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CUTTING EDGE

Cutting Edge: STAT Activation By IL-19, IL-20 and mda-7 Through IL-20 Receptor Complexes of Two Types[1]

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IL-10-related cytokines include IL-20 and IL-22, which induce, respectively, keratinocyte proliferation and acute phase production by hepatocytes, as well as IL-19, melanoma differentiation-associated gene 7, and AK155, three cytokines for which no activity nor receptor complex has been described thus far. Here, we show that mda-7 and IL-19 bind to the previously described IL-20R complex, composed by cytokine receptor family 2–8/IL-20Rα and DIRS1/IL-20Rβ (type I IL-20R). In addition, mda-7 and IL-20, but not IL-19, bind to another receptor complex, composed by IL-22R and DIRS1/IL20Rβ (type II IL-20R). In both cases, binding of the ligands results in STAT3 phosphorylation and activation of a minimal promoter including STAT-binding sites. Taken together, these results demonstrate that: 1) IL-20 induces STAT activation through IL-20R complexes of two types; 2) mda-7 and IL-20 redundantly signal through both complexes; and 3) IL-19 signals only through the type I IL-20R complex. *The Journal of Immunology,* 2001, 167: 3545–3549.

R ecently, a family of other cytokines with limited homology to IL-10 have been described (1). The first IL-10 homolog was called melanoma differentiation-associated gene 7 (mda-7)[3] because its expression was up-regulated during the in vitro differentiation of a melanoma cell line (2). Although this protein shows 22% amino acid identity with IL-10, it was not originally recognized as a secreted protein, and its biological activities remain poorly understood. The mouse ortholog of mda-7 was identified recently as a Th2-specific cytokine and called FISP,

for IL-4-induced secreted protein (3). Its rat counterpart, called mob5, was suggested to play a role in ras oncogene-mediated neoplasia (4).

The *IL10* and *MDA7* genes have been mapped on chromosome 1q31–32, in a region where two additional IL-10-related genes, *IL19* and *IL20*, also were located. Little is known about IL-19, except that this gene is expressed by LPS-activated monocytes (5). The biological activities of IL-20 have been studied by using transgenic mice overexpressing this cytokine. These mice are characterized by neonatal lethality with skin abnormalities, including aberrant epidermal differentiation reminiscent of psoriasis lesions in human (6). An IL-20R complex was described as a heterodimer of two orphan class II cytokine receptor subunits: corticotropin-releasing factor (CRF) 2–8, proposed to be renamed IL-20Rα, and DIRS1, designated IL-20Rβ (6).

In addition to the chromosome 1q31–32 cluster, two other IL-10-related cytokines, AK155 and IL-22, are located on human chromosome 12q15, near the IFN-γ gene. AK155 is known to be up-regulated by *Herpes saimiri* infection of T lymphocytes, but its activity and receptor remain unknown (7). IL-22 was described originally as an IL-9-inducible gene and called IL-TIF, for IL-10-related T cell-derived inducible factor (8). IL-22 activities include induction of the acute phase response in hepatocytes and are mediated through a heterodimeric receptor composed of the CRF2–9/IL-22R subunit and the β-chain of IL-10R (9–11). In addition to its cellular receptor, IL-22 binds to a secreted member of the class II cytokine receptor family, which was called IL-22BP, and appears to act as a natural IL-22 antagonist (12, 13).

Materials and Methods

Cell cultures and cytokines

HT-29 intestinal epithelial cells were grown in IMDM medium supplemented with 10% FCS, 0.55 mM L-arginine, 0.24 mM L-asparagine, and 1.25 mM L-glutamine. Human embryonic kidney (HEK) 293-EBV nuclear Ag cells were grown in DMEM medium supplemented with 10% FCS. IL-10 homologs were produced by transient expression in HEK293-EBNA cells by the Lipofectamine 2000 method (Life Technologies, Gent, Belgium). The coding sequences for mda-7, IL-19, and IL-22 were amplified by RT-PCR from RNA of T cells stimulated with anti CD3 Ab. The IL-20 coding sequence was amplified from skin RNA. These cDNAs were cloned into pCEP4 plasmid (Invitrogen, Groningen, the Netherlands) under the control of the CMV promoter. mda-7-Flag, IL-19-flag, IL-20-flag and IL-22-flag were generated from the pCEP4-cytokine constructs by mutating the STOP codon and introducing a sequence encoding a C-terminal flag: Gly-Gly-Gly-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys. The IL-22BP-Ig fusion cDNA was produced as described before (12). For Western blot analysis, 10 μl of HEK293 supernatant was mixed with Laemmli sample buffer and boiled for 5 min before SDS-PAGE and transfer onto a polyvinylidene difluoride membrane (Amersham, Arlington Heights, IL). The membrane

