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## **IL-2 Phosphorylates STAT5 To Drive IFN- $\gamma$ Production and Activation of Human Dendritic Cells**

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# IL-2 Phosphorylates STAT5 To Drive IFN- $\gamma$ Production and Activation of Human Dendritic Cells

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Human dendritic cells (hDCs) produce IL-2 and express IL-2R  $\alpha$ -chain (CD25), but the role of IL-2 in DC functions is not well defined. A recent study suggested that the main function of CD25 on hDCs was to transpresent IL-2 to activate T lymphocytes. Our results demonstrate the expression of the three chains of the IL-2R on hDCs and that IL-2 induces STAT5 phosphorylation. Interestingly, use of inhibitors of p-STAT5 revealed that IL-2 increases LPS-induced IFN- $\gamma$  through STAT5 phosphorylation. Finally, we report that IL-2 increases the ability of hDCs to activate helpless CD8<sup>+</sup> T cells, most likely because of IL-2-triggered IFN- $\gamma$  synthesis, as we previously described. For the first time, to our knowledge, we disclose that IL-2 induces monocyte-derived hDC's functional maturation and activation through IL-2R binding. Interestingly, our study suggests a direct effect of anti-CD25 mAbs on hDCs that may contribute to their clinical efficacy. *The Journal of Immunology*, 2014, 192: 5660–5670.

The cytokine IL-2, first known as T cell growth factor (1), is considered mainly synthesized by T cells in response to Ags. IL-2 is a four-bundle  $\alpha$ -helical cytokine promoting the development and homeostasis of thymic regulatory T cells (2) and activating memory T lymphocytes as well as B cells and NK cells.

IL-2R consists of three chains. The  $\beta$ -chain (CD122, shared with IL-2 and IL-15 receptors) and  $\gamma$ -chain (CD132, common to IL-2, -4, -7, -9, -15, and -21 receptors) transduce the signal, and the  $\alpha$ -chain (CD25) stabilizes the complex to create the high-affinity form of the receptor (3). CD25 expression is tightly regulated in target cells and greatly enhances the IL-2 signaling effect in cells expressing high levels of this molecule (4). IL-2 binding to CD25 triggers the grouping with IL-2R  $\beta$  and  $\gamma$ -chains, thus leading to signal transduction through STAT5, MAPK, and PI3K (5–8).

Anti-CD25 mAbs blocking the IL-2 binding site of CD25 (9) have been designed to inhibit T cell activation and are widely used in organ transplantation to prevent acute allograft rejection (10–12). Thus, their effects on human T lymphocytes have been extensively studied (13, 14). In contrast, their impact on human

dendritic cells (hDCs) has been seldomly investigated, and the role of IL-2 in hDCs has not been firmly established (15, 16). IL-2 secretion by DCs starts early after activation by TLR ligands or CD40L and is necessary to the optimal proliferation of T lymphocytes (17–19). We previously demonstrated that IL-2 could modulate several hDCs functions but did not investigate the precise mechanisms (15). CD25 was previously suggested to function as a transpresenter of IL-2 to T lymphocytes in hDCs (16).

In this study, we report on another mechanism involving a direct effect of IL-2 on hDC signaling. IL-2 greatly increases IFN- $\gamma$  production in hDCs by phosphorylating STAT5, thus leading to increased ability of hDCs to activate helpless CD8<sup>+</sup> T lymphocyte cytotoxicity.

## Materials and Methods

### Generation of human monocyte-derived DCs

PBMCs were obtained from cytophoresis of healthy volunteers who gave their informed written consent for use of blood. PBMCs were isolated by Ficoll Hypaque density-gradient centrifugation (Lymphoprep; AbCys), and monocytes were isolated by positive selection (Miltenyi Biotec; purity >95%) or obtained from elutriation (for Western blot experiments) (purity >97%). Monocytes were then plated in RPMI 1640 containing 10% inactivated FCS (Life Technologies), 50 IU/ml penicillin, 50 IU/ml streptomycin (Life Technologies), 2 mM L-glutamine (Life Technologies) (complete medium), 25 ng/ml recombinant human (rh)IL-4 (Miltenyi Biotec), and 333 IU/ml rhGM-CSF (Miltenyi Biotec). At day 6, immature hDCs were washed, and the hDC phenotype was characterized by staining for CD14 and DC-specific ICAM3-grabbing nonintegrin. For quantitative PCR (qPCR), immature hDCs were selected again by negative selection (Miltenyi Biotec; purity >98%). Then hDCs were stimulated for an additional 48 h with LPS (50 ng/ml; Invitrogen), TNF- $\alpha$  (20 ng/ml; R&D Systems), PGE<sub>2</sub> (1  $\mu$ g/ml; Merck), IL-6 (150 ng/ml; PeproTech), IL-1 $\beta$  (10 ng/ml; PeproTech), or TNF- $\alpha$  (50 ng/ml; PeproTech). At day 6, hDCs were treated with anti-CD25 (basiliximab; Novartis, 100  $\mu$ g/ml or the indicated doses), control Ab (rituximab; 100  $\mu$ g/ml or the indicated dose), anti-CD25 F(ab')<sub>2</sub> (100  $\mu$ g/ml), LY294002 (1  $\mu$ M), rapamycin (10  $\mu$ M), pimeozide (1  $\mu$ M; all Sigma-Aldrich), or N'-(11H-indolo[3,2-c]quinolin-6-yl)-N,N-dimethylethane-1,2-diamine (IQDMA; Calbiochem; 2  $\mu$ M) for 30 min before IL-2 (100 U/ml or the indicated doses; R&D Systems).

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Abbreviations used in this article: DC, dendritic cell; hDC, human dendritic cell; IQDMA, N'-(11H-indolo[3,2-c]quinolin-6-yl)-N,N-dimethylethane-1,2-diamine; LPS-hDC, LPS-treated hDC; qPCR, quantitative PCR; rh, recombinant human; TNF- $\alpha$ -hDC, TNF- $\alpha$ -treated hDC.

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### Isolation of CD8<sup>+</sup> T cells and allogeneic MLR

Allogeneic CD8<sup>+</sup> T cells were obtained from PBMCs of subjects unrelated to hDC donors by positive selection (Dynabeads; Invitrogen; purity >95%). For proliferation assay, lymphocytes were stained with 3  $\mu$ M CFSE (Sigma-Aldrich) in 5% FCS for 30 min at 37°C and washed three times in complete medium. At day 8, hDCs were washed and mixed with CD8<sup>+</sup> T cells at a 1:5 ratio. After 6 d of coculture, CFSE dilution, phenotype, and intracellular cytokine production were analyzed by FACS. In some experiments, anti-IFN- $\gamma$  Abs were added at days 1 and 4 of coculture (10  $\mu$ g/ml; R&D Systems).

### F(ab')<sub>2</sub> preparation

The F(ab')<sub>2</sub> fragment was prepared by use of a kit (Pierce Protein Biology Product; Thermo Scientific). The purity of F(ab')<sub>2</sub> was assessed by SDS-PAGE and then Coomassie brilliant blue staining.

### Cytotoxicity assay

Cytotoxicity of activated CD8<sup>+</sup> T lymphocytes was assessed with PBMCs from the same donors of hDCs. PBMCs were stained with 3  $\mu$ M CFSE in 5% FCS for 30 min at 37°C and washed three times in complete medium. At day 6, activated CD8<sup>+</sup> T lymphocytes were mixed with PBMCs at a 1:10 ratio for 7 h before being stained with 7-aminoactinomycin D (Sigma-Aldrich) and undergoing FACS analysis. Percentage specific lysis was normalized by basal lysis, determined by staining of PBMCs incubated alone, as follows: percentage of specific lysis = (100  $\times$  [percent sample lysis - percent basal lysis]) / (100 - percent basal lysis).

