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T cell Regulatory Plasmacytoid Dendritic Cells Expressing Indoleamine 2,3 Dioxygenase

David J. Kahler and Andrew L. Mellor

Immunotherapy & Cancer Centers, and Department of Medicine, Medical College of Georgia, 1120 15th St, Augusta GA

Abstract

Mature dendritic cells (DCs) are potent stimulators of T cells that recognize antigens presented by the DCs. In this chapter we describe mature DCs that suppress T cell responses to antigens they present due to expression of the intracellular enzyme indoleamine 2,3 dioxygenase (IDO). IDO-competent DCs are a subset of plasmacytoid DCs that can be induced to express IDO under certain inflammatory conditions in humans and mice. Though rare, IDO-expressing DCs acquire potent T cell suppressor activity that may predominate over the T cell stimulatory functions of all other antigen-presenting cells in physiologic environments due in part, to cooperation with regulatory T cells. Thus, IDO-expressing DCs are critical regulators of adaptive immunity that contribute to a wide range of inflammatory disease processes. As such, manipulating IDO expression in DCs using IDO inhibitors or IDO inducers offers considerable opportunities to improve immunotherapies in a range of clinically-significant disease syndromes.

1 Introduction

The notion that mature dendritic cells (DCs) induce clonal expansion and differentiation of T cells with effector functions is well established (Reis e Sousa 2006), as is the complementary notion that immature (or ‘alternately activated’) DCs induce weak or abortive T cell responses (Steinman et al. 2003; Morelli and Thomson 2007). In this chapter, we describe an under-appreciated aspect of DC immunobiology, the ability of some specialized DCs to promote active T cell suppression when fully mature. The mechanisms underlying active suppression by DCs are not well defined and are still under investigation, largely for technical reasons since assays to detect suppression mediated by DCs require careful optimization. Moreover, DCs specialized to mediate active T cell suppression represent a tiny minority of DCs that must be induced to acquire suppressive functions. Hence, these DCs are easy to overlook, unless cells from physiologic sources are treated and fractionated appropriately.

Despite their rarity, suppressive DCs may exert disproportionately potent effects in local tissue microenvironments by amplifying other regulatory mechanisms such as CD4⁺CD25⁺ regulatory T cells (Tregs) leading to dominant suppression that negates the T cell stimulatory functions of all other DCs and other antigen presenting cells (APCs) in the same local tissue milieu. One mechanism that has attracted considerable attention with regard to suppressive DCs involves expression of the intracellular enzyme indoleamine 2,3 dioxygenase (IDO) in specialized DCs, which acquire potent T cell suppressive functions as

a consequence. For the purposes of this review, we focus exclusively on the IDO mechanism due to recent progress in understanding how IDO activity in specialized DCs promotes active T cell suppression. Though other mechanisms may also cause DCs to acquire suppressive functions we focus on the IDO mechanism to illustrate some key points about DCs with regulatory functions that may be applicable to other mechanisms. Recent reviews summarize the extensive experimental evidence showing that IDO plays an important role in suppressing T cell immunity in murine models of infectious, autoimmune and allergic diseases, tumor growth, and survival of transplanted tissues and developing fetal expressing alloantigens (Mellor and Munn 2004; Fallarino et al. 2007). Indeed, cells expressing IDO, including but not limited to DCs, may create and maintain immune privilege in selected tissues under homeostatic or inflammatory conditions (Jaspersion et al. 2007; Mellor and Munn 2008). While the mechanistic basis of IDO-mediated effects on disease etiology is not fully understood, it is likely that IDO⁺ DCs make critical contributions to a range of chronic inflammatory syndromes, especially those in which T cells contribute to disease etiology. IDO-mediated T cell suppression by DCs may be clinically beneficial or detrimental according to which chronic inflammatory disease is under consideration. In cancer and chronic infectious diseases, lack of effective T cell immunity contributes to disease progression and persistence in otherwise immunocompetent individuals, suggesting that IDO⁺ DCs may contribute to disease etiology. In contrast, aberrant T cell regulation allows excessive responses to healthy tissues and innocuous stimuli (such as commensal microorganisms or allergens) leading to autoimmune and allergic diseases, and host responses to donor alloantigens leads to rejection of healthy transplants, unless globally immunosuppressive drugs are used to prevent rejection. In these syndromes, IDO activity in DCs may be beneficial by slowing disease progression or transplant rejection, and artificially enhancing IDO expression (for example in DCs) may help shift the balance towards effective T cell suppression and long term tolerance. After describing IDO biochemistry and molecular genetics, we summarize current knowledge about the identity of DCs competent to express IDO, and the signaling mechanisms that induce these specialized DCs to express IDO. Finally, we discuss the immunological significance of IDO-mediated T cell suppression by DCs, and how this knowledge might be exploited to improve immunotherapies to treat a range of chronic inflammatory diseases of clinical importance.

2 IDO Biochemistry

In this section we briefly describe the biochemistry of IDO including its substrates, downstream metabolites, and the cellular responses that occur following its activation. We identify compounds currently under investigation that reduce or inhibit IDO activity and end the section with a discussion of how an intracellular enzyme such as IDO might gain access to extracellular substrates and how decreased substrate is detected.

2.1 IDO Enzymology

Indoleamine 2,3-dioxygenase (IDO, EC1.13.11.17) is one of two conserved heme-containing enzymes in mammals that catalyze the initial and rate-limiting step in oxidative degradation of the essential amino acid tryptophan along the kynurenine pathway (Taylor and Feng 1991; Mellor and Munn 1999). IDO degrades substrates containing indole rings

such as (but not limited to) tryptophan (Fig. 1) and the neurotransmitter serotonin (Myint and Kim 2003). IDO gene expression is tightly regulated and is responsive to inflammatory signals such as interferons (IFNs). Hence, Toll-like receptor (TLR) ligands (such as LPS and CpGs) and other reagents that induce inflammation and IFN production also induce IDO expression.

Unlike IDO, tryptophan 2,3-dioxygenase (TDO, EC1.13.11.11) has substrate specificity for L-tryptophan only, is expressed exclusively in liver in response to glucocorticoids, and is thought to regulate dietary tryptophan intake to moderate tryptophan levels in serum (Salter et al. 1995; Liao et al. 2007). As no role for TDO in DCs has been described to date, we will not consider this enzyme further. As depicted in Fig. 1, in addition to catabolizing tryptophan cells expressing IDO consume reactive oxygen species (ROS) (Thomas and Stocker 1999), and generate downstream metabolites known collectively as kynurenines (Taylor and Feng 1991). Kynurenine itself is a stable metabolite produced by many IDO⁺ cells, and the presence of kynurenine in serum or tissue culture supernatants is commonly used to evaluate IDO activity in cells, though some cells possess enzymes that catalyze further degradation and may not release kynurenine. Thus, biochemical changes brought about by IDO activity include tryptophan withdrawal (depletion), reduced oxidative stress and production of downstream tryptophan metabolites (kynurenines). These changes may influence cellular functions in various ways to bring about some well-documented effects of IDO activity such as inhibiting the spread of infectious microorganisms in macrophages, and activating counter-inflammatory mechanisms that ameliorate pathology, as well as suppressing T cell responses and stimulating Treg suppressor functions that have been described more recently (Fig. 1). In addition to affecting other cells in the same microenvironment, the biochemical changes brought about by IDO activity in DCs may also modify the phenotype, functions and differentiation/maturation status of DCs themselves via cell autonomous mechanisms.