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[3] Abbreviations used in this paper: mda-7, melanoma differentiation-associated gene 7; CRF, cytokine receptor family; HEK, human embryonic kidney.

was probed with biotinylated anti-flag Ab (25 μg/ml) and with streptavidin-HRP (1/5000; Amersham). An ECL detection kit (Amersham) was used for expression of chemiluminescence. The chemiluminescence signal was detected and quantified with a Kodak (Rochester, NY) Digital Science Image Station 440CF. Anti-phospho-STAT3 Western blots were performed as described previously (8).

The DIRS1/IL-20Rβ cDNA was amplified by RT-PCR from K562 leukemia cells and cloned into pCEP4 plasmid. The IL-22R cDNA was amplified by RT-PCR from the HepG2 hepatoma cell line before cloning into the pEF-BOSpuro expression vector (14). The CRF2–8/IL-20Rα cDNA was amplified by PCR from a human placenta cDNA library (Clontech Laboratories, Palo Alto, CA), and cloned into the pCDEF3 plasmid. Anti-IL-10Rβ and anti-flag Abs were purchased from Peprotech (London, U.K.) and from Sigma (Bornem, Belgium), respectively. To produce anti-hIL-22R Abs, we transfected P815 mastocytoma cells with the hIL-22R cDNA in the pEF-BOS plasmid before injection into DBA/2 mice. After rejection of the tumors, the sera of these mice had high titers of neutralizing anti-hIL-22R Abs and were used at a 1/500 dilution.

Luciferase assays

The cytokine response was assessed by measuring luciferase production by cells transfected with the pGRR5 construct, (provided by Dr P. Brennan, Imperial Cancer Research Fund, London, U.K.). This construct contains five copies of the STAT-binding site of the FcγRI gene inserted upstream from a luciferase gene controlled by the TK promoter. Transfections of HT29 and HEK293 cells were performed as follows.

HT-29 cells were electroporated (10^7 cells in 400 μl, 250 V, 192 Ω, 1200 μF) with 15 μg of pGRR5 and 15 μg of each receptor cDNA, separately or in combination. Transfected cells were seeded in 96-well plates, incubated for 5 h at 37°C, and then preincubated, or not, for 1 h with anti-IL-22R antiserum (1/500) or with anti-IL-10Rβ Abs (6 μg/ml). Next, the cells were stimulated with each cytokine for 2 h. Luciferase activity was measured with the Luclite plus Assay System kit (Canberra-Packard, Meriden, CT) with a Top Count microplate scintillation counter (Canberra-Packard).

HEK293-EBNA cells were seeded in 24-well plates (Nunc, Roskilde, Denmark) for 24 h. Transfections were conducted by using the Lipofectamine method (Life Technologies, Gent, Belgium), with 500 ng of plasmid encoding IL-22R, IL-20Rβ, or IL-20Rα and with 100 ng of pGRR5. As an internal control, we used 100 ng of pRL-TK vector (Promega, Madison, WI) containing the *Renilla* luciferase gene under the control of the TK promoter. After 20 h, transfected cells were stimulated with cytokines, and 2 h later, cells were pelleted and lysed. Luciferase activity was monitored with the Dual-Luciferase Reporter Assay System kit (Promega).

IL-22BP interaction assays

Specific interactions between IL-22BP and cytokine-flag fusion proteins were assessed directly or indirectly by ELISA, as follows. Reacti-Bind Maleic Anhydride Activated Polystyrene plates (Pierce, Rockford, IL)

were coated overnight at 4°C with 12.5 μg/ml of anti-flag Ab in PBS. The plates were incubated 2 h at 37°C with 50 μl of cytokine-flag fusion proteins (HEK293 supernatants). A total of 10% of supernatant of IL-22BP-Ig was added for 2 h, and bound IL-22BP-Ig was detected by using anti-mouse IgG3 polyclonal Abs coupled to peroxidase (Southern Biotechnology Associates, Birmingham, AL). The enzymatic activity was measured as described previously (12). In the indirect assay, we tested the inhibitory effect of IL-10 homologs on the binding of IL-22BP to IL-22. For this purpose, IL-22BP-Ig (10%) was preincubated with IL-10 homologs 2 h before incubation with Reacti-Bind plates (Pierce) that had been coated with rIL-22 as described previously (12).

