto Monroy" del Consiglio Nazionale delle Ricerche, Palermo, Italy*
[b] *Marine Immunobiology Laboratory, Department of Biological Chemical Pharmaceutical Science and Technology, University of Palermo, Italy*
[c] *U.O.S.D. Laboratorio Specialistico Oncologia, Ematologia e Colture Cellulari per Uso Clinico, ARNAS Civico, Palermo, Italy*
[d] *Department of Microbiology and Immunobiology, Harvard Medical School, Boston, MA, USA*

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ABSTRACT

The selective modulation of immunity is an emerging concept driven by the vast advances in our understanding of this crucial host defense system. Invertebrates have raised researchers' interest as potential sources of new bioactive molecules owing to their antibacterial, anticancer and immunomodulatory activities. A LipoPolySaccharide (LPS) challenge in the ascidian *Ciona intestinalis* generates the transcript, *Ci*8 short, with *cis*-regulatory elements in the 3′ UTR region that are essential for shaping innate immune responses. The derived amino acidic sequence *in silico* analysis showed specific binding to human Major Histocompatibility Complex (MHC) Class I and Class II alleles. The role of *Ci*8 short peptide was investigated in a more evolved immune system using human Peripheral Blood Mononuclear Cells (PBMCs) as *in vitro* model. The biological activities of this molecule include the activation of 70 kDa TCR ζ chain Associated Protein kinase (ZAP-70) and T Cell Receptor (TCR) Vβ oligo clonal selection on $CD4^+$ T lymphocytes as well as increased proliferation and IFN-γ secretion. Furthermore *Ci*8 short affects $CD4^+/CD25^{high}$ induced regulatory T cells (iTreg) subset selection which co-expressed the functional markers TGF-β1/Latency Associated Protein (LAP) and CD39/CD73. This paper describes a new molecule that modulates important responses of the human adaptive immune system.

1. Introduction

The immune system is composedof a very complex and dynamic network of cell subsets and mediators that promote host defense from infectious agents or tumor cells and maintain immunological tolerance in the organisms (Brodin and Davis, 2017; Parkin and Cohen, 2001). The vertebrate immunity is classically divided into innate and adaptive immune systems that act in interdependent ways based on bidirectional cross-talk (Chaplin, 2010; Paul, 2011; Lanier and Sun, 2009). Innate immunity represents a conserved, complex and multi-pronged response to overcoming infection that is present in all complex host species. It is well known that the innate immune system not only provides the first line of defense in the immune response but can also induce and regulate many different adaptive immunity functions (Lanier and Sun, 2009; Iwasaki and Medzhitov, 2015). Many discoveries have been made in the field, but the mechanisms by which the innate immune system can control adaptive immunity remain to be fully clarified. Invertebrates produce a large number of bioactive molecules which have been proven to fulfill important immunological roles such as antibacterial or anticancer activities (Otero-González et al., 2010; Cheung et al., 2015; Suarez-Jimenez et al., 2012). A recent idea is to use invertebrates as a source of molecules with potential immunoregulatory activities to improve strategies for studying human immune system responses (Davis, 2008; Germain and Schwartzberg, 2011). The ascidian *Ciona intestinalis*, which is a descendant of the last common ancestor of all vertebrates, is a powerful model for studying innate immunity. However Ascidians occupy a key

Abbreviations: LPS, LipoPolySaccharide; PBMCs, Peripheral Blood Mononuclear Cells; MHC, Major Histocompatibility Complex; ZAP-70, 70 kDa TCR ζ chain Associated Protein kinase; TCR, T Cell Receptor; iTreg, induced regulatory T cells; LAP, TGF-β1/Latency Associated Protein; ITAMs, Immunoreceptor Tyrosine-based Activation Motifs; MAMPs, Microbe-Associated Molecular Patterns; CR-APA, Coding Region Alternative PolyAdenylation; RAG, Recombination Activating Genes; ARE, AU Rich Element; GAIT, interferon-γ-Activated Inhibitor of Translation element; IEDB, Immune Epitope Database and Analysis Resource

* Corresponding author at: Istituto di Biomedicina e di Immunologia Molecolare"Alberto Monroy" del Consiglio Nazionale delle Ricerche, Via Ugo La Malfa, 153, 90146, Palermo, Italy.
E-mail address: angela.bonura@ibim.cnr.it (A. Bonura).
URL: http://mailto:bonu1967@gmail.com (A. Bonura).
[1] These authors contributed equally to this work.

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phylogenetic position in chordate evolution and are considered the sister group of vertebrates (Zeng and Swalla, 2005; Delsuc et al., 2006). They are proto-chordates that possess an innate immune system, including inflammatory humoral and cellular responses (Shida et al., 2003). Challenge with Microbe-Associated Molecular Patterns (MAMPs), such as the Gram-negative LPS, induces inflammatory-like reactions in the pharynx (immunocompetent organ). These responses can induce several immunological phenomena, including the expression of characteristic innate immune genes and a repertoire of innate effectors (e.g., TLRs, TNF, IL-17s, TGF beta, complement components, and galectin-like) (Parrinello et al., 2008; Vizzini et al., 2016). Although a variety of different MAMPs induce *Ciona* TLRs, LPS does not activate TLR expression directly, as with TLR4 in vertebrates, suggesting the presence of alternative LPS sensors (Vizzini et al., 2015). In vertebrates, the development of the adaptive immune system is linked to the acquisition of the enzyme machinery encoded by Recombination Activating Genes (RAG) that provide the rearrangement of immunoglobulin (Ig) and TCR genes. Analysis of *Ciona intestinalis* genome sequences did not reveal the pivotal genes and molecules for adaptive immunity, such as MHC genes, TCRs, or dimeric Igs (Dehal et al., 2002; Azumi et al., 2003). Nevertheless, sequence analyses have allowed the recognition of two Ig domain-containing regions, key V regions, the essential feature of an Ig superfamily VC1-like core trait, presumptive proto-MHC regions scattered throughout the genome, and three types of genes with receptor-like V-C architecture (Du Pasquier, 2004; Du Pasquier et al., 2004). In a previous paper (Vizzini et al., 2013), we demonstrate that an LPS challenge induces the activation of Coding Region Alternative PolyAdenylation (CR-APA) mechanisms (Carpenter et al., 2014) responsible for the generation of a mRNA named *Ci*8 short, which is significantly enhanced in the inflammation process and strongly activated in immune cells. In the present study we determine the contribution of these innate immune-related molecule to act on specific aspects of human adaptive immune system functions by using PBMCs as an experimental model. We performed a detailed study by *in silico* analysis of *cis*-regulatory elements in the 3′ UTR mRNA region and deduced amino acid sequence. A *Ci*8 short synthetic peptide was used to set up an antigen-specific experimental system to analyze the effect on TCR activation mediated by the ZAP-70 checkpoint and explore the Vβ repertoire of $CD4^+$ and $CD8^+$ lymphocytes. Moreover, we evaluated different parameters of activation of immune cells including the proliferation rate of $CD16^+/CD56^+$ NK cells, $CD4^+$ and $CD8^+$ lymphocytes and secretion of different cytokines such as IFN-γ and IL-10. Finally, the *Ci*8 short peptide was used to study the phenotype and expression of functional markers of induced regulatory T cells (iTreg) such as CD25, TGF-β1 Latency-Associated Peptide (LAP), CD39 and CD73.

2. Materials and methods

2.1. Bioinformatic analysis

The Exon-Intron Graphic Maker (http://wormweb.org/exonintron) was used to create an image of the genomic organization of the *Ci*8 gene. To characterize the 3′UTR region, a computational analysis was performed using the Regulatory RNA Motifs and Elements Finder tool (http://regrna.mbc.nctu.edu.tw/html/prediction.html). Physical and chemical parameters, such as molecular mass and theoretical isoelectric point, were computed using the Prot-Param tool on ExPASy (http://www.expasy.org/tool/protparam/). The prediction of Major Histocompatibility Complex (MHC) binding affinity sites were studied using the immunological tool including the Immune Epitope Database and Analysis Resource (IEDB) (www.iedb.org). MHC Class I binding peptides prediction was performed using a Consensus method based on top two predictive methods, an Artificial Neural Network (ANN) and Stabilized Matrix Method (SMM). The output of prediction is: percentile rank (cut off 0.5) and ANN and SMM IC^{50} value, (peptides with IC50 values < 50 nM are considered high affinity, weakly binding peptides had IC50 value below 500 nM). MHC Class-II binding peptides prediction was performed using a consensus methods based on an approach which calculates the median rank of the top three predictive methods for each MHC class II molecules: Combinatorial library (Comb.lib), SMM-align (netMHCII-1.1) and NN-align (netMHCII-2.2).

