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Amino Acid Copolymers That Alleviate Experimental Autoimmune Encephalomyelitis In Vivo Interact with Heparan Sulfates and Glycoprotein 96 in APCs

Paul-Albert Koenig,* Eric Spooner,* Norio Kawamoto,† Jack L. Strominger,‡ and Hidde L. Ploegh*,*†

Multiple sclerosis (MS) is an autoimmune disease that affects the CNS. One approved treatment for relapsing forms of MS is YEAK, a random copolymer of the amino acids tyrosine, glutamic acid, alanine, and lysine. YEAK, a second-generation copolymer composed of tyrosine, phenylalanine, alanine, and lysine, is more successful in treating experimental autoimmune encephalomyelitis, a mouse model of MS. Although originally designed and optimized based on the autoantigen myelin basic protein (MBP) and the MBP-derived peptide MBP85-99 presented to the MS-associated class II MHC molecule HLA-DR2, YEAK and YFAK also stimulate cytokine and chemokine production in APCs that lack class II MHC products. How YEAK and YFAK copolymers interact with APCs remains enigmatic. We used biotinylated YEAK to affinity-purify YEAK-interacting proteins from RAW264.7 cells and tested APCs from mice deficient in several of the newly identified interactors for their capacity to secrete CCL22 in response to YEAK and YFAK. We propose that initial contact of YFAK with cells is mediated mainly by electrostatic interactions, and that interaction of YFAK with host proteins is strongly dependent on ionic strength. Cells deficient in enzymes involved in sulfation of proteins and proteoglycans showed strongly reduced binding of biotinylated YFAK. Lastly, cells stimulated with YFAK in the presence of heparin, structurally similar to heparan sulfates, failed to produce CCL22. We conclude that charge-dependent interactions of copolymers that alleviate MS/experimental autoimmune encephalomyelitis are critical for their effects exerted on APCs and may well be the main initial mediators of these therapeutically active copolymers.

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Abbreviations used in this article: App, amyloid β precursor protein; B4GALT7, xylosyltransferase β 1,4-galactosyltransferase; B3GAT3, β-1,3-glucuronyltransferase; BMDC, bone marrow–derived dendritic cell; BMDM, bone marrow–derived macrophage; EndoH, endoglycosidase H; GO, gene ontology; HSP, heat shock protein; HSPG, heparan sulfate proteoglycan; KO, knockout; LDL, low-density lipoprotein; MBP, myelin basic protein; MS, multiple sclerosis; PEG, polyethylene glycol; PNGaseF, peptide-N-glycosidase F; SA-PE, PE-conjugated streptavidin; SLC35B2, solute carrier family 35, member B2; TRIF, Toll/IL-1R domain-containing adaptor-inducing IFN-β.

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Materials and Methods

Copolymers and modification of copolymers

YEAK was obtained from Hanna Pharmaceuticals (Wilmington, DE). YEAK was synthesized as described in Kovalchin et al. (12). The random copolymers were biotinylated with a 2-fold molar excess (assuming an average molecular mass for the copolymers of 5500 [YEAK] and 7000 Da [YFAK]) of Sulfo ChroaLink Biotin reagent (Solulink, San Diego, CA) to 1 %. Labeling efficiency was determined spectroscopically to be approximately one biotin molecule per four YFAK molecules and one sulfated glycosaminoglycan, most likely through electrostatic interactions.
substitution with biotin to minimize structural alterations in the already quite heterogeneous copolymers.

Cells and cell lines

RAW264.7 cells were obtained from American Type Culture Collection (TIB-71). B702/30 NF-kB–GFP and E4.126 cells (18) were a gift from Brian Seed (Harvard Medical School, Boston, MA). Murine bone marrow–derived dendritic cells (BMDCs) were generated by culturing bone marrow cells in RPMI 1640 supplemented with 10% inactivated FCS, 0.005% (v/v) 2-ME, 0.1 mM nonessential amino acids, 1 mM pyruvate (Life Technologies), 1 ng/ml IL-4 (R&D Systems), and 1 ng/ml GM-CSF (R&D Systems) for 7 d.