Results

STAT activation by IL-10 homologs in class II cytokine receptor-transfected cells

To characterize the interactions between IL-10 homologs and receptors belonging to the class II cytokine receptor family, we expressed mda-7, IL-19, IL-20, and IL-22 as fusion proteins with a C-terminal flag sequence by transient transfection of HEK293 cells. Protein production was checked by Western blot with an Ab specific for the flag peptide (Fig. 1*A*). HEK293 cells secreted mda-7, IL-19, and IL-22 proteins with a heterogeneous MW of 23–30 kDa, most likely resulting from glycosylation. The IL-20-flag protein is secreted as a single band with a size of $\sim$18 kDa, suggesting that this cytokine is not glycosylated. Quantification of the chemiluminescence signal indicated that IL-19 and IL-22 were produced at a similar level, whereas IL-20 and mda-7 were produced 7-fold less.

These HEK293 supernatants were used to assess the interaction with class II cytokine receptors. A first set of experiments were performed in HT-29 cells, which endogenously express IL-22R and IL-10Rβ. STAT activation induced by IL-22 was monitored with the pGRR5 luciferase reporter (9). As shown in Fig. 1*B* (*top left*), these cells failed to respond to the other IL-10 homologs. When HT-29 cells were transfected with the IL-20Rβ cDNA, both mda-7 and IL-20 induced luciferase production. Interestingly, this effect was completely blocked by an anti-IL-22R antiserum, suggesting that mda-7 and IL-20 can activate STAT factors through a new IL-20R complex composed by IL-22R and IL-20Rβ (Fig. 1*B*, *bottom left*).

When cells were transfected with both IL-20Rα and IL-20Rβ cDNAs, they became responsive to mda-7, IL-20, and IL-19, and the luciferase production was not affected anymore by anti-IL-22R
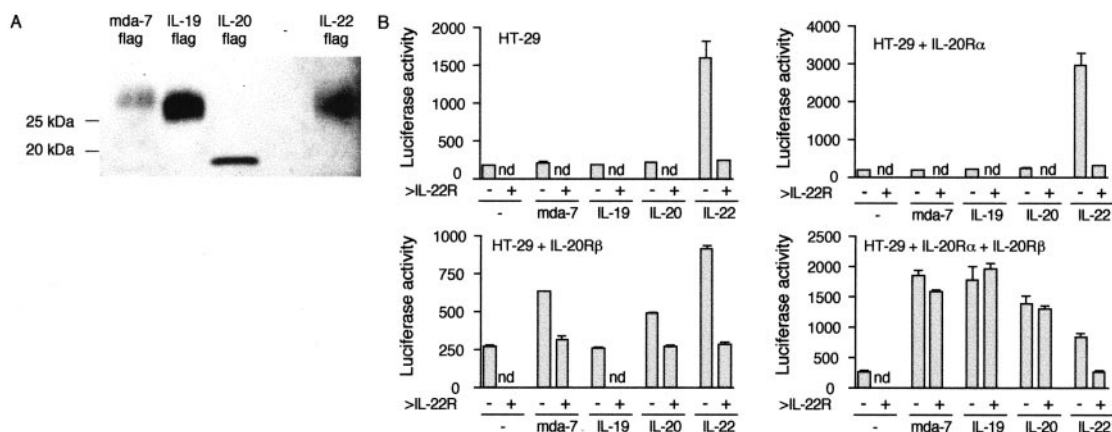