2.2 IDO Inhibitors

Table 1 lists selected reagents that inhibit IDO enzyme activity directly, or have indirect inhibitory effects on IDO expression or enzyme activity by cells. Several synthetic tryptophan analogues inhibit IDO enzyme activity (Peterson 1994), presumably by binding to the enzyme active site, which accepts the indole ring of substrates and substrate analogues (Sugimoto et al. 2006). For example, 1-methyl-tryptophan (1MT) is a competitive reversible IDO inhibitor commonly used in standard (cell-free) enzyme activity assays (Cady and Sono 1991; Sakurai et al. 2002; Travers et al. 2004; Ou et al. 2007). Surprisingly, the D-isomer of 1MT (D-1MT) was a less toxic, and more effective IDO inhibitor than the L-isomer of 1MT when tested in pre-clinical studies in mice as a method to block IDO activity in bioassays with indirect (but clinically relevant) readouts such as enhanced T cell stimulatory functions of DCs measured *ex vivo* and reduced tumor growth *in vivo*. D-1MT was also an effective IDO inhibitor in studies with human macrophages and DCs (Duluc et al. 2007; Hill et al. 2007b), though another report described results that were not consistent with this conclusion (Lob et al. 2007). Consequently, D-1MT is currently being evaluated as a potential tumor vaccine adjuvant treatment in cancer patients. It remains to be seen if the findings from pre-clinical studies are predictive of outcomes in human patients. This point notwithstanding, the

novel paradigm that IDO activity may facilitate resistance of tumors and some chronic infections to T cell immunity in humans is driving interest in developing novel and more effective IDO inhibitors for clinical applications.

Brassinin (3-(S-methyldithiocarbamoyl)aminomethyl indole), a natural plant product, and its synthetic derivative, 5-bromobrassinin (5-Br-brassinin) are novel compounds that also inhibit IDO activity directly (Banerjee et al. 2007). An indole ring was not necessary for effective IDO inhibition, and substitution of the S-methyl group with large aromatic groups yielded reagents with more potent inhibitory effects than 1MT, at least *in vitro* (Gaspari et al. 2006). A non-indolic competitive IDO inhibitor, NSC 401366 (imidodicarbonimidic diamide N-methyl-N'-9-phenanthrenyl-monohydrochloride) was identified using a novel yeast based assay in which human IDO was expressed in a *Saccharomyces cerevisiae* tryptophan auxotroph. In this assay, yeast growth was restricted by limiting access to free tryptophan and inhibition of IDO by reagents under investigation restored yeast growth (Vottero et al. 2006). Although little is known about the biological function of NSC 401366, the assay itself may prove useful for screening and identifying new compounds with IDO inhibitory properties.

Another group of compounds exhibiting antioxidant properties inhibit IDO expression indirectly by interfering with cofactors required for enzymatic activity or signaling molecules upstream of IDO expression. Epigallocatechingallate, rosmarinic acid (a-ocaffeoyl-3,4-dihydroxyphenyl-lactic acid), and p-coumaric acid displayed IDO inhibitory properties *in vitro* using murine bone marrow-derived dendritic cells (BMDCs) or CD11c⁺CD8α⁺ DCs harvested from tumor draining lymph nodes of mice injected with B16 melanoma cells (Youn et al. 2003; Jeong et al. 2007; Kim et al. 2007; Lee et al. 2007). These compounds inhibited IDO expression and function by blocking Cyclooxygenase 2 (COX2) or Prostaglandin E2 (PGE2) expression, reducing Signal Transducer and Activator of Transcription (STAT1) phosphorylation, and inhibiting the binding of activated STAT1 to the Interferon Regulatory Factor 1 (IRF1) promoter. Other antioxidants such as pyrrolidine dithiocarbamate, 2-mercaptoethanol, ebselen, and t-butyl hydroquinone have been reported to display IDO inhibitory properties (Thomas et al. 2001).

Some natural metabolites can also inhibit IDO activity. Nitric oxide (NO) inhibits IDO activity by binding irreversibly to the catalytic heme group (Thomas et al. 1994; Samelson-Jones and Yeh 2006). Exposure of recombinant human IDO to H₂O₂ *in vitro* caused oxidation of cysteine residues to sulfinic and sulfonic acids resulting in loss of enzymic activity but not tryptophan binding ability (Poljak et al. 2006). The peroxynitrite generator, 3-(4-morpholinyl) sydnonimine caused nitration of several tyrosine residues in recombinant human IDO leading to inactivation of IDO (Fujigaki et al. 2006). N-Chlorotaurine, the oxidation product of HOCL and intercellular taurine inhibits IDO in several murine models (Wirleitner et al. 2004).

Off target effects of two pharmacologic agents also inhibit IDO. The specific COX2 inhibitor Celecoxib reduced tumor associated IDO expression in a murine breast cancer model (Basu et al. 2006). Zebularine, a DNA methyl transferase inhibitor, was shown to induce IDO at high doses and inhibit IDO at low doses (Liu et al. 2007). Finally, some

compounds isolated from marine organisms have IDO inhibitory activity. Exiguamine A isolated from the marine sponge *Neopetrosia exigua* (Brastianos et al. 2006), and fractions from crude extracts of the marine hydroid *Garveia annulata* were potent inhibitors of IDO (Pereira et al. 2006).

2.3 Tryptophan Transport and Tryptophanyl tRNA Synthetase

Because IDO is an intracellular enzyme, tryptophan must be present inside cells, or be transported into IDO⁺ cells, to be degraded. This is an important consideration because T cells do not express IDO under physiologic conditions, and the ability of IDO⁺ DCs to suppress T cell responses by removing tryptophan from the cytoplasm of T cells therefore requires explanation. Human monocyte-derived macrophages with IDO-mediated T cell suppressive functions express sodium-independent, tryptophan-specific transporters that may be critical for their T cell regulatory functions (Seymour et al. 2006). Likewise, transporters may allow IDO⁺ DCs to acquire tryptophan from their immediate microenvironment. Speculatively, tryptophan transporters might be strategically distributed in immunologic synapses that form when T cells and DCs interact following recognition of cognate antigen, providing DCs with preferential access to free tryptophan from T cells. Such considerations, though technically difficult to verify (especially in physiologic settings) may explain why IDO⁺ DCs may not need to fully deplete tryptophan from tissue microenvironments to influence T cell responses.

IFN γ treatment caused IDO-dependent tryptophan depletion in tissues of tumor-bearing mice, and induced expression of tryptophanyl-tRNA synthetase, the only tRNA synthetase gene that is responsive to inflammatory stimuli (Burke et al. 1995). These findings prompted speculation that tryptophan ‘starvation’ might negatively impact cell growth in the vicinity of IDO⁺ cells; however, these effects did not fully explain how IFN γ slowed tumor growth in this experimental system. Another reason why tryptophan ‘starvation’ may not be the appropriate paradigm to explain IDO-mediated T cell suppression (Mellor and Munn 1999) emerged from evidence that T cells, in common with all cell types, detect reduced access to free amino acids via General Control Non-derepressible 2 (GCN2) kinase, which is triggered by increased binding of uncharged tRNAs to ribosomes (Dong et al. 2000). Thus, GCN2-kinase triggering may be highly sensitive to reduced tryptophan levels in the cell cytoplasm obviating the need to fully deplete tryptophan from cells or their immediate microenvironment to stimulate downstream responses, unless increased expression of tryptophanyl-tRNA synthetase protects cells from the anti-proliferative effects of tryptophan depletion (Burke et al. 1995).

3 IDO Molecular Genetics and Gene Expression

In this section we describe the molecular genetics of IDO and the recently identified IDO2 gene. We identify the tissues, cell types, and physiologic conditions under which IDO is expressed. Included in this section is a discussion of the growing list of agents and signaling pathways that have been identified to induce functional IDO.

3.1 IDO Genes

IDO genes are highly conserved in all mammalian species studied to date and they clearly evolved from ancestral genes related to the myoglobin gene. Recently, a second IDO gene (IDO2) was identified in mice and humans (Ball et al. 2007; Metz et al. 2007). In both species, IDO2 is closely linked to the previously identified IDO (IDO1) gene located in a syntenic region of chromosome 8. For the purposes of this review, we consider IDO as a single gene product, while acknowledging that IDO protein may be the product of IDO1, IDO2 or both genes. IDO1 and IDO2 genes exhibit similar but not identical patterns of regulated expression in response to inflammatory signals, and D-1MT inhibits enzyme activity of proteins encoded by IDO1 and IDO2 genes (Table 1). The biological significance of the two IDO genes is not yet clear, but some human ethnic groups possess relatively high frequencies (25-50%) of defective IDO2 alleles (Metz et al. 2007).