Radiolabeling and affinity purification experiments

For biosynthetic labeling, cells were fed overnight (~14–18 h) with 50 µl [35S]methionine/cysteine (1175 Ci/mmol; Perkin Elmer Life Sciences) per milliliter DMEM supplemented with 10% FBS. Cells were washed once in PBS, detached in PBS containing 5 mM EDTA, and washed once again in PBS. Cells were lysed in 12.5 mM HEPES pH 7.76, 50 mM NaCl (or as otherwise indicated in the text), 5 mM CaCl2, 2.5 mM MgCl2, 1% w/v digitonin, and “complete protease inhibitor” mixture tablets (Roche) for 1 h. Lysates were centrifuged for 20 min at 12,000 × g, and equal amounts of radioactive lysate (based on trichloroacetic acid–precipitable cpm) of soluble fractions (“cleared lysates”) were used for affinity purification experiments. YFAK-biotin or YEAK-biotin were added to the lysates for 3 h and captured with High Capacity NeutrAvidin beads (Pierce Bio- technologies) for 1 h. Beads were washed four times in lysis buffer with 0.1% digitonin. Polypeptides were either eluted in reducing SDS sample buffer or in glycoprotein denaturing buffer (New England Biolabs) for sub- sequent treatment with endoglycosidase H (EndoH) or peptide-glycosidase (App) (both obtained from New England Biolabs). EndoH and PNGaseF digestions were performed according to the manufacturer’s instructions.

Large-scale affinity purification experiments and mass-spectrometry analysis

Approximately 2 × 108 RAW264.7 cells per condition were lysed in digitonin lysis buffer as described earlier. Cleared lysates were incubated with ~250 µg biotinylated YFAK or YEAK for at least 4 h. Copolymers with interacting molecules were retrieved with 250 µl (bed volume) NeutrAvidin beads. After extensive washing in digitonin wash buffer (0.1% digitonin) beads were eluted by boiling in reducing SDS sample buffer containing 10 µM biotin. Samples were resolved on 10% SDS-polyacrylamide gels. Gels were stained with colloidal Coomassie blue, slices of individual lanes were excised, and proteins were digested with trypsin and analyzed by tandem mass spectrometry.

Gene ontology analysis

Gene ontology (GO) analyses were based on the list of 222 proteins (Supplemental Table I, section A) that were recovered in association with biotinylated YFAK from RAW264.7 lysates in both of two independent experiments. GO term enrichments for the different GO categories and p values plotted in Fig. 2 (right panels) were determined with the GeneGO MetaCore program (http://portal.genego.com/). To calculate the counts of enriched GO terms depicted in Fig. 2 (left panels), we performed GO analysis on a reduced set of GO categories (GO-slim) on mouse gene models available through the GO tools (http://go.princeton.edu).

Immunoblotting and Abs

RAW264.7 cells were lysed in digitonin lysis buffer for 1 h. Cell debris and nuclei were pelleted and discarded. YFAK-biotin was added to cleared cell lysates and incubated for another hour. YFAK-biotin was recovered by NeutrAvidin beads, and gp96 was recovered with a polyclonal anti-gp96 rabbit serum and protein G beads (Roche Diagnostics, Indianapolis, IN). Recovered proteins were separated by Tris-Glycine (for detection of gp96) or Tris-Tricine (to detect YFAK-biotin) SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes and (immuno)blotted with standard methods. YFAK-biotin was detected with streptavidin–HRP (GE Healthcare, Piscataway, NJ); gp96 was detected with the same polyclonal rabbit Ab that was used for immunoprecipitation.

