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Free IL-12p40 Monomer is a Polyfunctional Adapter for Generating Novel IL-12-Like Heterodimers Extracellularly

Kaveh Abdi[1,8], **Nevil J. Singh**[2], **Eric Spooner**[3], **Benedikt M. Kessler**[4], **Sergei Radaev**[5], **Larry Lantz**[6], **Tsan Sam Xiao**[7], **Polly Matzinger**[1,8], **Peter D. Sun**[8], and **Hidde L. Ploegh**[3]

[1]Laboratory of Cellular and Molecular Immunology National Institute of Allergy and Infectious Diseases, National Institutes of Health, 9000 Rockville Pike Building 4, Room 211 Bethesda, MD 20892

[2]University of Maryland School of Medicine Department of Microbiology & Immunology, 685 W Baltimore St., HSF1 Rm 380 Baltimore, Maryland 21201

[3]Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, MA 02142

[4]Target Discovery Institute, Nuffield Department of Clinical Medicine Oxford University, Roosevelt Drive Oxford OX3 7FZ UK

[5]Resources and Training Review Branch National Cancer Institute 9609 Medical Center Dr., Rm 7W634 Bethesda, Maryland 20892

[6]Research Technology Branch National Institute of Allergy and Infectious Diseases, National Institutes of Health Twinbrook II 12441 Parklawn Dr. Rockville, MD 20852

[7]Structural Immunobiology Unit, Laboratory of Immunology National Institute of Allergy and Infectious Diseases, National Institutes of Health 9000 Rockville Pike Building 4, Room 228B Bethesda, MD 20892

Abstract

IL-12p40 partners with the p35 and p19 polypeptides to generate the heterodimeric cytokines IL-12 and IL-23 respectively. These cytokines play critical and distinct roles in host defense. The assembly of these heterodimers is thought to take place within the cell, resulting in the secretion of fully functional cytokines. Although the p40 subunit alone can also be rapidly secreted in response to inflammatory signals, its biological significance remains unclear. Here, we show that the secreted p40 monomer can generate *de novo* IL-12-like activities by combining extracellulary with p35 released from other cells. Surprisingly, an unbiased proteomic analysis reveals multiple such extracellular binding partners for p40 in the serum of mice after an endotoxin challenge. We biochemically validate the binding of one of these novel partners—the CD5 antigen-like glycoprotein CD5L— to the p40 monomer. Nevertheless, the assembled p40-CD5L heterodimer does not recapitulate the biological activity of IL-12. These findings underscore the plasticity of secreted free p40 monomer, suggesting that p40 functions as an adapter which is able to generate

Phone: 301-435-5826, FAX 301-402-0284, kabdi@niaid.nih.gov.
[8]Present address: Laboratory of Immunogenetics National Institute of Allergy and Infectious Diseases, National Institutes of Health Twinbrook II 12441 Parklawn Dr. Rockville, MD 20852

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multiple *de novo* composites in combination with other locally available polypeptide partners, post secretion.

Introduction

IL-12 is a heterodimeric cytokine composed of p35 and p40 subunits (1). Secretion of biologically active IL-12 requires the coordinated expression of both genes, which are located on two different chromosomes, within the same cell (2). The p40 subunit also associates with p19 to form IL-23 (3). The prevailing view is that formation of these heterodimeric cytokines occurs within the same cell in endoplasmic reticulum with subsequent post-translational modifications including glycosylation that ultimately determines its secretion (4). Although, it has long been known that the p40 subunit is also secreted as a monomer as a homodimer (only in mice) in far excess of the heterodimer—in many diseases, in the absence of heterodimer—the biological significance of p40 monomer remains an enigma.

Large amounts of secreted p40 can be detected in response to many different kinds of infectious agents, such as bacteria (5), viruses (6), parasites (7), fungi (8), and many non-infectious stimulants (9-11) and allergens (12). p40 levels are also elevated in many disease states (13-15), and in individuals having undergone exhaustive exercise (16). Furthermore, *in situ* hybridization studies have demonstrated that p40 and p35 are expressed in two different anatomical sites in the spleens of mice challenged with LPS, suggesting that p40 and p35 may not always be co-synthesized by the same cells (17). In support of these studies, it has also been noted that the p35 and p40 cistrons are independently regulated transcriptionally (18), and we have shown that p40 can be secreted in the complete absence of IL-12 (19). COS or CHO cells transfected with mouse p40 secrete a monomeric (80-90%) form and also a disulfide-linked homodimer (HD) (10-20%) (20). It is well known that recombinant mouse p40HD, but not the monomer, act as an antagonist for IL-12 by binding to IL-12Rß1—the receptor shared by both IL-12 and IL-23 (21), and it has also been shown to be a chemoattractant for macrophages and dendritic cells (22). However, the physiological relevance of human p40HD is debated. Nevertheless, the role of p40 monomer and its function has never been addressed.

Based on these observations and the propensity of p40 to form at least two well-studied heterodimeric cytokines (IL-12 and IL-23), we have previously proposed that secreted p40 may bind (extracellularly) to additional, yet unidentified protein(s) (23). This hypothesis implied that free p40 monomer might have other functions in addition to being a part of IL-12 and IL-23—as eloquently summarized by Cooper and Khader (24). In this study, we test this hypothesis by examining if free p40 monomer can combine with proteins in its environment to generate *de novo* biological activities. To address this question, we used the formation of heterodimeric IL-12 as a model— both *in vitro* as well as *in vivo*.

The results described here demonstrate that first p40 monomer (but not the HD) in combination with p35 released from necrotic cells can generate IL-12-like activities. Second, IL-12-like activities could be recapitulated by combining p35 with serum-containing p40 from $p35^{-/-}$, but not $p40^{-/-}$ mice. Third, we have extracted multiple novel

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partners for the p40—including CD5L (the CD5 antigen-like protein, also known as AIM, SP-α, Api6), a glycoprotein that was found to copurify with p40 from the serum of $p35^{-/-}$ mice undergoing systemic inflammatory responses. These findings support a model wherein secreted p40 monomer functions as a pleiotropic adapter, capable of binding distinct partners extracellularly in order to mediate diverse biological activities.

Materials and Methods

Mice and reagents

C57BL/6 IL-12p40$^{-/-}$ and p35$^{-/-}$ mice (The Jackson Laboratory) were bred under SPF conditions and kept in AAALAC-accredited facilities at the NIH. Mice received (10μg) of LPS (Sigma) (i.v.). After 6hr, they were anesthetized and their blood was collected through intracardiacpuncture. Serum was separated using Microtainer tubes (BD Vacutainer System) and stored at −80°C. The Institutional Animal Care and Use Committee approved all studies. Anti-p40 mAbs (C17.8, C15.6) and anti-p35 (C18.2) and isotype control were purchased from BioXCell and BD Biosciences respectively. Recombinant CD5L, p40 HD and p40 monomer were purchased from R&D Systems and SBH Sciences (Natick, MA) respectively.

T cell proliferation

The IL-12-responsive mouse CD4^{+} T cell clone (2D6, a generous gift of Dr. Hiromi Fujiwara, Osaka University, Japan) was maintained in complete IMDM with 10-20ng/ml rIL-12 (Peprotech). These cells were stimulated at 3×10^{4} cells/well of U-bottom plate. PHA-activated human lymphoblasts were used for the determination of human IL-12-like activity as previously described (25). Proliferation of the mouse or human T cells was determined by adding 1μCi of [^{3}H] thymidine/well for the last 12-18 hrs of the 72-hour culture period. The data are expressed as the mean cpm ± SD of triplicate wells.

