1 | INTRODUCTION

The control of immune response is operated by specialized cells, solvable molecules and membrane-bound signals, which modulate the intensity of immune reactivity and preside over the maintenance of homeostasis. An imbalance between immunity and tolerance mechanisms can lead to pathological conditions, such as autoimmune diseases or neoplasia, characterized by excessive or deficient control of immune reactivity respectively.

Dendritic cells (DCs) are professional antigen-presenting cells with a key role in determining the outcome of the immune response, forcing naïve T cells into either activation or differentiation into regulatory T cells (Tregs). The components of the local microenvironment critically take advantage of the plasticity of DCs, resulting in phenotype changes. The tolerogenic molecules CTLA-4, TGF-β and interleukin 35 (IL-35) are particularly effective in turning otherwise immunogenic CD8α-DCs into tolerogenic cells. Reprogramming of a cell's phenotype involves an interplay between metabolic and immunological events known as cellular immunometabolism. In CD8α-DCs, the increased metabolism of specific amino acids and the subsequent production of regulatory metabolites critically contribute to the acquisition of a newly expressed suppressive phenotype. The amino acid degrading enzymes indoleamine 2,3-dioxygenase 1 (IDO1)
and arginase 1 (Arg1) are major components of immunometabolic pathways in DCs.\(^5\)

Interleukin-35 is a heterodimeric cytokine belonging in the IL-12 family. It powerfully dampens immune responses by suppressing T-cell proliferation and inducing the expansion of specific subsets of Tregs and regulatory B cells. Although elevated tissue and plasma levels of IL-35 are associated with a poor prognosis in many malignant tumours,\(^6\) the cytokine has a protective role in the prevention of autoreactivity in several experimental autoimmune models and in human autoimmunity as well.\(^7\)

IDO1 and Arg1 control tryptophan and arginine metabolism, respectively. IDO1 degrades the essential amino acid l-tryptophan to l-kynurenine. In T lymphocytes, l-tryptophan depletion activates an integrated stress response triggered by GCN2, inhibiting cell proliferation and inducing anergy by down-regulating TCR's \(\zeta\) chain.\(^8,9\) Moreover, l-kynurenine is an endogenous agonist of the Aryl Hydrocarbon Receptor, thus promoting the expansion of Treg cells and acting to up-regulate \(\text{Ido1}\) expression in a feedforward loop in DCs.\(^10\) Arg1 hydrolyses l-arginine into urea and l-ornithine, which is a substrate for ornithine decarboxylase (ODC), to produce polyamine pathway catabolites. Although l-arginine consumption by Arg1 is a well-known immunoregulatory mechanism at work in M2 macrophages and in myeloid-derived suppressor cells in many tumour settings,\(^11\) only recently has the immunosuppressive function of polypeptides been unveiled in DCs.\(^12\)

In the current study, we investigated the possible role of \(\text{Ido1}\) and Arg1 enzymes as potential immunometabolic effectors downstream of the tolerogenic action of IL-35Ig in splenic CD8\(^{α−}\) DCs.

## Materials and Methods

### 2.1 Mice

Eight- to ten-week-old female C57BL/6 mice were purchased from Charles River Breeding Laboratories and \(\text{Ido1}^{−/−}\) mice from the Jackson Laboratory. All in vivo studies were in compliance with National and Perugia University Animal Care and Use Committee guidelines.

### 2.2 Dendritic cell purification, transfection and treatment

Splenic DCs were fractionated using positive selection columns combined with CD11c and CD8 MicroBeads (Miltenyi Biotec, Germany).\(^13\) Purified CD8\(^{α−}\) DCs were transfected by DOTAP (Roche, USA) with IL-35Ig or control Ig gene constructs\(^3\) and incubated overnight before in vitro analysis or in vivo administration. Nω-hydroxy-nor-Arg (nor-NOHA; Bachem, Switzerland) 150 \(\mu\)mol/L was added 1.5 hours before transfection.

### 2.3 Real-time PCR and cytokine measurement

Real-time PCR analyses for mouse \(\text{Ido1}\), Arg1 and \(\text{Gapdh}\) were carried out using previously reported specific primers.\(^12\) Values were calculated as the ratio of the specific gene to \(\text{Gapdh}\) expression, as determined by the relative quantification method (\(Δ\Delta\text{CT}\); means ± SD of triplicate determination).\(^12\) Mouse TGF-\(\beta\) (Affymetrix, Santa Clara, USA), IFN-γ and IL-4 (Thermo Fisher Scientific, USA) ELISA kits were used to measure cytokines concentrations in culture supernatants.

## Results

### 3.1 Ectopic IL-35Ig induces in vitro Arg1, but not \(\text{Ido1}\), in DC\(_{35}\)

The ectopic expression of IL-35Ig, after transfection of the gene construct into murine splenic CD11c\(^{+}\)CD8\(^{α−}\) DCs, was previously demonstrated to confer powerful immunosuppressive properties on those cells. The presentation of diabetogenic autoantigen IGRP by DC\(_{35}\) in prediabetic NOD mice protected animals from the occurrence of overt diabetes by a long-lasting antigen-specific tolerance.\(^3\)

To interrogate the effector mechanisms underlying the immunosuppressive outcome of IL-35Ig transfection in DCs responsible for the long-term tolerance observed in vivo,\(^3\) we first analysed the immunometabolic programme acquired by DC\(_{35}\) in vitro after IL-35Ig transfection. As the increased expression of the amino acid degrading enzymes \(\text{Ido1}\) and/or Arg1 is a critical condition for the acquisition of suppressive functions by DCs, we investigated the possible induction of the two enzymes as a consequence of IL-35Ig ectopic...
expression. In a time course experiment, DC35 and control DC16g (i.e., transfected with Ig tag) were incubated for 6, 24 or 30 hours after transfection. Although Ido1 expression was similar in DC35 and DC16g over time, Arg1 was significantly increased in DC35 relative to DC16g at 24 hours (3.9-fold) and at 30 hours (2.2-fold) (Figure 1A). IFN-γ, Ido1, IL-4 and TGF-β, the most potent inducers of Ido1, Arg1 or both, respectively,12 were not differentially secreted by DC35 and DC16g in culture supernatants at 24 hours post transfection (Figure 1B). Therefore, besides the mere production of a tolerogenic cytokine, DC35 seems to be endowed with an additional suppressive immunometabolic effector mechanism, namely, the expression of Arg1 induced by ectopic IL-35Ig.