### ELISA of cytokine production assay

ELISA (eBioscience) was used to determine IFN- $\gamma$ , IL-12, IL-6, or TNF- $\alpha$  concentration in culture supernatant. Samples were tested in duplicate and quantified against standard curves. Optical densities were measured by use of an ELISA plate reader at 450-nm wavelength. The results are presented as the means of duplicate wells.

### Flow cytometry

A total of  $1 \times 10^5$  hDCs or CD8<sup>+</sup> T cells were incubated with fluorescent-conjugated Abs at 4°C for 30 min for extracellular staining. The Abs for CD25-allophycocyanin (clone M-A251; IgG1), CD83-FITC (clone HB15e; IgG1), CD86-PE Cy5 (clone 2331; IgG1), CCR5-allophycocyanin (clone 2D7/CCR5; IgG2a), CD107a-FITC (clone H4A3; IgG1), CD122-PE (clone TU27, IgG1, or Mik- $\beta$ 2; IgG2a), CD132-PE (clone AG184; IgG1), HLA-ABC-FITC (clone G46-2.6; IgG1), HLA-DR-allophycocyanin (clone G46-6; IgG2a), and isotype controls were from BD Biosciences; CD80-PE (clone MAB104; IgG1), CD8-FITC (clone B9.11; IgG1), DC-specific ICAM3-grabbing nonintegrin-PE (clone AZND1; IgG1), CD14-PE (clone RMO52; IgG2a), CD122-FITC (clone 2RB; IgG1), and isotype controls were from Beckman Coulter; and CCR7-PE (clone 150503; IgG2a) and isotype controls were from R&D Systems. For intracellular staining, cells were incubated with GolgiStop solution for the last 4 h and permeabilized with use of a Cytotfix/Cytoperm kit (BD Biosciences). IFN- $\gamma$ -allophycocyanin (clone B27; IgG1), granzyme B-FITC (clone GB11; IgG1), granzyme A-PE (clone CB9; IgG1), and isotype controls were from BD Biosciences. Cells were analyzed with use of a FACSCanto (BD Biosciences), and a proportion of marker-positive cells and geometric mean fluorescence intensity was determined by use of Diva software (BD Biosciences).

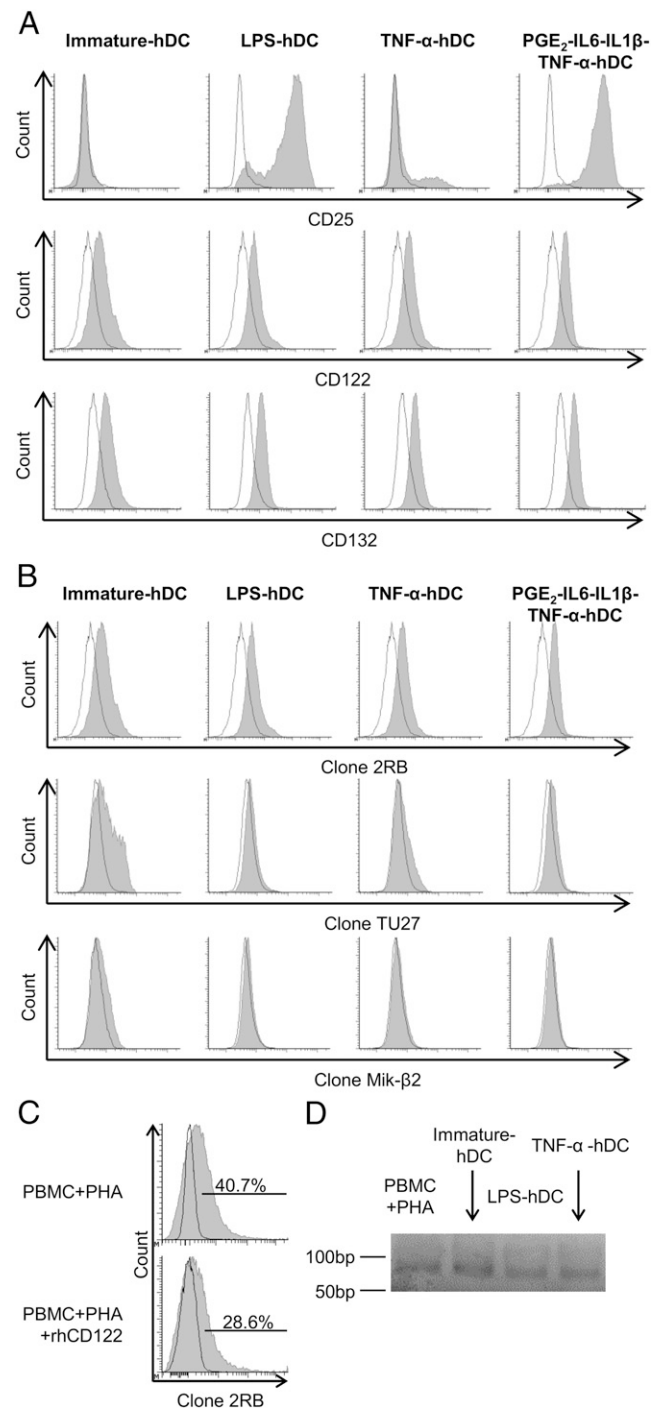
### Western blot analysis

At day 8, hDCs were washed and plated in complete medium for 3 h before treatment, and the maturation phenotype was analyzed by FACS. hDCs were washed at 4°C and lysed in Laemmli buffer (Bio-Rad). Cell extracts were separated by SDS-PAGE and blotted onto nitrocellulose membrane (Amersham Biosciences), which was incubated with Abs against phosphorylated AKT (p-Ser<sup>473</sup>, p-Thr<sup>308</sup>), total AKT, phosphorylated ERK (p-Thr<sup>202/204</sup>), total ERK, STAT5 (p-Tyr<sup>694</sup>), and total STAT5 (Cell Signaling Technology; STAT5a and STAT5b were from Invitrogen). Membranes were developed with the ECL detection system (GE Healthcare) with use of specific peroxidase-conjugated anti-IgG Abs (anti-goat IgG from Santa Cruz Biotechnology, anti-mouse IgG from GE Healthcare, and anti-rabbit IgG from Cell Signaling Technology). Band intensities were quantified by use of ImageJ (National Institutes of Health).

### PCR and qPCR

mRNA was isolated from hDCs and PBMCs by use of the RNeasy mini kit (Qiagen). An amount of 3.33  $\mu$ g total RNA was used to synthesize cDNA

(Invitrogen) after DNase treatment (Invitrogen). Sequences for primers were for CD122 (forward 5'-TTGAGAACCTTCGCCTGAT-3', reverse 5'-TAGTGGGAGGCTTGGGAGAT-3'), IFN- $\gamma$  (forward 5'-TAGCGGAT-



**FIGURE 1.** Mature hDCs express high-affinity IL-2R. **(A)** Surface expression of CD25, CD122, and CD132 on immature and mature hDCs (data are representative of three independent donors). Gray-filled histograms indicate stained hDCs, and open histograms represent the appropriate isotype controls. **(B)** Staining of immature and mature hDCs with three different Abs against CD122. Open histograms represent the appropriate isotype control (data are representative of two donors). **(C)** Staining of PHA-activated PBMCs with 2RB Ab against CD122 with or without rhCD122 competition. Gray-filled histograms indicate stained hDCs, and open histograms represent the appropriate isotype control (data are representative of two donors). **(D)** CD122 mRNA expression in PHA-treated PBMCs, immature hDCs, LPS-hDCs, and TNF- $\alpha$ -hDCs assessed by RT-PCR (data are representative of two donors).



