Ann. N.Y. Acad. Sci. ISSN 0077-8923

ANNALS OF THE NEW YORK ACADEMY OF SCIENCES
Issue: *Thymosins in Health and Disease*

Thymosin α1: the regulator of regulators?

Bonifazi Pierluigi,[1] Carmen D'Angelo,[1] Francesca Fallarino,[1] Silvia Moretti,[1] Teresa Zelante,[1] Silvia Bozza,[1] Antonella De Luca,[1] Francesco Bistoni,[1] Enrico Garaci,[2] and Luigina Romani[1]

[1]Department of Experimental Medicine and Biochemical Science, University of Perugia, Perugia, Italy. [2]Department of Experimental Medicine and Biochemical Science, University of Tor Vergata, Rome, Italy

Address for correspondence: Luigina Romani, M.D., Ph.D., Microbiology Section, Department of Experimental Medicine and Biochemical Sciences, University of Perugia, Via del Giochetto, 06122 Perugia, Italy. lromani@unipg.it

The peripheral immune system can promote either immunity or tolerance when presented with new antigens. Current knowledge withholds that populations of suppressor or regulatory T cells (T_{reg} cells) constitute a pivotal mechanism of immunological tolerance. The potential role of malfunctioning T_{reg} cells in chronic inflammatory immune and autoimmune diseases is well-documented. Learning how to successfully manipulate T_{reg} responses could result in more effective vaccines and immunomodulators. We have already shown that Thymosin α1 (Tα1), a naturally occurring thymic peptide first described and characterized by Allan Goldstein in 1972, by modulating signals delivered through innate immune receptors on dendritic cells, affects adaptive immune responses via modulation of Th cell effector and regulatory functions. We will discuss recent molecular mechanisms underlying the ability of Tα1 to activate or inhibit immune responses.

Keywords: thymosin; dendritic cells; IDO; regulatory T cells; infections

Introduction

The peripheral immune system can promote either immunity or tolerance when presented with new antigens. Dendritic cells (DCs) have a crucial role in determining immune outcomes by acquiring antigens, collating environmental cues, and then becoming cells that are either potent stimulators or suppressors of T-cell responses.[1] Current knowledge withholds that populations of suppressor or regulatory T cells (T_{reg} cells) constitute a pivotal mechanism of immunological tolerance.[2] Regulatory T cells are characterized by high-level surface CD25 and intracellular forkhead family transcription factor (FoxP3) expression. Diverse types of T_{reg} cells, with disparate and multiple functions have been discovered. Natural T_{reg} cells are selected by high-avidity interactions in the thymus, whereas inducible, adaptive $Foxp3^+CD4^+$ T_{reg} cells develop outside the thymus during chronic inflammation. Induced T_{reg} cells are essential in mucosal immune tolerance and in the control of severe chronic allergic inflammation, and most likely are one of the main barriers to the eradication of tumors, whereas natural T_{reg} cells are thought to prevent autoimmunity and raise the activation threshold for all immune responses. The potential role of malfunctioning T_{reg} cells in chronic inflammatory immune and auto immune diseases is well-documented.[3]

Given the recognized importance of T_{reg} cells in regulating immune responses, understanding their mechanism of action and interplay with other means of immunological tolerance, and their therapeutic exploitation is one major challenge in immunology. Increased comprehension of how T_{reg} cells exert their function holds the promise for therapeutic intervention by manipulating one of the most sophisticated features of immunity to either boost responses in cancer and microbial diseases or suppress those unwanted in autoimmunity and transplantation. In addition, learning how to successfully manipulate T_{reg} cell responses could result in more effective vaccines and immunomodulators.

Indoleamine 2,3-dioxygenase (IDO) is an enzyme that degrades the essential amino acid tryptophan.[4] The concept that cells expressing IDO can

doi: 10.1111/j.1749-6632.2010.05465.x

suppress T-cell responses and promote tolerance is a relatively new paradigm in immunology. IDO is a haeme-containing enzyme that catabolizes compounds containing indole rings, such as the essential amino acid tryptophan. IDO protein is encoded by a tightly regulated gene that is responsive to inflammatory mediators in a limited range of cell types. Enzymatic activity of IDO correlates with reduced T-cell-mediated responses in several experimental (mouse) systems, including models of autoimmune diseases, cancer, organ and tissue transplant rejection, and pregnancy.[5] Mature DCs that express functional IDO enzyme activity can be potent suppressors of T-cell responses *in vivo* and *in vitro*. The synthetic immunomodulatory reagent cytotoxic T lymphocyte antigen 4 (CTLA4)–immunoglobulin fusion protein is a potent inducer of IDO expression by DCs.[6] Regulatory T cells that express CTLA4 induce IDO-competent DCs to express IDO, indicating that $CTLA4^+$ T_{reg} cells use the IDO mechanism to suppress T-cell responses and promote tolerance. More speculatively, IDO-expressing DCs might promote the development of T_{reg} cells; if so, T_{reg} cells and IDO-competent DCs might cooperate to form a self-amplifying immunoregulatory network.[7]