3.2 IDO Gene Expression

IDO is expressed constitutively in tissues with large mucosal surfaces (lungs, GI tract, maternal-fetal interface), and may be a consequence of inflammation at mucosal surfaces under homeostatic conditions (Yamamoto and Hayaishi 1967; Keith and Brownfield 1985; Munn et al. 1998). IDO expression also occurs at sites of infection and tumor growth, and in chronically inflamed tissues associated with allergic and autoimmune disease syndromes (Friberg et al. 2002; Hayashi et al. 2004; Popov et al. 2006; Saxena et al. 2007). However, only a few cell types express IDO in these tissues. IDO is rarely, if ever, expressed by lymphoid cells under physiologic conditions, and IDO staining (assessed by immunohistochemical techniques) is largely confined to myeloid lineage cells (DCs, macrophages), and some stromal cells types such as epithelial cells lining blood vessels and some fibroblast-like cells. IDO⁺ cells always represent a small subset of any given cell type in tissues. Hence, lineage-specific factors that regulate the ability of cells to express IDO have not been defined and IDO appears to be regulated in large part by inflammatory cues (via IFNs) once cells have acquired competency to express IDO. It is important to emphasize that post-translational modifications, absence of co-factors such as hemin, and the presence of natural IDO inhibitors such as NO may compromise or inactivate IDO enzyme activity. Hence, caution should be exercised in interpreting data based solely on methods that detect IDO mRNA or protein (Thomas et al. 2001; Braun et al. 2005). Typically, IDO enzyme activity is measured using HPLC techniques to detect kynurenine, a stable downstream metabolite produced by certain IDO⁺ cell types. However, this method is not very sensitive, requiring relatively large numbers of cells, and relies on the assumption that IDO⁺ cells secrete kynurenine as the end product of oxidative tryptophan catabolism. For the specific purpose of measuring IDO-mediated effects in DCs, more sensitive (albeit indirect) methods to detect IDO activity involve measuring T cell stimulatory functions of DCs (see Sect. 6).

3.3 IDO Inducers

Interferons are potent inducers of IDO gene transcription in specialized cell types competent to express IDO (Taylor and Feng 1991; Mellor and Munn 1999). We describe DC subsets specialized to express IDO ('IDO-competent DCs') in Sect. 4. IFNs are a large family of cytokines, divided into three classes (types I, II and III), which are produced during rapid

innate immune responses to microbial infections, and at other sites of inflammation during normal tissue functions, or caused by extraneous factors such as tissue wounding and tumor growth (Pestka et al. 2004; Chelbi-Alix and Wietzerbin 2007). Human and murine IDO genes contain Interferon Stimulated Response Elements (ISRE) and Gamma Interferon Associated Sites (GAS) elements in the proximal promoter regions, which respond to type I and type II IFNs, respectively (Dai and Gupta 1990; Paguirigan et al. 1994; Sotero-Esteve et al. 2000; Decker et al. 2005).

The diversity of IFN α (type I) subtypes produced by cells at sites of infection or inflammation suggests that there is functional or cell-type specificity between subtypes, though little is known about such distinctions. Indeed, activation of different signaling pathways and responses were reported in DCs and T cells following *in vitro* treatment by different subtypes (Hilkens et al. 2003; van Boxel-Dezaire et al. 2006; Johnson and Scott 2007). For example, we observed IFN α 1-9 gene transcription in murine splenic CD19⁺ pDCs treated with CpGs or a soluble form of Cytotoxic T-Lymphocyte Antigen 4 (CTLA4-Ig) (to ligate TLR9 or B7) prior to IDO expression (Baban et al. 2005; Manlapat et al. 2007). In contrast to IFN α , which is produced by many cell types (including pDCs) during innate immune responses, IFN γ (type II) is produced (for the most part) by lymphoid cells such as activated CD4⁺ T cells during adaptive immune responses. Thus, DCs capable of expressing IDO may be responsive to a range of inflammatory cues at sites of tissue damage or infection, or in associated lymphoid tissues, that cause IFN release.

As depicted in Fig. 2, IFN type I (IFN $\alpha\beta$) and type II (IFN γ) induce IDO transcription via Janus Activated Kinase (JAK)/STAT signaling pathways that act on ISRE and GAS in IDO promoters, respectively (Platanias 2005). Under normal physiologic conditions few, if any, tissue DCs express IDO. However, when mice or splenocytes were treated with certain reagents that induce IDO (Table 2) a subset of DCs responded by expressing IDO and acquiring potent T cell suppressive functions. In many cases, the IDO inducers listed in Table 2 stimulated IDO expression indirectly by inducing IFN α production via MyD88/IRF7-dependent pathways that control IFN α expression in plasmacytoid DCs (pDCs). Thus, TLR ligands induced pDCs to express IFN α , which subsequently induced IDO-competent DCs to express IDO.

Similarly, soluble CTLA4 (CTLA4-Ig, a B7 ligand) induced IFN α production upstream of IDO, though in this case, IDO was also required upstream of IFN α production (Manlapat et al. 2007), revealing a cell (DC) autonomous feedback loop that presumably amplifies IDO expression via IFN α mediated signaling through its receptor (IFNAR). IFN γ also induced IDO expression in some DCs, such as CD8 α ⁺ DCs, formerly known as lymphoid DCs (Fallarino et al. 2002b). These findings provide insights into the complex positive and negative signaling mechanisms that restrict IFN-mediated IDO expression to specialized cells in DC and other cell populations, though more studies will be needed to understand how IDO expression and IDO enzyme activity are regulated at the level of gene expression and post-translation, respectively.

4 IDO-Competent DCs

In this section, we describe DCs that can be induced to express functional IDO and acquire potent T cell suppressor functions as a consequence. DC phenotypes are heterogeneous, varying between tissues and dependent on DC maturation status. Moreover, it is difficult to draw parallels between DCs in mice and humans making it hard to draw firm conclusions from cross-species comparisons. Consequently, the primary focus of this section is on murine IDO-competent DCs, which have been defined in more detail than their counterparts in humans.

4.1 Murine IDO-Competent DCs

Two major DC subsets expressing the ubiquitous DC marker CD11c have been identified in mouse spleen (Table 3A). Myeloid DCs (mDCs) express relatively high levels of CD11c but do not express B220 (CD45R) or mPDCA1 (CD317). Plasmacytoid DCs (pDCs) express both markers, but lower levels of CD11c than mDCs. Moreover, pDCs (but not mDCs) produce IFN type I in response to TLR ligation following microbial infection (Asselin-Paturel et al. 2001). IDO-competent DCs reside exclusively in the pDC subset, based on their morphologic appearance in tissues and on functional analyses of DC populations fractionated using the pDC specific-markers B220 and mPDCA1 (Fallarino et al. 2004; Baban et al. 2005; Fallarino et al. 2005; Fallarino et al. 2007). IDO-competent pDCs also exhibit phenotypes typical of mature DCs, expressing MHC and B7 molecules at relatively high levels compared to conventional pDCs and mDCs in spleen (Mellor et al. 2004). Surprisingly, IDO⁺ pDCs with T cell suppressor functions in tumor-draining lymph nodes (TDLNs) expressed the B cell marker CD19, a marker not previously associated with DCs (Munn et al. 2004a). Consistent with this finding, splenic IDO-competent pDCs also expressed CD19 (Baban et al. 2005; Mellor et al. 2005). CD19⁺ pDCs are a rare DC subset representing only ~5-10% of total DCs (~10-20% of pDCs) in each tissue and only ~0.1-0.2% of total splenocytes (Fig. 3B), so that each mouse spleen typically yields only ~10⁵ CD19⁺ pDCs. Conventionally, pDCs have been defined as CD19^{NEG}, suggesting that CD19⁺ pDCs represent a novel pDC subset that was over-looked, in part because CD19 is commonly used to segregate DCs from B cells. CD19⁺ pDCs resemble conventional (CD19^{NEG}) pDCs in many ways, such as the ability to produce IFN type I following TLR ligation (Mellor et al. 2005; Manlapat et al. 2007).

Nevertheless, CD19⁺ and CD19^{NEG} pDCs are functionally, as well as phenotypically, distinct pDC subsets as IDO-competent pDCs reside exclusively in the CD19⁺ pDC subset. For the purposes of the current discussion, the most important difference between rare CD19⁺ pDCs and the more abundant conventional (CD19^{NEG}) pDCs is the unique ability of CD19⁺ pDCs from spleen and TDLNs to mediate IDO-mediated T cell suppression. Splenic CD19⁺ pDCs also express CD8 α , CD80, CD86, and the chemokine receptor CCR6 (Mellor et al. 2004; Manlapat et al. 2007), but CD19⁺ pDCs do not express the conventional pDC marker 120G8 (Asselin-Paturel et al. 2003; Mellor et al. 2005).