2.2. Reagent

The *Ci*8 short peptide
1-MTSTVAIPQFFGNYPGVIPGSVPGGIP
CPIPGTMPPANVPIPTSANGVSYPTVPI
3.1 QVPIQLPVVPVGGGCYNE-73
was synthesized by Selleck Chemicals. *Ci*8 short peptide was resuspended at 10 μg/μl concentration in LAL Reagent Water (Lonza, BioWhittaker, USA), aliquoted and stored at −80 until use.

2.3. Human leukocyte specimens and cell lines

Freshly isolated Peripheral Blood Mononuclear Cells (PBMCs) were prepared by standard Ficoll (GE Healthcare Life Sciences) density gradient separation from blood of four healthy volunteer donors after obtaining written informed consent. The Prostatic Adenocarcinoma cell line PC-3 (NCI-PBCF-CRL1435 Prostate Adenocarcinoma) was purchased from American Type Culture Collection (ATCC). PBMCs and PC-3, except where otherwise indicated, were maintained in RPMI 1640 (Gibco™, Thermo Fisher Scientific) containing 10% FCS serum (Gibco™, Thermo Fisher Scientific), 100 U ml^{-1} penicillin/100 mg ml^{-1} streptomycin (Gibco™, Thermo Fisher Scientific), 1% Non-Essential Amino Acids (Gibco™, Thermo Fisher Scientific), and 1% sodium pyruvate (Gibco™, Thermo Fisher Scientific), at 37 °C in a humidified incubator with 5% CO_2.

2.4. Cell viability assay

The effect of the *Ci*8 short peptide on PC-3 and PBMCs cell growth was determined using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, Wisconsin, USA) according to the manufacturer's protocol. In brief, PC-3 cells were seeded at a concentration of 5×10^3 cells/well, while PBMCs was seeded at a concentration of 15×10^4 cells/well on 96-wells microtiter plates (Nunc, Roskilde, Denmark). PBMCs and PC-3 were treated with different concentrations of *Ci*8 short peptide (0,1 μl/ml, 1 μl/ml, 10 μl/ml and 100 μl/ml) and incubated at 37 °C in a humidified incubator with 5% CO^2 for 24 h and 48 h. Assays were performed in triplicates. The absorbance of the dissolved formazan was measured in an iMark™ Microplate Absorbance Reader (BioRad, Hercules, CA, USA) at 490 nm. Cell viability percentage was determined as the ratio between the absorbance of treated and control cells x 100.

2.5. Hemolysis assay

Heparinized blood samples were obtained from two healthy human subjects. *Ci*8 short peptide at concentrations of 1 μl/ml, 10 μl/ml and 100 μl/ml was added to an 8% human erythrocytes solution and incubated at 37 °C for 30 min. The samples were centrifuged at 2000 x g for 5 min and the supernatant absorbance was measured at 415 nm through the iMark™ Microplate Absorbance Reader (BioRad, Hercules, CA, USA) to determine the percentage of hemolysis. Hemolysis was induced with 1X PBS, and Triton X-100 1% solution was taken at 0% and 100%. Hemolysis percentage was determined as the ratio between the OD of treated cells and positive control cells x 100.

2.6. Flow cytometry

All staining were performed by adding the appropriate amount of the fluorochrome-conjugated antibody (according to antibody data sheet), and stained at 4 °C for 30–45 min in 100 μl of FACS buffer

(Phosphate Buffered Saline-PBS (Gibco™, Thermo Fisher Scientific) with 1% FCS serum (Gibco™, Thermo Fisher Scientific) and 0.1% sodium azide). Excess of antibodies were removed by two consecutive washings. Anti-human antibodies were used, purchased from BD Bioscience Heidelberg, Germany (BD), Beckman Coulter, Brea, CA, USA (BC) and Miltenyi Biotech, Bergisch Gladbach, Germany (MB): anti-CD3-FITC (clone UCHT1, BC), CD4 FITC (clone VIT4, MB), anti-CD4 PC5 (clone 13B8.2, BC), anti-CD4 APC (clone 13B8.2, BC), anti-CD8 Pacific Blue (clone B9.11, BC), anti-CD16 PC7 (clone 3G8, BC), anti-CD14 PerCp (Clone TÜK4, MB), anti-CD25 PC7 (clone B1.49.9, BC), anti-CD39 FITC (clone MZ18-23C8, MB), CD56 PC7 (BC), anti-CD73 PE (clone AD2, MB), 7AAD (BD), anti-LAP APC (clone CH6-17E5.1, MB), anti-ZAP70-PE (clone SBZAP, BC). Flow cytometry analysis were performed using BD FACSCalibur (BD Bioscience, Heidelberg, Germany), CyAn™ADP Analyzer (Beckman Coulter, Brea, CA, USA) and data were analyzed with FlowJo software (Tree star Inc., Ashland, OR, USA).

2.7. CFSE proliferation assay

Determination of PBMC proliferation, from four healthy donors, was conducted by use of 5 μM 5(6)-Carboxy-Fluorescein Diacetate Succinimidyl Ester solution (CFSE) (Molecular Probe, Eugene, OR, USA) as previously described (Bonura et al., 2007). CFSE labelled PBMCs were seeded at a concentration of 1×10^6 cells/ml, and incubated with *Ci*8 short peptide at the concentrations of 1 μg/ml and 10 μg/ml. Background proliferation was measured in the unstimulated sample. A minimum of 200,000 events were collected and acquired on a CyAn™ ADP Analyzer (Beckman Coulter, Brea, CA, USA). Analysis gates were set on proliferating lymphocytes by forward and side scatter profile and by the exclusion of $7AAD^+$ cells. The percentages of $CD4^+$, $CD8^+$ and $CD16^+/CD56^+$ proliferating cells were selected on a CD4 vs CFSE, CD8 vs CFSE and CD16/CD56 vs CFSE dot plots.

2.8. ZAP-70 intracellular staining

PBMC from four healthy donors were seeded at a concentration of 1×10^6 cells/ml, incubated with *Ci*8 short peptide at a concentration of 10 μg/ml and harvested at 24 h for intracellular determination of ZAP-70 expression. Cells were treated with PerFix-nc-kit (Beckman Coulter, Brea, CA, USA), according to the manufacturer's protocol optimized for PBMCs. A minimum of 50,000 events were acquired. A lymphocyte gate based on forward and side scatter properties was set: $CD3^+$ lymphocytes were selected on SSC vs CD3 dot plot, $CD3^+CD4^+$ and $CD3^+CD8^+$ lymphocytes were selected on a CD4 vs CD8 dot plot. The percentage value of $CD3^+CD4^+$ $ZAP\text{-}70^+$ cells and of $CD3^+CD8^+$ $ZAP\text{-}70^+$ was defined with a count vs ZAP-70 histogram.

2.9. Quantitative analysis of the TCR Vβ repertoire

The study the TCR repertoire flow cytometry analysis was performed using the IOTest® Beta Mark multianalysis tool (Beckman Coulter, Brea, CA, USA), which consists of fluorochrome conjugated monoclonal antibodies that identify 24 TCR Vβ subfamilies, covering about the 75% of the normal human T cells. Peripheral blood samples from four healthy donors were collected in tubes containing anticoagulant heparin and were stained within 2 h and used as a control. Subsequently, isolated PBMCs were seeded at a concentration of 1×10^6 cells/ml and treated with *Ci*8 short peptide at a concentration of 10 μl/ml and harvested at day 7. For each sample, 20 μl of a mixture of three distinct anti-Vβ monoclonal antibodies were added according to the manufacturer's protocol. A minimum of 50,000 lymphocyte events were acquired. A lymphocyte gate based on forward and side scatter properties was set. $CD4^+$ and $CD8^+$ lymphocytes were selected on a SSC vs CD4 or SSC vs CD8 dot plot. $CD4^+$ and $CD8^+$ lymphocytes TCR Vβ repertoire analysis was set on a FITC vs PE dot plot, which identified subpopulation clonality of different T cells.

