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Original Article

Preliminary findings on the absence of PEPITEM release in B cells isolated from Saudi donors: implications for expanded population studies

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Abstract: Background: Adiponectin (AQ) plays a role in regulating immune responses. Previous research indicates that B cells can affect T cell transmigration via the adiponectin-induced peptide PEPITEM in Caucasians. This study explores whether this mechanism is also applicable to Saudi populations, considering potential ethnic variations in immune response. Methods: We conducted unbiased peptidomic screen on B cells, NK cells, and monocytes isolated from the peripheral blood of male healthy Saudi donors. The cells were stimulated with AQ, and the secretion of PEPITEM and other peptides was assessed using liquid chromatography-mass spectrometry (LC-MS/MS). Flow cytometry was utilized to confirm the purity of isolated cell populations and to verify the expression of adiponectin receptors AR1 and AR2. Results: PEPITEM was not detected in the supernatants of AQ-stimulated B cells, NK cells, or monocytes. All three cell populations were isolated and purified with high purity, confirmed by flow cytometry showing AR1 and AR2 expression on the surface of these cells. Specifically, less than 47% of B cells expressed ARs, with AR1 at 12% and AR2 at 17%. AQ stimulation increased the number of identified peptides in B cells and monocytes but decreased peptide numbers in NK cells. Dimensionality reduction analysis demonstrated clear segregation of cell types, with strong reproducibility across technical replicates. Conclusion: The inability of B cells to release PEPITEM in response to AQ stimulation is an interesting finding and it needs more confirmatory tests and experiments, however; a hypothesis about the impact of predisposing factors, such as ethnicity could be formulated and tested in the future.

Keywords: Adiponectin, PEPITEM, immunometabolism, T cell recruitment, inflammation

Introduction

The immune cell interface has various environmental conditions, which in some cases affect their metabolic regulation, leading to potential transcriptional reprogramming of immune cells; however, when reprogramming is impaired, the risk of autoimmune disorder development is increased [1].

Immune cell activation results in the acquisition of new immune functions, such as cytokine

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production or proliferation, and immune cells are required to provide continuous metabolic adaptation to meet bioenergetics demands [2]. One of the activating molecules of immune cells is adiponectin (AQ), which stimulates B cells to release a naturally occurring mediator peptide, called PEPtide inhibitor of transendothelial migration (PEPITEM) that controls the recruitment of immune cells to inflamed tissues [3]. The role of cellular metabolism in immune cell function and differentiation has recently attracted a lot of attention. It has been shown that the treatment of NK cells with interleukin-2 and interleukin-15 increases glycolysis and oxidative phosphorylation (OXPHOS) pathways to improve their function [4]. There is highly regulated integration and interaction between the immune system and metabolism, as evident from accumulating data. For example, recent data indicate that T cell proliferation is influenced by nutrient uptake and utilization [5]. One mechanism by which immune cells respond to activating and proliferating signals is by modulating their metabolic activity; for example, T cells undergo metabolic reprogramming that leads to glucose and amino acid upregulation to improve their immune response [6, 7]. However, the mechanisms of intracellular metabolism and how these metabolic pathways modify the phenotype and activation of immune cells are poorly understood. The only aim of cellular metabolic reactions and pathways is biosynthesis and generation of ATP; however, recent discoveries and insights have shown that these reactions and pathways control and regulate immune cell function by metabolic reprogramming mechanisms [8].