AATGGAACCTCTT-3', reverse 5'-CTCCTTTTGGATCTCTTGGTC-3'), CD25 (forward 5'-ACCTGCTGATGTGGGGACTG-3', reverse 5'-TTG-AATGTGGCGTGTGGGAT-3'), GAPDH (forward 5'-CTGCACCAC-CAACTGCTTAG-3', reverse 5'-GTCTTCTGGGTGGCAGTAT-3'), and 18S (forward 5'-CGCCGCTAGAGGTGAAATTCT-3', reverse 5'-CGAACCTCCGACTTTCGTTCT-3') designed with use of Vector NTI (Invitrogen). RT-PCR was performed with the resulting cDNA using the CD122 primers. PCR was carried out with Thermo Cycler (Bio-Rad) at 95°C (4 min) and 40 cycles of 94°C (30 s), 59°C (30 s), 74°C (30 s), and 74°C (4 min). The PCR products were electrophoresed on 2% agarose gels containing 0.2 µg/ml ethidium bromide (Bio-Rad). qPCR involved use of LightCycler 480 (Roche) with SYBR Green (Invitrogen) at 95°C (2 min) and 50 cycles of 95°C (15 s), 60°C (30 s), 72°C (30 s), 72°C (5 min), and 4°C (5 min). Results were analyzed by use of LightCycler 480 software (Roche).

### Statistical analyses

Data are expressed as mean ± SD, or donor values and their mean are represented by a symbol. Analysis involved the paired nonparametric Wilcoxon test. Statistical significance was considered as  $p < 0.05$ .

## Results

### Mature hDCs express the three chains of IL-2R

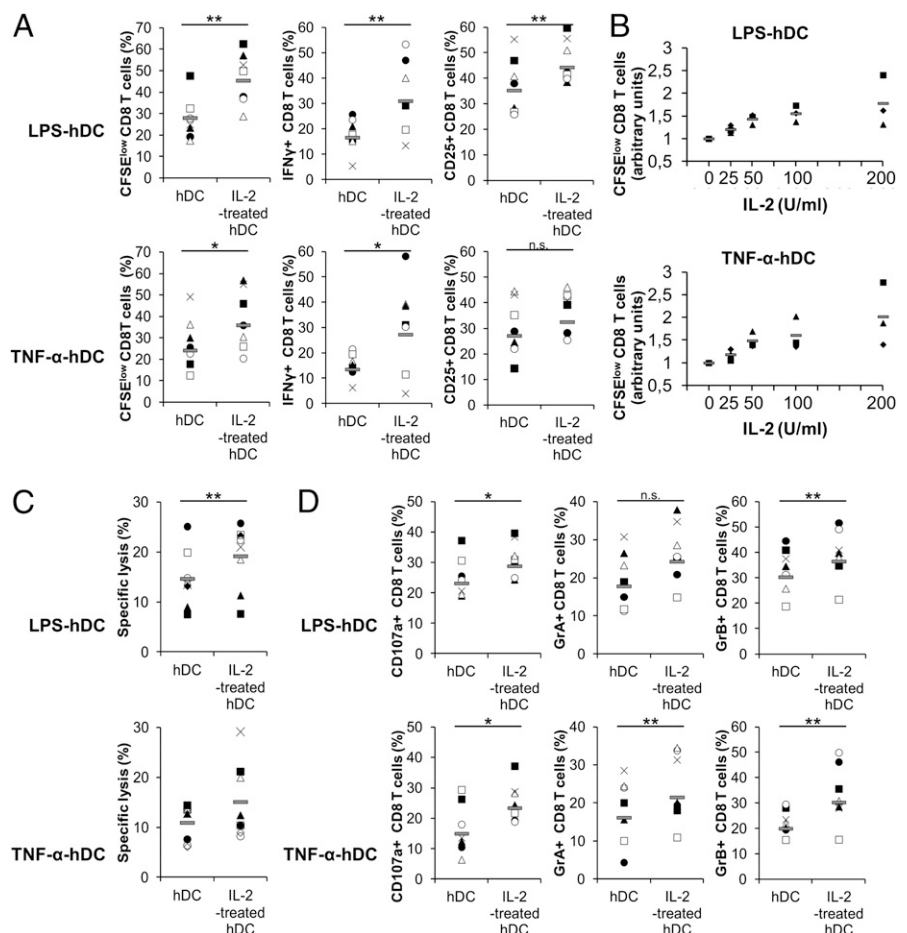
Whether hDCs express the three chains of IL-2R, in particular CD122 (15, 16, 20–23), remained controversial, so we explored CD132, CD122, and CD25 expression in immature hDCs and after 48-h maturation with different agents: LPS, TNF-α, and the mixture of inflammatory cytokines used by Wuest et al. (16) (PGE<sub>2</sub>, IL-6, IL-1β, and TNF-α). CD132 and CD122 were constitutively expressed on immature hDCs, and their expression was not modified with maturation (Fig. 1A). CD25 expression

was absent in immature hDCs, and LPS or the cytokine mixture strongly induced CD25 during cell maturation (Fig. 1A). In contrast, TNF-α weakly induced CD25 (Fig. 1B). Because of contradictory reports of the expression of CD122 by hDCs, we tested three commercially available Abs for CD122. As compared with 2RB, Mik-β2 and TU27 induced a lower shift of hDC or PBMC staining (Fig. 1B and data not shown). We therefore tested the specificity of 2RB Ab by rhCD122 competition in activated PBMCs. rhCD122 decreased the staining of anti-CD122 Ab (Fig. 1C), which demonstrates the specificity of the 2RB clone. Finally, RT-PCR of highly purified immature hDCs, LPS-treated hDCs (hereafter LPS-hDCs), or TNF-α-treated hDCs (hereafter TNF-α-hDCs) demonstrated transcription of the IL2RB gene (encoding CD122 protein) (Fig. 1D), which confirms CD122 expression in hDCs.

### IL-2 increases the capacity of hDCs to activate allogeneic CD8<sup>+</sup> T cells without affecting hDC surface maturation phenotype

Allogeneic CD8<sup>+</sup> T lymphocytes were incubated for 5 d with LPS-hDCs or TNF-α-hDCs with and without IL-2 pretreatment during maturation. IL-2-treated hDCs produced increased proliferation of CD8<sup>+</sup> T cells, as measured by CFSE dilution, which was dose dependent (Fig. 2A, 2B). Furthermore, IL-2-treated hDCs produced increased expression of membrane CD25 and intracellular IFN-γ in CD8<sup>+</sup> T cells (Fig. 2A, Supplemental Fig. 1). IL-2 increased the capacity of hDCs to activate the cytotoxic functions of allogeneic CD8<sup>+</sup> T cells, as assessed by specific lysis of PBMCs

**FIGURE 2.** IL-2 increases the ability of hDCs to activate allogeneic CD8<sup>+</sup> T lymphocytes. **(A)** Proliferation of CD8<sup>+</sup> T lymphocytes as assessed by CFSE (left panel), surface expression of CD25 (middle panel), and intracellular expression of IFN-γ (right panel) for CD8<sup>+</sup> T lymphocytes activated by mature hDCs with or without rhIL-2 pretreatment (100 U/ml) during maturation. Each symbol represents an experiment, and gray bars are mean of seven independent donors. CD8<sup>+</sup> T cells activated by LPS-hDCs (top panel) and TNF-α-hDCs (bottom panel). **(B)** Proliferation of CD8<sup>+</sup> T lymphocytes cultured with LPS-hDCs or TNF-α-hDCs pretreated with increasing doses of rhIL-2. Gray bars are the mean of three independent donors (data for untreated donor cells were normalized to 1). **(C)** Lysis of allogeneic PBMCs by CD8<sup>+</sup> T cells with hDCs presensitized with or without rhIL-2 treatment. Specific lysis =  $(100 \times (\text{percent sample lysis} - \text{percent basal lysis})) / (100 - \text{percent basal lysis})$ . Each symbol represents an experiment, and gray bars are mean of 8 independent donors. **(D)** Surface expression of CD107a (left panel) and intracellular expression of granzyme A (middle panel), and granzyme B (right panel) on CD8<sup>+</sup> T lymphocytes activated by mature LPS-hDCs (top panel) and TNF-α-hDCs (bottom panel) with or without rhIL-2 pretreatment. Gray bars represent the mean of seven donors. \* $p < 0.05$ , \*\* $p < 0.01$ , Wilcoxon analysis.