2.10. Cytokines secretion assay

PBMCs from four healthy blood donors were seeded at a concentration of 10×10^6 cells/ml in RPMI 1640 (Gibco™, Thermo Fisher Scientific) containing 10% AB serum (Euroclone, Milan, Italy), 100 U ml^{-1} penicillin (Gibco™, Thermo Fisher Scientific), 100 mg ml^{-1} streptomycin (Gibco™, Thermo Fisher Scientific), 1% Non-Essential Amino Acids (Gibco™, Thermo Fisher Scientific), and 1% sodium pyruvate (Gibco™, Thermo Fisher Scientific), at 37 °C in a humidified incubator with 5% CO^2. Cells were incubated with *Ci*8 short peptide at concentrations of 20 μg/ml for 16 h. The specific selection of IFN-γ and IL-10 producing cells was performed by using the IFN-γ Secretion Assay Cell Enrichment and Detection Kit (PE) (Miltenyi Biotech, Bergisch Gladbach Germany) and the IL-10 Secretion Assay Cell Enrichment and Detection Kit (PE) (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions. IFN-γ and IL-10 positive cell fractions were magnetically sorted and stained with the appropriate set of antibodies. Immediately before flow cytometry acquisition, Propidium Iodide (PI) (0.5 mg/ml, Sigma-Aldrich, St. Louis, MO, USA) was added to each sample in order to distinguish live and dead cells. A minimum of 100,000 events was collected. A lymphocyte gate based on forward and side scatter properties was set. Dead cells and monocytes were excluded on a PI vs CD14 dot plot. The $cytokine^+/CD4^+$ cells were selected on a cytokine vs CD4 dot plot. For each sample we evaluated Coefficient of Variation (CV) as a statistical parameter.

2.11. Studying T regulatory cells subpopulation

PBMCs from four healthy donors were seeded at a concentration of 1 × 106cells/ml, treated with *Ci*8 short peptide at a concentration of 10 μl/ml, and harvested at days 6, 7 and 8. A minimum of 100,000 events was collected. Analysis gates were set on lymphocytes using forward and side scatter profiles. The gate to define the $CD4^+CD25^{high}$ population was set according to the level of expression of CD25 on $CD4^{neg}$ T cells (Baecher-Allan et al., 2001). The percentage value of $CD4^+CD25^{high}CD39^+CD73^+$ was defined on a CD39 vs CD73 dot plot; the relative CD39 MFI and CD73 MFI variations were evaluated. The percentage value of $CD4^+CD25^{high}LAP^+$ cells was selected on a count vs LAP histogram and the relative LAP MFI variation was evaluated. The percentage value of $CD4^+CD25^{high}CD39^+CD73^+$ LAP^+ cells was defined with a count vs LAP histogram and the LAP MFI variation was evaluated.

2.12. Statistical analysis

The statistical significance of the differences between data sets was assessed by a two-tailed unpaired Student's *t*-test, unless stated otherwise. The results are expressed as the mean values ± SD. Differences with a P value < 0.05 are marked with asterisks. ns, not significant; *P < 0.05; **P < 0.01; ***P < 0.001.

3. Results

3.1. LPS challenge in the ascidian Ciona intestinalis induces a CR-APA mechanism that generates the transcript Ci8 short with GAIT and ARE cis-regulatory elements in the 3′ UTR region.

In a previous paper, we reported that LPS challenge in *Ciona intestinalis* induces a CR-APA event in the annotated *Ci*8 gene (Vizzini et al., 2013). This gene contains five exons and four introns (Fig. 1A) encoding for the following two alternative transcripts: *Ci*8 long and a short variant named *Ci*8 short derived by an intronic polyadenylation signal within the first intron (Fig. 1B). To study the structural elements of 3′ UTR involved in posttranscriptional regulation, we performed an *in silico* analysis using the REGRNA 2.0 database (Chang et al., 2013). In the 3′UTR of *Ci*8 short, we identified different *cis*-regulatory elements

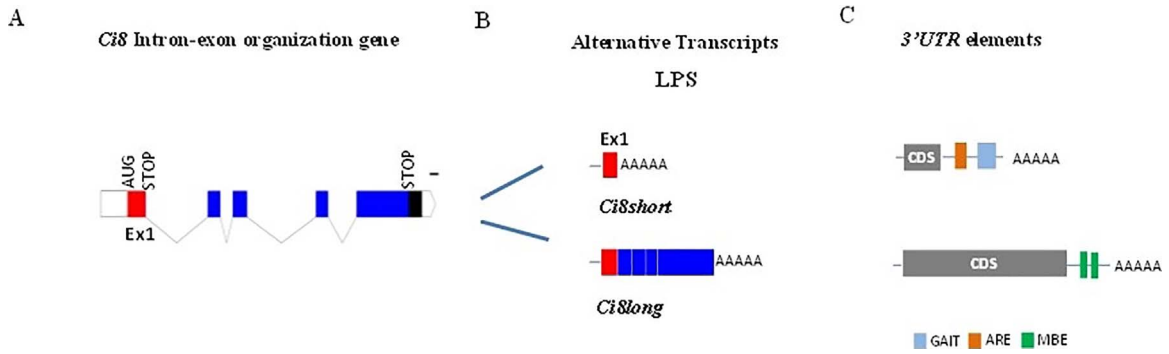

Fig. 1. LPS challenge in the ascidian *Ciona intestinalis* induces a Coding Region Alternative PolyAdenylation (CR-APA) event in the annotated *Ci*8 gene. An intronic polyadenylation signal within the first intron leading to the transcription of a variant named *Ci*8 short. A. Intron-exon organization of the *Ci*8 gene (ENSCING00000009651): the thin lines represent the four introns and the open boxes indicate the five exons. Exon 1 (Ex1) is shown as a red open box. Exons from 2 to 5 are shown as blue opened boxes. B. LPS challenge leading to the transcription of a shorter mRNA, *Ci*8 short, including the first exon 1 (red open box) C. *Ci*8 short and *Ci*8 long transcripts show different 3′ UTR *cis*-regulatory element organization. *Ci8* short present a GAIT element (Interferon-γ-Activated Inhibitor of Translation, light blue open box) and ARE element (AU Rich Elements, orange open box). *Ci8* long 3′ UTR containing MBE (Musashi Binding Element, green open boxes). CDS (Coding DNA Sequence). (For the interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

essential for shaping innate immune responses. In particular, we detected an AU Rich Element (TTATATTTATTTG) (ARE) (Fig. 1C) and an Interferon-γ-Activated Inhibitor of Translation element (TAAATGTTG-TTTGTGTTAAAGCATTTA) (GAIT). ARE elements are major regulators of the mRNA stability of early and transient transcripts that are induced in LPS-stimulated immune responses. In addition to ARE, GAIT elements have an important role in gene-specific translational control in innate immunity. These elements result in the absence of the *Ci*8 long 3′ UTR region, where Musashi Binding Elements (MBEs) (Fig. 1C) are instead identified as being involved in mRNA translational control during cell cycle progression. However *Ci*8 short transcript is significantly enhanced during the inflammatory process in the early hours (1–12) of the LPS challenge compared to *Ci*8 long transcript (Vizzini et al., 2013) and for all of these results we must assume a possible role of *Ci*8 short in controlling both the magnitude and duration of the inflammatory response in *Ciona intestinalis*.

3.2. In silico analysis of Ci8 short physical-chemical and immunological properties

An *in silico* analysis of *Ci*8 short deduced amino acidic sequence was performed using the Expasy ProtParam server (Gasteiger et al., 2005) to deduce physical-chemical properties and the Immune Epitope Database and Analysis Resource (IEDB) (Peters et al., 2005; Vita et al., 2015) to predict the possible MHC Class I and Class II binding affinity sites. *Ci*8 short mRNA codifies for a peptide of 73 AA with a predicted molecular size of 7.3 kDa, a pI of 4.00 and a total number of negatively charged residues (Asp + Glu = 1). Within the 73 AA deduced sequence, a sequence from 55 to 63 AA was identified with good binding prediction (Percentile rank 0.1) for MHC Class I Alleles as HLA-A*02:06 and from 42 to 50 AA for HLA-A*01:01 (Percentile rank 03), (Table 1 supplementary). The MHC Class II binding peptide prediction describes the following two different putative binding sequences: a peptide sequence between AA 49–67 (Percentile rank 1.45/2.03) that can bind to the HLA-DRB4*01:01 Allele and a sequence at position AA 4–22 that can recognize HLA-DRB1*13:02 (Percentile rank 3.41/3.85) (Table 2 supplementary). These data highlight that there are specific amino acid short peptides that can bind to different human HLA class I and HLA class II alleles.