CHO-p35 necrotic cell lysate SN

CHO cells expressing the IL-12p35 or p40 subunit or mock-transfected cells were used as previously described (26). IL-12p35 or mock-transfected CHO cells were grown to confluence, harvested by trypsinization, washed 2 times with complete medium and adjusted at 1×10^{6} or 2×10^{6} cells/ml respectively in complete medium and then frozen at −80°C and thawed immediately at 37°C. The lysate was spun at 400xg for 10 min and the SN was filtered through a 0.22μm PVDF syringe filter unit and kept at −80°C as a source of p35LSN.

Cytokine measurements

The concentrations of IL-12p40 and IFNγ in CSN were determined using cytokine-specific ELISA (R&D Systems) or IFNγ-secreting cells was measured by ELISPOT (MABTECH) using titrated numbers of C57BL/6 splenocytes. The spots were enumerated by an independent contractor (ZellNet Consulting).

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Affinity purification of recombinant and serum p40

HEK293 T cells expressing moue p40 were seeded into a hollow fiber cell system following the manufacturer's instructions (FiberCell Systems Inc.). The CSN obtained was affinity-purified over 1 ml HiTrap NHS-activated HP column, which had been previously coupled with anti-p40 mAb C17.8 following the manufacturer's instructions (GE Healthcare Life Sciences).

Immunoprecipitation

For immunoprecipitations, C17.8 or C15.6 anti-p40 and anti-CD5L mAbs were covalently linked to Dynabeads M-280 following the manufacturer's instructions (Invitrogen). Anti-p40 or CD5L coupled beads were incubated for 1h on ice with rp40 (10μg), rCD5L (10μg) or the mixture of both, which had been pre-incubated over night at 37°C in 20mM Tris, 140mMNaCl, 1mMCaCl, and 1mMMgCl pH 7.4. The bead–protein complexes were washed 3times with 50mM Tris/150mM NaCl, 50mM/Tris 200mM NaCl pH8.2 and 2times with PBS before being eluted with 30μl of 50mM Glycine pH2.5 at which time 4x sample buffer was added to each tube and heated at 70°C for 10 min before loading into the gel.

Sample preparation for 1D gel mass spectrometry

Eluted proteins were separated by SDS-PAGE and visualized by silver staining. Gel bands were excised divided into ∼2 mm squares and washed overnight in 50% methanol/water. The samples were washed once more with 47.5/47.5/5 % methanol/water/acetic acid for 2 hours, dehydrated with acetonitrile and dried in a speed-vac. Reduction and alkylation of disulfide bonds was then carried out by the addition of 30 μl of 10 mM dithiothreitol (DTT) in 100 mM ammonium bicarbonate for 30 minutes to reduce disulfide bonds. The resulting free cysteine residues were subjected to an alkylation reaction by removal of the DTT solution and the addition of 100 mM iodoacetamide in 100 mM ammonium bicarbonate for 30 min to form carbamidomethyl cysteine. Samples were then sequentially washed with aliquots of acetonitrile, and 100 mM ammonium bicarbonate, acetonitrile and dried in a speed-vac. The bands were enzymatically digested by the addition of 300 ng of trypsin in 50 mM ammonium bicarbonate to the dried gel pieces for 10 min on ice. Depending on the volume of acrylamide, excess ammonium bicarbonate was removed or enough was added to rehydrate the gel pieces. The samples were digested overnight at 37°C with gentle shaking. The resulting peptides were extracted by the addition of 50 μl of 50 mM ammonium bicarbonate with gentle shaking for 10 min. The SN was collected in a 0.5 ml conical autosampler vial. Two subsequent additions of 47.5/47/5/5 acetonitrile/water/formic acid with gentle shaking for 10 minutes were performed then the supernatant was added to the 0.5 ml autosampler vial. Organic solvent was removed and the volumes were reduced to 15 μl using a speed vac for subsequent analyses.

Chromatography and peptide Separations

The digested extracts were analyzed by reversed phase HPLC using Dionex RSLCnano pumps and autosampler and a ThermoFisher Orbitrap Elite mass spectrometer using a nano flow configuration. A 20 mm × 180 μm column packed with 5μm Symmetry C18 material (Waters) using a flow rate of 15 μl per minute for two minutes was used to trap and wash

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peptides. These peptides were eluted onto the analytical column, which was a self-packed with 3 μm Jupiter C18 material (Phenomenex) in a fritted 10 cm × 75 μm fused silica tubing pulled to a 5 μm tip. The gradient was isocratic 1% A Buffer (1% formic acid in water) for 1 minute 250 ml min^{-1} with increasing B buffer (1% formic acid in acetonitrile) concentrations to 40% B at 22 minutes. The column was washed with high percent B and re-equilibrated between analytical runs for a total cycle time of approximately 50 minutes.

Mass spectrometry

The mass spectrometer was operated in a dependant data acquisition mode where the 10 most abundant peptides detected in the Orbitrap using full scan mode with a resolution of 240,000 were subjected to daughter ion fragmentation in the linear ion trap. A running list of parent ions was tabulated to an exclusion list to increase the number of peptides analyzed throughout the chromatographic run.

MS data analysis

Peptides were identified from the MS data using SEQUEST algorithms. A species-specific database generated from NCBI's non-redundant (nr.fasta) database and concatenated to a database of common contaminants (keratin, trypsin, etc) was used to gather data. The resulting data was then loaded into Scaffold (Proteome Software). A minimum of two peptides and a peptide threshold of 95% and protein threshold of 99% were used for identification of peptides and protein positive identifications.

2D-difference in-gel electrophoresis (2D-DIGE)

Affinity purified p40 obtained from $p35^{-/-}$ serum was analyzed by a service contractor (Applied Biomics Hayward, CA) as following.

Sample preparation for 2D gel

The sample's buffer was exchanged with 2D lysis buffer (30 mM Tris-HCl, pH 8.8, containing 7 M urea, 2 M thiourea and 4% CHAPS) using 3 kDa MWCO spin columns.

Minimal CyDye labeling

To 30 μg of proteins, 1μl of diluted Cy5 (1:5 diluted with DMF from 1 nmol/μl stock) was added followed by vortexing and incubation on ice in the dark. After 30 min, 1μl of 10 mM Lysine was added to each sample, vortexed and incubated on ice in the dark for additional 15 mins. Cy5 labeled sample were mixed with 2× 2D sample buffer (8 M urea, 4% CHAPS, 20 mg/ml DTT, 2% pharmalytes and trace amount of bromophenol blue) 100 μl destreak solution and Rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mg/ml DTT, 1% pharmalytes and trace amount of bromophenol blue) to 350 μl for the 18 cm IPG strip. Labeled sample was loaded into strip holder.

IEF and SDS-PAGE

After loading the labeled samples into the strip holder, strip was face down and 1.5 ml of mineral oil was added on top of strip. The IEF was done following the manufacturer's instructions (Amersham BioSciences) in dark at 20C. Then, the IPG strips were incubated in

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equilibration buffer 1 (50 mM Tris-HCl, pH 8.8, containing 6 M urea, 30% glycerol, 2% SDS, trace amount of bromophenol blue and 10 mg/ml DTT) for 15 minutes. The strips were rinsed in the equilibration buffer 2 (50 mM Tris-HCl, pH 8.8, containing 6 M urea, 30% glycerol, 2% SDS, trace amount of bromophenol blue and 45 mg/ml Iodacetamide) for 10 minutes with slow shaking. The IPG strips were then rinsed in the SDS-Gel running buffer before being transferred into the SDS-gel (12% SDS-gel prepared using low florescent glass plates) and sealed with 0.5% (w/v) agarose solution (in SDS-gel running buffer).

Image scan and data analysis

Image scans were carried out immediately following SDS-PAGE using Typhoon TRIO (GE Healthcare). The scanned images were then analyzed by Image QuantTL software (GE-Healthcare) and were subjected to in-gel analysis and cross-gel analysis using DeCyder software version 6.5 (GE-Healthcare).