FACS analysis

Cells were detached in PBS containing 5 mM EDTA, washed in cold PBS containing 2% (v/v) BSA (FACS buffer), and incubated with the indicated concentrations of biotinylated copolymers for 30 min on ice. Cells were washed with FACS buffer and incubated on ice with 0.5 µg/ml streptavidin–PE (Invitrogen, Carlsbad, CA) for another 30 min. After washing with FACS buffer, cells were analyzed with a FACSCalibur flow cytometer (BD Biosciences). FACS data were analyzed using FlowJo software (Tree Star, Ashland, OR).

ELISA

ELISA assays were done as described previously (12). In short, 500,000 cells were stimulated in 250 µl medium containing different concentrations of stimulant. For experiments shown in Fig. 7, 500,000 RAW264.7 cells or 100,000 BMDCs were stimulated for 4 h in serum-free medium. LPS (from E. coli 055:B5) was obtained from Sigma-Aldrich. CCL2 ELISA kits were purchased from R&D Systems and used according to the manufacturer’s protocol.

Mice

Wild-type C57BL/6 mice were obtained from The Jackson Laboratory. Animals were housed at the Whitehead Institute for Biomedical Research and maintained according to protocols approved by the Massachusetts Institute of Technology Committee on Animal Care. Bones from gp96+/− animals were provided by Zhai Li (Medical University of South Carolina, Charleston, SC); bones from CD91+/− and CD91−/− LDLr−/−–deficient animals were provided by Dudley Strickland (University of Maryland School of Medicine, Baltimore, MD). Bones from MyD88−/− Toll/IL-1R domain-containing adaptor-inducing IFN-β (TRIF)−/−–deficient animals were provided by Kate Fitzgerald (University of Massachusetts Medical School, Worcester, MA) and from amyloid β precursor protein (App)−/− animals by Tracy Young-Pearse (Harvard Medical School).

Results

Copolymers elicit CCL22 secretion in RAW264.7 cells and in mouse BMDCs

The copolymer YFAK was designed to optimize the YEAK copolymer for treatment of experimental autoimmune encephalomyelitis. The immunosuppressive effect of YEAK has been ascribed to its binding to class II MHC molecules, which would then interfere with the interaction of MHC class II–autoantigenic peptide complexes and autoreactive TCRs (3). Glutamic acid was replaced by phenylalanine to improve binding to class II MHC molecules, based on the known binding motifs of the autoantigenic peptide myelin basic proteins 85–99 (MBP85–99) to HLA-DR2 (DRB1*1501) (3). Only recently was attention drawn to class II MHC–independent effects of the YFAK and YEAK copolymers on innate immune cells, such as monocytes, macrophages, and dendritic cells (12, 15–17). We confirmed the earlier observation that exposure to YFAK and YEAK stimulates RAW264.7 cells (Fig. 1A) and BMDCs (Fig. 1B) to secrete the Th2 and regulatory T cell chemotactractant CCL22. Attempts to identify an innate immune receptor using various knockout (KO) and mutant cells, as well as blocking Abs, have been unsuccessful (17).

Biotinylation of YFAK and YEAK

To obtain insight into the molecular mechanism of action of YFAK and YEAK on APCs, we sought to identify interacting partners of the copolymers. We prepared biotinylated versions of either copolymer by chemical modification at lysine residues, using a reagent that includes a biotin portion, a (polyethylene glycol)3 linker, a bis-aryl hydrazone chromophore, and an amine-reactive succinimidyl ester. Unreacted and hydrolyzed labeling reagents were separated from the copolymers by size-exclusion chromatography (shown for YFAK in Fig. 2A). The presence of the aryl hydrazone chromophore in the copolymer peaks indicated successful biotinylation. After electrophoretic separation by Tris-Tricine SDS-PAGE and transfer to polyvinylidene difluoride membranes, the biotinylated copolymers were readily detected with streptavidin-HRP (shown for YFAK-biotin in Fig. 2B), confirming successful conjugation. Consistent with their inherent heterogeneity, both copolymers presented themselves as a diffuse smear over a range of apparent molecular weights. To avoid significant alteration of
the immunological properties of the copolymers, we established conditions that resulted in substoichiometric modification. Capitalizing on the bis-aryl hydrazone chromophore’s absorption at λ = 354 nm, labeling efficiency was determined to be ∼0.31 or 0.17 mol biotin/mol YFAK or YEAK, respectively.