4.1.1 Fractionation and Phenotypic Analyses of IDO-Competent pDCs—Two methods are widely used to fractionate DCs and DC subsets based on their affinity (or lack

thereof) for specific monoclonal antibodies (mAb). Magnetic Activated Cell Sorting (MACS) is a rapid technique whereby cells labeled with mAb conjugated to magnetic beads are passed through a magnetic field to either enrich or deplete labeled cells from cell suspensions. In Fig. 3A, we show a typical dataset in which a standard MACS procedure yielded a cell population containing ~36% CD11c⁺ cells (DCs) from a starting population of splenocytes containing only 7% DCs, representing a 5-fold enrichment for DCs. Note that carry over of CD11c^{NEG}CD19⁺ and CD11c^{NEG}CD19^{NEG} cells (mostly B and T cells, respectively) is significant, possibly due to interactions between DCs and T or B cells. Also, some cells in the CD11c⁺ enriched population fall in the auto-fluorescent region (AF region, colored orange in Fig. 3A) and may not be DCs.

Some investigators have reported significantly higher DC enrichment rates (up to 99% DCs) using more sophisticated procedures such as depleting T or B cells using CD5 or CD19 (Honda et al. 2005) or multiple rounds of MACS selection using mPDCA1 or CD8α following CD11c enrichment (Fallarino et al. 2004; Fallarino et al. 2005). In our experience, 'purified' populations of IDO-competent CD19⁺ pDCs cannot be obtained via MACS techniques because they are extremely rare (even amongst DCs), and low recovery rates after each round of enrichment means that it is not feasible to use such methods to isolate IDO-competent CD19⁺ pDCs. Moreover, sophisticated pre-enrichment techniques designed to deplete contaminating T or B cells using CD8α or CD19 also remove CD19⁺ pDCs. These technical considerations aside, and for the reasons discussed in Sect. 4.1, MACS is still a useful technique for enriching CD19⁺ pDCs from total splenocytes for further use in *in vitro* assays and IHC staining analyses (Mellor et al. 2004; Baban et al. 2005; Mellor et al. 2005).

Fluorescence Activated Cell Sorting (FACS) is the second method by which DCs and DC subsets can be fractionated from total splenocytes. In this technique mAbs conjugated to fluorescent molecules allow specific DC subsets to be identified and sorted based on combinations of surface marker expression. However, careful optimization of enrichment protocols and sorting strategies is necessary as CD19⁺ pDCs can be easily overlooked or inadvertently discarded during sorting procedures.

A common problem encountered during FACS is the presence of auto-fluorescent cells (AF) which complicate DC sorting because DCs display high forward and side (light) scatter (FSC, SSC) properties similar to naturally occurring AF cells. To address this problem, many investigators set precise initial sorting gates (based on FSC and SSC properties) to avoid collecting 'false positive' AF cells. While such gating techniques succeed in 'cleaning up' sorted cell populations, this approach can generate misleading outcomes because critical DC subsets may also be gated out with AF cells. An example of this problem is shown in Fig. 3B using the pre- and post-MACS enrichment fractions shown in Fig. 3A to visualize the location of distinct DC subsets. Datasets in the upper panels of Fig. 3B show analyses of the light scatter (FSC, SSC) and phenotypic analyses (CD11c vs CD19) of total (pre-MACS) and DC-enriched (post-MACS) splenocytes, using precise a FSC and SSC analytic gating strategy designed to avoid AF cells. This strategy eliminates almost all AF cells from DC populations, which contain large cohorts of CD19^{NEG} mDCs and conventional pDCs (CD11c^{HIGH} & CD11c^{LOW}, highlighted in blue & green, respectively), though contaminating lymphoid cells were also present.

However, the much rarer CD19⁺CD11c^{HIGH} pDCs (highlighted in red) of major interest from the perspective of IDO-competent DCs were almost completely excluded by this restricted gating strategy. Thus, use of restricted sort gates will completely exclude IDO-competent pDCs from DC populations sorted by flow cytometry. In contrast, use of a much wider light scatter gate to analyze DC subsets revealed that CD19⁺ pDCs were enriched ~25-fold following MACS. The key point is that CD19⁺CD11c^{HIGH} pDCs were not excluded in this analysis because CD19⁺ pDCs have relatively high light scatter properties compared to other DCs. Indeed, the pDC and mDC populations, as well as CD19⁺ pDCs lie outside the region containing AF cells. Moreover, the common practice of using restrictive gating procedures to assess DC enrichment can lead to artificially inflated enrichment factors since restricted gating ignores the actual presence of contaminating cells in sorted populations. In our studies on IDO-competent pDCs, we have consistently used FACS techniques to fractionate DC subsets prior to performing functional experiments to detect IDO-mediated T cell suppression by sorted DC subsets. In our hands, this approach circumvented some of the pitfalls associated with MACS separation techniques, and resulted in better enrichment of specific DC subsets.

4.1.2 Functional Analyses of IDO-Competent pDCs—A key attribute of CD19⁺ pDCs is the potency of their T cell suppressor functions when induced to express IDO. We developed novel methods to measure the T cell suppressor functions of small numbers of sorted DCs. Briefly, sorted DCs were co-cultured in Mixed Lymphocyte Reactions (MLRs) with T cells of defined antigen-specificity from T cell receptor (TCR) transgenic mice and T cell proliferation was assessed using standard procedures. Parallel cultures containing IDO inhibitor (1MT) or excess tryptophan were included to determine if these manipulations rescued T cell responses suppressed by IDO⁺ DCs due to IDO inhibition or preventing tryptophan depletion, respectively. Using this approach, we showed that FACS sorted splenic (CD11c^{HIGH}) DCs co-expressing B220, CD8α or CD19 contained IDO⁺ pDCs after mice were pre-treated with B7 (CTLA4-Ig) and TLR9 (CpG) ligands to induce IDO (Mellor et al. 2004; Baban et al. 2005; Mellor et al. 2005).

In contrast, CD11c^{HIGH} DCs that did not express these phenotypic markers (sorted from the same mice treated with IDO inducers) stimulated robust T cell proliferation that was not further enhanced by adding IDO inhibitor or excess tryptophan. For example, FACS sorted CD19^{NEG} splenocytes from mice treated with IDO inducers stimulated robust T cell proliferation, indicating that the T cell suppressive functions of CD19⁺ pDCs predominated over the stimulatory functions of all other DCs (and other APCs) in unfractionated splenocyte populations, even though CD19⁺ pDCs represent only a minor DC subset relative to CD19^{NEG} pDCs, which comprise 90-95% of all splenic DCs. Similar results were obtained with sorted DC subsets from TDLNs of melanoma bearing mice, showing that tumor growth induced IDO-competent pDCs to express functional IDO in TDLNs (Munn et al. 2004a).

The key point is that the presence of DCs with T cell stimulatory functions was not apparent unless rare CD19⁺ pDCs were removed, even when IDO⁺ pDCs were minor fractions amongst total DCs. This finding implies that the presence of residual or ‘contaminating’ CD19⁺ pDCs may result in selected DC populations possessing IDO-dependent T cell

suppressive functions, even though the majority of DCs in such populations do not express IDO. Hence, caution should be exercised when assigning IDO-dependent suppressive functions to all DCs present in unfractionated (or even fractionated) DC populations. Moreover, a few CD19⁺ pDCs expressing IDO may establish and maintain suppressive tissue microenvironments, even though they are far outnumbered by other DCs. Hence, the presence of CD19⁺ pDCs, and whether or not they are induced to express IDO, has profound implications for the outcome of T cell responses to antigenic stimuli following inflammation. In our experience, IDO-dependent T cell suppressive functions are more difficult to detect in DCs cultured from bone marrow or blood precursors, though some studies have reported such observations, especially amongst cultured human DCs (see Sect. 4.2).

