Diabetes Induced Alterations in Murine Vitreous Proteome Are Mitigated by IL-6 Trans-Signaling Inhibition

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Purpose. Diabetic retinopathy (DR) is a microvascular complication caused by prolonged hyperglycemia and characterized by leaky retinal vasculature and ischemia-induced angiogenesis. Vitreous humor is a gel-like biofluid in the posterior segment of the eye between the lens and the retina. Disease-related changes are observed in the biochemical constituents of the vitreous, including proteins and macromolecules. Recently, we found that IL-6 trans-signaling plays a significant role in the vascular leakage and retinal pathology associated with DR. Therefore, in this study, comprehensive proteomic profiling of the murine vitreous was performed to identify diabetes-induced alterations and to determine effects of IL-6 trans-signaling inhibition on these changes.

Methods. Vitreous samples from mice were collected by evisceration, and proteomic analyses were performed using liquid chromatography–tandem mass spectrometry (LC-MS/MS).

Results. A total of 154 proteins were identified with high confidence in control mice and were considered to be characteristic of healthy murine vitreous fluid. The levels of 72 vitreous proteins were significantly altered in diabetic mice, including several members of heat shock proteins, 14-3-3 proteins, and tubulins. Alterations in 52 out of 72 proteins in diabetic mice were mitigated by IL-6 trans-signaling inhibition.

Conclusions. Proteomic analysis of murine vitreous fluid performed in this study provides important information about the changes caused by diabetes in the ocular microenvironment. These diabetes-induced alterations in the murine vitreous proteome were mitigated by IL-6 trans-signaling inhibition. These findings further support that IL-6 trans-signaling may be an important therapeutic target for the treatment of DR.

Keywords: diabetic retinopathy, IL-6 trans-signaling, vitreous fluid, proteomics, LC-MS/MS

Increased retinal vascular permeability in diabetic retinopathy (DR) has been linked to inflammatory processes and the release of proinflammatory cytokines, including members of the interleukin family. IL-6 is a pleiotropic cytokine known to be elevated in patients with DR and associated with DR pathology. Importantly, IL-6 signaling occurs via two different mechanisms: the classical and trans-signaling pathways. Classical signaling is mediated by the membrane-bound IL-6 receptor; in contrast, IL-6 trans-signaling uses a soluble form of the IL-6 receptor (sIL-6R) and is primarily proinflammatory. Existing anti–IL-6 therapies inhibit both classical and trans-signaling together. However, IL-6 trans-signaling can be selectively inhibited using sgp130Fc, a fusion protein consisting of the endogenous IL-6 trans-signaling inhibitor soluble gp130 (sgp130) and the Fc region of IgG1. Our lab has previously demonstrated that sgp130Fc can decrease inflammation, restore oxidative balance, and prevent endothelial barrier dysfunction in retinal endothelial cells.

Vitreous humor is a gel-like biofluid in the posterior segment of the eye between the lens and the retina. Composed primarily of water, vitreous also contains a variety of macromolecules, both native to the vitreous and from neighboring tissues. Because of the proximity of the vitreous to the retina and the contribution of retinal vasculature to the maintenance of the vitreous body, disease-related retinal changes can alter the protein composition of the vitreous. Several proteomic studies have been conducted on human vitreous fluid, providing important and clinically relevant insight into DR pathogenesis. However, controlled murine studies play a crucial role in the preclinical evaluation of novel therapies, and studies examining murine vitreous are very limited. This is largely due to the small vitreous volume, and murine samples are often pooled for analysis. Recent technological advancements in mass spectrometry (MS) have improved proteomic profiling in small sample volumes, providing a new method for...
evaluating the effects of novel DR therapies on the ocular microenvironment, including the murine vitreous proteome.

The purpose of this study was to identify the protective effects of IL-6 trans-signaling inhibition on the diabetic mouse vitreous proteome using advanced MS technology. The vitreous proteomes of healthy, diabetic, and diabetic mice treated with sgp130Fc were characterized. Murine vitreous samples were processed and analyzed individually, without pooling, resulting in increased power to detect the variation in biological replicates. Our analyses identified vitreous proteomic changes associated with diabetes, as well as a large set of proteins that normalized in response to sgp130Fc treatment, providing novel insight into the role of IL-6 trans-signaling in the pathogenesis of DR.