**Copolymers bind to the surface of RAW264.7**

We next examined the ability of the biotinylated copolymers to bind to intact cells. RAW264.7 cells were incubated for 30 min at 4˚C with different concentrations of YFAK-biotin (0.01, 0.1, 1, and 10 μg/ml) and then stained with PE-conjugated streptavidin (SA-PE). We observed concentration-dependent binding of YFAK-biotin to the cell surface, with maximal binding at 10 μg/ml among the concentrations tested in this study (Fig. 2C, right panel). Binding was specific, because neither SA-PE alone nor hydrolyzed ChromaLink reagent plus SA-PE gave any signal above background (Fig. 2C, left panel).

**Affinity purification using biotinylated copolymers**

Given that YFAK can stimulate RAW264.7 cells to secrete CCL22 and that YFAK-biotin binds to RAW264.7 cells, we next sought to identify proteins capable of binding to YFAK-biotin. We prepared digitonin extracts of [35S]Met/Cys-labeled RAW264.7 cells. YFAK-biotin was added to the lysate and subsequently recovered with NeutrAvidin-conjugated beads. As shown in Fig. 2D, neither NeutrAvidin beads alone nor nonbiotinylated YFAK recovered significant amounts of protein from the cell lysates. In contrast, both biotinylated YFAK and YEAK retrieved a diverse set of coprecipitating proteins. The overall pattern of recovered proteins was similar for both copolymers, although some unique bands were present for each condition.

We reasoned that APCs might detect YFAK by means of a cell-surface receptor(s) that would acquire N-linked sugar modification(s) while passing through the secretory pathway. To distinguish proteins with N-linked glycan(s) from proteins without them, we treated the samples with the N-glycosidase PNGaseF. Indeed, some of the interaction partners of YFAK were sensitive to PNGaseF (Fig. 2E). The most intense signal under the conditions used was a glycoprotein that migrated slightly below the 100-kDa marker (Fig. 2E, asterisk).

Next, we analyzed the polypeptides recovered in complex with YFAK-biotin and YEAK-biotin by mass spectrometry to establish their identity. We performed two independent experiments with...
YFAK-biotin from RAW264.7 cell lysates. For subsequent analysis, we created a list of proteins recovered in both experiments (Supplemental Table I). Furthermore, proteins for which the coverage, number of unique peptides, or number of total peptides recovered in the control sample (NeutrAvidin beads only) was ≥20% than in the experimental sample (YFAK-biotin added) were deleted from the list. Applying these criteria, we obtained a list of 222 interactors of YFAK-biotin for which we performed a GO term enrichment analysis for the three GO categories “cellular component,” “biological process,” and “molecular function” (Fig. 3, Supplemental Table I). The most significantly enriched molecular function was “RNA binding” \( (p = 2.6 \times 10^{-29}) \), followed by “aminoacyl-tRNA ligase activity,” “ligase activity, forming aminoacyl-tRNA and related compounds,” and “ligase activity, forming carbon-oxygen bounds” \( (p = 1.3 \times 10^{-20} \text{ for each}) \). In terms of absolute numbers, “ion binding” and again “RNA binding” were the most abundant GO terms in the “molecular function” category.

