Immunobiology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

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*

ARTICLE INFO

Keywords:
Human PBMCs
Adaptive immunity
T cells

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.

http://dx.doi.org/10.1016/j.imbio.2017.10.024
Received 10 May 2017; Accepted 8 October 2017

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