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SUPPLEMENTAL RESULTS AND DISCUSSION

Assembly of IL-12 and IL-23 heterodimers

mono-DCs, pretreated or not with IFN-γ, were stimulated with LPS and biosynthetically labeled with [^{35}S]methionine. Supernatants were immunoprecipitated with a mixture of anti–IL-12/IL-23 p40 mAbs (C11.79 and C8.6) and analyzed by SDS-PAGE under nonreducing conditions (Fig. S1 A, left). Supernatants from DCs pretreated with IFN-γ or not revealed two major bands at 35–40 kD, corresponding to glycosylation forms of free IL-12 p40 chains (22). In contrast, LPS-induced, IFN-γ–primed cells also produced IL-12 heterodimers (75-kD band) and three additional bands of 61, 57, and 51 kD. To identify the nature of the intermediate bands immunoprecipitated by the anti-p40 antibodies, IL-12 p75 was first depleted from the supernatants using the IL-12 p35–specific 12H4 mAb. Samples were split into two equal parts, immunoprecipitated with a mixture of the 512 and 187 mAbs that recognize the p19 chain of IL-23, or with a mixture of antibodies that recognize the IL-12/23 shared p40 chain (Fig. S1 A, right). The anti-p19 mAbs immunoprecipitated three bands of 61, 57, and 51 kD corresponding to the intermediate bands immunoprecipitated by the anti-p40 mAbs in addition to the two major free p40 chain bands. The use of anti-p19 mAbs for immunoprecipitation of supernatants and cell lysates derived from [^{35}S]methionine-labeled, monocyte-enriched PBMCs primed with IL-4 and IFN-γ and stimulated with LPS revealed essentially the same three bands immunoprecipitated from cell lysates and supernatants (Fig. S1 B, left) and similar to those observed in mono-DCs. Each band immunoprecipitated from cell lysates and from supernatants was cut from the gel, eluted, reduced, alkylated, and resolved under reducing conditions in SDS-PAGE (Fig. S1 B, right). Material immunoprecipitated with the anti-p19 mAbs (Fig. S1 B, right) revealed three bands of 44, 41, and 34 kD, representing differently glycosylated forms of p40 (22), and a fourth band of ∼20 kD. Superimposable migration patterns were shown by the species immunoprecipitated by anti-p40 (C11+C8) mAbs (not depicted).

Two-dimensional peptide mapping was performed with the p35 and p33 bands derived from supernatant immunoprecipitated with the anti-p35 mAb and with the p20, p41, and p34 bands derived from supernatant immunoprecipitated with the anti-p40 mAbs after exhaustive preclearing of the IL-12 heterodimer with the anti-p35 antibody (Fig. S1 C). The unique pattern of spots derived from the 20-kD as compared with the 41- and 34-kD bands (two glycosylation forms of IL-12/23 p40) or to the 33- and 35-kD bands (two glycosylation forms of IL-12 p35) clearly demonstrated that the IL-23 p19 polypeptide chain is unrelated to the IL-12 p40 and p35 chains, respectively. Thus, the p61, p57, and p51 bands immunoprecipitated in nonreducing conditions by either the anti-p40 or the anti-p19 mAbs were identifiable as the p19 chain of IL-23 covalently linked to all of the glycosylation variants of the IL-12/23 p40 chain.

Glycosylation is not involved in the posttranslational regulation of IL-23

We previously demonstrated a fundamental role for *N*-linked glycosylation of IL-12 p35 in the secretion of the p75 heterodimer (22). To assess whether *N*-linked glycosylation plays a similar role in IL-23 secretion, IFN-γ–primed [^{35}S]methionine-labeled mono-DCs were treated or not with tunicamycin (TM) and stimulated with LPS. Secretion of IL-12 p75, IL-23, and free p40 was evaluated in supernatants by immunoprecipitation. As shown in Fig. S2 (left), TM-treated cells secreted <6% of the IL-12 heterodimer, which migrated as deglycosylated molecules at 65 instead of 75 kD. In cells immunoprecipitated with the C11+C8 mAb mixture, TM treatment reduced the secretion of p36 and p39 components of free p40 bands, which migrated as deglycosylated molecules as a major band of 34 kD, to ∼40%. In contrast, the major 57-kD component of IL-23 migrated at 54 kD in amounts comparable to those in untreated cells. To confirm the identity of the deglycosylated p54 band as the major p57-untreated form of IL-23, single bands were cut, eluted, reduced, and alkylated and resolved in SDS-PAGE under reducing conditions (Fig. S2, right). The 57-kD band from untreated cells appeared as a heterodimer composed of the p19 chain of IL-23 associated to the 44-, 41-, and 34-kD canonical chains of p40, superimposable on the bands derived from the p40 chain of the IL-12 heterodimer and from the free forms of untreated cells. The 54-kD band derived from TM-treated cells appeared as two major bands of 38 and 31 kD, superimposable on the bands derived from the deglycosylated p34 component of free p40 of TM-treated cells. The associated p19 chain remained unmodified in its migration pattern when secreted by either untreated or TM-treated cells. As a comparison, the migration patterns of the IL-12 p75 band derived from control cells and the corresponding p65-eluted band derived from TM-treated cells are depicted (Fig. S2, right). These data indicated that inhibition of *N*-linked sugar adduction using TM at concentrations that did not affect IL-12 or IL-23 biosynthesis drastically inhibits IL-12 heterodimer secretion but does not affect IL-23 secretion, thus excluding a relevant role of *N*-linked glycosylation in secretion of IL-23 (23). The lack of *N*-linked glycosylation consensus sequences (2) explains the unmodified migration pattern of p19 after TM treatment. Moreover, the unaltered amount of secreted IL-23 and the reduced amount of free p40 in TM-treated cells indicates that *N*-linked glycosylation of p40 is not crucial for secretion of IL-23, suggesting a greater role for p19 regulation than that of p40 in determining the amount of IL-23 secretion.