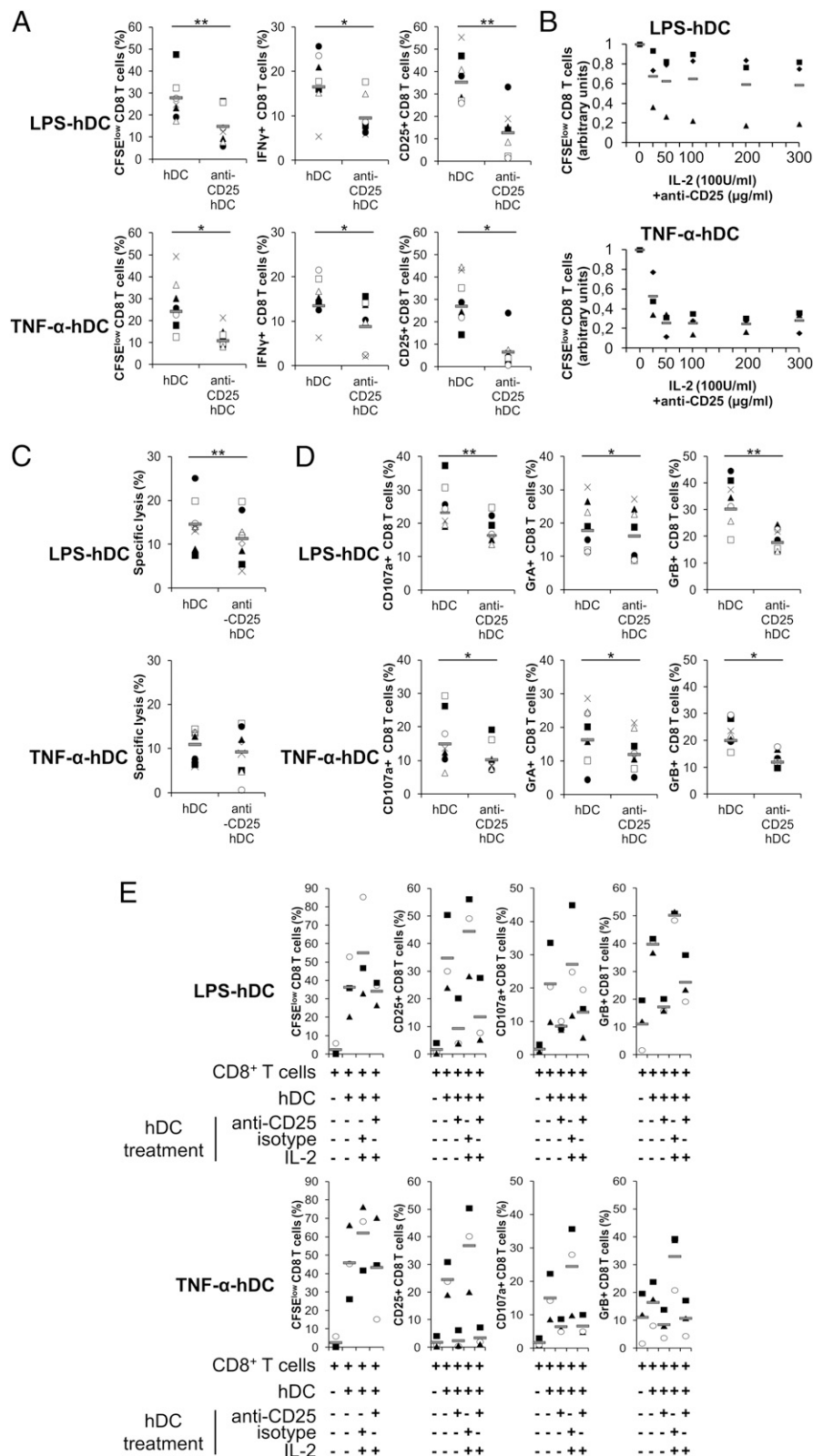


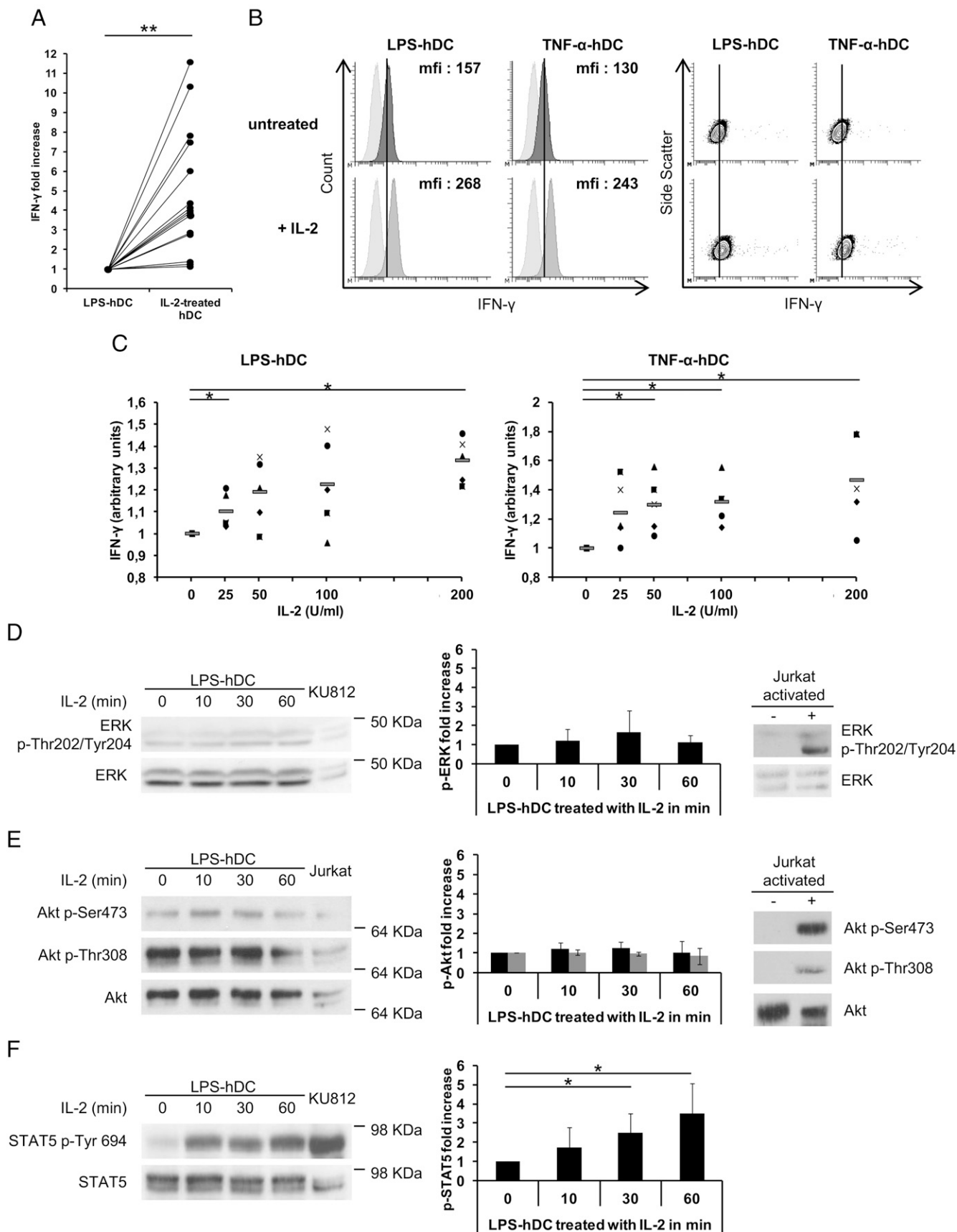
autologous to the donor of hDCs or expression of CD107a, granzyme A, or granzyme B (Fig. 2C, 2D).