**Copolymers interact with gp96**

Among the interacting proteins identified in this article, one prominent hit consistently identified in the two independent affinity purification experiments and with known involvement in immune signaling was gp96 (also known as endoplasmic, GRP94, heat shock protein [HSP] 90 β, or ERp99). Fig. 4A shows the sequence coverage from one such experiment: 45 unique gp96-derived peptides were recovered, which corresponds to a sequence coverage of 49%. Coprecipitated gp96 was detected also by immunoblot after retrieval of YFAK-biotin–bound materials and vice versa (Fig. 4B), confirming the interaction of YFAK with gp96. PNGaseF sensitivity of gp96 (Fig. 4B) is consistent with previous data, showing that gp96 is a glycosylated protein with five potential N-glycosylation sites (20, 21), not all of which are used. We confirmed these results by a similar affinity purification experiment using YFAK-biotin on lysates from the gp96-deficient B cell line E4.125 (18) and its parental gp96-proficient line B70Z/3 (Fig. 4C). Only the latter yielded a positive signal for gp96.

**CCL22 secretion in cells deficient for interactors of YFAK and YEAK**

Next, we tested whether the absence of some of the proteins identified as interactors of YFAK-biotin has consequences for CCL22 production in APCs. We generated BMDCs from gp96-deficient bone marrow and stimulated cells with different concentrations of YFAK, YEAK, or the TLR4 agonist LPS. As expected, wild-type BMDCs secreted elevated amounts of CCL22 in response to LPS (Fig. 5A, lower panel) (12). In contrast, gp96^−/−^ cells did not secrete additional CCL22 upon LPS stimulation, consistent with gp96’s role as an essential chaperone for most
FIGURE 4. YFAK interacts with gp96. (A) Exemplary sequence coverage of gp96 coprecipitated with biotinylated YFAK from RAW264.7 cell lysate. Top, Schematic map of gp96 (white bar) and recovered peptides (light grey, overlaps in dark grey); (bottom) amino acid sequence of gp96 with recovered peptides (light grey, overlaps in dark grey). (B, top) Anti-gp96 immunoblot of coprecipitates with YFAK-biotin from RAW264.7 cell lysate; (bottom) streptavidin and anti-gp96 blot of immunoprecipitates with an anti-gp96 rabbit serum or preimmune serum from the same rabbit (“pre’”). (C) Autoradiograph of coprecipitating proteins with biotinylated YFAK from [35S]Met/Cys-labeled B70Z/30 (gp96-proficient) and E4.126 (gp96-deficient) cell lysate. F, PNGaseF treated; H, EndoH treated; TCL, total cell lysate.

Another interactor identified in the affinity purification experiments with biotinylated copolymers was CD91 (also known as LRP1). Several structurally diverse ligands have been identified for CD91, including various HSPs such as gp96 (28, 29), calreticulin, and HSP90 (29), but also Pseudomonas aeruginosa exotoxin A (30), receptor-associated protein, α2-macroglobulin (31), lipoprotein lipase, and others (32). Some immunomodulatory HSPs, such as gp96, mediate Th cell polarization through CD91 (33). Heparan sulfate is important for binding of gp96 to CD91 (34). YFAK binds to both CD91 and some of its ligands (e.g., gp96 and HSP90; Supplemental Table I), and as will be shown later, binding of YFAK to cells is mediated mainly by heparan sulfate. Thus, we asked whether YFAK or YEAK might also signal through CD91 to facilitate CCL22 secretion by APCs. We stimulated CD91-deficient bone marrow-derived macrophages (BMDMs) from CD91<sup>−/−</sup> LysM-Cre mice with YFAK or YEAK. However, we saw no difference in the amounts of CCL22 secreted (Fig. 5C). Because the functions of CD91 and the LDL receptor partly overlap (35), we also examined BMDMs from LDLr<sup>−/−</sup>CD91<sup>−/−</sup> LysM-Cre mice. CCL22 secretion by these double-KO cells was unaffected as well (data not shown). We conclude that neither LDLr nor CD91 are essential for CCL22 secretion in response to YFAK and YEAK.