Thymosin α1 (Tα1) is a naturally occurring thymic peptide first described and characterized by Goldstein *et al.*[8] Although the peptide is produced in small amounts in several peripheral lymphoid and nonlymphoid tissues, the highest concentrations of Tα1 are found in the thymus. Prepared as a 28mer synthetic amino-terminal acylated peptide, Tα1 is in clinical trials worldwide for the treatment of some viral infections, either as a monotherapy or in combination with IFN-α. Additional indications are some immunodeficiencies, malignancies, and HIV/AIDS.[9] The mechanism of action of the synthetic polypeptide is thought to be related to its immunomodulating activities, centered primarily on the augmentation of T-cell function. However, mechanistically, Tα1 has shown an action beyond its effect on T lymphocytes to include an ability to act as an endogenous regulator of both the innate and adaptive immune systems.

In this review, we discuss current knowledge of molecula mechanisms by which Tα1 may affect immunity and tolerance in various experimental settings.

Thymosin α1 activates dendritic cells for antimicrobial immunity

Tα1 strongly upregulated the expression of Toll-like receptors (*TLR2*) and *TLR9* by murine DCs and activated NF-κB and JNK/p38/AP-1 pathways.[10,11] Studies with human DC confirmed this finding and further showed that Tα1 upregulated the expression of *TLR2* by myeloid or conventional DC (cDC) and of *TLR9* by plasmacytoid dendritic cells (pDC). Induction of *TLR2* or *TLR9* expression by DC by Tα1 was associated with a distinct activation program in both murine and human DC subsets. Tα1 induced the production of IL-12p70 in cDC and IL-10 in pDC. Production of IL-12p70 was reduced in $TLR2^{-/-}$ mice, whereas IL-10 was reduced in $TLR9^{-/-}$ mice. The production of both cytokines was severely reduced in DC lacking the myeloid differentiation factor 88 (MyD88), an adaptor protein essential for the production of cytokines upon signaling by all the TLR family members. Therefore, consistent with the finding showing that Tα1 directly signaled through TLR9 and potentiated ligand-induced TLR2 signaling by TLR-transfected HEK293 cells,[10] these data indicate that Tα1 activates DC subsets through distinct TLR and involves MyD88.

The immunomodulatory effects of Tα1 on DCs correlated with a therapeutic effect of the peptide in experimental fungal or viral infections.[12] Administration of Tα1 to mice with *Aspergillus fumigatus* resulted in a state of full protection to the fungus as revealed by the increased survival after the infection that paralleled the reduced fungal growth, the promotion of IFN-γ-producing Th1 cells, and the inhibition of IL-4-producing Th2 cells. Despite the fact that Tα1 increased the recovery and functional activity of effector macrophages and neutrophils, the myeloid functional recovery could not *per se* account for the efficacy of Tα1. In fact, recovery from neutropenia alone, as by treatment with granulocyte colony stimulating factor, was not sufficient to mediate a degree of antifungal resistance comparable to that obtained with Tα1.[10] Therefore, the achievement of a state of full protection to the fungus required the coordinated action of innate effector cells and protective Th1 responses, an activity successfully promoted by Tα1.[10,12] Consistent with the *in vitro* data on TLR expression, the therapeutic efficacy of Tα1 *in vivo* was dependent on MyD88

signaling activated by TLR2 and partially by TLR9. Therefore, despite a degree of redundancy in the TLR usage, the MyD88-dependent signaling pathway is essential in the antifungal activity of Tα1 *in vitro* and *in vivo*.

The ability of Tα1 to modulate DC functioning through TLR9 correlated with an effect on Cytomegalovirus sensing by DCs *in vitro* and *in vivo*.[13] The antiviral effect of Tα1 led to the activation of IRF7 and the promotion of the IFN-α/IFN-γ-dependent effector pathway in pDC via the TLR9-dependent viral recognition sensing. In infection, Tα1 decreased the viral load in both susceptible and resistant mice with primary MCMV infection and induced the expansion of cytolytic $NK1.1^+$ cells, IFN-γ-producing $CD8^+$ or $CD4^+$ T cells, and the production of IL-12p70, IFN-α, IFN-γ, and IL-10.[13] Together, these data indicate that Tα1 affects both the innate and adaptive antimicrobial immune responses *in vivo*.