FIGURE 1. Human IL-10 homolog production and activity. *A,* The cDNAs encoding mda-7, IL-19, IL-20, and IL-22 tagged with a flag sequence were transfected in HEK293 cells. After 4 days, supernatants were analyzed by Western blotting with an Ab raised against the flag peptide. *B,* HT-29 cells were transfected with the pGRR5 luciferase construct with or without IL-20Rα and IL-20Rβ as indicated. Cells were preincubated 1 h with or without anti-IL-22R antiserum (1/500) before stimulation with 1% of HEK293 supernatants. Luciferase activity was monitored 2 h later and is expressed in arbitrary units.
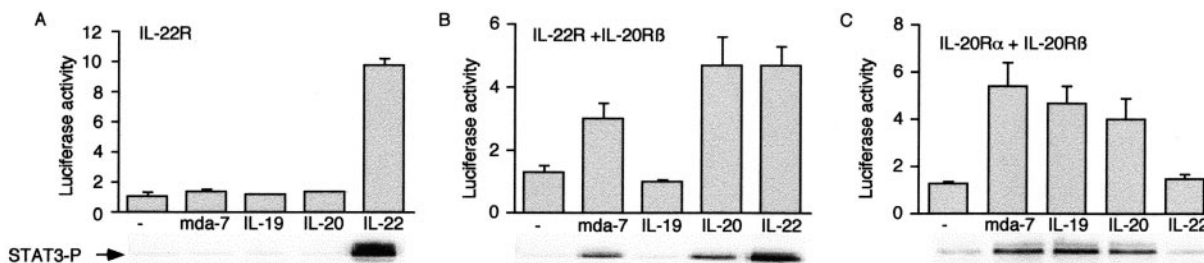
FIGURE 2. Activity of IL-10 homologs in HEK293 cells. HEK293 cells were transfected with the pGRR5 luciferase construct and cDNAs encoding IL-22R (*A*), IL-22R and IL-20Rβ (*B*), or IL-20Rα and IL-20Rβ (*C*). Cells were stimulated with 10% of mda-7, IL-19, IL-20, or IL-22 supernatant. Luciferase activity was monitored 2 h later. The results are normalized by using *Renilla* luciferase as an internal control. Western blot analysis for STAT3 phosphorylation was performed after 15 min of stimulation.

Abs (Fig. 1*B*, *bottom right*), indicating that this activity was independent from this chain. Finally, on transfection with the IL-20Rα cDNA alone, we failed to detect any response to mda-7, IL-19, and IL-20 (Fig. 1*B*, *top right*), confirming that IL-20Rβ is required for this process.

To characterize further the different types of receptor complexes, we used HEK293 cells, which express endogenous IL-10Rβ but not IL-22R. Untransfected HEK293 cells did not respond to any IL-10 homolog (data not shown). When the IL-22R cDNA was transfected, only IL-22 induced luciferase production and STAT-3 phosphorylation (Fig. 2*A*). Cells transfected with IL-22R and IL-20Rβ responded not only to IL-22 but also to IL-20 and mda-7 (Fig. 2*B*), whereas IL-20Rβ alone did not confer any cytokine responsiveness (data not shown). Transfection of both IL-20Rα and IL-20Rβ cDNAs allowed for STAT activation by mda-7, IL-19, and IL-20, but not IL-22 (Fig. 2*C*). No response was observed in cells transfected with the IL-20Rα cDNA alone (data not shown). In all cases, luciferase induction correlated with phosphorylation of STAT-3, as analyzed by Western blotting (Fig. 2). Similar results were obtained with HEK293 supernatants containing the wild-type cytokines.

Comparison of IL-20 and mda-7 sensitivity conferred by both types of IL-20R complexes

The observation that two different receptor complexes allowed for the response to IL-20 and mda-7 raised the possibility that each complex would respond preferentially to one cytokine. To test this hypothesis, we analyzed the response of HT-29 cells, transfected either with IL-20Rβ alone or both IL-20Rα and IL-20Rβ, to different dilutions of mda-7, IL-19, and IL-20 supernatants. When both IL-20Rα and IL-20Rβ were transfected, mda-7 and IL-20 dilutions showed a similar dose-response curve, indicating a similar sensitivity to both cytokines (Fig. 3, *bottom*). The activity of IL-19, but not those of mda-7 and IL-20, could be detected with 0.1% of supernatant, in agreement with the higher concentration of IL-19 supernatants. When only IL-20Rβ was transfected, HT-29 cells showed a better response to mda-7 at nonsaturating dilutions (1% and 0.1% supernatant), indicating that this type of complex is more sensitive to mda-7 (Fig. 3 *top*). Similar results were obtained in HEK293 cells (data not shown).