3.3. Ci8 short peptide induces the expression of ZAP-70 on human T lymphocytes

The T cell epitope prediction shows putative *Ci*8 short binding with MHC class I and II for different peptide sequences and alleles. To study *Ci*8synthetic peptide immunological characteristics, we selected human PBMCs as an *in vitro* system. First, we demonstrate that the peptide did not show cytotoxic activity in PBMCs and Prostate Adenocarcinoma (PC-3) cells after 24 and 48 h stimulation. The peptide demonstrated the same lack of cytotoxic activity at a higher concentration and did not induce hemolytic activity on Red Blood Cells (RBCs) (supplementary Fig. 1 for details). To investigate possible effects of the peptide on the human immune system, we analyzed ZAP-70 expression, a key signaling component of antigen recognition mediated by TCR. Flow cytometric analysis was performed on $CD3^+CD4^+$ and $CD3^+CD8^+$T lymphocytes to evaluate the percentage of $ZAP\text{-}70^+$ cells and ZAP-70 Mean Fluorescence Intensity (MFI) values. The cells were incubated with *Ci*8 short peptide for 24 h and unstimulated samples were used as a negative control. Fig. 2A and B shows the histograms representative of one experiment where the ZAP-70 MFI variation is evident for $CD3^+CD4^+$ and $CD3^+CD8^+$ T lymphocytes, respectively. Fig. 2C summarizes ZAP-70 MFI values for all donors and shows a trend for increased ZAP-70 in $CD3^+CD4^+$ and $CD3^+CD8^+$ cells in the presence of *Ci8* short peptide in comparison to the negative control. Furthermore, the peptide induces a statistically significant increase only for $CD3^+CD4^+ZAP70^+$ cells (*P*-value = 0.048) (Fig. 2D). Together these results demonstrate that *Ci*8 peptide increases the expression of ZAP-70 tyrosine kinase particularly on $CD4^+$ T lymphocytes.

3.4. Ci8 short modulates specific TCR Vβ in $CD4^+$ T lymphocytes

T-cell mediated immune responses are characterized by activation and subsequent clonal expansion of antigen-specific cells. T lymphocyte repertoire variations can be evaluated by flow cytometric quantification of TCR Vβ family subgroups after antigen stimulation. Therefore, we used a panel of 24 monoclonal antibodies that stains approximately 75% of the entire T repertoire to study the selective responses of each Vβ subgroups which were evaluated in all donors on $CD4^+$ and $CD8^+$ cells in control samples without stimulation and in cultures in presence of *Ci*8 short peptide for seven days. Figs. 2 and 3 supplementary data show the gating strategy for 24 TCR Vβ clone analysis in a representative experiment on $CD4^+$ and $CD8^+$ lymphocytes. Fig. 3A and B shows the clonograms that summarize the patterns of Vβ family expression. Selective expansion of $CD4^+$ and $CD8^+$ of specific Vβ subgroups was considered significant when the% of Vβ in the stimulated cultures was elevated at statistically significant levels compared to the control group. All of the means and *P*-value data are reported in Supplementary Table 3 for $CD4^+$ and $CD8^+$ T subsets. Significant results were obtained for Vβ20 (*P*-values = 0.005), Vβ2 (*P*-values = 0.003) and Vβ21.3 (*P*-values = 0.002) families. In addition, we also observed

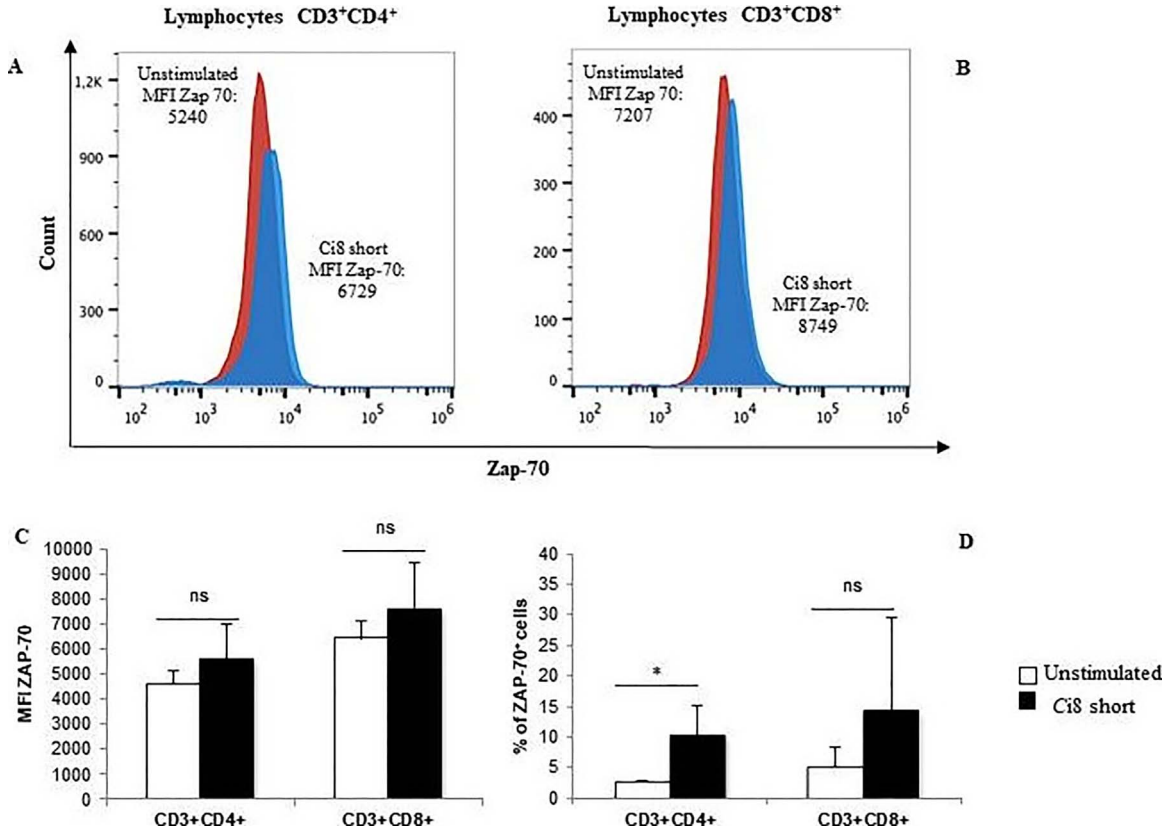

Fig. 2. *Ci*8 short peptide induces the activation of ZAP-70 tyrosine kinase on T lymphocytes.
A and B. Representative histograms of ZAP-70 in $CD3^+CD4^+$ and $CD3^+CD8^+$ cells after a 24 h stimulation with *Ci*8 short peptide at 10 μg/ml (blue histograms) and an unstimulated sample (red histograms). **C.** Histograms show MFI values of ZAP-70 expression described as the mean values ± SD of four independent experiments for cultures without stimuli (white boxes) and cultures treated with *Ci*8 short (black boxes) for $CD3^+CD8^+$ and $CD3^+CD4^+$T Lymphocytes. D. Histograms of% $ZAP\text{-}70^+$ cells of $CD3^+$-$CD4^+$-$ZAP\text{-}70^+$ and $CD3^+$-$CD8^+$-$ZAP\text{-}70^+$ cell for unstimulated samples (white boxes) and *Ci*8 short samples (black boxes). Data are shown as the mean value ± SD and are representative of four independent experiments. Statistical significance was determined using Student's *t*-tests (two-tailed): ns, not significant; *P < 0.05. (For the interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

an increase for TCR Vβ16 (*P*-values = 0.031), Vβ13.1 (*P*-values = 0.026), Vβ8 (*P*-values = 0.020), Vβ14 (*P*-values = 0.028) and Vβ7.2 (*P*-values = 0.033). For the $CD8^+$ subset we could detect a *P*-value = 0.007 for the Vβ14 clone (Fig. 3B) and *P*-values = 0.031 for Vβ16. Taken together, these data suggest that *Ci*8 short peptide induces an oligoclonal response in TCR Vβ20, Vβ2 and Vβ21.3 families primarily in peripheral $CD3^+CD4^+$T lymphocytes. In $CD3^+CD8^+$ lymphocytes, *Ci*8 short peptide induces a response only for TCR Vβ14.