Controlling leukocyte recruitment and transmigration plays a crucial role in inflammatory response initiation and resolution. In some cases, inappropriate leukocyte recruitment is prolonged, eventually leading to pathological conditions, such as diabetes, autoimmune diseases, and atherosclerosis [9-11]. Our previous work showed that B cells stimulated by AQ secrete PEPITEM, which effectively inhibits inappropriate and prolonged T cell transmigration, ameliorates the symptoms, and inhibits disease progression [3]. Moreover, exogenous treatment with PEPITEM restored PEPITEM control of T cell trafficking and inhibited T cell recruitment into inflamed tissues in animal models [3]. During pathological conditions, for many various cellular metabolic modifications, the B cell-AQ stimulation pathway could become dysregulated, which in turn leads to the inability to release PEPITEM and subsequently leads to prolonged and unwanted T cell transmigration, which has been supported by strong evidence [3, 9-11]. AQ is the most abundant hormone derived from adipocytes, and recent data have shed some light on its homeostatic function in immune cells or metabolic cells through its immunomodulatory effects [12]. AQ plays a key role in the progression of inflammation and metabolic disorders. Therefore, the role of AQ in immune modulation and disease progress is important for our understanding of disease progression and normal homeostasis. Innate immunity mediators, such as natural killer (NK) cells and monocytes, have been suggested to mediate cellular metabolic pathways, such as the regulation of energy and glucose homeostasis. Additionally, the literature suggests that AQ exerts its effects on energy consumption and insulin sensitivity through a dependent innate immune response [13].

Studying the effect of predisposing factors, such as racial and ethnic backgrounds, will help clarify the role of causative factors, such as genetics or environment, as well as evaluate the efficacy of novel drugs and optimize dosing for a certain population. The impact of racial differences in disease development has been increasingly supported by many studies; for example, the US database analysis has shown that the progression of chronic kidney disease at advanced stages in black people is four times faster than that in Caucasians [14, 15]. Moreover, racial differences are an important factor in evaluating drug response in clinical trials. Aggarwal et al. studied the impact of ethnicity on the tolerability of PI3K/mTOR inhibitors and concluded that the drug has a higher metabolic impact on Asian patients and that these patients need more studies to optimize their doses [15]. In autoimmune diseases, another study has shown that gender and ethnic differences play a significant role in the development of autoimmune diseases; nevertheless, the underlying mechanism remains unclear [16]. Another retrospective study in the UK, a cohort study of 4.4 million subjects, concluded that ethnicity has a significant impact on the development of allergic diseases and autoimmune disorders [17].

Here, we aimed to investigate the ability of B cells, NK cells, and monocytes to release PEPITEM in response to AQ stimulation. Such findings could lead to the determination of novel therapeutic strategies in inflammation modulation, both in the context of vascular biology and classical clinical immunology.

Methods and materials

Cell preparation and isolation protocol for different cell types were optimized to one workflow to control any variability related to different isolation methods specific to each peripheral blood mononuclear cells (PBMCs) subtype.

Human subjects: sample collection

Fresh blood was collected from male Saudi healthy donors (20-35 years old and BMI < 25) recruited from the College of Medicine at King Saud bin Abdulaziz University for Health Sciences (KSAU-HS), Riyadh, KSA. This study was approved by the institutional review board (RC18/004/R). The phlebotomy procedure was performed by a certified phlebotomist, and donors were asked to fill out a mandatory consent form. Fresh blood was used for isolation and purification protocols, whereas serum samples were collected and stored at -80°C for enzyme-linked immunosorbent assay (ELISA). The sample size is guided by the variance from our published work, where we determined the right dosage of adiponectin and the proper incubation time [3]. Moreover, Randomization was not required for this study.

Three blood samples were obtained from 3 different donors and from each sample three experimental samples (B cells, NK cells, and monocytes) were isolated. Thereafter, two sets from each experimental sample (AQ stimulated and non-AQ stimulated) were prepared. Finally, from each set, two analytical replicates.