Spot picking and Trypsin digestion

The spots of interest were picked up by Ettan Spot Picker (GE Healthcare) based on the in-gel analysis and spot picking design by DeCyder software. The gel spots were washed a few times, and digested in-gel with modified porcine trypsin protease (Trypsin Gold, Promega). The digested tryptic peptides were desalted by Zip-tip C18 (Millipore). Peptides were eluted from the Zip-tip with 0.5 μl of matrix solution (a-cyano-4-hydroxycinnamic acid, 5 mg/ml in 50% acetonitrile, 0.1% trifluoroacetic acid, 25 mM ammonium bicarbonate) and spotted on the MALDI plate.

Results

p35 released from necrotic CHO cells combines with p40 to generate biologically active IL-12

To examine whether p40 is able to pair with other partners after being secreted from cells, we started with IL-12, a heterodimer composed of p35 and p40. Although it is currently thought that the heterodimer forms only within the cell that produces the p35 and p40 subunits, the converse hypothesis—that extracellular combination of p40 and p35 can generate IL-12— as not yet been examined. While various cells constitutively express p35 transcript, expression of p40 gene is restricted to antigen presenting cells (APCs) (20). Moreover, secretion of p35 independent of p40 has never been reported. We first evaluated if p35 can be released upon necrotic cell death, and if this might then combine with locally produced p40 from activated APCs, using CHO-cells transfected with p35 as a model system. Culture supernatants from these CHO cells (CSN) or the necrotic lysate obtained by freeze/thaw (Lysate supernatant, LSN) were combined with p40 in vitro. The successful generation of *de novo* IL-12-like activity was measured using a bioassay with the IL-12-dependent CD4 T cell clone (2D6), which proliferates in response to IL-12 (27). Figure 1 A shows that the LSN from freeze/thawing the CHO-p35 cells (p35LSN) was able to combine with CSN of CHO-p40 to induce significant proliferation of 2D6 cells compared to the individual subunits ($p<0.0001$), which had no detectable IL-12-like activity (Fig. 1A and B). Furthermore, proliferation of 2D6 in the presence of recombinant p40 (rp40) was dose-

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dependent when combined with p35LSN (Fig. 1B). We confirmed that the synergistic effect of p35LSN with rp40 was due to p35 by attempting to block the functional activity of IL-12 using a p35-specific monoclonal antibody (C18.2) (Fig. 1C). This antibody completely abrogated the activity obtained by combining p35LSN with p40. To further validate our data, we exploited a previously reported observation that a hybrid heterodimer consisting of human p35 (hp35) and mouse p40 was inactive on mouse cells, due to the incompatibility of the p35 subunit (28). Therefore, we combined the LSN from CHO cells expressing the hp35 subunit with mouse rp40 (mrp40). This combination was not active on mouse cells, although it was able to enhance the proliferation of PHA-activated human lymphoblasts (Fig. 1D). Finally, we examined the ability of rp40+p35LSN to induce IFNγ (a benchmark for the biological function of IL-12). We stimulated human PBMCs with hp35LSN alone or in combination with human recombinant p40 (hrp40). Figure 1E demonstrates that the combination of hrp40+hp35LSN induced large amounts of IFNγ—comparable to that elicited by recombinant IL-12 - in the culture SNs. These data show that a functional IL-12 heterodimer is being generated by combining p35 with p40. It is well established that structural association involving disulfide-link between p35 and p40 subunits forms the IL-12 heterodimer. We therefore wondered if the structural signature of such an association is also preserved in the extracellular assembly. Using an IL-12-specific ELISA, we therefore confirmed that p35 and p40 could associate extracellularly and generate IL-12-Like activities (Fig. 1F). Taken together, these data suggest that both mouse and human secreted free p40 has the ability to generate fully active IL-12 heterodimer *de novo* in concert with p35 extracellularly and in a species-specific manner.

Purified p40 monomer but not the homodimer generates de novo IL-12 activity

Previous studies have shown that CHO or COS cells expressing the mouse p40 subunit of IL-12 secrete a mixture of both monomeric and disulfide-linked HD forms of this protein (21). The p40HD binds to IL-12Rß1 on both high (K_d 5pM) and low (K_d 15nM) affinity sites and competes with IL-12 (21). In contrast, p40 monomer, which is the predominant species, is 50-100-fold less active for the receptor binding than p40HD (21). We extended these observations to HEK293T cells and found that similar to CHO cells, HEK293T transfected with p40 predominately secreted p40 in the form of monomer (39-43kDa) (Fig. 1G).

Although p40 is usually secreted as a monomer and homodimer (HD exists only in mouse) in much larger amounts than the heterodimeric IL-12 both *in vivo* and *in vitro* (19), the biological role of the free p40 monomer is not known. Nevertheless, it is an evolutionarily conserved innate response, found in both mice and humans. In this context, we examined whether the extracellular adapter activity we observed was a property of the monomer or the p40HD. We tested purified rp40HD or monomer for their ability to generate IL-12-like activity in the presence of p35LSN. Figure 1H shows that the combination of p40HD plus p35LSN had no IL-12-like activity, whereas the monomeric form combined with p35LSN induced proliferation of 2D6 cells. Therefore, although both p40 monomer and p40HD have been detected in mouse peritoneal macrophages and microglia cells (29), the monomeric form preferentially pairs up extracellularly with p35 to generate IL-12-like biological activity. In fact, p40HD is also capable of suppressing IL-12 activity generated by the

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combination of p35LSN and p40 monomer (Fig. 1I). It is therefore likely that the ratio of monomer to p40HD is a critical determining factor for the adapter like activity of the free p40 monomer.

Serum p40 will generate de novo IL-12-like activity when combined with p35LSN

Since the above experiments used reductionist models to demonstrate that recombinant p40 can combine extracellularly with p35, we asked if this could also happen during a physiological inflammatory response. As we have previously demonstrated (19), copious amounts of p40 can be detected in the serum of mice injected intravenously with the bacterial endotoxin LPS (Fig. 2A). To test if the serum p40 obtained from LPS-challenged mice has the ability of generating *de novo* IL-12-like activities in combination with p35LSN, we harvested serum from either $p35^{-/-}$ (which contains p40 but no IL-12) or $p40^{-/-}$ (as a control), and tested them for IL-12-like activity, either alone or combined with p35LSN. The combination of $p35^{-/-}$ serum plus p35LSN generated *de novo* IL-12-like activity, while $p40^{-/-}$ serum had no effect (Fig. 2B). These data suggest that secreted serum p40 generated during endotoxemia, can act in concert with p35 released by necrotic cells to generate IL-12-like activity.

To further confirm these data, we purified and characterized the free p40 from the serum of $p35^{-/-}$ mice that had been challenged with LPS using immuno-affinity column. We first confirmed the presence of p40 by ELISA, which showed that it was rapidly eluted from the column at low pH in fractions 3-6 (Fig. 2C). Interestingly p40 isolated from mouse serum revealed a surprising degree of molecular pleiotropy, when resolved by SDS-PAGE (Fr# 4, 5, and 6), followed by silver staining (Fig. 2D). First, in agreement with what has been shown for *in vitro* derived rp40 (from CHO, COS or HEK293T cells), serum p40 was predominantly composed of the monomeric form (Fig. 2D lane 7). We verified and confirmed the presence of p40 in fractions five and six by amino-terminal sequencing (data not shown). In fraction six, p40 migrated with an observed molecular mass of ∼40 to 45kDa under non-reducing conditions with a minor faint band migrating at ∼96kDa (suggestive of the p40HD, Fig. 2D lane 7), which disappeared upon further reductions (Fig. 2D lane 8). Under reducing conditions, the p40 bands shifted upward, and migrated at ∼45 to 52kDa (Fig. 2D, four bands, lane 8). However, in fraction four under-reducing conditions there were multiple weak bands ∼38 to 49kDa and strong band migrating at ∼52 to 60kDa with a higher molecular weight band at ∼98kDa (Fig. 2D lane 3). Moreover, upon reductions, multiple bands could be detected migrating at ∼38 to 50-kDa, mirrored the bands in fraction 5 (where p40 usually migrates) (Fig. 2D compare lane 4 with 6). Also, under reducing conditions, both the ∼52 to 60kDa and the ∼98kDa bands shifted upward yielding ∼64kDa and ∼180kDa (Fig. 2D lane 4) bands. Therefore, we are able to report for the first time, that p40 isolated from serum has surprisingly different characteristics from the classical *in vitro*-derived rp40.