Another method has been employed extensively by Grohmann and colleagues to assess IDO-dependent T cell suppressive functions of DCs, and involves adoptive transfer of DCs (pulsed with a peptide antigen) into mice and evaluating subsequent Delayed Type Hypersensitivity (DTH) responses by the classical immunological method of measuring foot-pad swelling (Grohmann et al. 2001a; Grohmann et al. 2001b; Grohmann et al. 2003; Fallarino et al. 2007). This method has the advantage of measuring the actual immunizing and tolerizing properties of specific DC populations and fractionated DC subsets using a well-accepted *in vivo* model system. However, this experimental approach has the disadvantage of being an indirect measure of IDO-mediated T cell suppression, and this method may be prone to a number of extenuating circumstances that affect eventual outcomes. For example, it is unclear how to interpret observations based on treating small cohorts of donor DCs with IDO inhibitor before adoptive transfer, which then leads to enhanced DTH responses because the recipient mice contain a large excess of DCs and other cells, such as regulatory T cells, that are capable of influencing T cell responses. Though such outcomes may occur due to loss of direct T cell suppressive potential by treated DCs in recipient mice, it is also possible that IDO inhibitor modifies the viability or differentiation status of DCs, leading to completely different DTH outcomes not necessarily linked to loss of IDO activity.

Differences in the way that IDO-dependent T cell suppression is evaluated and in the way that DCs are prepared and fractionated make it difficult to compare results from one laboratory to another. This issue notwithstanding, there is now general agreement that murine IDO-competent DCs have plasmacytoid morphology, reside in the pDC subset, and produce IFN α when exposed to several ligands with IDO inducing properties (Table 2 and Fig. 2A). However, reported requirements for either IFN type I versus type II signaling to induce IDO expression in splenic DCs may (a) reflect key, biologically significant differences or (b) merely arise from differences in technical approaches (Fallarino et al. 2002b; Baban et al. 2005). Regarding the distinctive phenotype of IDO-competent pDCs, the discovery that CD19⁺ pDCs from spleen and TDLNs were the only DC subset capable of mediating IDO-dependent T cell suppression represents corroborating evidence from two independent physiologic situations that IDO-competent DCs reside exclusively amongst the CD19⁺ pDC subset in mice.

4.2 Human IDO-Competent DCs

Unlike their counterparts in mice, human IDO-competent DCs cannot be isolated directly from tissues. Instead, IDO⁺ human DCs have been characterized by immunohistochemical analyses of tissue biopsy samples and by analyzing the functional (T cell suppressive) status of DCs cultured from peripheral blood mononuclear cells (PBMCs). Variations between individual blood donors and DC preparation protocols complicate comparative data analyses between different laboratories. Consequently, there is little consensus, and lingering controversy about the identity and significance of human IDO-competent pDCs. These points notwithstanding, IDO⁺ cells with plasmacytoid morphology (in some cases expressing DC markers) have been found in biopsies from a range of clinical tumors, in sentinel LNs draining sites of tumor growth and in granulomas caused by *Listeria* infection (Lee et al. 2003; Munn et al. 2004a; Munn and Mellor 2006; Popov et al. 2006), prompting speculation that IDO⁺ cells may be DCs with T cell suppressor functions that inhibit anti-tumor immunity in cancer patients and help mediate an immunological ‘standoff’ between host and pathogen during chronic *Listeria* infections.

In early studies, functional analyses revealed that the majority of human DC cultures contained at least some DCs that suppressed T cell proliferation *ex vivo* via IDO, since adding IDO inhibitor significantly enhanced T cell responses (Hwu et al. 2000; Munn et al. 2002). Functional IDO activity was associated exclusively with DCs expressing the human pDC marker CD123 and the chemokine receptor CCR6 (Table 3B). Another group reported findings that were inconsistent with these initial reports (Terness et al. 2005). However, a recent report identified a human DC subset expressing IDO after culturing pBMCs with inactivated HIV-1, IFN type I or IFN type II (Boasso and Shearer 2007); DCs induced to express IDO by these agents expressed surface CD4, CD123, and BDCA2. In summary, the consensus is that human IDO-competent DCs can be detected amongst cultured PBMCs, provided that investigators adhere strictly to using specific protocols to prepare DCs and induce them to express IDO (Munn et al. 2005a). If correct, these findings suggest that there are particular requirements for differentiation and survival of human IDO-competent DCs in culture, which may have critical implications for clinical use of cultured human DCs as vaccine adjuvants to stimulate anti-tumor immunity or, conversely, to suppress allo-responses to transplanted cells and tissues by recipient T cells.

4.3 IDO Induction in IDO-Competent pDCs

As key roles for IDO⁺ DCs in disease progression have emerged from studies on murine models of human disease syndromes, it became important to identify physiologic mechanisms and artificial reagents that induce IDO-competent pDCs to express functional IDO. Reagents that induce DCs to express functional IDO were listed in Table 2 and, as discussed above, IFNs are positive regulators of IDO gene transcription via the JAK/STAT signaling mechanisms depicted in Fig. 2B (Platanias 2005). However post-translational factors also influence the ability of DCs to express functional IDO. For example, IFN γ induced splenic CD8 α ⁺ and CD8 α ^{NEG} DCs to express IDO protein, but only CD8 α ⁺ DCs acquired T cell suppressive functions (Grohmann et al. 2001a; Fallarino et al. 2002b). Most synthetic IDO inducing reagents listed in Table 2 bind to specific molecules expressed by DCs, including soluble CTLA4 (CTLA4-Ig), CD200-Ig and CpG-oligonucleotides, which

ligate B7 (CD80/86) CD200-receptor and TLR9 respectively (Grohmann et al. 2002; Mellor et al. 2003; Fallarino et al. 2004; Mellor et al. 2005). These reagents stimulated DCs to produce IFN γ , which then induced IDO expression, presumably via autocrine or paracrine signaling.

The biological significance of these molecular interactions which cause IDO-competent pDCs to express IDO are not clear and is the subject of ongoing research. However, B7 ligation leading to IDO induction may be linked to cooperative interactions between IDO-competent pDCs (which express B7 molecules) and Tregs, many of which express surface CTLA4. Indeed, Treg suppressor functions may depend on CTLA4 $\rightarrow$ B7 signaling from Tregs $\rightarrow$ pDCs (Fallarino et al. 2004; Mellor et al. 2004). The biological significance of IDO induction following ligation of TLRs is less obvious, though this response may be linked to counter-regulation of T cell responses during bacterial sepsis (Wingender et al. 2006).

Using mice with defective IFN signaling due to ablation of IFN type I (IFNAR) and type II (IFN γ R α) receptors we found that IFN type I signaling was obligatory upstream of IDO following B7 ligation and TLR9 ligation (using CTLA4-Ig and CpGs, respectively), because IFNAR (but not IFN γ R α) ablation abolished IDO induction in CD19 $^{+}$ pDCs (Baban et al. 2005) (Mellor et al. 2005). On further investigation, we confirmed that IFN α (but not IFN γ) induced selective STAT1 activation and functional IDO expression in splenic CD19 $^{+}$ pDCs. Requirements for IFN type I signaling to induce IDO were not consistent with previous reports that IFN γ induced IDO in DCs, (Grohmann et al. 2001a; Fallarino et al. 2002b) though different methods were used to prepare DCs, assess the requirements for IFN signaling and evaluate DC suppressor functions.

More recently, we discovered an obligatory requirement for cell autonomous IDO (and GCN2) upstream of IFN α expression by CD19 $^{+}$ DCs following B7 ligation (Manlapat et al. 2007). The ability of CTLA4-Ig reagents to induce functional IDO expression depends on the structure of the Fc (Ig) domain and on the mouse strain used, suggesting that additional factors contribute to signaling processes that induce IDO following B7 ligation. In our experimental systems, a cytolytic isoform of CTLA4-IgG2a induced IDO expression in most mouse strains tested (except B6 mice), whereas a closely related non-cytolytic isoform (with mutated Fc binding and C1q binding domains) did not induce IDO (Mellor et al. 2004), suggesting that functionalities in the Fc domain were required to induce IDO. Moreover, a CTLA4-Ig3 isoform induced IDO in spleen of B6 mice, but failed to induce IDO in other mouse strains such as CBA mice (unpublished data).