We tested the ability of a therapeutic mAb anti-CD25 to modulate hDC functions and found that proliferation of CD8<sup>+</sup> T lymphocytes and expression of CD25 and IFN- $\gamma$  in CD8<sup>+</sup> T cells were reduced with anti-CD25 Ab as compared with no

treatment (Fig. 3A). Anti-CD25 dose-dependently inhibited the IL-2-mediated increase in capacity of TNF- $\alpha$ -hDCs to activate CD8<sup>+</sup> T cells (Fig. 3B). We also tested CD8<sup>+</sup> T lymphocyte cytotoxicity induced by anti-CD25-treated hDCs. Ag-specific cytotoxicity as well as the expression of CD107a and granzyme A and B were reduced with anti-CD25 pretreatment of hDCs

**FIGURE 3.** Anti-CD25 Ab decreases hDC capacity to activate CD8<sup>+</sup> T lymphocytes. **(A)** Proliferation of CD8<sup>+</sup> T cells (left panel), surface expression of CD25 (right panel), and intracellular expression of IFN- $\gamma$  (middle panel) on CD8<sup>+</sup> T lymphocytes activated by mature LPS-hDCs (top panel) and TNF- $\alpha$ -hDCs (bottom panel) treated with or without anti-CD25 (100  $\mu$ g/ml) during maturation. Each symbol represents an experiment. Gray bars are the mean of seven donors. **(B)** Proliferation of CD8<sup>+</sup> T lymphocytes cultured with LPS-hDCs (top panel) or TNF- $\alpha$ -hDCs (bottom panel) pretreated with rhIL-2, and increasing doses of anti-CD25 Ab hDC were treated with during the maturation process (pretreatment). Gray bars are the mean of three independent donors (data for IL-2-treated cells were normalized to 1). **(C)** Percentage of specific lysis induced by CD8<sup>+</sup> T cells cultured with hDCs pretreated with or without anti-CD25 Ab as assessed in Fig. 2C. Each symbol represents an experiment, and gray bars are mean of eight independent donors. **(D)** Surface expression of CD107a (left panel) and intracellular expression of granzyme A (middle panel) and granzyme B (right panel) in CD8<sup>+</sup> T lymphocytes activated by mature LPS-hDCs (top panel) and TNF- $\alpha$ -hDCs (bottom panel) pretreated with or without anti-CD25 only during maturation. Gray bars are the mean of seven donors. **(E)** Proliferation of CD8<sup>+</sup> T cells (left panel), expression of CD25 (middle left panel), CD107a (middle right panel), and granzyme B (right panel) on CD8<sup>+</sup> T lymphocytes activated or not by LPS-hDCs (top panel) or TNF- $\alpha$ -hDCs (bottom panel) pretreated with or without anti-CD25 and rhIL-2 during maturation. Each symbol represents an experiment. Gray bars are the mean of three donors. \* $p$  < 0.05, \*\* $p$  < 0.01, Wilcoxon analysis.





**FIGURE 4.** IL-2 increases IFN- $\gamma$  synthesis and STAT5 phosphorylation in hDCs. **(A)** ELISA of IFN- $\gamma$  secretion by LPS-hDCs in culture supernatants with or without rhIL-2 treatment during maturation. Each symbol represents one donor, with data from 15 donors (data for untreated donor cells were normalized to 1). **(B)** Intracellular staining of IFN- $\gamma$  in LPS-hDCs or TNF- $\alpha$ -hDCs with or without rhIL-2. Black histograms represent untreated cells, dark gray rhIL-2-treated cells, and light gray the isotype control (data are representative of five independent donors). **(C)** Intracellular IFN- $\gamma$  staining of LPS-hDCs (*left panel*) or TNF- $\alpha$ -hDCs (*right panel*) with increasing doses of rhIL-2. Each symbol represents an experiment, and (*Figure legend continues*)

Table I. IL-2 does not affect proinflammatory cytokine secretion by hDCs

	IL-12 (pg/ml)	IL-6 (pg/ml)	TNF- $\alpha$ (pg/ml)
LPS-hDC	1,008.0 $\pm$ 1,428.3	11,360.4 $\pm$ 5,683.8	2,637.5 $\pm$ 998.4
IL-2-treated hDC	877.1 $\pm$ 1,241.8	11,657.2 $\pm$ 6,443.0	3,814.1 $\pm$ 3,032.3
LPS-hDC versus IL-2-treated hDC	$p > 0.05$	$p > 0.05$	$p > 0.05$

IL-12, IL-6, and TNF- $\alpha$  secretion assessed by ELISA in culture supernatants of LPS-hDCs treated or not with IL-2 during maturation. Mean (pg/ml)  $\pm$  SD of 17 (IL-12) or 6 (TNF- $\alpha$  and IL-6) donors. Wilcoxon analysis.

(Fig. 3C, 3D). Of note, even in the absence of exogenous rhIL-2, anti-CD25 pretreatment inhibited the allostimulatory capacity of hDCs as assessed by specific lysis and CD107a and granzyme B expression by CD8<sup>+</sup> T cells (Fig. 3C, 3D), suggesting the presence of endogenous IL-2 in the coculture, presumably produced by allogeneic CD8<sup>+</sup> T lymphocytes. Moreover, pretreatment of hDCs with anti-CD25 Abs reduced the IL-2-mediated increase in capacity of hDCs to activate CD8<sup>+</sup> T cells (Fig. 3E). Finally, IL-2 or anti-CD25 treatment of immature or mature hDCs did not affect the maturation-induced hDC surface phenotype (CD83, CD25, CCR7, CCR5, CD80, CD86, HLA-A, -B, -C, and HLA-DR expression; Supplemental Fig. 2) or the expression of CD40, programmed cell death ligand 1, ILT3, and ILT4 (data not shown).

#### IL-2 increases the mature hDCs production of IFN- $\gamma$ via STAT5 signaling

As we previously described (24), helpless CD8<sup>+</sup> T cell activation requires IFN- $\gamma$  production by hDCs. Thus, we assessed the synthesis of IFN- $\gamma$  in IL-2-treated hDCs. IL-2 dose-dependently and strikingly increased IFN- $\gamma$  secretion from maturing hDCs (Fig. 4A–C). In contrast, IL-2 did not affect IL-12, IL-6, and TNF- $\alpha$  secretions by hDCs (Table I).