IL-20Rβ cannot substitute for IL-10Rβ in IL-22 signaling

The finding that IL-22R can associate not only with IL-10Rβ as described previously, but also with IL-20Rβ raised the possibility that the complex of IL-20Rβ with IL-22R could mediate an IL-22 response. Because IL-10Rβ is ubiquitously expressed, we could not address this question by direct transfection, but the role of IL-10Rβ was assessed with an anti-IL-10Rβ Ab. As shown in Fig.

4, this Ab could block the IL-22 activity both in control HT-29 cells and in cells transfected with the IL-20Rβ cDNA, indicating that IL-20Rβ cannot substitute for IL-10Rβ when the latter chain is not accessible to IL-22. The same Ab did not affect the activity of mda-7 or IL-20 in the same cells (data not shown).

mda-7, IL-19, and IL-20 do not bind IL-22BP

IL-22BP has been shown to bind IL-22 (12, 13), but nothing is known concerning its ability to bind other IL-10 homologs. The fact that this soluble receptor exhibits the same degree of homology with the extracellular domains of IL-22R and IL-20Rα prompted us to test the hypothesis that IL-22BP could also bind IL-20. In a first set of experiments, we tested the ability of the IL-10 homologs to compete for the binding of IL-22BP to insolubilized IL-22. Microtiter plates were coated with rIL-22 and incubated with an IL-22BP-Ig fusion protein in the presence of IL-10 homologs. The interaction between IL-22 and IL-22BP was detected with an anti-Ig Ab. As shown in Fig. 5*A*, only IL-22 supernatants were able to block IL-22BP binding. To directly assay the
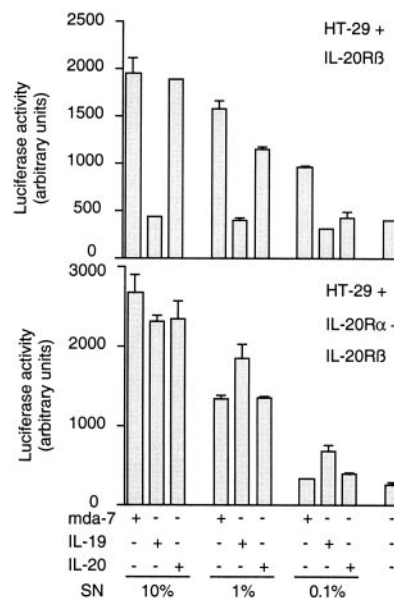

FIGURE 3. Comparison of IL-20 and mda-7 sensitivity with both types of IL-20R complexes. HT-29 cells were transfected with the pGRR5 luciferase construct and the cDNAs encoding IL-20Rβ alone (*top*), or IL-20Rα and IL-20Rβ (*bottom*). Cells were stimulated with different dilutions of mda-7, IL-19, and IL-20 supernatants for 2 h before measuring luciferase activity.
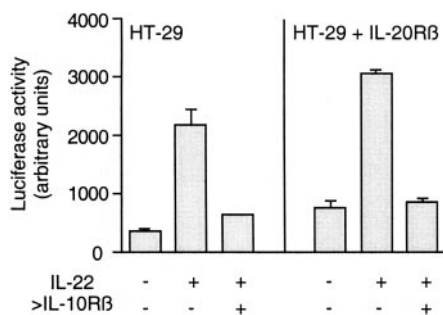
FIGURE 4. IL-20Rβ cannot substitute for IL-10Rβ in IL-22 signaling. HT-29 cells were transfected with the pGRR5 luciferase construct and the IL-20Rα cDNA. Cells were preincubated 1 h with anti-IL-10Rβ Ab before stimulation with 10% supernatant from IL-22- or mock-transfected HEK293. Luciferase activity was monitored 2 h later.

FIGURE 6. Schematic representation of IL-22R and IL-20R complexes.

interaction between IL-10 homologs and IL-22BP, we coated microtiter plates with anti-flag Ab before incubation with flag-tagged IL-10 homologs. IL-22BP-Ig was added, and interaction was checked with an anti-Ig Ab. As shown in Fig. 5*B*, only IL-22 was able to bind IL-22BP-Ig, and no other IL-10 homolog showed the same activity.