Indeed serum p40 migrates differently from rp40HD under reducing conditions (Fig. 2D compare lane 2 with lane 8), suggesting that naturally occurring p40 is composed of more complex isoforms than previously appreciated. Faced with this complex biochemistry of serum p40 we used a high-resolution 2D-DIGE analysis coupled with mass spectrometer to

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further confirm that p40 is composed of multiple species. We identified nine spots migrating at ∼40 to 66kDa within three different zones: the first zone (spots 1-3) appeared as three spots at ∼42kDa with pI ranging from ∼6.5 to 7.2, the second zone with four spots, migrating at ∼50kDa with the pI ranging from ∼5.9 to 6.5 (Fig. 2E spots 4 to 7). The third zone included two spots migrating at ∼66kDa with the pI of ∼5.9 and 6.1 (Fig. 2E spots 8 and 9).

This is the first time that serum p40 has been purified and characterized from the serum—or for that matter, without the help of over-expression or *in vitro* modifications. These data suggest that within each band that was resolved in the 1-D SDS-PAGE, there are multiple isoforms of p40 with distinct pI. The biological significance of p40 having multiple species is not clear, but this pleiotropy offers a potential structural template for our biological findings that p40 is a polyfunctional adapter for the formation of extracellular cytokine(s).

Multiple partners associate with p40 in the serum of $p35^{-/-}$ mice

Our observation that free p40 can combine with p35 outside the cell, together with p40's ability to generate immunologically potent cytokines with other polypeptides (such as p19), raised the intriguing possibility that perhaps multiple binding partners may associate with free p40 monomer to generate distinct biological activities. We decided to use an unbiased proteomic strategy to evaluate this hypothesis by affinity-purifying p40 (and perhaps other accompanying protein(s) from the serum of $p35^{-/-}$ mice that have been challenged with LPS, and as a control group, serum from LPS-challenged $p40^{-/-}$ mice was used in parallel. We reasoned that those proteins that bind to the anti-p40 column from the $p35^{-/-}$ mice but not from the $p40^{-/-}$ serum might be a stringent strategy to identify binding partners for p40 (Fig. 3).

Equal volumes of serum from both $p35^{-/-}$ and $p40^{-/-}$ control mice (pooled from ∼135 mice) that were injected with LPS were separately applied onto an anti-p40 column and after stringent washes, the columns were eluted by linear pH gradient. The presence of p40 in each fraction was confirmed by p40-specific ELISA (we were unable to detect IL-23 from the serum of $p35^{-/-}$ mice), which indicated that the majority of p40 eluted at low pH in later fractions (Fig. 4).

The fractions from both $p35^{-/-}$ and $p40^{-/-}$ serum were resolved by SDS-PAGE and after silver staining, multiple intense bands migrating at molecular mass of ∼10 to 80kDa (Fr# 49-60) appeared in the $p35^{-/-}$ but not in $p40^{-/-}$ serum (Fig. 4 B to E). These bands were excised from each gel and processed for identification by liquid chromatography-tandem mass spectroscopy (LC-MS/MS). Although, the majority of these bands were absent in the $p40^{-/-}$ serum, we excised similar regions corresponding to the bands that were detected in the gel from serum of $p35^{-/-}$ mice (Fig. 4B and 4C compare to D and E).

A data based search revealed multiple proteins that were co-purified with p40, which were either highly enriched in the serum of $p35^{-/-}$ or were completely absent in the serum of $p40^{-/-}$ mice. The identity of the top 20 proteins revealed by the data mining, which had resulted in 8 or more peptides are summarized in Table 1. It should be noted that the other most commonly reported binding partner for p40—namely p19—is not identified herein, since

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IL-23 is not produced in response to the LPS stimulus in this model system. The large number of MS-based protein identified allowed us to generate for the first time, a catalog of binding partners for p40 obtained from the serum of mice. This supports the idea that p40—rather than being a narrow participant in an IL-12 or IL-23 dependant immune response—is a broad based adaptor for multiple proteins. Preliminary analysis did not reveal significant sequence homology between these proteins.

Combination of CD5L plus p40 does not recapitulate the biological activity of IL-12

Finally, we evaluated one of the binding partners—CD5L—and asked if it could associate with p40 outside the cell. CD5L is a 352 amino acid with an apparent molecular mass of ∼54kDa secreted glycoprotein belonging to the macrophages' scavenger receptor cysteine-rich domain superfamily, which is also known as SP-α, CT-2, AIM or API6 for which there is little functional information available. To confirm this interaction, we obtained recombinant CD5L (rCD5L) from a commercial source and either mixed equal concentration of rp40 with rCD5L or incubated them individually at 37°C. After incubation, they were retrieved by immunoprecipitation (IP), a benchmark method commonly used for the verification of protein-protein interactions. The eluted proteins were resolved in 1-D SDS-PAGE and subjected to silver staining. The bands were excised and processed for identification by MS. Figure 5A shows that elutes from immunoprecipitation migrated at 43 to 52kDa and at 51kDa where individual protein were migrated, which was confirmed by MS to be p40 and CD5L, respectively, in lanes 1 and 2 and a mixture of both in lane 3 and 4 where both proteins were mixed and mAb was added to each protein and used separately for co-IP. Then we asked if the heterodimers of p40 and CD5L have similar biological activity as IL-12. We examined this by either adding rp40 or rCD5L alone or in combination to various numbers of mouse splenocytes (as a positive control we added IL-12) and then measured the IFNγ production using ELISPOT. Consistent with the established data, only IL-12 was able to induce IFNγ from these cells when compared to the combination of rp40+rCD5L (Fig. 5B). These data suggest that although p40 associates with CD5L in the serum of mice, its functional consequence is distinct from that of IL-12. Perhaps p40-CD5L complex might have other biological function(s), which remain to be defined.

Discussion

Since the discovery of IL-12 in the early 1990s, it was observed that cells expressing both subunits of IL-12 (p40 and p35) also secrete p40 by itself, often in vast excess relative to the heterodimer. The physiological significance of the copious amount of IL-12p40 secreted from various cells, especially in its monomeric form, has been a longstanding puzzle. Our data not only offer a potential answer to this question, but also offers an explanation as to why resolving its functional roles has been challenging. We show that the secreted p40 monomer has the ability to combine with multiple partners extracellulary to potentially generate several distinct cytokines. While this promiscuity has interesting implications for the localized control of innate immunity, it also means that p40 subunit of IL-12/IL-23 is a multifunctional protein, depending on its binding partner—underlining the complexity of this family of cytokines. In addition, our data also reveal multiple biochemical isoforms of p40 *in vivo*, which can potentially facilitate this plasticity.

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While most cytokines are made from single cistrons, the IL-12 family represents a relatively rare case of a heterodimer—assembled from polypeptides encoded on two different chromosomes (2). This raises a teleological question about the nature of selective pressures that have maintained such an unlinked association over a vast evolutionary time. The idea that p40 is instead a pleiotropic adapter allows us to expand this perspective. Instead of a selective association with p35 or p19, it is able to sense a wide range of secreted binding partners (Fig. 6). The rules of engagement for each partner are also likely to be complex, since we do not observe any sequence motifs or homologies between the proteins we have identified from mouse serum.