The molecular basis for these disparities is currently unknown, but they may have important implications for the use of soluble CTLA4 in clinical settings, especially as human versions of CTLA4-Ig tested in mice and in the clinic did not induce IDO expression in mice, but may do so in (at least) some humans. We have also detected strain-specific disparities in IDO inducing properties of different sequence classes of CpGs, which ligate TLR9 (Table 4). Furthermore, CpGs exhibit sequence, strain, and dose specificity with respect to IFN α production (Blackwell and Krieg 2003; Vollmer et al. 2004a; Vollmer et al. 2004b; Abel et al. 2005; Roberts et al. 2005; Booth et al. 2007; Martinson et al. 2007). While type B and C CpG sequences induced IFN α and IDO in most strains tested, type A CpGs only induced

IFN α expression in DCs from BALB/c mice. Again, the molecular basis of these disparate responses to TLR ligands is unknown. The route of CpG administration was also critical since injecting CpGs into the tail vein induced IDO while intra-peritoneal injection of the same amount of CpGs did not induce IDO (Mellor et al. 2005; Wingender et al. 2006).

The mechanistic basis for the failure of IDO inducers to stimulate IDO expression in all DCs was investigated by Orabona and colleagues who reported that Suppressor of Cytokine Signaling 3 (SOCS3) and the molecular adapter DAP12 functioned as negative regulators of IDO transcription in most splenic DCs (Orabona et al. 2005a; Orabona et al. 2005b). CD28-Ig also induced IFN γ -dependent IDO expression through B7-1/B7-2 engagement but only in SOCS3-deficient pDCs (Fallarino et al. 2004; Fallarino et al. 2005). Treating pDCs with a soluble glucocorticoid-induced tumor necrosis factor receptor (GITR-Ig) induced IFN α -dependent IDO expression due to ligation of GITR ligand on pDCs via a non-canonical NF- κ B mechanism, identifying a novel IDO induction pathway in DCs (Grohmann et al. 2007).

Human pDCs also expressed IDO following treatment with B7 ligands. Monocyte-derived DCs showed functional IDO expression following ligation of B7-1/B7-2 molecules on DCs by CTLA4/CD28 expressed by activated CD4⁺ T cells (Munn et al. 2004b). In another study PBMCs expressing CD4 produced B7-dependent, functional IDO in response to CTLA4-Ig, but not CD28-Ig treatment (Boasso et al. 2005). Human (CD4⁺, CD123⁺, BDCA-2⁺, BDCA-4⁺) pDCs exposed to infectious or inactivated HIV expressed IDO, which was independent of IFN type I or type II signaling, but required the viral gp120 and cellular CD4 interaction (Boasso et al. 2007).

In summary, complex signaling mechanisms induce IDO-competent pDCs to express IDO, and prevent IDO up-regulation in other cells, including other DC subsets. The fact that IDO induction is tightly regulated and occurs only in minor DC subsets implies that there may be a critical need to regulate IDO induction and tailor this response to particular inflammatory conditions that prevail (or are induced) in some tissue microenvironments.

5 IDO-Mediated T cell suppression

In this section, we discuss how pDCs acquire potent and dominant T cell suppressor functions when induced to express IDO. IDO activity in DCs may influence how T cells respond to antigen stimuli in several ways. IDO may bring about cell autonomous changes in DCs themselves that (a) reduce their ability to stimulate clonal expansion of effector T cells and (b) enhance their ability to suppress T cell responses. Though emerging evidence supports this hypothesis (Hill et al. 2007a; Manlapat et al. 2007) it is not known how IDO activity modifies DC functions. Alternatively, T cells and Tregs may respond to activation signals from antigens expressed by IDO⁺ DCs differently when T cells and Tregs experience the biochemical changes caused by IDO activity in DCs (Fig. 4). In the remainder of this section we discuss how IDO enzyme activity in DCs affects T cells and Tregs.

5.1 T cell Suppression by IDO⁺ pDCs

Two basic hypotheses have been proposed to explain how IDO activity in DCs suppresses T cell responses when T cells are activated. First, T cells may sense and respond to reduced

levels of free tryptophan and second, T cells may be sensitive to tryptophan metabolites produced by IDO⁺ pDCs (Mellor and Munn 2004; Fallarino et al. 2007). There is evidence in support of each hypothesis, and these mechanisms are not mutually exclusive, though most studies were performed *ex vivo* and therefore it is not yet clear if findings are applicable to physiologic situations. APCs (macrophages and DCs) expressing IDO cause activated T cells to undergo cell cycle arrest and apoptosis, and induced anergy if T cells survived (Munn et al. 1996; Munn et al. 1999; Hwu et al. 2000; Grohmann et al. 2001a; Munn et al. 2002; Munn et al. 2005b). Initial studies revealed that robust T cell proliferative responses to IDO⁺ APCs were restored by adding IDO inhibitor or excess tryptophan (Munn et al. 1999), suggesting that failure of T cells to undergo normal responses was due to active suppression by APCs, and was not a consequence of 'weak' T cell stimulation, which has been associated with immature APCs (Hackstein 2004).

The finding that excess tryptophan rescued T cell proliferation provided a hint that tryptophan depletion was a key biochemical change that affected the way T cells responded to antigenic stimulation because adding excess tryptophan would increase consumption of ROS and production of downstream tryptophan metabolites. Consistent with this interpretation, human and murine T cells are highly sensitive to reduced access to free tryptophan during activation in the absence of APCs (Lee et al. 2002). T cells activated in chemically defined tryptophan-free medium underwent cell cycle arrest prior to S-phase (DNA synthesis). In contrast, T cells activated in medium without isoleucine and leucine began to incorporate thymidine, but stopped shortly after S-phase started, consistent with cessation of protein translation due to amino acid starvation. Thus, rather than tryptophan 'starvation', T cells appear to sense reduced access to tryptophan and trigger a response that shuts down cell cycle progression.

What mechanisms could account for such a response? The mammalian target of rapamycin (mTOR) and general control non-derepressible-2 (GCN2) kinase are two ribosome based mechanisms that sense and trigger cellular responses when cell access to amino acids is limited. Munn and colleagues performed comprehensive gene expression profile analyses that revealed a number of cell cycle control genes whose transcription rates were up-regulated significantly in activated T cells following abortive cell cycle arrest due to tryptophan deprivation (relative to control T cells) (Munn et al. 2005b). One such gene encodes CCAAT/enhancer-binding protein homologous protein (CHOP, aka GADD153), which is under strict transcriptional control by GCN2-kinase and has pro-apoptotic effects on cells (Harding et al. 2003; Wek et al. 2006; Puthalakath et al. 2007). To test the hypothesis that IDO-activity in DCs triggered GCN2-kinase activation in T cells, the effects of IDO⁺ pDCs on T cells from GCN2-deficient (GCN2-KO) mice were evaluated. T cells from GCN2-KO mice responded normally to IDO⁺ pDCs showing that intact GCN2 was essential for T cell susceptibility to the cell cycle inhibitory effects of IDO activity in DCs, and confirming that IDO-mediated effects on T cells were GCN2-dependent (Munn et al. 2005b). GCN2-kinase senses reduced access to amino acids when uncharged tRNA molecules bind to ribosomes (Fig. 4), which triggers GCN2-kinase activation and leads to a cascade of downstream responses known as the cellular integrated stress response (ISR) to amino acid withdrawal, which lead to cell cycle arrest and cessation of most (but not all) protein translation in cells (Dong et al. 2000; Harding et al. 2003). Interestingly, L-arginine

depletion also induced GCN2-dependent T cell cycle arrest, suggesting that myeloid-derived suppressor cells (MDSCs), which express L-arginase, may also exploit the GCN2 mechanism to suppress T cell immunity in tumor microenvironments where MDSCs congregate (Rodriguez et al. 2007).