FIGURE 5. CCL22 secretion upon YFAK and YEAK stimulation is not altered in APCs from gp96-deficient, MyD<sup>88</sup>−/−TRIF<sup>−/−</sup>, or LRP-deficient mice. (A) Gp96-deficient BMDCs (one experiment, n = 1), (B) MyD88<sup>−/−</sup>TRIF<sup>−/−</sup> BMDCs (DKO; mean values ± SEM of two independent experiments, n = 1 per genotype and experiment), and (C) LRP-deficient BMDMs (mean values ± SEM of two mice per genotype, one experiment) were stimulated for 24 h with 12.5, 25, 50, or 100 μg/ml YFAK or YEAK (upper panels), and CCL22 in the culture supernatants was measured by ELISA. Gp96-deficient and MyD88<sup>−/−</sup>TRIF<sup>−/−</sup> double-deficient cells were stimulated with 1, 10, 100, or 1000 ng/ml LPS as well (lower panels in (A) and (B)).
We further identified the App as an interactor of YFAK. App is a cell-surface receptor best known as the precursor for different amyloid β species implicated in Alzheimer’s disease. Although App is expressed on myeloid cells, not much is known about the function of App in these cells. App comprises an intracellular domain presumed capable of signal transduction. Thus, we asked whether App is involved in the signaling cascade(s) triggered by YFAK or YEAK. We stimulated BMDCs from App−/− mice and measured CCL22 in the cell culture supernatant. Again, we saw no difference in secretion of CCL22 between BMDCs from wild-type control mice and App-KO mice (data not shown), excluding an essential role for App in this signaling pathway.

**YFAK binds to heparan sulfate proteoglycans**

Because lysine residues are a key component of YFAK, we reasoned that the observed interactions might be mediated by electrostatic interactions. To examine this possibility, we first tested whether binding of YFAK-biotin to target proteins is disrupted by raising of ionic strength. Recovery of most, if not all, of the proteins gradually decreased with increasing salt concentrations in the lysis buffer (Fig. 6A), consistent with the notion that YFAK binds through electrostatic interactions. Does YFAK also bind to negatively charged heparan sulfate proteoglycans (HSPGs)? To test this, we performed cell binding assays with three recently described KO cell lines that lack functional β-1,3-glucuronyltransferase 3 (B3GAT3), xylosylprotein β 1,4-galactosyltransferase (B4GALT7), or solute carrier family 35, member B2 (SLC35B2) (36). These enzymes are involved in sulfation of heparan sulfate precursors and other macromolecules. Although the parental Hap1 cell line bound readily detectable amounts of YFAK-biotin (Fig. 6B, top panel), binding to the KO lines with the earlier mentioned gene disruptions was much reduced (Fig. 6B, left column). YFAK binding was restored in cells in which the function of the disrupted genes had been reconstituted by transduction with an intact cDNA version of the disrupted gene (Fig. 6B, right panels), but not in cells reconstituted with cDNAs that encoded catalytically inactive mutants (Fig. 6B, middle panels). We conclude that YFAK binds to sulfated molecules on the cell surface via electrostatic interactions. We have not been able to demonstrate CCL22 production by the parental Hap1 line or its mutant derivatives under any condition of stimulation.

To determine whether the interaction of YFAK with HSPGs has functional consequences, we stimulated RAW264.7 cells or BMDCs with YFAK in the presence or absence of heparin, a highly sulfated glycosaminoglycan structurally similar to but distinct from HSPGs (Fig. 7). We reasoned that if binding of YFAK to HSPGs is important to prompt APCs to eventually secrete CCL22, then preventing this interaction with a competitor such as heparin for HSPGs to bind to YFAK should reduce CCL22 production. Indeed, coinubation with YFAK and heparin abolished or markedly decreased CCL22 secretion in RAW264.7 cells (Fig. 7A) or BMDCs (Fig. 7B), respectively.

**Discussion**

We designed experiments to explore the molecular details of how random amino acid copolymers that alleviate experimental autoimmune encephalomyelitis in vivo interact with cells of the innate immune system, notably macrophages and dendritic cells. By making use of biotinylated versions of these copolymers, we used an affinity purification strategy to recover interacting proteins. In this manner, we identified proteins that interact with YEAK and YFAK, and defined sulfated glycosaminoglycans as a major cell-surface structure necessary for interaction of YFAK with cells.