We next explored pathways leading to IFN- $\gamma$  transcription after IL-2 binding to its receptor. The main pathways involved in IL-2 transduction signals in T lymphocytes, such as JAK/STAT, MAPK, and PI3K–Akt, differ by lymphocyte subset (25, 26). Thus, we used Western blot analysis to examine these distinct pathways in LPS-hDCs, an inflammatory condition inducing high CD25 expression in hDCs. IL-2 did not induce phosphorylation of ERK or Akt (Fig. 4D, 4E). In addition, we analyzed the effects of two inhibitors of the PI3K–Akt pathway: a PI3K-specific inhibitor (LY294002) and a mammalian target of rapamycin C1 inhibitor (rapamycin). Although these inhibitors decreased the LPS-induced IFN- $\gamma$  production, neither inhibited the capacity of IL-2 to increase IFN- $\gamma$  production by hDCs (Supplemental Fig. 3A). In addition, IL-2 increased the ability of inhibitor-treated hDCs to activate CD8<sup>+</sup> T cells (Supplemental Fig. 3B).

In contrast and importantly, IL-2 induced an immediate increase in phosphorylation of STAT5 in mature hDCs (Fig. 4F). Although the STAT5 phosphorylation intensity and kinetics varied among donors (Supplemental Fig. 4A), the increased phosphorylation was always evident after 30 min of treatment and was sustained for several hours (data not shown). Of note, LPS-hDCs expressed

both forms of STAT5, STAT5a, and STAT5b, and IL-2 treatment did not affect their protein levels (Supplemental Fig. 4B). Anti-CD25 mAb could significantly reduce the phosphorylation of STAT5 when added 30 min before IL-2, which confirmed the specificity of the STAT5 activation (Fig. 5A). Furthermore, anti-CD25 Ab dose-dependently decreased IFN- $\gamma$  production in hDCs, as assessed by intracellular staining or ELISA (Fig. 5B–D). Blocking anti-CD25 Abs inhibited 55 and 100% of the IL-2-induced IFN- $\gamma$  secretion by LPS-DC and TNF- $\alpha$ -DC, respectively. Likewise, the mean inhibition of IL-2-induced IFN- $\gamma$  staining in LPS-DC and TNF- $\alpha$ -DC was 51 and 76%, respectively. Finally, use of the F(ab')<sub>2</sub> fragment had the same effect as anti-CD25 Ab, which negates any role for the Fc portion of the Ab (Fig. 5E).

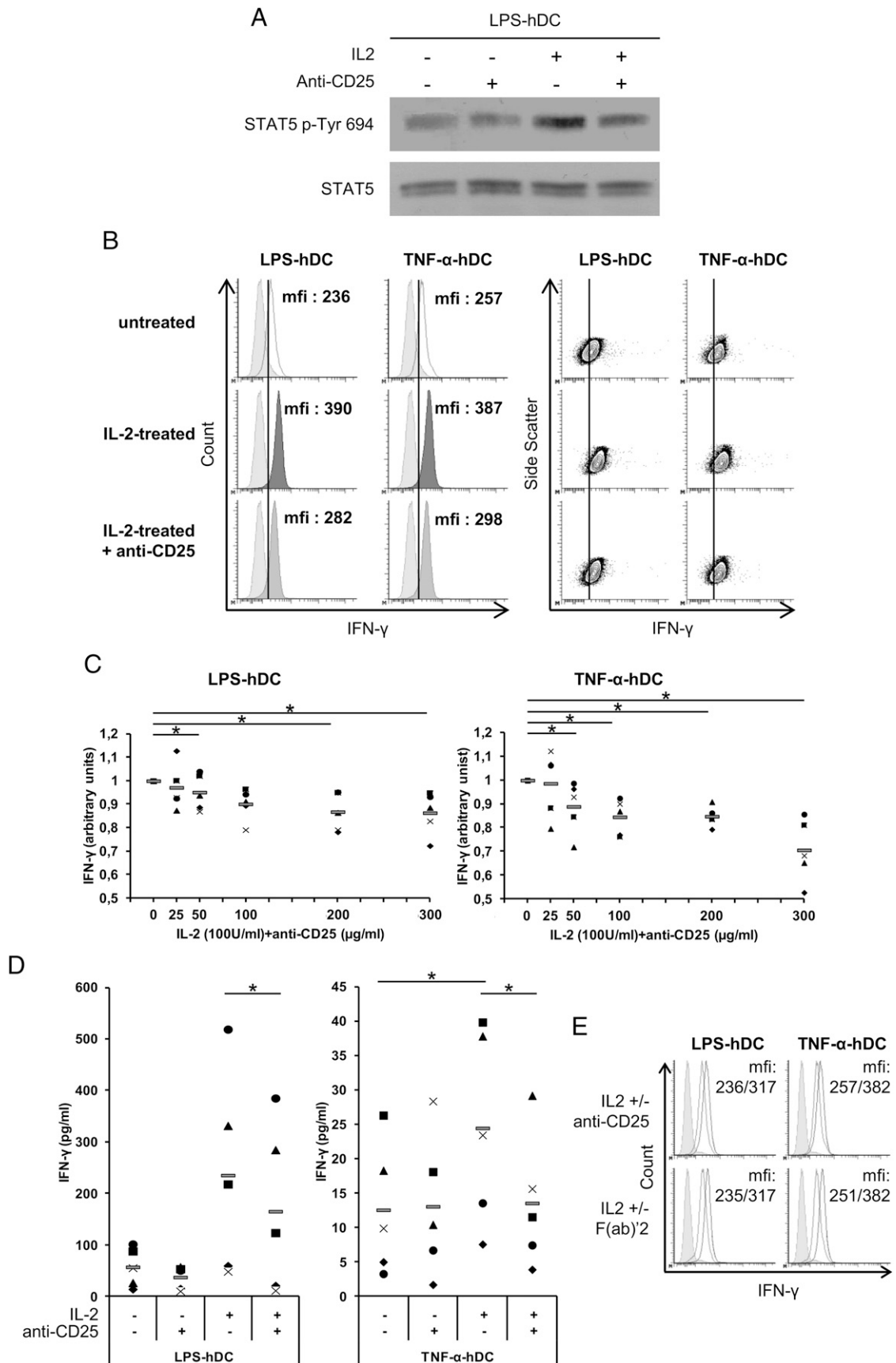
STAT5 can upregulate the promoter activity of both IL2RA (27, 28) and IFNG (29) genes (encoding CD25 and IFN- $\gamma$  protein, respectively) in T cells. We analyzed whether IL-2-dependent STAT5 phosphorylation also induced the transcription of CD25 and IFN- $\gamma$  in highly purified LPS-hDCs with IL-2 treatment with and without p-STAT5 inhibitors [pimozide (30) and IQDMA (31)]. Both inhibitors decreased STAT5 phosphorylation responding to IL-2 without impacting cell viability (Fig. 6A and data not shown). IL-2 greatly increased CD25 mRNA level, which was almost totally abrogated by the inhibitors (inhibition of 85.3  $\pm$  22.6 and 92.5  $\pm$  3.6% for pimozide and IQDMA, respectively) (Fig. 6B). Similar results were obtained for IFN- $\gamma$  (inhibition of 85.2  $\pm$  27.3 and 88.1  $\pm$  11.7%, respectively). Moreover, the p-STAT5 inhibitors decreased IFN- $\gamma$  protein synthesis, which implies that IL-2, by activating STAT5, is directly involved in IFN- $\gamma$  production (Fig. 6C). Thus, IL-2 increases the production of IFN- $\gamma$  by mature hDCs through STAT5 signaling. Finally, we explored connection between the increase of IFN- $\gamma$  secretion induced by IL-2 pretreatment and further CD8<sup>+</sup> T cell activation and cytotoxicity. As shown in Fig. 7, *left panel*, the increase of CD8<sup>+</sup> T cell proliferation was only slightly and not significantly inhibited by blocking IFN- $\gamma$  during coculture. In contrast, the increase of CD8<sup>+</sup> cytotoxicity induced by IL-2 pretreatment of LPS-hDC was abolished (Fig. 7, *right panel*). Thus, these results together indicate that IL-2/STAT5-mediated IFN- $\gamma$  increase licensed the hDC to activate cytotoxic functions in helpless CD8<sup>+</sup> T cells.