**METHODS**

**Animal Studies**

All animal studies were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Diabetes was induced in 8-week-old male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME, USA) by intraperitoneal injection of 65 mg/kg streptozotocin (Sigma, St. Louis, MO, USA) in sodium citrate buffer.
Vitreous Proteomic Changes in Diabetic Mice

(0.05 M, pH 4.5) once daily for 5 days. Mice were considered diabetic when glucose levels were consistently above 250 mg/dL, and both blood glucose and body weight were assessed continually for the duration of the study. After 8 weeks of hyperglycemia, diabetic mice were randomly assigned to untreated or sgp130Fc treatment groups. Treated mice received 5 mg/kg sgp130Fc (Mouse sgp130Fc Chimera Protein; R&D Systems, Minneapolis, MN, USA) by intraperitoneal injection twice weekly for the last 2 weeks of study. At the end of the treatment period, all mice (control, diabetic, and diabetic treated with sgp130Fc) were euthanized for sample collection. Eyes were dissected and vitreous bodies extracted as previously described by Skeie et al. Briefly, a linear incision was made in the cornea, and lens-vitreous tissue was extracted by gently applying pressure to the external surface of the sclera. To isolate vitreous fluid, tissue was washed in 20 µL PBS and centrifuged in Costar Spin-X 0.22-mm centrifuge tube filters (Corning, Inc., Corning, NY, USA) at 14,000 × g for 15 minutes at 4°C. The vitreous was collected as filtrate and stored at −80°C until analysis.

Proteomic Profiling by Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS)

Following protein digestion, vitreous samples were analyzed using an Ultimate 3000 RSLC nano system (Thermo Scientific, Waltham, MA, USA) coupled to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific). Raw MS peptide data were analyzed using Proteome Discoverer (v1.4; Thermo Scientific) and searched against the Uniprot protein database using TurboSequest. Parallel reaction monitoring (PRM) was used to confirm the findings of discovery proteomic analyses by targeting and quantifying specific peptides of interest. Additional details for LC-MS/MS methodology are provided in the supplemental materials and methods section.

Statistical and Bioinformatics Analyses

All statistical analyses were conducted using the R Project for Statistical Computing (v3.2.5, R Foundation for Statistical Computing, Vienna, Austria; www.r-project.org). Peptide spectral match (PSM) count data for the identified proteins were log2 transformed and differential expression analyses were conducted using the “edgeR” package. False discovery rate (FDR)-adjusted P values <0.05 were considered significant. In order to identify biological processes, cellular components, and molecular functions, a functional annotation analysis was conducted using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) Bioinformatics Resources v6.8. Similarly, Ingenuity Pathway Analysis (IPA) software was used for network analysis to display the interactions between proteins of interest.

RESULTS

Proteomic Constituents of Healthy Murine Vitreous

A total of 443 unique proteins were identified in all mouse vitreous samples, which is comparable to the number of proteins identified in other studies. Of these, 154 proteins were identified with high confidence in healthy control mice (present in at least three of six control animals, a distinction not possible with pooled samples) and were considered to be characteristic of healthy murine vitreous fluid. The top 50 proteins identified in the healthy murine vitreous are listed in Table 1, and a complete list of all the 154 proteins available in Supplementary Table S1. The most abundant proteins found in murine vitreous are several crystallin isoforms, including γ-crystallin C (average PSM = 1561), γ-crystallin B (average PSM = 1431), γ-crystallin D (average PSM = 797), β-crystallin S (average PSM = 613), γ-crystallin E (average PSM = 608), β-crystallin B2 (average PSM = 584), β-crystallin A4 (average PSM = 542), β-crystallin A1 (average PSM = 262), β-crystallin B3 (average PSM = 189), γ-crystallin A (average PSM = 179), β-crystallin A2 (average PSM = 183), α-crystallin A (average PSM = 155), and β-crystallin B1 (average PSM = 131). Other abundant vitreous proteins include albumin (average PSM = 359), hemoglobin subunit β1 (average PSM = 141), phosphatidylethanolamine-binding protein 1 (average PSM = 88), and phosphoglycerate kinase 1 (average PSM = 64). Functional annotation analysis revealed that two highly enriched molecular functions in the vitreous proteins are structural constituents of the eye and cadherin binding involved in cell-cell junctions (Fig. 1A). The major biological
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metabolism (Fig. 2E). PI3K-Akt signaling, glycolysis/gluconeogenesis, and carbon biosynthesis of antibiotics, biosynthesis of amino acids, ways associated with the proteins altered in diabetic vitreous Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, mitochondrial, and protein complexes (Fig. 2D). The major cellular components include cytoplasm, extracellular exosomes, extracellular space, myelin sheath, axons, cell-cell adherens junctions, and neuronal projections (Fig. 1C).