We generated biotinylated copolymers to affinity-purify interacting proteins from RAW264.7 cell lysates. We found overlapping sets of proteins to interact with either copolymer. GO enrichment analysis for the “biological process,” “molecular function,” and “cellular component” categories revealed diverse functions assigned to the hits. The most significantly enriched biological processes were “cellular macromolecule metabolic process” and “gene expression,” and the most significantly enriched molecular function was “RNA binding.” Although not further investigated in this study, it is possible that the copolymers exert their effects by interfering with cellular events involved in generation or processing of (maybe specific) mRNAs and/or events involved in translation. It is unlikely that the copolymers gain access to the cytosol to exert their effect, given their size and charge, unless one were to postulate an endosomal escape route sufficiently efficient to allow delivery of the biologically active component to cytosolic targets. Regardless, the list presented in this article might serve as a resource for further
studies on downstream cellular targets of the amino acid copolymers investigated in this study.

We were primarily interested in molecules displayed at the cell surface as possible copolymer targets, because this could identify a cell-surface receptor or receptors that are capable of activating APCs in a manner that would program Th cells to a tolerogenic and/or anti-inflammatory response (as described for YEAK in Ref. 16). First, we focused on gp96 as the major interactor identified. Gp96 has been assigned an endogenous immunomodulatory function. Despite its C-terminal ER retention motif (KDEL), gp96 is found on the cell surface, a feature that appears to be phylogenetically conserved (37), although the cell biological mechanisms that underlie such surface display remain obscure. Gp96 participates in both adaptive and innate immune responses by eliciting class I MHC–restricted CD8+ T cells specific for the antigenic peptides it chaperones (38–40), or by directly activating cells of the innate immune system to secrete cytokines (41, 42). Not only gp96, but also other immunogenic HSPs dictate Th cell responses by stimulating APCs in a CD91-dependent manner (33). Furthermore, it was proposed that gp96 functions as a Th2-specific costimulatory molecule (43). In this article, we identify gp96 as a strong interactor of YEAK and YFAK. We confirmed this association by reciprocal precipitation, followed by immunoblotting in RAW264.7. We used a gp96-deficient cell line to further validate these results. However, gp96 is not essential for CCL22 production as shown by the analysis of gp96-deficient BMDCs. Secretion of CCL22 in response to copolymers occurs also in the absence of gp96. This finding implies that other proteins reliant on gp96 chaperone activity for their native fold (reviewed in Ref. 44) are also dispensable for CCL22 induction by YEAK or YFAK. These include most integrin receptor pairs except α5β1, α6β1, and αIIbβ3 (24), at least four members (LDLR, LRp6, Sorl1, and LRP8) of the LDL receptor family (25), and all TLRs except TLR3 (45). Moreover, the interaction we observed for YFAK and LRp (CD91) is not required for CCL22 induction in BMDCs, again as verified by experiments in a CD91−/− background.

Experiments with MyD88/TRIF double-KO BMDCs showed that neither of these two proteins is essential for CCL22 secretion stimulated by YEAK or YFAK. This result excludes any nonredundant role of MyD88, TRIF, any of the TLRs, IL-18R, IL-1R, and potentially other as yet unidentified MyD88 and/or TRIF-dependent pathways. Many TLRs and C-type lectin receptors have also been excluded by a recent study (17).