## Discussion

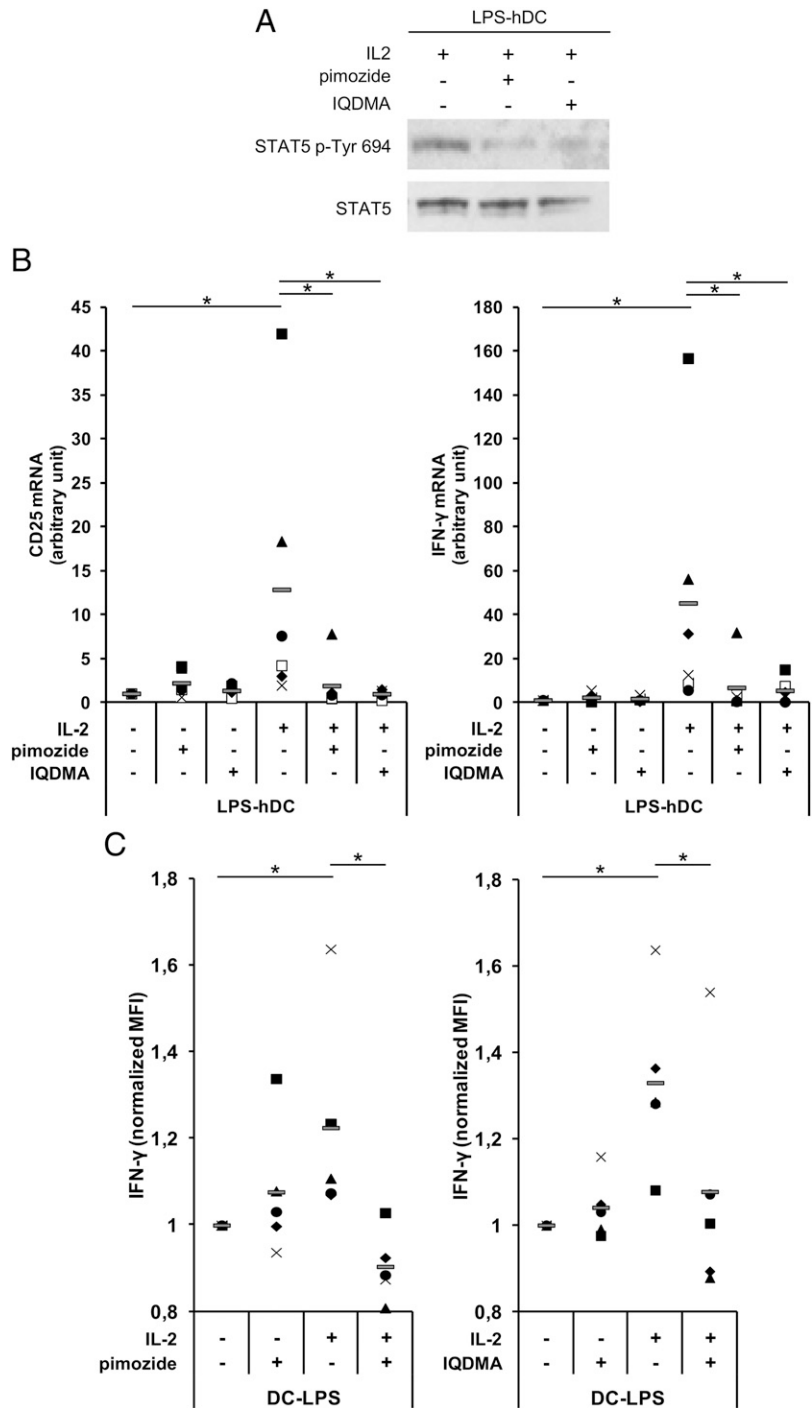
Human and mouse DCs produce IL-2 (17–19) and upregulate CD25 during maturation (15, 20, 32, 33), but the role of hDC-derived IL-2 and the effect of IL-2 on hDC activity are not well

gray bars are the mean of five independent donors. (D) Western blot analysis of ERK phosphorylation in LPS-hDCs in response to 100 U/ml rhIL-2 added after 3 h of GM-CSF and IL-4 deprivation (*left panel*), quantification of ratio of ERK p-Tyr<sup>202/204</sup> to ERK level (*middle panel*), and positive control on Jurkat cells activated or not (*right panel*). Data for untreated donor cells were normalized to 1. Data are mean  $\pm$  SD from three donors. Phosphorylation of Akt (p-Ser<sup>473</sup> and p-Thr<sup>308</sup>) (E) and STAT5 (p-Tyr<sup>694</sup>) (F) with the same protocol. Data are mean  $\pm$  SD from four (E) or five (F) experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , Wilcoxon analysis. mfi, mean fluorescence intensity.





**FIGURE 5.** Anti-CD25 Ab inhibits IL-2–induced IFN- $\gamma$  production. **(A)** Phosphorylation of STAT5 in anti-CD25–treated LPS-hDCs with rhIL-2 treatment (data are representative of three donors). **(B)** Intracellular staining of IFN- $\gamma$  in LPS-hDCs or TNF- $\alpha$ -hDCs pretreated with rhIL-2 with or without anti-CD25 blocking Ab. Empty histograms represent untreated cells, black histograms represent rhIL-2–treated cells, dark gray rhIL-2 and anti-CD25 treatment, and light gray the appropriate isotype control (data are representative of five donors). **(C)** Intracellular staining of IFN- $\gamma$  in LPS-hDCs (*left panel*) or TNF- $\alpha$ -hDCs (*right panel*) pretreated with rhIL-2 and increasing doses (*Figure legend continues*)

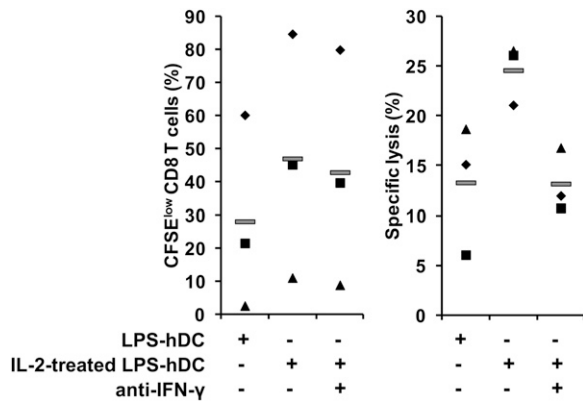


**FIGURE 6.** IL-2 induces the transcription of both CD25 and IFN- $\gamma$  genes via phosphorylation of STAT5. **(A)** Western blot analysis of STAT5 phosphorylation in LPS-hDCs in response to 100 U/ml rhIL-2 with or without treatment with pimozide or IQDMA (STAT5 inhibitors). Data are representative of two independent donors. **(B)** RT-PCR analysis of mRNA levels of CD25 (left panel) and IFN- $\gamma$  (right panel) in LPS-hDCs with and without pimozide or IQDMA and/or rhIL-2 treatment for 4 h. Each symbol represents one experiment, and gray bars are the mean of six independent donors (data for untreated donor cells were normalized to 1). **(C)** Normalized mean fluorescence intensity (MFI) of intracellular IFN- $\gamma$  staining in LPS-hDCs with and without pimozide or IQDMA and/or rhIL-2 treatment. Gray bars are the mean of five independent donors (data for untreated donor cells were normalized to 1). \* $p < 0.05$ , Wilcoxon analysis.