**FIGURE 1** Arg1 but not Ido1 transcript is induced in vitro in DCs expressing ectopic IL-35Ig. A, Real-time PCR analysis of Ido1 and Arg1 transcripts in splenic DCs transfected with the IL-35Ig single chain gene construct (DC35) or Ig tail control (DC16g). Data (means of three experiments using triplicate samples) represent the fold change expression of Ido1 and Arg1 transcripts in DC35 normalized to the expression of Gapdh and reported as relative to results in DC16g for each time-points. Dotted line denotes a fold change = 1. *P < 0.05, **P < 0.01 (Student’s t test). B, Secretion of IFN-γ, IL-4 and TGF-β in supernatants of DC35 or DC16g 24 h after transfection. n.d. = not detectable. Results are the mean ± SD from three different experiments (Student’s t test).

3.2 | Arg1 is required for the tolerogenic effect of DC35 in vivo

To confirm the selective involvement of Arg1 (Figure 1A) in the suppressive mechanisms activated by IL-35Ig in DC35 and to further verify if either of the two enzymes might act as tolerogenic effector of the cytokine, DC35 lacking either Ido1 (Ido1−/− DC35) or Arg1 (nor-NOHA-treated DC35) was assayed in vivo for their ability to inhibit antigen-specific immune response. In DTH experiments, 2 weeks after mice sensitization with the HY-peptide–loaded DCs, the induction of immune reactivity vs tolerance was evaluated through an intrafootpad challenge of the HY antigen, according to an established protocol3,14 (Figure 2A). Wild-type DC35 (wt DC35) were able to prevent the immunogenic DTH response, otherwise observed in the DC16g control group (Figure 2B). Likewise, the loss of Ido1 function in DC35 (Ido1−/− DC35) did not modify the unresponsiveness to skin test following wt DC35 administration. On the contrary, Arg1 inhibition in DC35 by the specific catalytic inhibitor nor-NOHA reverted the immunosuppressive effect on untreated DC35 and resulted in a significant footpad weight increase upon skin test challenge, similar to the nor-NOHA-treated DC16g control group (Figure 2C). Therefore, skin test experiments excluded the involvement of Ido1 and rather depicted Arg1 enzyme as a relevant DC35 effector triggering tolerogenic mechanism in vivo.

Moreover, regulatory T-cell populations induced in vivo by sensitization with HY-pulsed DC35 and locally recalled by i.p. boost on day +14 with the same peptide were investigated by flow cytometry in MLN (Figure 2A). Interestingly, in accordance with a previous study on the protective effect of DC35 in autoimmune diabetes,3 an increased percentage (8.6%) of CD25+CD39+ T cells, rather than Foxp3+ T cells, was observed in DC35-sensitized group relative to the nor-NOHA-treated DC16g control group (Figure 2D and E). These data confirm that DC35 presented HY peptide in a tolerogenic manner and triggered a suppressive response mediated by Arg1 activation and involving CD25+CD39+, rather than Foxp3+, regulatory T cells.

4 | DISCUSSION

The immunosuppressive role of IL-35 has been observed and confirmed in many different studies,6 so that this member of the IL-12 family belongs in the small group of cytokines capable of suppressing the immune response. A new aspect of IL-35 contribution to immune regulation is the possible effect of this cytokine on the expression of amino acid degrading enzymes, and therefore on their immunosuppressive function. The Arg1 induction we found in DC35 appears to be an event related to the autocrine/paracrine action of ectopic IL-35Ig and independent from the production in culture supernatant of either IL-4 or TGF-β, two main inducers of Arg1 in DCs.12 A potential mechanism (still to be explored) underlying the increased expression of Arg1 in DC35 could be the activation of the STAT3 transcription factor, already known to be phosphorylated along the IL-35 signalling pathway in both T and B cells15 and to directly bind multiple sites of the Arg1 promoter in
myeloid-derived suppressor cells. On the contrary, Ido1 expression resulted unaffected by IL-35Ig in DC35, similar to the results in a study on monocyte-derived DCs treated with recombinant IL-35. However, the early induction of Arg1 in vitro by IL-35Ig in DC35 may not exclude the late involvement of IDO1 in vivo, according to the documented relay pathway between the two enzymes.

The finding that Arg1 is a downstream effector of IL-35 has immunological relevance for several aspects. In IL-35-producing DCs (i.e., DC35, and most likely IL-35+ DCs, as well) Arg1 induction might
represent a local amplification loop of tolerance, targeting more precisely those T cells that interact with such suppressive DCs in the immunological synapsis. Moreover, the translational potential of a cell therapy with DC35 loaded with a specific autoimmune peptide is confirmed and reinforced by the new data of Arg1 involvement in IL-35 tolerogenic effect. Finally, IL-35 is emerging as an important target in tumour immunotherapy because of its inactivation could lead to the inhibition of Arg1, one of the most important immune checkpoints allowing tumour immune escape.

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**CONFLICTS OF INTEREST**

The authors confirm that there are no conflicts of interest.

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