The concept of an extracellular adapter for *de novo* generation of a cytokine is novel. However, our findings are consistent with a previous study that examined the requirements for IL-12 secretion from COS cells. This study demonstrated that a deep hydrophobic pocket, which is a docking site in the p40 subunit, is crucial for the heterodimerization with p35 or p19 (30). In figure 6, X could be p35, p19 or any other protein that can adopt the conformation established in the two crystal structures (30, 31). Although, IL-12 is a disulfide-linked heterodimeric cytokine, which uses the cysteine 177 in p40 to form disulfide bond with cysteine 74 in p35 (30), surprisingly, mutation of cysteine 74 to serine in the p35 did not affect the levels of bioactive IL-12 that was formed and secreted in the culture supernatants of COS cells (30). Most importantly, the crystal structure of IL-12 has revealed a protruding centrally located arginine in the p35 subunit as a key-binding determinant with p40's deep hydrophobic pocket containing aspartic acid buried at its base. This has shown to be a critical (“hotspot”) interface for creating an interlocking topography essential for the formation of IL-12 heterodimer (30). Furthermore, although, p19 and p35 have limited sequence homology but, they have distinct biological function when paired with p40; comparative structural analysis of both IL-12 (p35 + p40) and IL-23 (p19 + p40) have indicated that the critical “hotspot” (the central arginine and hydrophobic pocket) is maintained in the two complexes (31). These data suggest that the presence of disulfide bond only ensures the stability of IL-12/IL-23 heterodimer and is not essential for the formation of IL-12 or its subsequent shuttling to the secretory vesicles of the cell. Perhaps, we can deduct from these structural data combined with our data that the presence of “hotspot” coupled with appropriate conformational changes that usually takes place when proteins interact with each other is sufficient for the formation of heterodimer(s) and induction of distinct biological functions.

In fact, the crystal structure of IL-12p40 subunit resembles the extracellular domain of a class 1 cytokine receptor (30). It contains a highly conserved WSXWS motif in D3 domain and a hydrophobic pocket, which is ideally suited for association with a broad range of partner(s) (Fig. 6). Moreover, additional domains such as a conserved RGD and a highly conserved heparin-binding site are also present in p40 sequenced from multiple species (K. Abdi and A. Gittis unpublished observations). This broad template of structural entities on the receptor-like surface of p40, although not used by p35 or p19 offer a broad range of interface for interactions with various ligands.

Although secretion of monomeric p40 is well documented, export of p35 to the extracellular milieu is thought to be strictly dependent on the presence of p40 subunit (32). Furthermore,

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many cell types constitutively express p35 transcript, whereas only those cells that secrete IL-12 express the p40 gene (33). This raises the important question of the source of binding partner(s) that p40 (as an extracellular adapter) can associate with. In our case, p35 was provided from lysate of necrotic p35-expressing CHO cells. An analogous source *in vivo* can be cells that are undergoing necrotic cell death in the local tissues. Indeed many molecules released from such cells have shown to be important messengers in the immune system such as alarmins or DAMPs (34, 35). Our model would predict that rapidly secretion of free p40 monomer (found in both human and mouse), from APCs in response to PAMPs and/or DAMPs, will generate a specific heterodimeric cytokine if it encounters an appropriate partner(s) released by a necrotic cell locally. Moreover, our data suggests that p35, which is expressed by many cell types, if released by necrotic cell death into such a milieu could pair with free p40 to generate a local IL-12 response.

Finally, it is tempting to speculate that during an inflammatory response the secretion of p40 monomer by APCs within various tissues, might allow generation of distinct heterodimeric cytokines locally. This is consistent with the idea that various tissues significantly modulate local immunity (36, 37). Thus, the novel interactions we have described for p40 monomer have the potential to extend our understanding of p40's role beyond the current paradigm of intracellularly generated IL-12 and IL-23.

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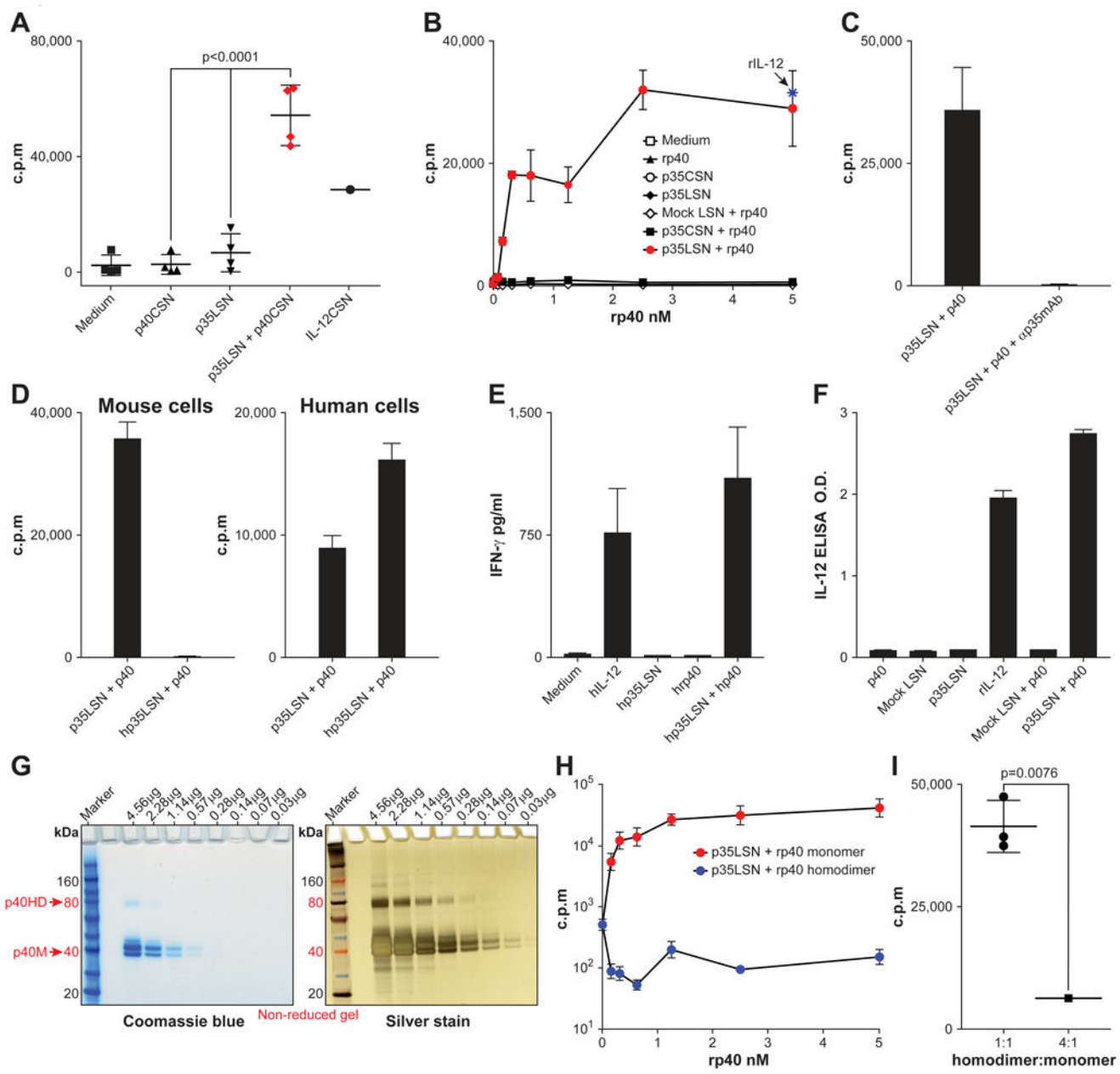

Figure 1. p40 associates with p35 extracellularly to generate IL-12-like activities