Thymosin α1 activates dendritic cells for IDO-mediated immune tolerance

The induction of tolerance is critical for the maintenance of immune homeostasis. DCs not only play a key role in the induction of immune responses but also in the induction and maintenance of immune tolerance.[1] Tryptophan catabolism and kynurenines play a crucial role in the induction of peripheral tolerance.[4,5] IDO is widely expressed in a variety of human tissues as well as in macrophages and in DC, and is induced in inflammatory states by IFN-γ and other proinflammatory cytokines. TLR9 stimulation may lead to the activation of the tryptophan catabolism pathway in DC,[14] and IDO is a molecular signature of tolerogenic pDC.[15] Tα1 was found to induce IDO activity in DCs through TLR9 and type I Interferon receptor signaling.[16] IDO blockade prevented the activation of IL-10–producing $CD4^+$ T cells, while sparing that of IFN-γ-producing cells, a finding confirming the causal link of IDO with priming for IL-10–producing T cells. Consistent with the finding that TLR9 stimulation can promote pDC-mediated generation of $CD4^+CD25^+$ T_{reg} cells,[17] $CD4^+CD25^+$ T cells induced by Tα1-treated DC expressed FoxP3. Therefore, Tα1 induced $CD4^+CD25^+T_{reg}$ cells through the activation of a TLR9-dependent immunosuppressive pathway of tryptophan catabolism. Studies on experimental hematopoietic stem cell transplantation (HSCT) and respiratory allergy established a proof-of-concept principle that the induction of immune tolerance via IDO-induced T_{reg} cells could be exploited for immunoregulatory therapy with Tα1 or Tα1-conditioned DCs. Treatment with Tα1 or the infusion of Tα1-treated DC resulted in pathogen clearance, prompt resolution of inflammatory pathology, and prevention of graft-versus host disease in HSCT.[16] Similarly, Tα1 given either prophylactically, during the sensitization phase or therapeutically, at the elicitation phase, attenuated signs of inflammatory allergy in a murine model of fungal allergy by promoting the tolerogenic program of lung DC and priming for $CD25^+$ T_{reg} cells inhibiting allergic Th2 responses.[12]

Balancing immunity and tolerance by Tα1: the regulator of regulators

Learning how to successfully manipulate T_{reg} cell responses could result in more effective vaccines by boosting responses in cancer and microbial diseases and immunomodulatory strategies to suppress unwanted responses in autoimmunity and transplantation.[2,3] By affecting the balance between immunogenic and tolerogenic DCs through TLR exploitation, Tα1 fulfills the requirement of a promising adjuvant candidate for strategies aimed at the control of inflammation, immunity, and tolerance in a variety of clinical settings (Fig. 1). The real challenge will be to discover molecular pathways underlying these apparently opposite activities of Tα1 on the immune system. Our own unpublished studies would suggest an ability of Tα1 to affect the balance between inflammatory/antiinflammatory NF-κB pathways in DCs. NF-κB is a family of seven structurally related transcription factors that play a central role in the stress response and inflammation by controlling gene network expression.[18,19] Although the NF-κB subunits are ubiquitously expressed, their actions are regulated in a cell type- and stimulus-specific manner, allowing for a diverse spectrum of effects. Recent molecular dissection of its mechanisms for activation has shown that NF-κB can be induced by the so-called "canonical" and "noncanonical" pathways, leading to distinct patterns in the individual subunits activated and downstream genetic responses produced. Although much

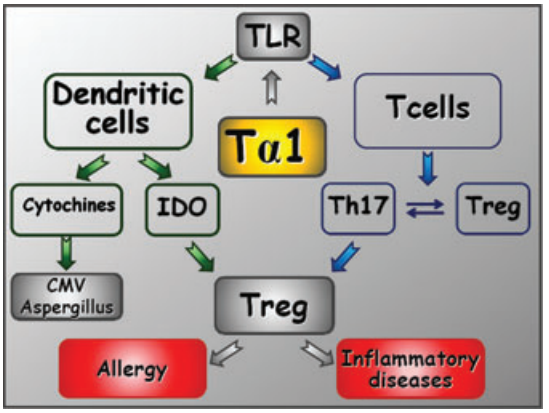

Figure 1. Balancing immunity and tolerance by Tα1. A view of the possible actions of Tα1 on dendritic cells through TLR exploitation eventually leading to the induction of antimicrobial Th priming and tolerance via the IDO/T_{reg} axis. The ability of Tα1 to affect the skewing of Th1/Th17 cell activation through a direct action on polyclonal-stimulated T cells *in vitro* (unpublished observations) is also shown. IDO, indoleamine 2,3-dioxygenase; T_{reg}, regulatory T cells, TLR, Toll-like receptors.

attention has been focused on the pro-inflammatory signaling of canonical NF-κB, recent data indicate that noncanonical NF-κB could have opposing roles, limiting canonical NF-κB activity, inducing IDO, and controlling inflammation.[7] Thus, the intersection between canonical and noncanonical signaling pathways may be crucial in promoting an optimally protective response balanced between inflammation and tolerance by Tα1. This unanticipated finding could have significant implications in immune regulation by Tα1, including the development of T_{reg} cell responses.