Isolation kits and AQ stimulation

B cells, NK cells, and monocytes were isolated from fresh blood using immunomagnetic negative selection kits for B cells, NK cells, and monocytes (EasySep™ Human B Cell Isolation Kit (Cat 19674)), EasySep™ Human NK Cell Isolation Kit (Cat 19665), EasySep™ Human Monocytes Isolation Kit (Cat 19669), (STEMCELL Technologies, Vancouver, Canada), according to the manufacturer's protocols. In summary, undesired cells were labeled with antibodies and easySep direct rapidSpheres particles and were separated using an EasySep magnet. Briefly, 7 mL of heparinized fresh blood was collected and added to 14 mL Falcon® round-bottom tubes (Cat 38008). Next, isolation cocktails and RapidSpheres (50 µL/mL sample) were vortexed and transferred into Falcon tubes and incubated for 5 min at room temperature (RT). D-phosphate-buffered saline-free Ca++ and Mg++ (Cat 37350) were then transferred to the top of the tube with gentle pipetting. The tube was then placed into the big easy magnet (STEMCELL Technologies (Cat 18001)) without a lid and incubated for 5 min. After incubation, only RapidSpheres were added, and the tubes were again placed in the magnet. Finally, a third separation step was performed, and the cell population was purified and stored until use. The NK cell and monocyte isolation methods were similar to the previously mentioned steps, with the only difference being that the isolation cocktail varied based on the isolation kit used. Another difference is the monocyte isolation kit, which recommends using 1-3 mL of fresh blood and phosphate-buffered saline containing 1 mM EDTA medium. For AQ stimulation, the isolated populations, approximately 2-5 × 10^5 B cells, NK cells, and monocytes were incubated in the presence or absence of AQ at 15 µg/mL for 1 h under agitation. The mixture was then centrifuged at 1,800 rpm for 8 min, and the supernatant was stored for mass spectrometry analysis.

Flow cytometry

To analyze the purity of the isolated populations, flow cytometry was performed. PBMCs (1.5 × 10^6) were washed with phosphate-buffered saline by centrifugation at 1,500 rpm for 7 min, followed by the addition of 2 µL of FcR blocking reagent (Miltenyi Biotec, (Cat 130-059-901)) to prevent non-specific binding of antibodies, and the conjugated markers CD19 for B cells, CD56 for NK cells, and CD14 for monocytes (Thermo Fisher Scientific, Waltham, MA USA) (25-0199-42, 12-0567-42, 17-0149-42). An additional experiment was performed to measure the surface expression of AQ receptors 1 and 2 (AR1 and AR2, respectively) in each population (phoenix pharmaceuticals, Cat G-001-23, G-001-44). After incubation for 30

min at 4°C, the samples were washed twice and resuspended in 300 µL flow cytometry staining buffer (STEMCELL Technologies, Vancouver, Canada).

Mass spectrometry analysis

We used an unbiased peptidomics screen to detect PEPITEM in the supernatant of purified isolated subsets following stimulation with or without AQ. After B cells, NK cells, and monocytes were incubated with AQ, the peptides were purified from the supernatant. Purification was performed using a C18 solid-phase extraction (SPE) column (Supelco, DSC-18/Sigma Aldrich Darmstadt, Germany (Cat 52602-U)) as previously published [18]. The columns were preconditioned with 1 mL 0.1% trifluoroacetic acid (TFA) in acetonitrile and allowed to flow under gravity. Subsequently, the column was equilibrated with 0.1% TFA/water and the sample, adjusted to 0.1% TFA, was added to the column. Finally, the column was washed with 1 mL of 0.1% TFA/water, and the peptides were eluted using 0.1% TFA/acetonitrile (1 mL). The eluted peptide samples were analyzed using a nano LC-MS/MS (nano liquid chromatography-tandem mass spectrometry) as previously described (35). Briefly, the samples were injected into an Ekspert nano LC 425 (Eksigent, Dublin, CA, USA) coupled with a TripleTOF 5600 (Sciex, Concord, Canada). Peptide separation was achieved using a C18 nano LC column (3C18-CL, 75 µm × 15 cm, Eksigent, USA) with an elution gradient, 0-1 min, 5% B; 1-60 min, 5-30% B; 60-66 min, 30-80% B; 66-74 min, 80% B; 74-85 min, 80-5% B; and 85-100 min, 5% B using mobile phase A (0.1% HCOOH in water) and mobile phase B (0.1% HCOOH in Acetonitrile). Data-dependent acquisition was used to acquire the MS and MS/MS spectra. Peptide data processing for peptide identification was performed using ProteinPilot Software (v. 4.5, Sciex). MS2 data files were searched using the UniProt Proteome Humans database (downloaded on October 29, 2019).