(**A**) 2D6 T cells were cultured (3×10^4 cells/well) in the presence of medium alone, CSN from CHO cells expressing the p40 subunit only, or 50μl/well p35LSN or a mixture of both. As a positive control, CHO CSN expressing both subunits (IL-12) were also used in a final volume of 200μl. Incorporation of ^{3}H-thymidine was measure in the last 16hrs of the 72hr culture. Results from 4 independent experiments using ANOVA with Dunnetts multiple comparison $p<0.0001$. (**B**) same as (A) except purified rp40 was used in the presence or absence of mock-transfected CHO cells (mock) or CSN from CHO cells expressing the p35 or p35LSN. (**C**) same as (B) except anti-p35 specific mAb was added to the mixture of rp40 plus p35LSN at the final concentration of 10μg/ml. (**D**) same as (C) except mouse or human

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p35LSN was used with human PHA-activated T lymphoblasts. (**E**) ELISA measuring IFNγ released by human PBL (3×10^4/well) in the presence or absence of hrp40 alone or in combination with hp35LSN and hIL-12 was used as positive control. Data are expressed as the mean ± SD of triplicate well from 3 independent experiments. (**F**) ELISA specific for IL-12 heterodimer measuring mouse rp40 monomer, CHO mockLSN or CHO p35LSN alone or mixed with rp40 monomer, which were kept overnight at 37°C in the presence of 5% CO^2 and then tested by ELISA. As a positive control, rIL-12 was used. (**G**) SDS-PAGE of titrated mouse purified rp40 from HEK293 transfected T cells - Coomassie and silver staining. (**H**) same as (B) except purified rp40 homodimer and monomer were used. (**I**) same as (A) except rp40 monomer or homodimer at the ratio of 1:1 or 4:1 was added to p35LSN. Data are expressed as the mean of triplicate well from 3 independent experiments using Student's T-test.

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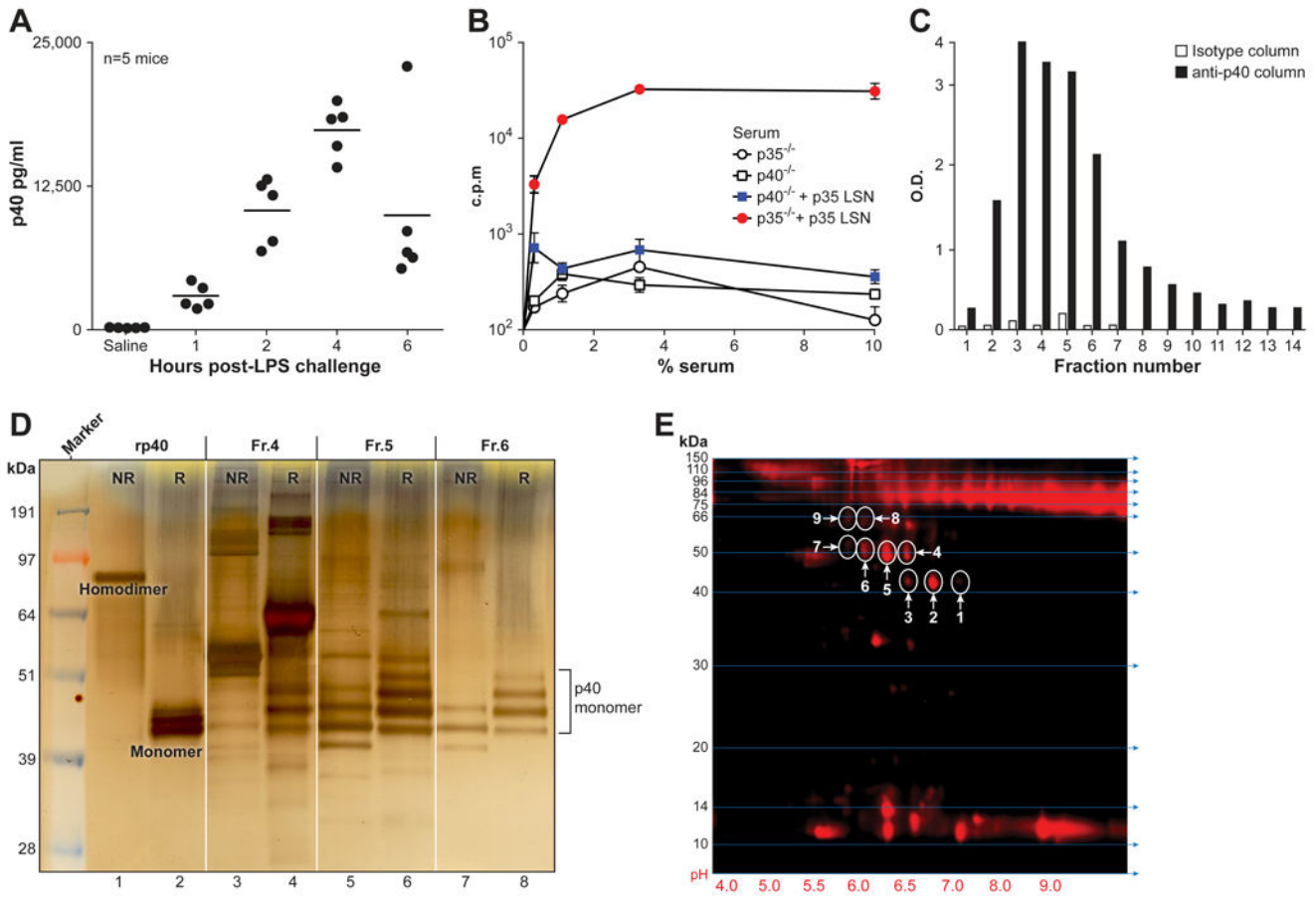

Figure 2. Serum p40 acts in concert with p35SN to generate IL-12-like activity

(**A**) ELISA specifically measuring serum p40 from $p35^{-/-}$ mice (n=5) that were challenged i.v. with 10μg of LPS. (**B**) same as (Fig. 1B) instead of using rp40, serum from either $p35^{-/-}$ or $p40^{-/-}$ mice that had been challenged with LPS was mixed with p35LSN in 2D6 proliferation assay. These data are expressed as the mean ± SD of triplicates wells. (**C**) ELISA measuring affinity purified p40 from serum of $p35^{-/-}$ mice, which is eluted with low pH into various fractions. (**D**) same as (C) Silver stain SDS-PAGE of fractions eluted from anti-p40 affinity column under non-reducing (NR) and reducing conditions. (**E**) same as (C) 2D-DIGE of serum p40 obtained from $p35^{-/-}$ mice under reducing conditions. Similar results were obtained from 3 independent experiments.