Though excess tryptophan completely abrogates the T cell suppressor functions of IDO⁺ pDCs, certain downstream tryptophan metabolites enhance T cell apoptosis, and modify how T cells differentiate into effector T cells (Fallarino et al. 2002a; Frumento et al. 2002; Terness et al. 2002), suggesting that production of toxic metabolites may also contribute to IDO-mediated suppression. A recent study revealed that the downstream metabolite 3-Hydroxyanthranilic acid (HAA), which induces T cell apoptosis, inhibits NF- κ B activation by binding to PDK1, an essential mediator of CD28-induced NF- κ B activation in T cells activated by TCR engagement (Hayashi et al. 2007). Though it is not clear if IDO⁺ pDCs produce sufficient HAA to affect T cell responses under physiologic conditions, administering HAA to mice alleviated experimentally induced asthma, providing support for the notion of using natural or synthetic tryptophan metabolites to treat chronic inflammatory disease syndromes with T cell involvement such as experimental autoimmune encephalitis (EAE), a murine model of multiple sclerosis (Platten et al. 2005). However, it is not known if natural tryptophan metabolites produced by IDO⁺ cells also have palliative effects on chronic inflammatory diseases in physiologic settings.

5.2 IDO-Mediated Treg Differentiation and Activation

Though purified (sorted) IDO⁺ pDCs suppress T cells that activate in response to antigens they present, the fact that IDO⁺ pDCs constitute only a small fraction (~10% in mouse spleen and TDLNs) suggests that their suppressive functions ought to be subordinate to the stimulatory functions of other DCs in physiological microenvironments. Moreover, it is highly unlikely that T cells would preferentially recognize and respond to their cognate antigens displayed by the small subset of IDO-competent DCs, rather than the majority of other DCs present in inflamed tissue microenvironments. Hence, the ability of rare IDO⁺ pDCs to mediate dominant suppression requires an explanation that does not rely exclusively on direct T cell suppression by IDO⁺ pDCs themselves. Indeed, dominant suppression is not simply an artifact of *in vitro* culture systems because resident IDO⁺ pDCs completely blocked T cell mediated destruction of splenic tissues following adoptive transfer of allo-specific T cells into mice treated with CTLA4-Ig to induce IDO (Mellor et al. 2003).

These considerations suggest that IDO⁺ pDCs activate other T cell suppressive mechanisms. One candidate mechanism is CD4⁺CD25⁺ Tregs, which are potent T cell suppressors in a range of clinically relevant syndromes, even though they also constitute only a minor subset (~5-10%) of CD4⁺ T cells, a fact that contributed to Tregs being overlooked as sources of T cell suppression for some time (Sakaguchi 2005; Shevach et al. 2006). Key points about Tregs relevant to their potential interactions with IDO⁺ pDCs are (a) the majority of Tregs are unique amongst T cells in expressing CTLA4 stably at the cell surface; (b) the proportions of effector T cells and Tregs that emerge from antigen-driven encounters with DCs depends on the functional (maturation) status of DCs (Mahnke et al. 2007); and (c)

peripheral Tregs normally do not exhibit constitutive suppressor activity and 'resting' Tregs must be activated to acquire suppressor functions (Thornton et al. 2004).

As discussed previously, surface CTLA4 expression by Tregs provides a natural physiologic counterpart to the ability of synthetic soluble CTLA4 (CTLA4-Ig) to induce IDO expression in DCs by ligating B7 molecules (Grohmann et al. 2002; Mellor et al. 2003). Experimental evidence supporting this notion reveals that CTLA4→B7 interactions between Tregs and IDO-competent (but IDO^{NEG}) DCs induce IDO expression in DCs (Fallarino et al. 2003; Mellor et al. 2004). Indeed, the T cell suppressive functions of cloned CTLA4⁺ Tregs were completely dependent on DCs having an intact IDO1 gene and were blocked by adding anti-CTLA4 mAb (Mellor et al. 2004). If verified, the requirement for CTLA4→B7 interactions to stimulate DCs to express IDO may have critical implications for understanding how anti-CTLA4 mAbs enhance T cell immunity to tumors in ongoing clinical trials in cancer patients (Peggs et al. 2006).

Immature DCs and certain DC subsets from some tissues (such as the GI tract) are more effective than the majority of DCs in promoting Treg differentiation. Little is currently known about mechanisms that influence how DCs promote Treg rather than effector T cell development. However, IDO activity in DCs may be one mechanism that favors Treg over effector T cell development, and experimental evidence supporting this hypothesis has emerged from several studies. For example, Fallarino et al. reported that IDO⁺ DCs enhanced *in vitro* Treg differentiation from naïve CD4 precursors via the combined effects of GCN2-dependent tryptophan depletion and tryptophan metabolites (Fallarino et al. 2006). Moreover, some promising new therapies to treat chronic inflammatory diseases in which enhanced Treg functions are likely to be beneficial may work, at least in part, by inducing DCs to express IDO. Thus, soluble CD40-Ig and CTLA4-Ig may facilitate allograft survival by inducing DCs to express IDO and promoting Treg differentiation, as well as blocking co-stimulation (Grohmann et al. 2002; Mellor et al. 2003; Guillonnet et al. 2007; Hill et al. 2007b). However, these synthetic reagents with immunosuppressive properties may also induce other cell types to express IDO that help suppress allograft rejection.

Even if the ability to promote increased Treg differentiation to control T cell responses is an important attribute of IDO⁺ pDCs, this may not be sufficient to create suppressive microenvironments because mature Tregs are functionally quiescent and require additional activation signals (via the TCR) to stimulate suppressor functions (Thornton et al. 2004). Nevertheless, Tregs are constitutively activated and possessed potent suppressor functions when isolated from TDLNs of mice bearing melanomas, suggesting that tumor growth created physiologic conditions in which Tregs received activation signals. Consistent with this notion, IDO⁺ pDCs from TDLNs activated quiescent splenic Tregs to acquire potent suppressor functions *ex vivo*, and this response was mediated via GCN2 since adding excess tryptophan and ablating GCN2 in Tregs blocked IDO-induced Treg activation (Sharma et al. 2007). In this experimental system, the suppressor functions of IDO-activated Tregs were dependent on the PD-1 pathway because antibodies that blocked interactions between PD-1 and its ligands (PD-L1 and PD-L2) abrogated suppression completely. In contrast, splenic Tregs activated by standard mitogenic anti-CD3 treatment were less potent suppressors, and blockade of the PD-1 pathway had no effect on suppressor functions. Hence, dependence on

PD-1 was a unique feature of IDO-activated Tregs. It remains to be seen if IDO⁺ pDCs are also responsible (at least in part) for activating Tregs in other chronic inflammatory syndromes in which Tregs possess constitutive suppressor activity such as local sites of *Leishmania* infections (Peters and Sacks 2006), the GI-tract (Mrass and Weninger 2006) and the maternal-fetal interface during pregnancy (Hunt 2006), to name only a few syndromes in which pDCs expressing IDO may actively suppress T cell responses.

6 Summary and Conclusions

In this chapter, we describe subsets of pDCs specialized to express IDO, which acquire potent T cell suppressor functions when induced to express IDO. The discovery of IDO⁺ pDCs is generating considerable interest in manipulating IDO expression to improve clinical outcomes in a wide range of chronic inflammatory syndromes, including cancer, chronic infections, autoimmune disease and transplantation. Clinical use of IDO inhibitors to enhance anti-tumor immunity is the subject of ongoing experimental trials, based on encouraging evidence that such treatments work in murine tumor models. If successful in humans, the use of IDO inhibitors to block IDO-mediated T cell suppression may be applicable to chronic infectious diseases in which T cell hypo-responsiveness is a major contributory factor to disease progression and persistence. Experimental evidence is also emerging in support of the hypothesis that increased IDO activity will suppress host T cell immunity allo-grafts following transplantation of healthy donor tissues. Hence, the ability of some DCs to express IDO offers considerable opportunities to improve immunotherapies in a wide range of disease syndromes that affect many people.