Dimensionality reduction using peptide binary profile data

Once the peptides were identified by MS analysis of different cell types from different subjects, we created a binary (0: absent; 1: present) matrix for the set of unique peptides across different samples. Peptides with > 1.3 "unused score" were considered for further analysis. The purpose of this study was to examine peptide profiles across different samples under different treatment conditions. Principal component analysis (PCA) of such binary data is known as logistic PCA. We used the R package "logisticPCA" to analyze the peptide presence/absence profiles across different samples. We chose the rank 3 solution (k = 3) because it provided a better separation of different subjects.

ELISA

Human total AQ levels were measured in the serum using the Quantikine ELISA kit (R/D Systems) according to the manufacturer's protocol. Fresh blood was collected in yellow top chemistry tubes and spun at 2,500 rpm for 15 min. The serum was then separated from the blood using a pasture pipette, and the concentration was measured according to the manufacturer's protocol. All reagents, working standards, and samples were prepared according to the manufacturer's protocol. Briefly, 100 µL of the assay diluent RD1W was added to each well. Then, 50 µL of each standard, control, or sample was added to the designated well, and an adhesive strip was used to cover the plate and incubated for 2 h at RT, as previously mentioned. The wells were then washed three times before 200 µL of human AQ conjugate was added to each well. An adhesive strip was used to cover the plate, which was then incubated for 2 h at RT. After that, three washing steps were performed before adding 200 µL of substrate solution to each well, followed by incubation for 30 min at RT in the dark. Finally, 50 µL of stop solution was added to each well, followed by plate reading using a microplate reader, where the optical density was measured and AQ serum level was measured against a calibration curve.

Statistical analysis

All experiments were repeated three times, independently from a minimum of three biological replicates. Data are means ± SEM (N = 3), and the differences between groups were determined by using an independent sample t-test with a Bonferroni post hoc test. *P*-value less than 0.05 were considered significant. Probability values of less than 0.05 were considered significant (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$).