3.5. Human $CD4^+$ T lymphocytes proliferate in response to Ci8 short peptide

In our system, we demonstrated the engagement of TCR and ZAP-70 tyrosine kinase activation; we next asked whether the peptide does increase the proliferation rate of innate and adaptive human immune cells involving $CD4^+$, $CD8^+$ and $CD16^+/56^+$ lymphocytes. For eight days human PBMCs from four healthy donors were incubated in presence of two different concentrations of *Ci*8 short (1 and 10 μg/ml) or without *Ci*8 short as a negative control (Bonura et al., 2007); the proliferation rate was studied using CFDA-SE staining at 6, 7 and 8 days (Supplementary Fig. 4 for gating strategy). Fig. 4A shows the dot plots of a representative experiment where $CFSE^+/CD4^+$ cells increase at days 6 and 7 after antigen stimulation and with higher values at 10 μg/ml. The same experiments were performed to study the proliferative response of $CD8^+$ lymphocytes and the $CD16^+/CD56^+$ NK cell compartment. For $CD8^+$ lymphocytes we could no detect any difference in the proliferation rate in three out of four subjects (Fig. 4A the dot plots of a representative experiment at day 6) in comparison to the control group. The analysis performed on $CFSE^+/CD16^+/CD56^+$ cells demonstrated that there are no significant changes in the percentage of proliferating cells in response to the *Ci*8 short (Fig. 4A the dot plots of a representative experiment at day 6). Fig. 4B summarizes the $CD4^+$ proliferation response for all donors and describes a statistically significant increase in $CFSE^+/CD4^+$ T cells compared to the unstimulated sample at day 6 (*P*-value = 0.04) and day 7 (*P*-value = 0.04) when cells were stimulated with 10 μg/ml of peptide. In summary, our results suggest that the TCR activation drives $CD4^+$ T lymphocytes mainly to a proliferative response, but no significant data were obtained for $CD8^+$ cells and NK cells.

3.6. Ci8 short induces IFN-γ secretion by $CD4^+$ T lymphocytes

Next, we analyzed whether the treatment with *Ci*8 short changes the cytokine profile in our experimental system. In particular, we have focused on the effect of antigen on IFN-γ and IL-10 investigating human T cells by using an affinity matrix technology, which allows the enrichment of viable $cytokine^+$ cells and analyses cells down to frequencies of 0.01–0.1% (rare population) both as a% of positive cytokine-secreting cells and as MFI. For each sample, we evaluated the Coefficient of Variation (CV) as a parameter; in all experiments, CVs are < 5. Flow cytometric analysis performed on live PBMCs show an interesting increase in the IFN-γ^+ MFI value in three out of four subjects 16 h post-stimulation compared to the negative control (Fig. 5A). To understand which subsets of PBMCs can produce IFN-γ, positive fractions were stained with anti-CD4 antibody after the enrichment procedure (supplementary Fig. 5 for gating strategy). All donors

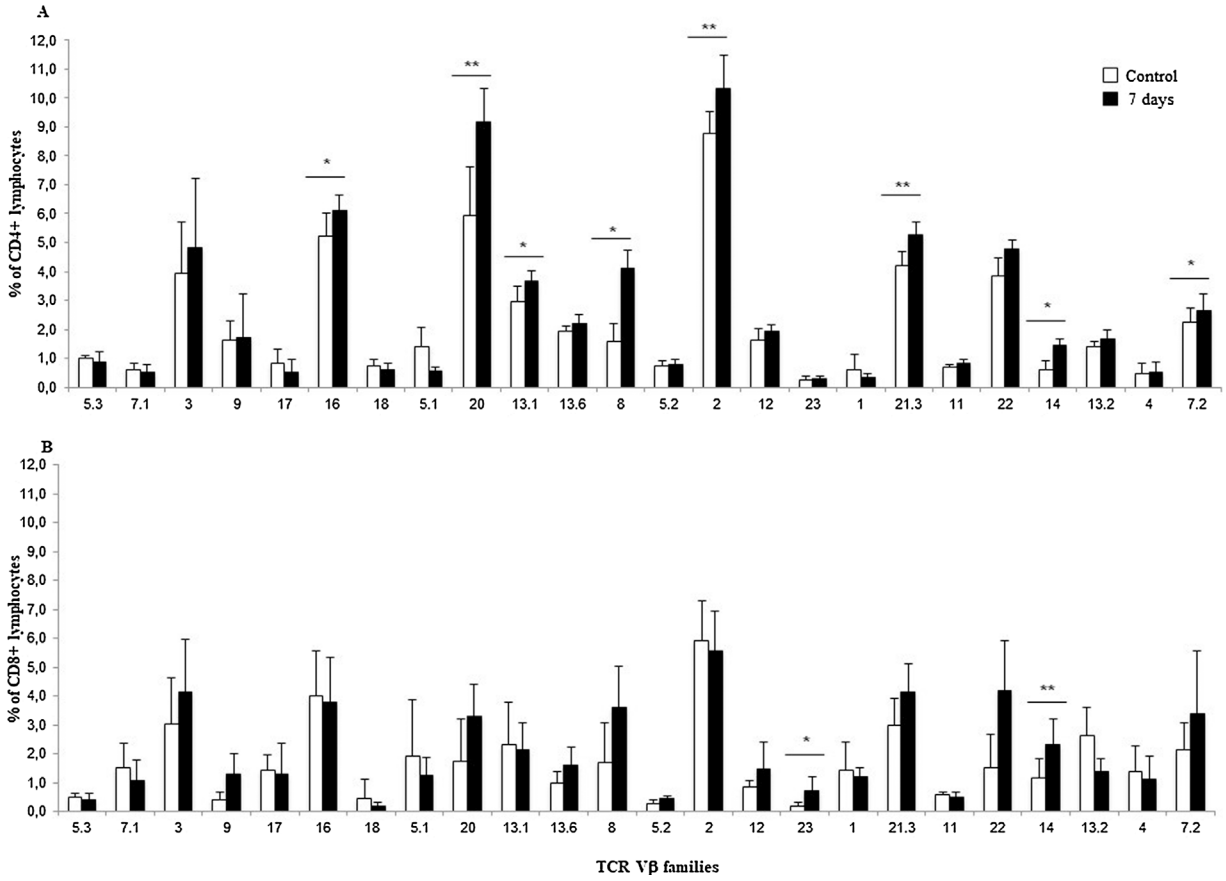

Fig. 3. T Cell Receptors Vβ repertoire on $CD4^+$ and $CD8^+$ lymphocytes stimulated with *Ci*8 short peptide. A. Clonogram of TCR Vβ repertoire on $CD4^+$ lymphocytes. The% of cells positive for specific Vβ clones detected for different times after *Ci*8 short peptide stimulation at 7 days (black boxes) and in cultures without stimuli (control, white boxes) are described as the mean values ± SD of four independent experiments. Statistical significance was determined using Student's *t*-tests (two-tailed): *P < 0.05, **P < 0.01. B. Clonogram of TCR Vβ repertoire on $CD8^+$ lymphocytes. The% of cells positive for specific Vβ clones detected at 7 days after *Ci*8 short peptide stimulation at 7 days (black boxes) and in cultures without stimuli (control, white boxes) are described as the mean value ± SD of four independent experiments. Statistical significance was determined using Student's *t*-tests (two-tailed): *P < 0.05.

demonstrated an increase in $IFN\gamma^+$ $CD4^+$ cells compared to the unstimulated sample; Fig. 5B summarizes $CD4^+$ $IFN\gamma^+$ cell percentage values for all donors and indicates a positive, but not statistically significant, trend. With the same experimental set-up, we examined whether *Ci*8 short peptide can induce IL-10 secretion, but we could not observe any significant change in IL-10 MFI values between the non-stimulated and stimulated sample in all subjects. Furthermore, *Ci*8 peptide induces IL-10 secretion by both $CD4^-$ and $CD4^+$ T lymphocytes (data not shown).