Vitreous Proteomic Changes in Diabetic Mice

Vitreous protein levels in diabetic mice were compared to healthy mice. A total of 72 proteins were significantly altered in diabetic mice as compared to healthy controls (Table 2). The proteins with the largest change in vitreous from diabetic mice are tubulin β5 (Tubb5: 42-fold), 14-3-3 protein-ε (Ywhae: 38-fold), tubulin β2A (Tubb2a: 37-fold), 14-3-3 protein-θ (Ywhaq: 35-fold), lengsin (Lgsn: 32-fold), glutamine synthetase (Glul: 32-fold), tubulin α-ε (Eno2: 38-fold), tubulin β2 (Tubb2: 37-fold), 14-3-3 protein-θ (Ywhaq: 35-fold), and rhodopsin (Rho: 28-fold) (Fig. 2A). Molecular functions associated with the altered proteins include protein binding, nucleotide binding, polypeptide/RNA binding, cadherin binding, GTP binding, GTPase activity, and protein kinase binding (Fig. 2B). Enriched biological processes are protein folding, glycolytic processes, visual perception, eye development, lens development, glycolytic processes associated with vitreous proteins include visual perception, eye development, lens development, glycolytic processes, and response to peptide hormones (Fig. 1B). The major cellular components include cytoplasm, extracellular exosomes, extracellular space, myelin sheath, axons, cell-cell adherens junctions, and neuronal projections (Fig. 1C).

Inhibition of IL-6 Trans-Signaling Prevents Vitreous Proteomic Changes in Diabetic Mice

Sgp130Fc treatment was used for selective inhibition of IL-6 trans-signaling in diabetic mice. Inhibition of IL-6 trans-signaling prevented the changes in 52 out of the 72 vitreous proteins that were significantly altered in diabetic mice (Table 2), including all of those identified by upstream regulator analysis. The bar plots of nine highly upregulated proteins in diabetic mice that were normalized to control levels by sgp130Fc treatment are shown in Figure 4. These proteins include aldehyde dehydrogenase cytosolic 1 (Aldh1a7), alpha-1-antitrypsin 1-2 (Serpina1b), Apoa1, C3, γ-enolase (Eno2), vimentin (Vim), and two serine protease inhibitors (Serpina1, Serpina3) (Fig. 3B).

Confirmation of Vitreous Proteomic Alterations Using PRM Analysis

PRM analysis was performed to confirm the increased abundance of these nine proteins in diabetic mice vitreous (Fig. 5). Aldh1a7 (6.71-fold), Serpina1b (10.78-fold), Apoa1 (515.5-fold), C3 (209.3-fold), Eno2 (10.51-fold), Scgb2b20 (6.27-fold), Serpina3m (12.05-fold), Tuba1b (59-fold), and Tubb4b (54.47-fold) were all upregulated in diabetic mice as compared to healthy control animals (Table 3). PRM analysis also confirmed that all these proteins were not significantly upregulated in diabetic animals treated with sgp130Fc.

Discussion

Diabetic retinopathy is a microvascular complication that is a leading cause of visual disability and blindness in diabetic patients. Increasing evidence suggests that the IL-6 pathway plays a prominent role in DR pathogenesis and interactions between IL-6 and endothelial cells regu-
late recruitment of leukocytes and expression of inflammatory proteins. A soluble form of the IL-6R is generated by limited proteolysis and alternative splicing. In cells lacking membrane bound IL-6 receptor, the IL-6/sIL-6R complex associates with the ubiquitously expressed signal transducing protein gp130, initiating dimerization and intracellular signaling (IL-6 trans-signaling).