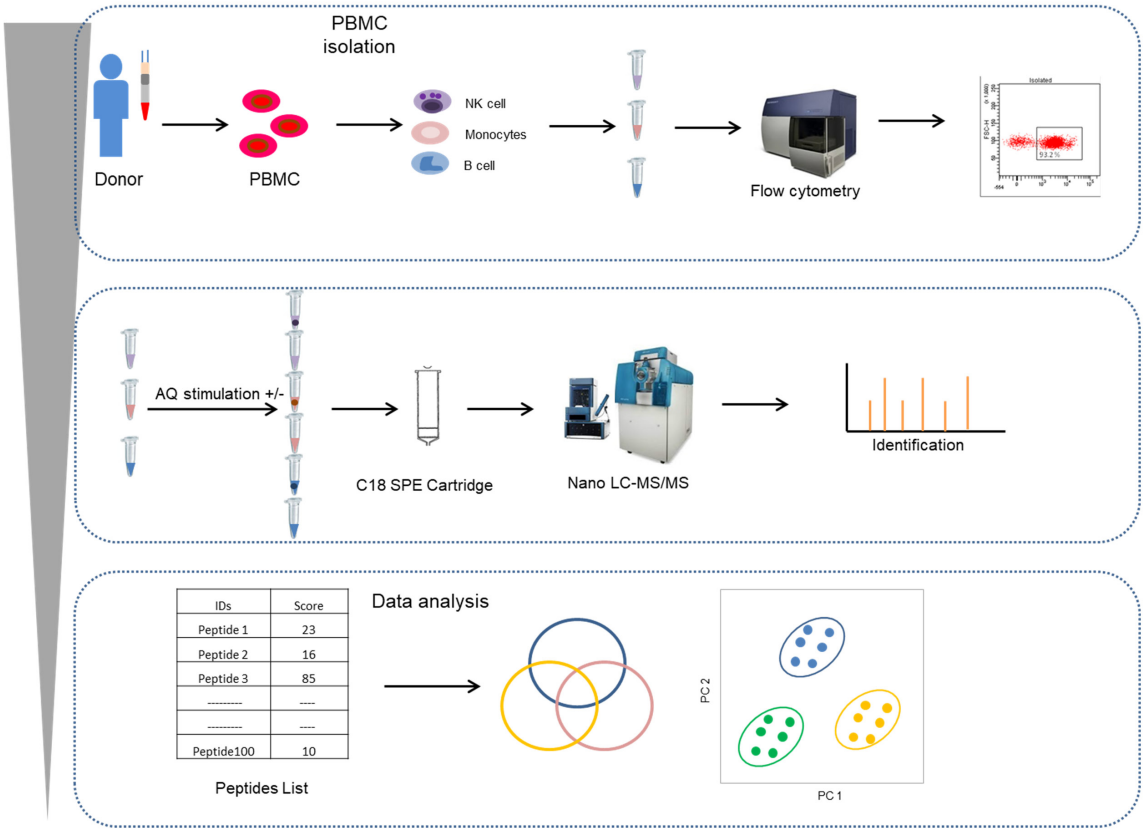

Figure 1. Overview of the experimental design. B cell, NK cell, and monocyte were isolated from fresh peripheral blood using immunomagnetic negative selection. Flow cytometry was use to Validate the purity of isolated populations. For mass spectrometry analysis, supernatant of purified B cells, NK cells, and monocytes were stimulated with or without adiponectin and then purified using solid phase extraction (SPE). Peptides samples were then subjected to Nano LC-MS/MS. Peptides identification was generated using ProteinPilot software and data analysis was performed using R package.

Results

Here, we aimed to determine whether there were any alterations in the responses of B cells, NK cells, and monocytes to AQ stimulation. Specifically, we investigated the release of PEPITEM from B cells following AQ stimulation. An experimental workflow was employed to assess PEPITEM release after AQ stimulation. The workflow involved stimulating these cells with adiponectin (AQ) and measuring their ability to release PEPITEM. A schematic of the workflow is shown in **Figure 1**. B cells, NK cells, and monocytes were isolated from fresh peripheral blood of three healthy Saudi donors using immune-magnetic negative selection. Flow cytometry was used to validate the purity of the isolated populations. The results showed that all three cell populations were isolated and purified with high purity, as determined by the expression of specific cell surface markers (Figure S1). Additionally, flow cytometry was used to detect the expression of AR1 and AR2 on the surface of isolated cells. The analysis confirmed the expression of ARs as shown in **Figures 2A**, **2B**, and **3**, respectively. The unbiased peptidomic analysis was employed. However, prior to this, the identity of the AQ protein standard that would be utilized for stimulation must be verified. This was performed by injecting the AQ protein standard into liquid chromatography-mass spectrometry (LC-MS/MS). The results of this analysis validated the identity of the AQ protein standard (Figure S2).

Next, the validity of the LC-MS/MS method used to detect PEPITEM was assessed. This was done by pooling all three cell samples into one, spiking with the PEPITEM standard as a positive control sample, and injecting into LC-MS/MS. The resulting MS1 spectrum exhibited PEPITEM (m/z 774.37) with a retention