defined (34). In this study, we demonstrated for the first time, to our knowledge, that STAT5 phosphorylation by IL-2 has a key role in the activation of human monocyte-derived DCs. Our results are in line with a previous study by Naranjo-Gómez et al. (33),

who reported that treatment of plasmacytoid hDCs with IL-2 increased the secretion of several proinflammatory cytokines. However, these authors did not analyze the signaling downstream the IL-2R in plasmacytoid hCs. In our study, we showed that

of anti-CD25. Each symbol represents one experiment, and gray bars are the mean of five independent donors. **(D)** ELISA of IFN- $\gamma$  secretion in culture supernatant by LPS-hDCs treated with and without rhIL-2 (100 U/ml) and/or anti-CD25 (100  $\mu$ g/ml). Each symbol represents one donor, and gray bars are the mean of five independent donors. **(E)** Intracellular staining of IFN- $\gamma$  in LPS-hDCs and TNF- $\alpha$ -hDCs treated with and without rhIL-2 and/or anti-CD25 or F(ab')<sub>2</sub> of anti-CD25. Open dark histograms are rhIL-2-treated hDCs; open gray histograms are rhIL-2 plus anti-CD25- or rhIL-2 plus F(ab')<sub>2</sub>-treated hDCs; and gray histograms are the isotype control (data are representative of two donors). \* $p < 0.05$ , Wilcoxon analysis. mfi, mean fluorescence intensity.



**FIGURE 7.** Anti-IFN- $\gamma$  Abs decreased the cytotoxic activity of allogeneic CD8<sup>+</sup> T cells induced by IL-2-treated hDCs. Proliferation (*left panel*) and specific cytotoxicity (*right panel*) of CD8<sup>+</sup> T lymphocytes activated by allogeneic mature LPS-hDCs pretreated with or without IL-2 (100 U/ml) during maturation and cocultured with or without anti-IFN- $\gamma$  Abs (10  $\mu$ g/ml). Each symbol represents an experiment. Gray bars are the mean of three donors.

mature monocyte-derived DCs expressed the three chains of IL-2R. IL-2R  $\gamma$ -chain was constitutively expressed by both immature and mature DCs, as we and others reported (15, 23). Moreover, in contrast to a recent report (16), we found that monocyte-derived hDCs also expressed the  $\beta$ -chain. The discrepancy might be due to the different Abs used in studies. Indeed, in comparing staining by three different CD122-specific Abs, two weakly stained hDCs and PBMCs, and only one, 2RB, produced intense staining of hDCs. The decreased staining with the addition of rhCD122 demonstrated the specificity of the 2RB clone. Furthermore, bone marrow-derived mouse DCs show transcription of the CD122 gene (22) and CD34<sup>+</sup>-derived hDCs can express the three IL-2R chains (21). These data are consistent with our findings of the expression of CD122 in hDCs and therefore the high-affinity form of the receptor in mature hDCs.

In addition, we demonstrated that although IL-2 does not modify the cell-maturation phenotype, it increases the ability of hDCs to activate helpless allogeneic CD8<sup>+</sup> T cells alloctotoxicity function. These results are in line with the concept of a dichotomy between the membrane expression of maturation markers and the activation status of DCs (35). Interestingly, we found that hDCs showed increased IFN- $\gamma$  production in response to IL-2. IL-15 was found to have a similar role in bone marrow-derived DCs (36, 37). This common property might be because IL-2R and IL-15R share the  $\beta$ - and  $\gamma$ -chains (38). However, the mechanisms involved in this IL-15 effect were not studied. IL-15 could induce IFN- $\gamma$  in DCs (39), and the absence of the  $\beta$  or  $\gamma$ -chains in mouse DCs abrogated IFN- $\gamma$  secretion by these cells (40). Furthermore, in the presence of IL-2, human monocytes can differentiate into IFN- $\gamma$ -producing DCs (41) and human plasmacytoid DCs can secrete more proinflammatory cytokines (33). Therefore, this positive regulation of IFN- $\gamma$  production in DC appears to be common to IL-2 and IL-15. Our data with anti-IFN- $\gamma$ -blocking Abs suggest that an IL-2-triggered IFN- $\gamma$  production in hDCs positively regulated cytotoxic function in CD8<sup>+</sup> T cells. IFN- $\gamma$  synthesis by human and mouse DCs after microbial stimuli or cytokine stimulation is well documented (42–45). Previous studies showed that the hDC-derived IFN- $\gamma$  regulated the production of other cytokines in hDCs (45) and played a pivotal role in conferring to hDCs the ability to activate alloctotoxic function in CD8<sup>+</sup> T cells (24). Interestingly, our data with anti-IFN- $\gamma$  blocking Abs suggest that regulation of

cytotoxicity of CD8<sup>+</sup> T cells by hDC-derived IFN- $\gamma$  appeared to be rather independent of their proliferation. Of note, such regulation of cytotoxic function independent of cellular proliferation has been previously reported in CD8<sup>+</sup> T cells (46–48).

We demonstrated for the first time, to our knowledge, that IL-2 induces the phosphorylation of STAT5 in hDCs. This result is in sharp contrast with a recent study that did not reveal IL-2R transducing STAT5 phosphorylation in monocyte-derived hDCs, which led the authors to conclude that the function of CD25 in hDCs was for transpresentation of IL-2 to T lymphocytes (16), a mechanism well described for IL-15. This discrepancy might be due to technical aspects, because the authors did not starve cells in GM-CSF before assessing STAT5 phosphorylation, which is known to be highly induced by GM-CSF and IL-4 in hDCs (49, 50). Most of the studies with IL-15 analyzed the transpresentation aspects of IL-15/IL-15R $\alpha$ , but the signal transduced by  $\beta$ - and  $\gamma$ -chains (composing IL-15R and IL-2R) in DCs has been rarely investigated. However, one study showed a direct effect of IL-15, mediated by  $\beta$ - and  $\gamma$ -chains, on apoptosis of mouse DCs (51). In agreement, other groups described increased production of proinflammatory cytokines by DCs cultured with IL-15 (36, 37, 39). These data strongly suggest that the  $\beta$ - and  $\gamma$ -chains, expressed in DCs, transduce signals in response to IL-15 or IL-2.

We demonstrate that IL-2 could not signal through ERK or PI3K–Akt pathways in hDCs, contrary to what was reported for effector T lymphocytes (2). Nonetheless, such preferential signaling has been described in regulatory CD4<sup>+</sup> T cells, with the PI3K–Akt pathway not activated by IL-2R (25, 52). The distinct signaling patterns between effector and regulatory T lymphocytes result from the upregulation of phosphatase and tensin homolog in regulatory T cells that negatively regulates PI3K signaling (53). Determining whether phosphatase and tensin homolog is active in DCs would be of interest to explain the lack of PI3K activation.

Finally, we used inhibitors of p-STAT5 and demonstrated that IL-2-induced STAT5 phosphorylation boosts IFN- $\gamma$  synthesis in hDCs. This result suggests a functional STAT5-binding site in the promoter of the IFN- $\gamma$  gene in hDCs, which is consistent with previous results from PBMCs, NK, and CD4<sup>+</sup> T cells (29, 54). Moreover, we demonstrate that a clinically used anti-CD25 mAb could block STAT5 activation and IFN- $\gamma$  production, which suggests new therapeutic perspectives to specifically target this receptor in hDCs.

In summary, we demonstrate that IL-2 induces IFN- $\gamma$  synthesis through STAT5 phosphorylation in hDCs, for increased capacity to activate helpless CD8<sup>+</sup> cytotoxic T cells. IL-2R $\alpha$  production by hDCs has been reported to mediate transpresentation of IL-2 to lymphocytes (16). Our study assigns a new and crucial role of this receptor in activating hDCs that highlights new perspectives for specific immunotherapy.

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## Disclosures

The authors have no financial conflicts of interest.

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