Studies have shown that IL-6 is upregulated in the vitreous of patients with type 2 diabetes.33 Also, levels of sIL-6R have been found to be increased in the vitreous of patients with proliferative DR as compared to nondiabetics.34 Using experimental models, we and others have shown that IL-6 trans-signaling plays an important role in endothelial barrier dysfunction, inflammation, and oxidative stress in DR and other diabetic complications.5,8,11,35,36 In this study, we sought to gain further insight into the role of IL-6 trans-signaling in the ocular microenvironment in a murine model of DR through proteomic analysis of vitreous fluid following systemic inhibition of IL-6 trans-signaling with sgp130Fc.
**FIGURE 3.** Network analysis of vitreous proteins altered in diabetic mice. IPA software was used to identify interactions among the vitreous proteins altered in diabetic mice (A). Nodes in the network represent proteins, and edges represent known protein-protein interaction. Nine proteins, including Hsp90aa1, Hsp90ab1, Hspa8, Tuba1a, Vcp, Vim, Ywhae, Ywhaq, and Ywhaz, are the key hubs of this network having more than 15 interactions. (B) Upstream regulator analysis revealed known connections between IL-6 and other proteins, including Apoa1, C3, Eno2, Serpina1, Serpina3, and Vim.
Vitreous Proteomic Changes in Diabetic Mice

**Figure 4.** Relative PSM counts of proteins upregulated in vitreous of diabetic mice. The relative PSM counts of nine candidate proteins, including Aldh1a7, Serpina1b, Apoa1, C3, Eno2, Scgb2b20, Serpina3m, Tuba1b, and Tubb4b, are shown in three different groups (control, diabetes, diabetes with sgp130Fc treatment). There was a significant upregulation in the levels of these proteins in the vitreous of diabetic mice, and inhibition of IL-6 trans-signaling mitigated these changes.

The vitreous is a transparent, highly aqueous extracellular matrix overlaying the retina. Due to close proximity to the retina and the breakdown of the blood-retinal barrier, the proteomic composition of the vitreous can be altered by pathologic retinal changes. Understanding these changes in vitreous biochemical composition due to the progression of retinal disease may be beneficial in the development of preventative measures and therapies. While murine studies are a critical part of preclinical therapeutic evaluation, vitreous sample collection and analysis from mouse models are difficult due to a very small vitreous body. However, the technological revolution during the past decade has helped in overcoming this limitation. In this study, we performed comprehensive proteomic profiling of the mouse vitreous humor using the latest LC-MS/MS technology to evaluate the effects of IL-6 trans-signaling inhibition using sgp130Fc. To the best of our knowledge, this is the first study to evaluate the effects of IL-6 trans-signaling inhibition on the diabetic vitreous proteome.

Our analysis identified 443 unique proteins, of which 154 were characteristic of healthy murine vitreous. These findings are similar to a previous murine vitreous proteomic profiling study that identified 675 unique vitreous proteins in samples pooled from eight eyes. The most abundant proteins identified in our analysis are crystallins, which are structural proteins primarily expressed in the lens, cornea, and retina. Previous proteomic profiling studies have also shown that crystallins are the most abundant proteins in the vitreous humor. We also identified fatty acid binding protein 5 (Fap5), acyl-CoA-binding protein (diazepam binding inhibitor, Dbi), phosphoglycerate kinase 1 (Pgk1), phos-
Vitreous Proteomic Changes in Diabetic Mice

**Figure 5.** Confirmation of vitreous proteomic alterations using PRM analysis. Differential abundance of nine candidate proteins (Aldh1a7, Serpina1b, Apoa1, C3, Eno2, Scgb2b20, Serpina3m, Tuba1b, and Tubb4b) in three groups (control, diabetes, diabetes with sgp130Fc treatment) was confirmed using PRM high-precision mass spectrometry analysis.