3.7. Ci8 short peptide induced the expression of LAP^+, $CD39^+/CD73^+$ on $CD4^+CD25^{high}$ lymphocytes

Next we investigated if stimulation of PBMCs (n = 4 donors) increased the $CD4^+CD25^{high}$ subsets. Fig. 6A shows the gating strategy used for identifying these cells in agreement with Bacher-Allan et al. (Baecher-Allan et al., 2001). $CD4^+CD25^{high}$ positive cell percentage increased significantly at day 8 (*P*-value = 0.015), compared to *Ci*8 short peptide stimulated samples and the negative control (Fig. 6B). The expression of high levels of CD25 is a useful and necessary marker but is not sufficient for identifying complex and heterogeneous cell populations, such as human *in vitro* induced T reg. Hence, this study focused on two different inhibitory pathways for iTreg mediated mechanisms, such as LAP and CD39/CD73. Thus, we exploit a hierarchical gating strategy based on the selection of $CD4^+CD25^{high}$ T cells and then CD39/CD73 co-expression to evaluate the percentage of double positive cells. Fig. 6C shows the dot plots of a representative experiment indicating an increase in the number of $CD39^+CD73^+$ in PBMCs challenged with antigen. This is true from day 6–8, but with a clear peak of response at day 7. In this subject, the % $CD39^+CD73^+$ increased to 22.5% in the *Ci*8 short sample compared to the unstimulated cells. The expression of CD39 and CD73 markers was performed and the complete data analysis for all donors is reported in supplementary Fig. 6 (A% $CD4^+CD25^{high}CD39^+CD73^+$ cells; B and C for CD39 MFI and CD73 MFI, respectively). Flow cytometric analysis showed that the peptide induces a significant increase in the number of $CD4^+CD25^{high}$ cells expressing the two functional markers at day 6 (*P*-value = 0.014) and day 7 (*P*-value = 0.034). Furthermore, MFI value trends for CD39 (Supplementary Fig. 6 B) and CD73 (Fig. 6C) were analyzed, but a statistically significant increase was only found for CD73 MFI at day 6 (*P*-value = 0.0037) and day 7 (*P*-value = 0.037). Therefore, we next examined whether $CD4^+CD25^{high}CD39^+CD73^+$ cells that increased after the antigen challenge, also co-express LAP. In Fig. 6D, the histograms of one representative experiment show the number of positive cells that co-express the three functional markers studied here, i.e., the value of LAP MFI in $CD4^+CD25^{high}CD39^+CD73^+LAP^+$ cells. The difference between the unstimulated sample (below) and the sample stimulated with *Ci*8 short (above) are described for every day. For this subject, we detected a notable increase in $CD4^+CD25^{high}CD39^+CD73^+LAP^+$ cells at all time points but with particularly significant data at day 6 and 8. As conducted for the CD39 and CD73 functional Treg analysis, we examined the expression of the LAP marker on $CD4^+CD25^{high}$ cells to understand if the TGF-β1 immunosuppressive pathway had a role in *Ci*8-mediated mechanisms *per se*. The data are reported in supplementary Fig. 6D and E.

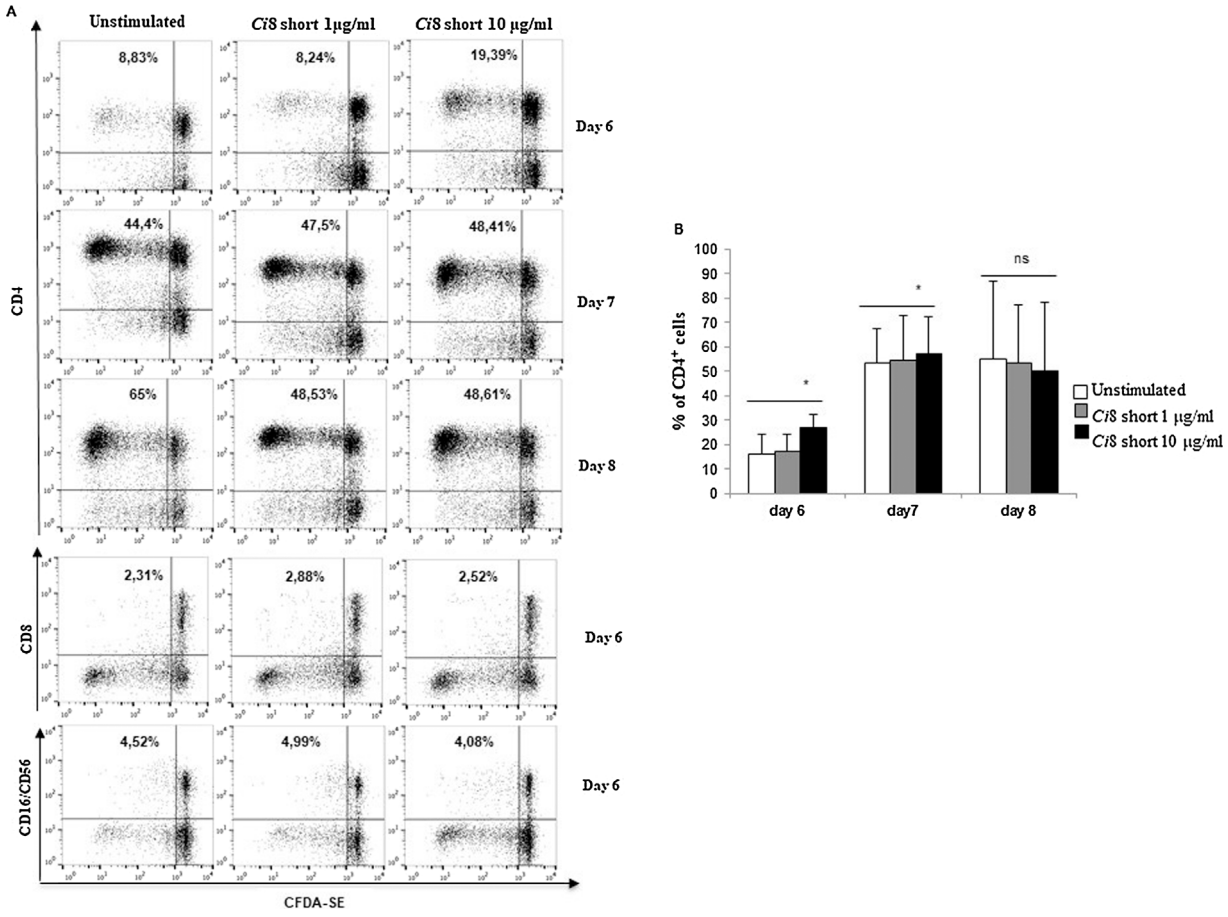

Fig. 4. *Ci8* short induces $CD4^+$ T cell proliferation. Proliferation assay of 5(6)-Carboxy-Fluorescein DiAcetate Succinimidyl Ester (CFDA-SE) labelled PBMCs stimulated with 1 and 10 µg/ml *Ci*8 short peptide at indicated time points. **A.** Flow cytometric dot plots of one representative out of four individuals. Values inside the plots indicate the percentage of $CFSE^+/CD4^+$- $CFSE^+/CD8^+$- $CFSE^+/CD16^+/CD56^+$cells. B. Histograms show the percentage of $CD4^+$ proliferating cells as the mean values ± SD of four independent experiments (unstimulated sample, white box; cells treated with 1 µg/ml *Ci*8 short, gray box; cells treated with 10 µg/ml *Ci*8 short, black box). Statistical significance was determined using Student's *t*-tests (two-tailed): ns, not significant; *P < 0.05.