List of Abbreviations

1MT	1-methyl-tryptophan
HAA	3-Hydroxyanthranilic Acid
APC	Antigen Presenting Cells
AF	Auto Fluorescent
BMDCs	Bone Marrow-Derived Dendritic Cells
CHOP	CCAAT/enhancer-binding protein homologous protein
COX2	Cyclooxygenase 2
CTLA4	Cytotoxic T-Lymphocyte Antigen 4
DTH	Delayed Type Hypersensitivity
DCs	Dendritic Cells
D-1MT	D-isomer of 1MT
EAE	Experimental Autoimmune Encephalitis
FACS	Fluorescence Activated Cell Sorting

FSC	Forward Scatter
GAS	Gamma Interferon Associated Sites
GCN2	General Control Non-derepressible 2
GITR	Glucocorticoid - Induced Tumor Necrosis Factor Receptor
IFN$\alpha\beta$	IFN type I
IFNγ	IFN type II
IHC	Immunohistochemistry
IDO	Indoleamine 2,3 dioxygenase
ISR	Integrated Stress Response
IFNAR	Interferon Alpha Receptor
IRF	Interferon Regulatory Factor
ISGF3	Interferon Stimulated Gene Factor 3
ISRE	Interferon Stimulated Response Elements
IFNs	Interferons
JAK	Janus Activated Kinase
L-1MT	L-isomer of 1MT
MACS	Magnetic Activated Cell Sorting
mTOR	mammalian Target of Rapamycin
MLRs	Mixed Lymphocyte Reactions
mAb	Monoclonal Antibodies
MS	Multiple Sclerosis
MDSCs	Myeloid - Derived Suppressor Cells
mDC	Myeloid Dendritic Cell
NO	Nitric Oxide
PBMC	Peripheral blood mononuclear cells
pDC	Plasmacytoid Dendritic Cell
PGE2	Prostaglandin E2
ROS	Reactive Oxygen Species
Tregs	Regulatory T cells

SSC	Side Scatter
STAT	Signal Transducer and Activator of Transcription
SOCS3	Suppressor of Cytokine Signaling 3
TCR	T cell receptor
TLR	Toll-like receptor
TDO	Tryptophan 2,3-dioxygenase
TDLNs	Tumor Draining Lymph Nodes

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Fig. 1.
IDO Biochemistry & Biology. Overview of the cellular responses to IDO activation resulting from decrease in tryptophan and reactive oxygen species and increased production of kynurenines. See text for details.

Fig. 2.

Potential signaling events downstream of TLR and B7 ligation that induce IDO-competent pDCs to express IDO. **A.** TLR4, TLR9 and B7 ligands stimulate IFN α transcription in pDCs via the distinct signaling pathways depicted (see text for details). **B.** IFN α signaling through IFNAR induces formation of ISGF3 complexes containing activated STAT1-STAT2 heterodimers and IRF9 that act on ISRE motifs in IDO promoters. In contrast IFN γ induces IDO via STAT1-homodimers that bind to GAS elements. STAT-dependent signaling is suppressed by SOCS3 and DAP12 in most DCs.

Fig. 3. Analysis of MACS Enriched CD11c⁺ CD19⁺ DC Populations. A. Five-fold enrichment of CD11c⁺ DCs resulting from typical MACS procedure. AF cells shown in orange. B. Effects of strategic light scatter gating techniques to eliminate AF cells. Upper panels indicate elimination of CD19⁺ IDO-competent DCs (shown in red). Application of wider light scatter gates reveals the location of CD19⁺ pDCs. Conventional pDCs shown in green and myeloid mDCs shown in blue.

Fig. 4. IDO-mediated effects on T cells and Tregs. Tryptophan catabolism and the resulting metabolites produce different cellular responses in effector and regulatory T cells. See text for details.

Table 1

Reagents that inhibit or reduce enzyme IDO activity

Mode of Action	Reagent (inhibitor)	Reference
Competitive Inhibitor	1-Methyl-Tryptophan	(Cady and Sono 1991) (Agaugue et al. 2006) (Hou et al. 2007) (Metz et al. 2007)
	Brassinin	(Gaspari et al. 2006) (Banerjee et al. 2007)
	NSC 401366	(Vottero et al. 2006)
Antioxidants	Epigallocatechingallate	(Jeong et al. 2007)
	Rosmarinic acid	(Lee et al. 2007)
	p-Coumaric acid	(Kim et al. 2007)
	Pyrrolidine dithiocarbamate	(Thomas and Stocker 1999) (Thomas et al. 2001)
Metabolites	Nitric Oxide	(Thomas et al. 1994) (Samelson-Jones and Yeh 2006) (Thomas et al. 2007)
	Hydrogen Peroxide	(Poljak et al. 2006)
	Peroxynitrite	(Fujigaki et al. 2006)
	N-chlorotaurine	(Wirleitner et al. 2004)
Pharmacologic	Celecoxib	(Basu et al. 2006)
	Zebularine	(Liu et al. 2007)
Marine Derived Compounds	Exiguamine A	(Brastianos et al. 2006)
	Garveia annulata	(Pereira et al. 2006)

Table 2

Reagents that induce IDO activity

Agent	Ligand	Receptor	Reference
Cytokines	IFN α / β	IFNAR1, IFNAR2	(Baban et al. 2005)
	IFN γ	IFNG1, IFNGR2	(Yasui et al. 1986)
	IL-10/TGF β	IL-10R ^a	(Finger and Bluestone 2002) (Munn et al. 2002)
Fusion Proteins	CTLA4-Ig	B71/2 (CD80/86)	(Mellor et al. 2003) (Mellor et al. 2004) (Fallarino et al. 2004) (Baban et al. 2005) (Fallarino et al. 2005) (Manlapat et al. 2007)
	CD28-Ig ^b	B71/2 (CD80/86)	(Fallarino et al. 2005)
	CD200-Ig	CD200R	(Fallarino et al. 2004) (Fallarino et al. 2005)
TLR Ligands	GITR-Ig	GITRL	(Grohmann et al. 2007)
	Anti B71/2 mAb	B71/2 (CD80/86)	(Munn et al. 2004b)
	LPS	TLR4	(Jung et al. 2007) (Penberthy 2007)
	Resiquimod,R848	TLR7	(Furset et al. 2007)
	CpG	TLR9	(Mellor et al. 2005) (Wingender et al. 2006) (Manlapat et al. 2007)
PGE2			(Braun et al. 2005) (von Bergwelt-Baildon et al. 2006)
HDAC Inhibitors SAHA/ITF2357			(Reddy et al. 2007)

^aIL10 (and TGF β) sustains IDO expression in IFN γ activated mature DCs.^bIn conventional pDCs treated with siRNA to silence SOCS3.

Table 3A

Phenotypic analyses of murine DC subsets

DC subset ^a	% Total DCs*	CD11c	B220 (CD45R)	CD8α	CD19	120G8	CCR6	B71/2 (CD80/86)	References
Myeloid (mDCs)	~50%	high	–	–	–	–	Int.	Inducible	(Shortman and Naik 2007)
Plasmacytoid (pDCs)	~40%	low	+	+	–	+	low	Inducible	(Asselin-Paturel et al. 2003)
IDO-competent (CD19 ⁺) pDCs	5–10%	high	+	+	+	–	high	high	(Mellor et al. 2004) (Mellor et al. 2005) (Baban et al. 2005) (Manlapat et al. 2007)

^aBased on analyses of murine splenic DCs.

Table 3B

Phenotypic analyses of human DC subsets

DC Subset ^b	CD11c	CD123	CCR6	CD4	BDCA2	References
Myeloid (mDCs)	+	-	?	+	-	(Dzionek et al. 2000) (Nair et al. 2004)
Plasmacytoid (pDCs)	-	+	?	+	+	(Dzionek et al. 2000) (Patterson et al. 2001) (Hochrein et al. 2002) (Janke et al. 2006) (Cravens et al. 2007) (Grage-Griebenow et al. 2007)
IDO-competent (CD19 ⁺) pDCs	-	+	+	+	+	(Munn et al. 2002) (Boasso et al. 2005) (Boasso et al. 2007)

^bBased on analyses of human DCs derived from pBMCs; leukocyte lineage (lin) negative (null for CD3, CD14, CD19, CD56) and HLA-DR, CD4 or CD33 positive.

Table 4

Strain-specific responses to three classes of TLR9 ligands (CpGs)

Mouse strain	Type A (2336)		Type B (1826)		Type C (2395)	
	IFN α^a	IDO b	IFN α	IDO	IFN α	IDO
C57BL/6/J	nt ^c	-	+	+	+	+
CBA	nt	-	+	+	nt	+
BALB/c	+	-	+	+	-	+
129	nt	-	+	-	+	+

^aIFN α production assessed *ex vivo* (ELISA) following 5 hrs culture of CD11c⁺ AMACS enriched DCs with indicated ligand.

^bIDO expression assessed (IHC) 24 hrs treatment *in vivo* (50-100 μ g/mouse, i/v).

^cnot tested.