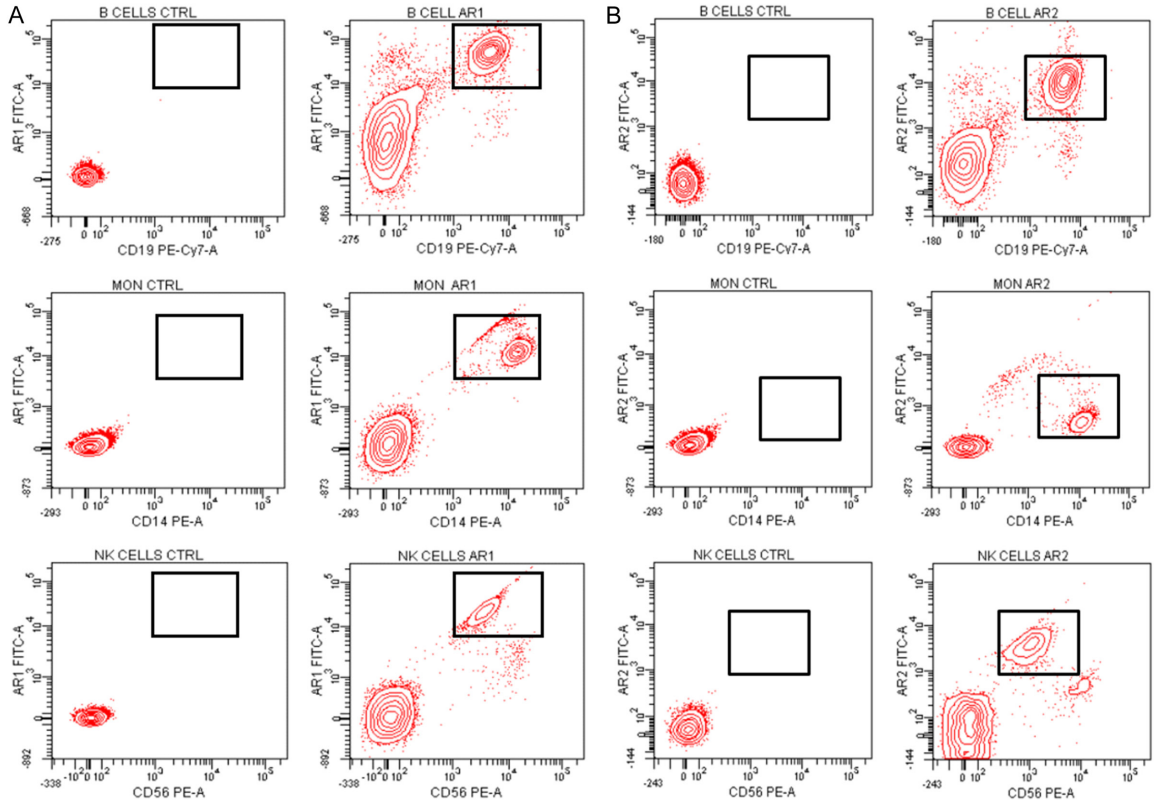

Figure 2. Flow cytometry analysis. A. Representative dot plots of surface expression of adiponectin receptors 1 (AR1). Flow cytometry analysis was performed to measure the surface expression of AR1 in B cells, NK cells, and monocytes. AR1 was measured based on the isotype control for each subset. PBLs were isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. A CD19 antibody was used to identify B cells, CD56+ for natural killer (NK) cells, and CD14+ for monocytes (Mono). B. Representative dot plots of surface expression of adiponectin receptors 2 (AR2). Flow cytometry analysis was performed to measure the surface expression of AR2 in B cells, NK cells, and monocytes. PBLs were isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. A CD19 antibody was used to identify B cells, CD56+ for natural killer (NK) cells, and CD14+ for monocytes. Data are means ± SEM (N = 3), and the differences between groups were determined by using an independent sample t-test. Probability values of less than 0.05 were considered significant (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001).

time of 29 min as shown in **Figure 4**. Moreover, the MS2 spectrum, which shows the fragment ions for PEPITEM, confirmed this detection. The results are presented in Figure S3. Subsequently, unbiased peptidomics analysis was performed on each cell sample. The results showed that the number of identified peptides in each cell sample varied (**Table 1**). The dataset that contained all peptides identification information including peptide sequences and confidence score can be found in Table S1. AQ stimulation was found to increase the number of identified peptides in B cells and monocytes, but decreased the number of peptides in NK cells.

Interestingly, PEPITEM was not present in any of the supernatants of the subsets. Then, we investigated the number of unique peptides in each cell type in at least two subjects and found that each cell type has a unique peptide signature, as shown in Table S2.