**Table 3.** Confirmation of Changes in the Vitreous Humor Proteome Using PRM Analyses

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</tr>
<tr>
<td>Q03734</td>
<td>Serpina3m</td>
<td>Serine protease inhibitor A3M</td>
<td>12.05</td>
<td>0.0382</td>
<td>23.13</td>
<td>0.0790</td>
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<tr>
<td>P05213</td>
<td>Tuba1b</td>
<td>Tubulin alpha-1B chain</td>
<td>59.0</td>
<td>0.0360</td>
<td>27.67</td>
<td>0.3364</td>
</tr>
<tr>
<td>P08372</td>
<td>Tubb4b</td>
<td>Tubulin beta-4B chain</td>
<td>54.47</td>
<td>0.0481</td>
<td>27.67</td>
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*Diabetic versus controls.
†Diabetic with sgp130Fc treatment versus controls.
phatidylethanolamine binding protein 1 (Pepbp1), carboxic anhydrase 2 (Ca2), peroxiredoxin 5 (Prdx5), and hemoglobin subunit beta 1 (Hbb-b1) among the most abundant proteins in healthy murine vitreous fluid. Our analysis also revealed a large number of intracellular proteins and proteins associated with extracellular exosomes, as previously reported in both mouse and human vitreous studies.

Comparison of the vitreous proteome of healthy mice to that of diabetic mice revealed a higher abundance of proteins in diabetic vitreous than in control. This could possibly be due to increased vascular leakage and breakdown of the blood-retinal barrier commonly seen in DR. Furthermore, it is also possible that diabetes-induced retinal damage released retinal proteins into the vitreous, thereby increasing protein content. We confirmed that vitreous samples were free of cellular contaminants by hematoxylin and eosin (H&E) staining (data not shown). Interestingly, protein abundance was decreased in the vitreous of diabetic mice after treatment with sgp130Fc. While this offers support to the vascular leakage hypothesis, as sgp130Fc has been shown to have protective effects on endothelial barrier function,5 it is also possible that any changes to vascular permeability are instead concordant with an overall decrease in retinal inflammation. Our data demonstrate the protective effects of IL-6 trans-signaling inhibition on the diabetic vitreous proteome, although further studies are needed to delineate the exact mechanism(s) underlying this response.

The most upregulated proteins in diabetic vitreous humor are tubulin beta-5 chain (Tubb5), 14-3-3 protein epsilon (Ywhae), tubulin beta-4B (Tubb4b), gamma-enoalase (Eno2), and tubulin beta-2A chain (Tubb2a). Both beta-tubulins40 and Eno241 have been previously identified in proteomic analyses of diabetic animal models. Tubb5 is a major component of microtubules known to be upregulated in retinopathy.52 Ywhae plays a role in the cellular response to heat stress and regulation of apoptotic signaling and is known to be expressed in murine photoreceptors.53 Two proteins downregulated in the vitreous of diabetic mice as compared to controls are major urinary protein 4 (Mup4) and 8 (Mup8). MUPS are part of the lipocalin family, which are involved in communication and regulate glucose and lipid metabolism. Others have reported decreased Mup4 expression in diabetes,43 while Mup8 levels are reportedly decreased45 or unchanged.46 Despite significant changes in the abundance of these proteins in diabetic mouse vitreous, their significance to DR pathology remains unclear.

Inhibition of IL-6 trans-signaling with sgp130Fc prevented diabetes-induced proteomic changes in 52 out of the 72 vitreous proteins. Since the effects of IL-6 trans-signaling in vitreous fluid have not previously been characterized, we performed IPA analyses to identify any known interactions between proteins in this subset. Network and upstream regulator analyses revealed a highly interconnected network of regulatory relationships among these proteins. Vimentin, an intermediate filament protein identified as one of the key regulatory hubs in the network, plays a role in retinal response to injury and is upregulated in DR.47,48 IL-6 promotes epithelial-mesenchymal transition through vimentin upregulation in a STAT3-dependent manner.49 Vimentin is also known to play a role in the regulation of Notch signaling and angiogenesis.50

Other protein hubs identified by network analysis include several heat shock proteins (HSPs), including Hsp90β, Hsp90α, and Hsp8. Hsp90α and β are members of the Hsp90 family of proteins and serve as critical molecular chaperones during protein synthesis.51,52 Hspa8 is a member of the Hsp70 family of proteins and plays roles in protein folding, chitin-mediated endocytosis, and ubiquitin-mediated protein degradation.53 HSPs are important for the cellular response to damage or injury,54 and Hsp70 specifically has been implicated in both DR and the retinal damage response.55,56 While IL-6 has been associated with several HSPs,57,58 this interaction in the context of DR has not yet been described.