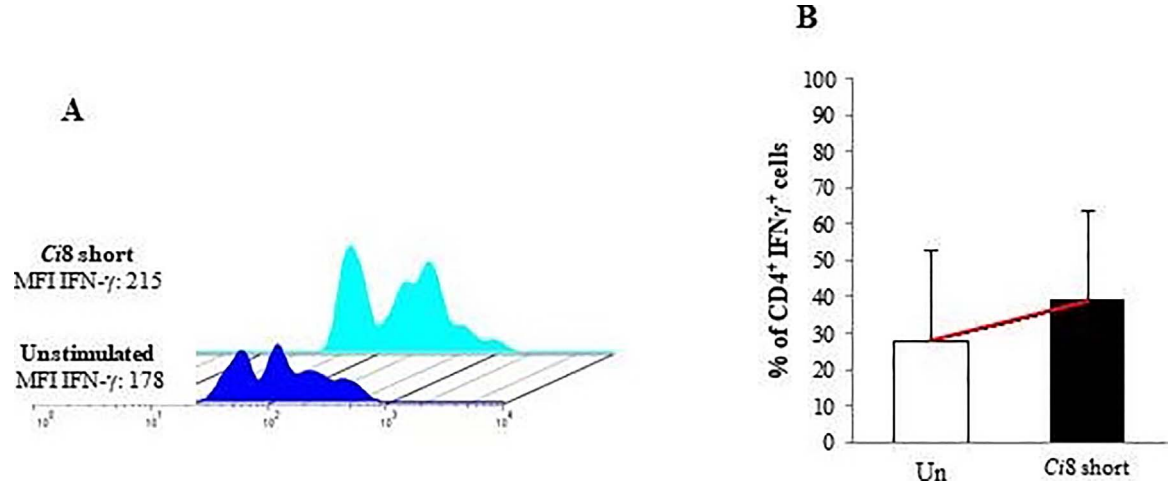

Fig. 5. *Ci*8 short induces IFN-γ secretion in $CD4^+$T lymphocytes. PBMCs from n = 4 donors were tested for IFN-γ production using a cell-surface affinity matrix technology for the analysis and enrichment of cytokine-secreting antigen-specific cells. A MFI IFN-γ^+ unstimulated PBMCs (blue histogram) and *Ci*8 stimulated PBMCs (light blue histograms) from one representative subject. B Histograms of mean value data ± SD for $CD4^+$IFN-γ^+ cells (white box unstimulated sample values, black box antigen induced). The trend line is in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

$CD4^+CD25^{high}$ LAP^+ cells showed a significant increase at day 6 (*P*-value = 0.038) and day 8 (*P*-value = 0.023) and LAP MFI at day 6 (*P*-value = 0.012) and day 8 (*P*-value = 0.021) (Supplementary Fig. 6D and F). The flow cytometric analysis indicates an increase in $CD4^+CD25^{high}CD39^+CD73^+LAP^+$ cell percentage at day 6 (*P*-value = 0.014), 7 and day 8 (Fig. 6E). There is no statistically significant increase in CD39 MFI and CD73 MFI values in the stimulated samples compared to the unstimulated ones (data not shown). LAP

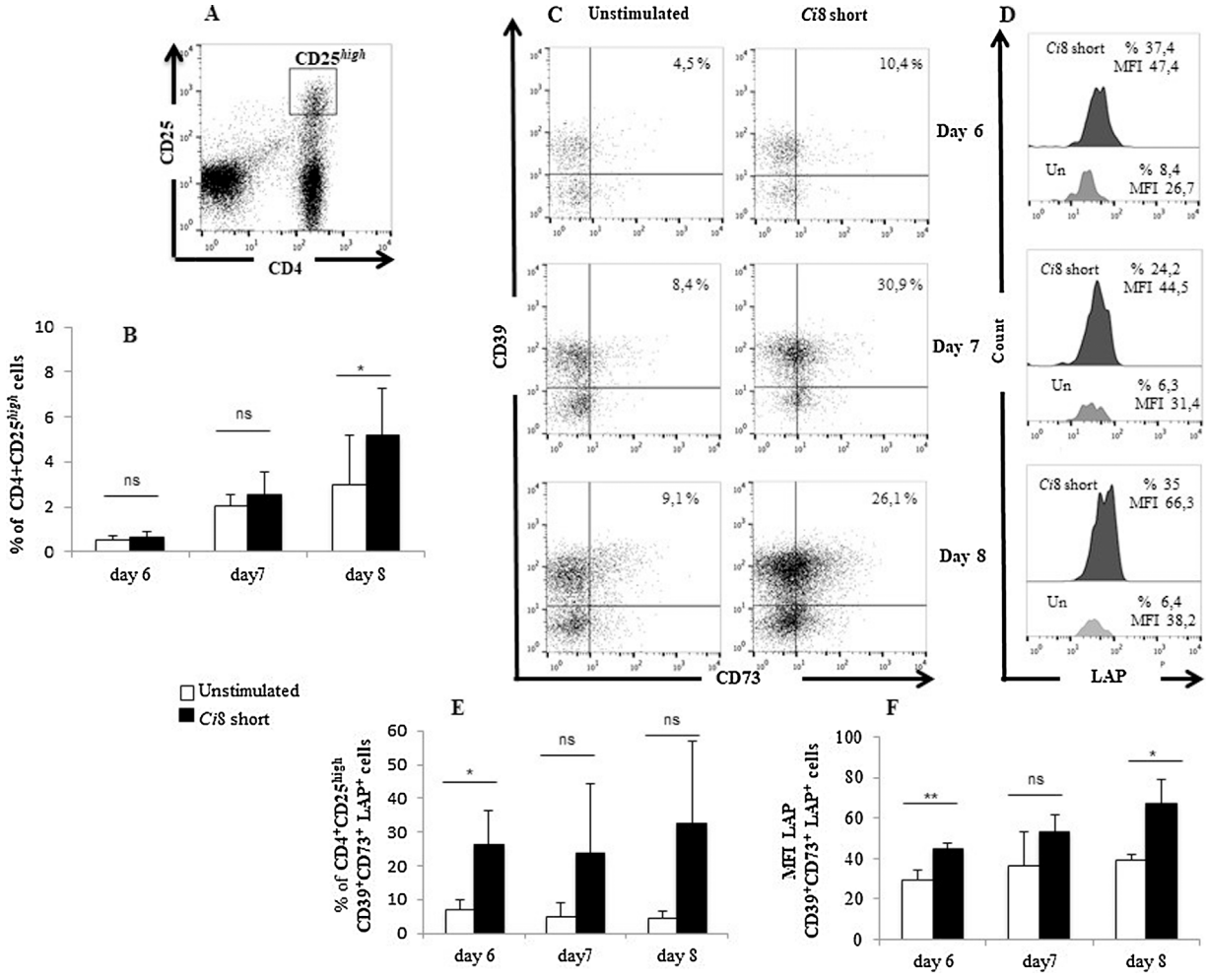

Fig. 6. *Ci*8 short peptide induces the expression of CD39, CD73 and LAP on $CD4^+CD25^{high}$ lymphocytes.

A. Dot plot showing the gating strategy of the multiparametric flow cytometry analysis utilized for the selection of the $CD4^+CD25^{high}$ cell population. B. Histograms showing the percentage of $CD4^+CD25^{high}$ cells of PBMCs unstimulated (white box) and incubated with 10 μg/ml *Ci*8 short peptide (black box) at six, seven and eight days after antigen stimulation. Values indicate mean values ± SD of four independent experiments. Statistical significance was determined using Student's *t*-tests (two-tailed): ns, not significant; *P < 0.05. C. Representative experiment dot plots showing the multiparametric flow cytometry analysis of unstimulated PBMCs and PBMCs incubated with 10 μg/ml *Ci*8 short peptide from day six to eight. Numbers inside the graphs indicate the percentage of the $CD39^+CD73^+$ double positive cell population. D. Histograms show the multiparametric flow cytometry analysis of unstimulated PBMCs and PBMCs incubated with 10 μg/ml *Ci*8 short peptide from day six to eight. Numbers inside the graphs indicate the percentage of the $CD39^+CD73^+LAP^+$ cell population and the MFI value of LAP on the $CD39^+CD73^+LAP^+$ cell population. Dot plots and histograms shown are representative of four individuals. E.Histogram showing the percentage of $CD4^+CD25^{high}CD39^+CD73^+LAP^+$ cells in unstimulated PBMCs (white box) and PBMCs incubated with 10 μg/ml *Ci*8 short peptide (black box) from day six to eight. The values represent the mean ± SD of four independent experiments. Statistical significance was determined using Student's *t*-tests (two-tailed): ns, not significant; *P < 0.05.