Discussion

Sharing receptor subunits is a well-known feature within class I cytokine receptors and has allowed to define subfamilies based on the involvement of subunits such as βc, gp130, and IL-2Rγ. Among the class II cytokine receptors, the only example of a shared receptor so far was the IL-10Rβ chain, which is involved in both IL-10 and IL-22 signaling (9–11). In this paper, we show that IL-22R and DIRS1/IL-20Rβ are also shared by different receptor
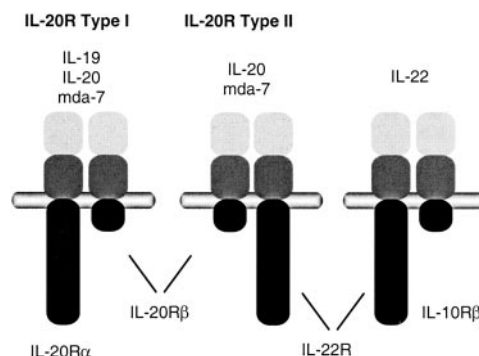

FIGURE 5. IL-22BP interacts specifically with IL-22. *A,* Plates were coated with rIL-22 before addition of IL-22BP-Ig preincubated with IL-10 homologs. Specific interactions were detected with rabbit polyclonal anti-Ig Abs. *B,* Plates were coated with anti-flag Ab. Supernatants containing flag-tagged IL-10 homologs were added before incubation with IL-22BP-Ig. Specific interactions were detected with a rabbit polyclonal anti-mouse Ig.

complexes. The IL-20Rβ subunit can associate either with IL-20Rα, leading to a functional receptor for IL-19, IL-20, and mda-7 (type I IL-20R complex). IL-20Rβ also can associate with the IL-22R subunit and lead to a functional receptor for IL-20 and mda-7, but not for IL-19 (type II IL-20R complex), as schematically represented in Fig. 6. Additional experiments are needed to determine which of these chains serve as an actual ligand binding component or as a Jak-recruiting subunit. Alternatively, these receptor subunits may be expressed as preassociated complexes at the surface of the cells.

IL-20-transgenic mice show neonatal lethality and skin abnormalities, including thickened epidermis and expression of markers of hyperproliferation (6). Our observations strongly suggest that IL-19 and mda-7 can have a similar activity. Interestingly, IL-19 acts only on type I IL-20R and should therefore recapitulate only partly IL-20 activities. By contrast, IL-20 and mda-7 seem to behave similarly regarding both complexes. Noticeably, expression of the rat ortholog of mda-7 seems to be up-regulated during wound healing, a process that definitely involves keratinocyte proliferation (15).

Although mda-7 was originally identified several years ago (2), its activities and mode of action remain poorly understood. This protein was reportedly expressed intracellularly and was shown to induce apoptosis in certain tumor cell lines by an unknown mechanism (16, 17). On transfection of the mda-7 cDNA in HEK293 cells, we found most of the protein in the supernatant, indicating that it can be secreted, at least in this cell type. Secretion of the rat and mouse orthologs of mda-7 in various cell types also has been reported (3, 4). Together with our observation that exogenous mda-7 binds to the IL-20R complexes, these data support the hypothesis that mda-7 acts as a paracrine or autocrine factor. However, it remains possible that mda-7 might be expressed either as a cytoplasmic protein, inducing cell growth inhibition and apoptosis, or as a secreted protein acting on various cell types through IL-20R complexes.

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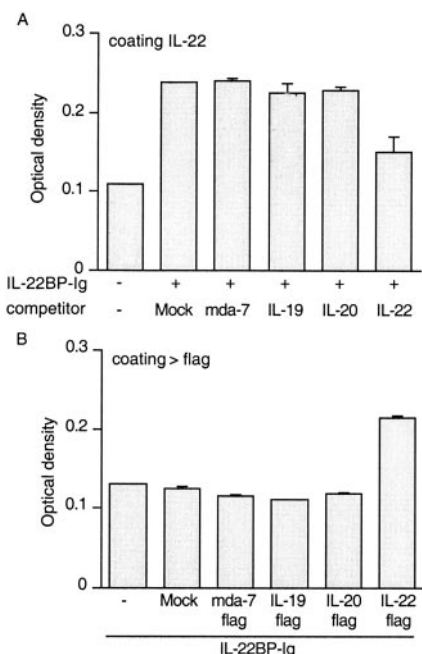
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