We also found several 14-3-3 family proteins that were upregulated in the vitreous of diabetic mice, including Ywhab, Ywhae, Ywhag, Ywhaq, and Ywhaz. These proteins constitute five out of the seven members of a highly conserved protein family responsible for regulating the activity of phosphoproteins and modulate several signaling pathways, including those related to metabolism, cell proliferation, and stress responses.59,60 The 14-3-3 proteins have been shown to be crucial for the cellular response to insulin signaling.61 While members of this protein family have been reported to be overexpressed in DR,62,63 their significance to disease pathology and relationship to IL-6 trans-signaling remains unclear.

The five other proteins identified by upstream regulator analysis (Apoa1, C3, Eno2, Serpina1, and Serpina3) are increased in vitreous from diabetic mice and have predicted activation by IL-6. These proteins were normalized to control levels in diabetic mice after sgp130Fc treatment. Apoa1 is a well-known biomarker of DR and is elevated in the serum, retina, vitreous, and tears of patients with DR.64,65 Its expression is known to be regulated by IL-6.66 Similarly, complement C3 and the acute phase response protein α-1-antitrypsin 1-2 (Serpina1b) are known to be elevated in diabetic vitreous15,65 and regulated by IL-6.66–68 Eno2 has also been associated with DR,41 and as a neuron-specific isoform, the presence of this protein in vitreous could indicate neuronal damage. Regulation of serpina3m (alpha-1-antichymotrypsin) by IL-6 has been well characterized in some cell types,69 but to our knowledge, this protein has not previously been associated with DR.

Several tubulin proteins, including Tuba1a, Tuba1b, Tubb2a, Tubb4b, and Tubb5, were highly upregulated in diabetic mice, and this change was prevented by inhibition of IL-6 trans-signaling. Tubulin is a major cytoskeletal component regulated by inflammatory signaling,70 and tubulin dysfunction can disrupt cell proliferation and differentiation.71 Tubulin α1B (Tuba1b) has been shown to be increased in the vitreous of patients with DR,72 and this increase may be due to increased endothelial cell proliferation during angiogenesis.73 While the significance of many specific dysregulated proteins remains unclear, the restoration of normal protein abundance in vitreous fluid following sgp130Fc treatment emphasizes the importance of IL-6 trans-signaling to DR pathogenesis. As previously mentioned, IL-6 is known to regulate several of the proteins identified in our study, but it is also possible that the observed proteomic changes are the indirect consequence of retinal tissue damage due to inflammation or oxidative stress, both of which are known to be associated with IL-6 trans-signaling.51,53 Corticosteroids and nonsteroidal anti-inflammatory drugs have shown benefits in patients with DR by reducing leukocyte recruitment into the retinal vasculature.74–76 and as leukocyte recruitment is often linked to IL-6 trans-signaling,77 it is likely that this...
mechanism is a major contributor to the effectiveness of sgp130Fc. The use of un pooled samples offers an advantage in being able to distinguish between biological replicates for statistical purposes, but it appears to be less sensitive for the detection of low-abundance proteins. Qualitative comparison of our proteomics data from control mice with that of a similar study by Skeie and Mahajan29 showed similar vitreous protein composition for highly abundant proteins but greater disparities in low-abundance proteins. To confirm that the elevated protein in diabetic vitreous was not due to contamination from retinal tissue, we verified the aces
dous protein composition for highly abundant proteins but greater disparities in low-abundance proteins. To confirm that the elevated protein in diabetic vitreous was not due to contamination from retinal tissue, we verified the acel-


8. Rose-John S. IL-6 trans-signaling via the soluble IL-6 recep-


25. Raunyiar N. Parallel reaction monitoring: a targeted experi-