F. Histogram showing the MFI value of LAP on $CD4^+CD25^{high}CD39^+CD73^+LAP^+$ cells. The values represent the mean ± SD of four independent experiments. Statistical significance of the difference between experimental groups was determined using Student's *t*-test: ns, not significant; *P < 0.05 **P < 0.01.

MFI value increased from day 6 (*P*-value = 0.005) to day 8 (*P*-value = 0.027) (Fig. 6F). All these results indicate that *Ci*8 short is capable of inducing a $CD4^+CD25^{high}$ T cell population characterized by the expression of phenotypic and functional markers, such as CD39/CD73 and LAP.

4. Discussion

Marine invertebrates produce a large number of unique and structurally diversified natural products which represent an important source of molecules exhibiting a wide range of bioactivities such as anticancer, antiviral, antifungal, and antibacterial properties (Otero-González et al., 2010; Cheung et al., 2015; Suarez-Jimenez et al., 2012). An LPS challenge in *Ciona intestinalis* induces a CR-APA event encoding for a *Ci*8 short transcript that is strongly up-regulated in immune cells and present the 3′ UTR mRNA peculiar *cis*-regulatory elements essential for innate immune responses. All this leading to the hypothesis that *Ci*8 short could have immunological activity. In these studies an *in silico* analysis was performed to evaluate whether the peptide can be recognized by the human immune system demonstrating that there are different HLA binding regions that are spread throughout the protein sequence, and, therefore, we chose to first characterize the entire protein regarding its possible role using human PBMCs as an *in vitro* system (Davis, 2008; Germain and Schwartzberg, 2011). Also it would be interesting to define exactly the binding sites for different alleles and study the more specific epitope mapping. Our data highlight that there are putative MHC binding sequences, and this is necessary for recognition by T cells but not sufficient to define and demonstrate immunogenicity of a *Ci*8 short peptide. The selective recognition of any antigenic peptides binding with MCH (pMHC) by specific TCR requires very complex and not fully known mechanisms based on spatio/temporal integration of a panoply of triggers (Valitutti et al., 2010). Since 1995, the kinetic proof-reading model was described, postulating that TCR/pMHC binding triggers a signaling cascade with several sequential intermediates, but this is only true for stronger ligands. The perfect assembly of the whole signaling cascade results in TCR activation and

finally in productive signal transduction (McKeithan, 1995). Despite numerous and intensive investigations, we still do not understand how the TCR transduces signals across the plasma membrane, a process referred to as TCR triggering. Three basic mechanisms have been proposed, involving aggregation, conformational change or segregation of the TCR upon binding to the pMHC ligand (Anton van der Merwe, 2001). The first question is whether the *Ci*8 short peptide can exceed the threshold of TCR activation (Rachmilewitz and Lanzavecchia, 2002). The time frame of T cell activation encompasses molecular events occurring within seconds and sustained for hours or days up to the acquisition of functional properties. In this context, the kinetics of different experiments has been developed. One of the earliest biochemical events detected following TCR engagment is certainly ZAP-70 activation, a critical checkpoint in this complex pathway (Chan et al., 1992; Wang et al., 2010). In the resting state of T-cells, ZAP-70 is distributed throughout the cytoplasm; following T cell engagement, the earliest event is the phosphorylation of the Immunoreceptor Tyrosine-based Activation Motifs (ITAMs) that promote recruitment and subsequent activation of ZAP-70. Recent studies indicate that this event also helps to release ZAP-70 from its autoinhibited conformation (Hermiston et al., 2002; Klammt et al., 2015) and this triggers downstream events of the T cell signaling cascade. We demonstrated a clear increase in the ZAP-70 MFI value on $CD3^+CD4^+$and $CD3^+CD8^+$ T lymphocytes related to PBMCs treated without antigen and a comparison between the percentages of ZAP-70 positive cells indicates that there is an increase in $CD4^+$ lymphocytes. The T cell activation based on pMHC/TCR-specific interaction results in the clonal expansion of T cells. The study of the human TCR repertoire has become an important tool in studies of the immunological mechanisms in various physiological and pathological conditions(Gran et al., 1998), but the analysis of variable gene segment families is very complex (Arden et al., 1995; Davis and Bjorkman, 1988). For example, for the β locus, 65 Vβ segments have been described and functional TCRβV families are grouped into 24 families based on greater than 75% nucleotide sequence identity (Rowen et al., 1996). The most functionally relevant analysis of TCR usage is the detection of the membrane surface proteins by flow cytometry. Our data strongly suggest that there is statistically significant increase in the percentage of $CD4^+$ T lymphocytes bearing TCR Vβ2, TCR Vβ8 and TCR Vβ20 in PBMCs primarily after *Ci*8 short challenge. For $CD8^+$ repertoire, a statistically significant increase is only obtained by the TCR Vβ14 clone. Then, the binding of TCR to specific pMHC triggers distinct signaling events leading to T cell proliferation, cytokine production and immunoregulation (Guy et al., 2013). TCR has a central role in the control of T cell proliferation but the signaling pathway remains obscure and there are many difficulties for fully understanding these complex pathways. We investigated the *Ci*8 molecule capabilities that drive the proliferative response and the results obtained demonstrate that induction of $CD4^+$ T lymphocyte mainly proliferates but interestingly no significant data were obtained for $CD8^+$ and $CD16^+/CD56^+$ cells. The TCR-driven pathways that lead to proliferation appear separable to the secretion of cytokines, another event that by immune cells defines a significant part of their functional activity (Guy et al., 2013). Our hypothesis is that *Ci*8 short cytokines production is mediated by Effector Memory T cells (T_{EM}) (Farber, 2014) that can produce these immediately after antigen challenge with a rapid recall mechanism (Scheffold et al., 2000; Han et al., 2012; Campbell et al., 2010). These events are also mediated by TCR engagement and high ZAP-70 tyrosine kinase expression levels. There are encouraging results on the role of IFN-γ in *Ci*8-mediated mechanisms especially by $CD4^+$ memory T lymphocytes and this in agreement with Farber et al. (Farber, 2014) who demonstrated that the T_{EM} subset has the highest proportion IFN-γ producing cells. Effector T cell stimulation by specific antigens leads to an array of functional events optimally tailored to provide an appropriate immune response that includes T cell selection, proliferation and cytokine production by antigen specific T cells. However, another way to respond to the antigen challenge is to activate peripheral tolerance mechanisms (Li Mo and Rudensky, 2016; Zhu and Shevach, 2014; Vignali et al., 2008). The central role of different Treg subsets (Abbas et al., 2013) in the immune response is unquestioned, but their immunophenotypic characterization, especially in the human system, is very difficult to define. The identification of the IL-2 receptor (CD25) as a marker for natural Treg cells dates to 1995 (Sakaguchi et al., 1995) and the up-regulation of CD25 expression is a signature feature of human Treg. We demonstrate a statistically significant increase in a specific and rare $CD4^+CD25^{high}$ population after antigen stimulation and for these reasons we studied the expression of molecules crucial for the delineation of functionality *in vitro* inducing Treg cells (Abbas et al., 2013). In the human immune system, it is difficult to determine the more specific markers for defining *in vitro* Treg. We also studied LAP (Andersson, 2008; Tran, 2009), CD39 (Borsellino et al., 2007; Dwyer et al., 2010) and CD73 (Deaglio et al., 2007; Schuler et al., 2014) as functional markers especially because they are not expressed constitutively on resting and expanded Treg but are rapidly induced on activated Treg. In this paper, we describe not only an increased number of positive cells that co-express these functional markers but a clear MFI positive shift after antigen challenge, especially for CD73 and LAP expression. The coexistence of two crucial regulatory pathway molecules in $CD4^+CD25^{high}$ *Ci*8 short-induced cells encourages us to implement the studies to answer the questions about mechanisms of actions of this iTreg subset. In conclusion, all the experimental evidence that has accumulated thus far supports the idea that *Ci*8 short is directly involved in many important events of TCR-mediated immunity but the role of *Ci*8 short has begun to be studied but its exact functions in the adaptive immune system requires further exploration. Moreover, we could demonstrate that *Ci*8 short improves $CD4^+$ immune responses allowing us to hypothesize that can be used as a really useful tools to study the immunological conditions in which the $CD4^+$ T lymphocyte responses are reduced.

5. Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.imbio.2017.10.024.

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