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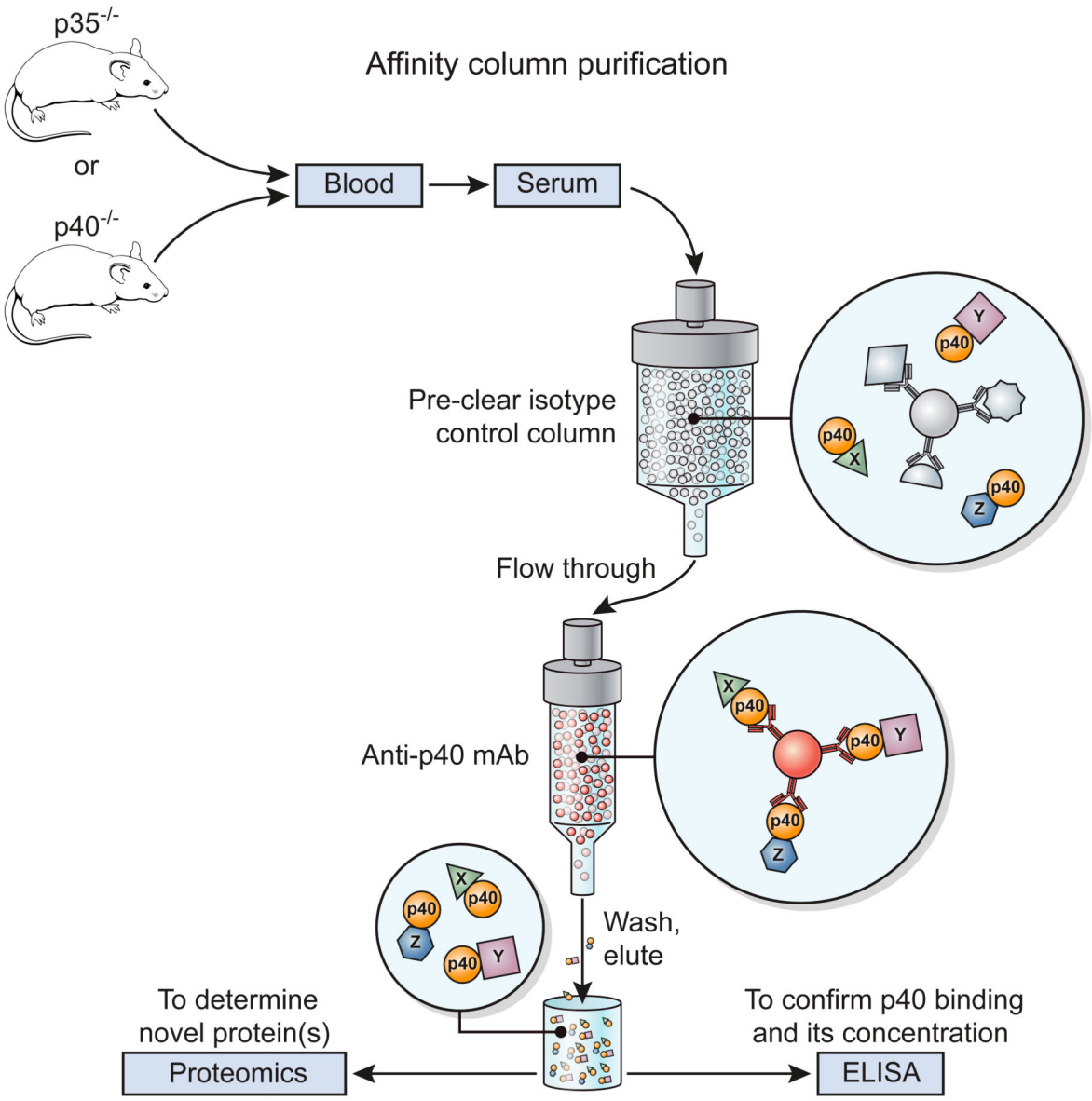

Figure 3.

Schematic presentation of affinity purification of p40-binding proteins from the serum of $p35^{-/-}$ or $p40^{-/-}$ mice.

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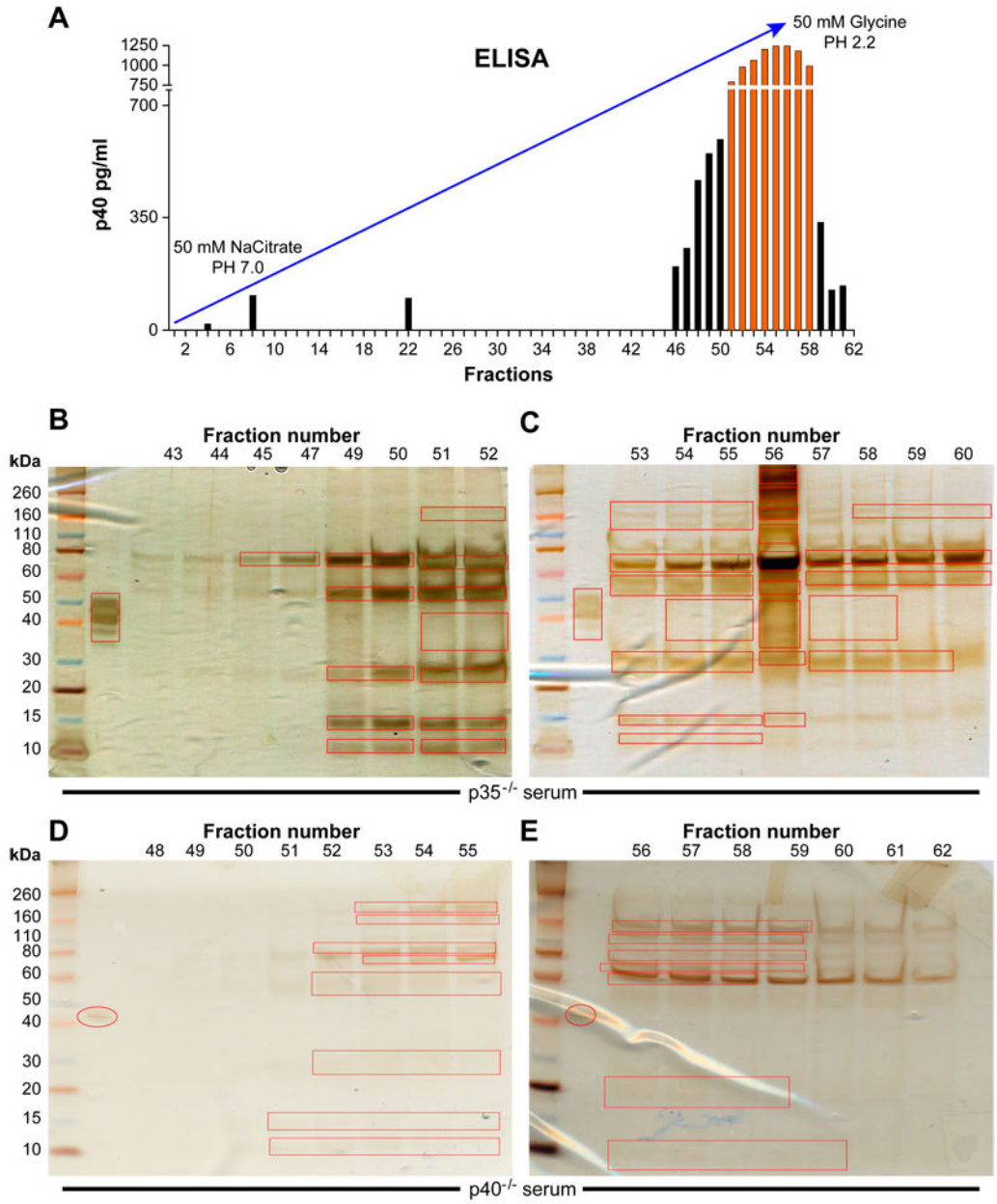

Figure 4. Affinity purification of p40 from p35$^{-/-}$ or p40$^{-/-}$ mice serum

(**A**) Serum from p35$^{-/-}$ or p40$^{-/-}$ mice (30ml) was diluted 1:10 with PBS containing a cocktail of protease inhibitors and filtered through a 0.45μm filter before loading onto anti-p40 (clone C17.8) affinity column (2ml volume) with the flow rate of 0.3ml/min at 4°C. The column was washed with 50ml PBS, 50ml of 50mMTris/150mMNaCl pH 8.0, 50 ml of 50mMTris/500MNaCl pH 8.0 and 50 ml of 50mMNaCitrate pH7.0 with the flow rate of 2ml/min. The column was eluted with the gradient of 50mMNaCitrate pH7.0 to 50mM Glycine pH2.2 into various tubes containing 100μl of 1MTris pH8.0. (**B & C**) Fractions were resolved by SDS-PAGE under reducing conditions and then Silver stained (**D & E**) same as (B & C) except serum from p40$^{-/-}$ mice was used. Bands were excised for MS analyses are marked by red rectangles.