Acknowledgments

We thank Dr. Cristina Massi Benedetti for digital art and editing. The original studies conducted in the authors' laboratory were supported by the Italian Projects PRIN 2007KLCKP8˙004 (to LR), 2007XYB9T9˙001 (to SB), and 2007KLCKP8˙005 (to FB), and by the Project 2008.021.338 from Fondazione Cassa di Risparmio di Perugia.

Conflicts of interest

The authors declare no conflicts of interest.

References

1. Steinman, R.M., D. Hawiger & M.C. Nussenzweig. 2003. Tolerogenic dendritic cells. *Annu. Rev. Immunol.* **21:** 685–711.
2. Mills, K.H. 2004. Regulatory T cells: friend or foe in immunity to infection? *Nat. Rev. Immunol.* **4:** 841–855.
3. Bour-Jordan, H. & J.A. Bluestone. 2009. Regulating the regulators: costimulatory signals control the homeostasis and function of regulatory T cells. *Immunol. Rev.* **229:** 41–66.
4. Grohmann, U., F. Fallarino & P. Puccetti. 2003. Tolerance, DCs and tryptophan: much ado about IDO. *Trends Immunol.* **24:** 242–248.
5. Mellor, A.L. & D.H. Munn. 2004. IDO expression by dendritic cells: tolerance and tryptophan catabolism. *Nat. Rev. Immunol.* **4:** 762–774.
6. Fallarino, F., R. Bianchi, C. Orabona, *et al.* 2004. CTLA-4-Ig activates forkhead transcription factors and protects dendritic cells from oxidative stress in nonobese diabetic mice. *J. Exp. Med.* **200:** 1051–1062.
7. Grohmann, U., C. Volpi, F. Fallarino, *et al.* 2007. Reverse signaling through GITR ligand enables dexamethasone to activate IDO in allergy. *Nat. Med.* **13:** 579–586.
8. Goldstein, A.L., A. Guha, M.M. Zatz, *et al.* 1972 Purification and biological activity of thymosin, a hormone of the thymus gland. *Proc. Natl. Acad. Sci. USA* **69:** 1800–1803.
9. Goldstein, A.L. & M. Badamchian. 2004. Thymosins: chemistry and biological properties in health and disease. *Expert Opin. Biol. Ther.* **4:** 559–573.
10. Romani, L., F. Bistoni, R. Gaziano, *et al.* 2004. Thymosin alpha 1 activates dendritic cells for antifungal Th1 resistance through toll-like receptor signaling. *Blood* **103:** 4232–4239.
11. Zhang, P., J. Chan, A.M. Dragoi, *et al.* 2005. Activation of IKK by thymosin alpha1 requires the TRAF6 signalling pathway. *EMBO Rep.* **6:** 531–537.
12. Romani, L., F. Bistoni, C. Montagnoli, *et al.* 2007. Thymosin alpha1: an endogenous regulator of inflammation, immunity, and tolerance. *Ann. N.Y. Acad. Sci.* **1112:** 326–338.
13. Bozza, S., R. Gaziano, P. Bonifazi, *et al.* 2007. Thymosin alpha1 activates the TLR9/MyD88/IRF7-dependent murine cytomegalovirus sensing for induction of anti-viral responses *in vivo*. *Int. Immunol.* **19:** 1261–1270.
14. Fallarino, F. & P. Puccetti. 2006. Toll-like receptor 9-mediated induction of the immunosuppressive pathway of tryptophan catabolism. *Eur. J. Immunol.* **36:** 8–11.

15. Orabona, C., P. Puccetti, C. Vacca, *et al*. 2006. Toward the identification of a tolerogenic signature in IDO-competent dendritic cells. *Blood* **107:** 2846–2854.
16. Romani, L., F. Bistoni, K. Perruccio, *et al*. 2006. Thymosin alpha1 activates dendritic cell tryptophan catabolism and establishes a regulatory environment for balance of inflammation and tolerance. *Blood* **108:** 2265–2274.
17. Moseman, E.A., X. Liang, A.J. Dawson, *et al*. 2004. Human plasmacytoid dendritic cells activated by CpG oligodeoxynucleotides induce the generation of CD4+CD25+ regulatory T cells. *J. Immunol.* **173:** 4433–4442.
18. Hayden, M.S. & S. Ghosh. 2004. Signaling to NF-kappaB. *Genes Dev*. **18:** 2195–2224.
19. Bonizzi, G. & M. Karin. 2004. The two NF-kappaB activation pathways and their role in innate and adaptive immunity. *Trends Immunol*. **25:** 280–288.