Given the physicochemical properties and chemical heterogeneity of the copolymers investigated in this study, and in view of the negative results summarized earlier, we hypothesized that interactions of copolymers with their targets might be mediated by electrostatic and polar interactions and, therefore, be of a less specific nature. YFAK is composed of tyrosine, phenylalanine, alanine, and lysine in a molar ratio of Y:F:A:K 1:1.2:23.5:6.0 (amino acid input mixture used for copolymer synthesis), which results in a strong net positive charge. YEAK (Y:E:A:K 1:1.5:4.5:3.6) is also positively charged, although not as strongly as YFAK, because glutamic acid residues contribute negative charges and will partly compensate for the charged lysine residues. In our affinity purification experiments with biotinylated copolymers in radiolabeled cell lysates, we found that the amount of recovered proteins correlated inversely with the sodium chloride concentration in the buffers used, arguing in favor of our hypothesis. The interaction of streptavidin with biotinylated DNA is not affected by high sodium chloride concentration (46). We reasoned that the positively charged copolymers might interact with negatively charged structures on the cell surface, such as sulfated proteoglycans and sialylated proteins or lipids. Indeed, YFAK did not bind to cells lacking functional SLC35B2 (3′-phosphoadenosine 5′-phosphosulfate transporter I) and bound much more weakly to cells lacking B3GAT3 (glucuronyltransferase I) or B4GALT7 (galactosyltransferase I). B3GAT3- and B4GALT-deficient cells entirely lack heparan sulfates but are able to produce other sulfated protein species. Cells deficient for 3′-phosphoadenosine 5′-phosphosulfate transporter I retain ~5–10% of heparan sulfate levels compared with wild-type cells but produce hardly any proteins with other sulfate modifications (36). Thus, YFAK predominantly binds to cells through electrostatic interactions. HSPGs account for the majority of these interactions, because binding to B3GAT3- and B4GALT-null cells is greatly reduced. However, because binding to B3GAT3- and B4GALT-null cells is less completely abolished, whereas binding to SLC35B2 (PAPS transporter)-deficient cells is almost completely absent, YFAK also binds to other sulfated molecules (obvious candidates are sulfated tyrosines or chondroitin sulfate). Charge-based interactions of YFAK with target cells is functionally relevant because cells stimulated with YFAK in the presence of the competitor heparin do not produce CCL22.

The exact role of this interaction remains to be defined. (Co) receptor(s) for YFAK might be sulfated themselves. Once bound to the cell surface, YFAK might trigger a conformational change of and/or cross-link sulfated proteoglycans and/or associated receptors, upon which cells get activated and eventually secrete, for example, CCL22. The identification of such a receptor(s) will help to understand how the class of immunomodulating copolymers investigated in this study is sensed by a cell and which “downstream” signaling events occur. HSPGs might also serve as scavengers that capture YFAK and deliver it to a cellular compartment that is appropriately equipped to sense YFAK and

**FIGURE 7.** Heparin treatment reduces CCL22 secretion in response to YFAK. (A) CCL22-ELISA of supernatant of RAW267.4 cells stimulated for 4 h with 50 μg/ml YFAK in the presence (black bars) or absence (white bars) of 100 μg/ml heparin in serum-free medium. (B) CCL22-ELISA of supernatant of BMDCs stimulated for 4 h with 50 μg/ml YFAK in the absence (white bars) or presence (black bars) of 12.5, 25, or 50 μg/ml heparin (titration indicated by black wedges below graph) in serum-free medium. n.d., Not detected.
transmit a signal to elicit CCL22 production. Interestingly, YFAK and YEAK are similar in nature to cationic cell-penetrating peptides. Although the mechanism of translocation of cell-penetrating peptides to the cytosol is still a matter of debate, there is agreement that they interact in a first step with cellular heparan sulfates and negatively charged components of the phospholipid bilayer (reviewed in Ref. 47). By analogy, YFAK and YEAK might follow a similar path, gain access to the cytosol/nucleus and/or other organelles, and act there on their target molecule(s). Possibly these are components of the translational machinery, the most enriched “biological process” among the interaction partners identified in this study. In summary, we identify cellular interaction partners of YFAK and YEAK. For some of the identified receptors, we tested functional relevance for CCL22 production in BMDCs or BMDMs. Although none of the tested interactors proved to be essential or play a nonredundant role for CCL22 production, the list of interactors presented in this article provides a basis for further investigations on the molecular mechanism of YFAK and YEAK.

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Disclosures

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