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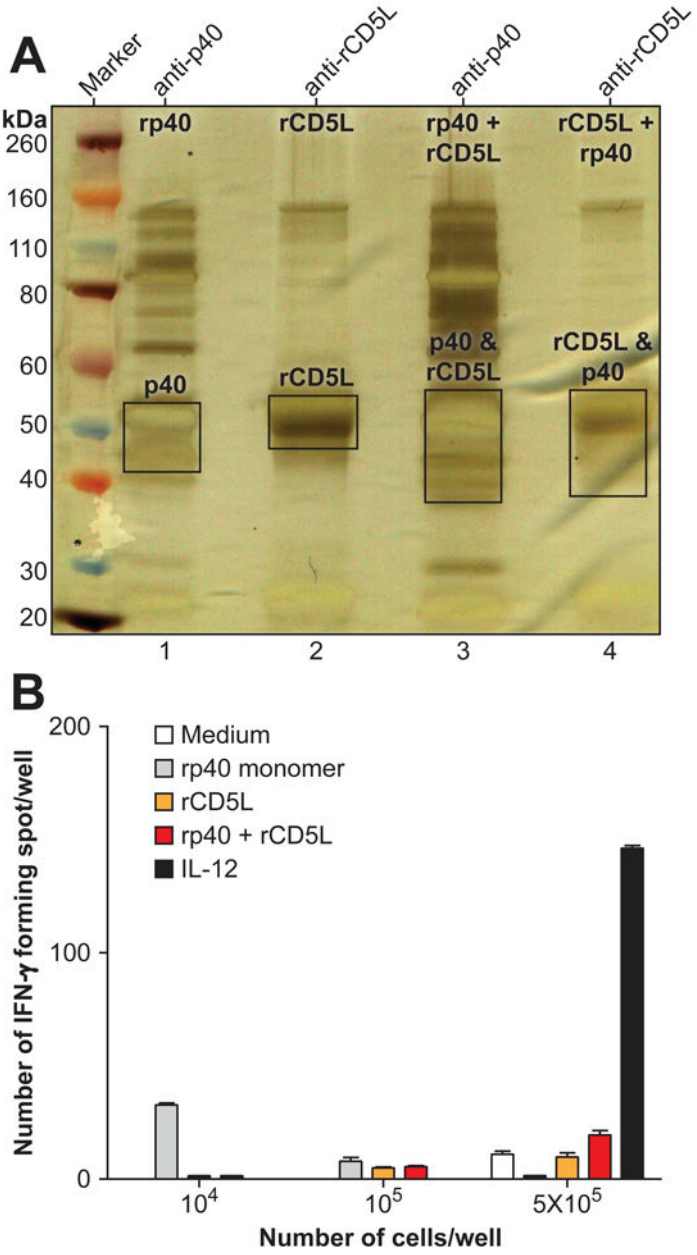

Figure 5. Coimmunoprecipitaion of CD5L with p40

(**A**) Recombinant CD5L, p40, or the mixture of both at 10μgml were incubated over night at 37°C in a 96-well Polypropylene U-bottom plate in final volume of 200μl/well. Proteins were retrieved by immunoprecipitation using magnetic beads (Dynal M-280) that were covalently linked to mAbs specific to p40 (C17.8) or CD5L. Beads were washed stringently with high salt buffer (500mM) and low salt buffer pH8.0 before being eluted with 10mM Glycine pH 2.0. Eluates were resolved by SDS-PAGE. After silver staining, protein bands were excised and processed for MS analysis. (**B**) B6 splenocytes were cultured in a 96-well IFNγ ELISPOT plate in the presence or absence of rp40, rCD5L or a mixture of both, rIL-12

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was used as a positive control. These data are expressed as the mean ± SD of triplicates wells. Similar results were obtained from 2 independent experiments.

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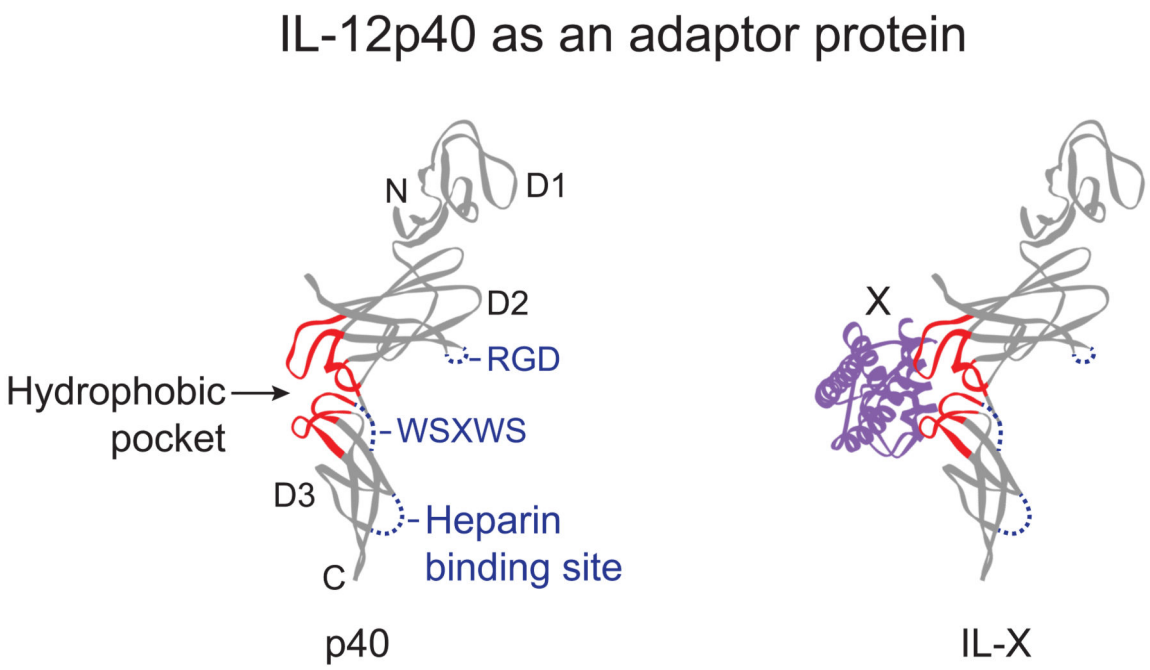

Figure 6.
Schematic of p40 structure showing the highly conserved RGD and heparin bind domains also the hydrophobic pocket that is a “hotspot” for p35, p19 or other proteins shown by X.

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Table 1

Summary of proteins copurified with p40 from the serum of p35$^{-/-}$ mice identified by tandem mass spectrometry

Multiple proteins bind to p40 in the serum of p35$^{-/-}$ mice. Proteins identified by tandem mass spectrometry analysis from affinity column experiments – after sorting based on the protein identification and selecting out those that appeared with high score in the p35$^{-/-}$ serum are shown. Similar results were obtained from 3 independent experiments.

Protein identified	Accession no.	Predicted MW (Da)	No. of peptides
CD5 antigen-like	160358823	38837	25
Clusterin	214010170	51623	22
Lipopolysaccharide-binding protein	113865991	53022	19
Interleukin-12 subunit beta (p40)	6680397	38211	17
Histidine-rich glycoprotein	226958456	60401	16
Glia-derived nexin	6678099	44179	15
Hypothetical protein LOC16061	309262797	37448	12
Mannose-binding protein C	6754656	25941	12
Serum amyloid A-1 protein	6677843	13762	12
Vitamin K-dependent protein Z	13385306	44275	12
Mannan-binding lectin serine protease	148226524	79916	10
Alpha-1-antitrypsin	357588427	48765	10
Resistin-like gamma	225007619	12526	10
Pentraxin-related protein PTX3	113930726	41785	10
Antithrombin-III	18252782	51971	10
Kininogen-1	12963497	47868	9
Vitamin K-dependent protein	13385306	44275	9
Hyaluronan-binding protein	22122613	57289	9
Murinoglobulin-1	31982171	165192	9
Immunoglobulin J chain	170172530	18002	8