Comparative analysis and dimensionality reduction were conducted using peptide binary profile data across different subjects, different cell types, stimulated or unstimulated by AQLogistic principal component analysis revealed cell-type specific signature of unique peptides after merging the biological and technical replicates, as shown in **Figure 5**. However, with all biological samples (S1, S2, S3) and technical replicates (R1 and R2), the logisticPCA produced similar segration plot showing different cell-types segregated (Figure S4). Furthermore, this figure is demonstrating a

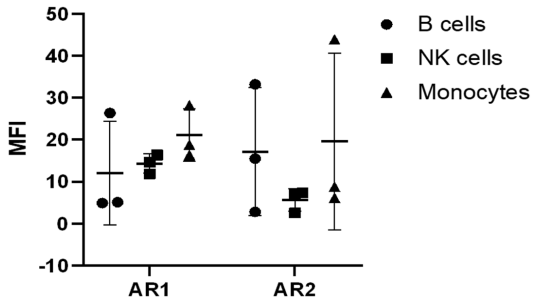

Figure 3. Percentage of the surface expression of adiponectin receptors 1 and 2 (AR1, AR2) on B cells, NK cells, and monocytes. AR1 was measured based on the isotype control for each subset. PBLs were isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. A CD19 antibody was used to identify B cells, CD56+ for natural killer (NK) cells, and CD14+ for monocytes (Mono). Data are means ± SEM (N = 3), and the differences between groups were determined by using an independent sample t-test. Probability values of less than 0.05 were considered significant (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001).

good reproducibility as the technical replicates are close on the two-dimensional plot.

Discussion

The interaction of AQ and immune cells is essential for our understanding of the disease progression, normal homeostasis, and in immune modulation. Our previous work shown that B cell, upon AQ stimulation, secrets PEPITEM which modulates T cell transmigration [3]. Here we wanted to check if there are any alterations in the response of B cell, NK cell, and monocyte to AQ stimulation; specifically, we aimed to investigate the release of PEPITEM from B cell after AQ stimulation. Based on the same rational, since NK cell and Monocyte express ARs on their surface just like B cells, we want to check if PEPITEM or other novel peptides will be released from them after AQ stimulation using unbiased peptidomic analysis to identify any secretomes from the purified subsets.

After excluding all possible technical issues, the inability to release PEPITEM could be attributed to immunometabolic alterations in B cells. Immune responses, such as B cell activation and proliferation, are heavily linked to dynamic modifications of cellular metabolism in B cells; hence, any noticeable changes in response, such as the inability to release PEPITEM, could be attributed to changes in cellular metabolism, which has been supported by the literature [19-22]. Additionally, advances in immunometabolic research in the last decade have suggested a strong relationship between inflammation and metabolism, and the regulation of immune cell functions by intracellular metabolism [20]. Moreover, due to environmental factors such as intracellular signals or cytokines, immune cells endure intense reprogramming, which could lead to the dysregulation of immune cell functions in diseases [22]. B cells are known to be key players in the AQ-PEPITEM pathway because of PEPITEM release after AQ stimulation; therefore, they exhibit anti-inflammatory properties, which is supported by the identification of regulatory B cells characterized by their anti-inflammatory properties [23]. Based on our findings, the lack of PEPITEM release could indicate that B cells underwent cellular metabolic modifications that led to the exhibition of pro-inflammatory properties, such as the absence of PEPITEM release in response to AQ stimulation. Literature suggests that the cytokine microenvironment and intracellular and extracellular metabolic signals determine the fate of B cells with anti-inflammatory and pro-inflammatory properties [23]. Two well-known B cell metabolic pathways are linked to adipocyte immune cell inflammation, which can be further investigated: glycolysis, which is essential for growth, and clonal expansion mediated by B cell antigen receptors [24]. The second mechanism is OXPHOS, which is important for B-cell differentiation and growth [24, 25]. Furthermore, Keppel et al. discovered that the activation signal of IFN-γ changes the metabolic requirements of immune cells [26]. In conclusion, AQ stimulation could provide another activation signal, and as a result, the metabolic requirement of stimulated B cells would change.

As mentioned earlier, PEPITEM inhibits T-cell transmigration, which ameliorates disease progression [3]. Therefore, if PEPITEM is not released for any reason, prolonged T-cell transmigration would eventually lead to a chronic inflammation state. This hypothesis can explain the soaring epidemic of diabetes in Saudi Arabia, which is ranked as the second highest rate of diabetes in the Middle East and the seventh highest in the world by the World Health Organization [27]. Further research and replication studies are required to confirm this hypothesis.

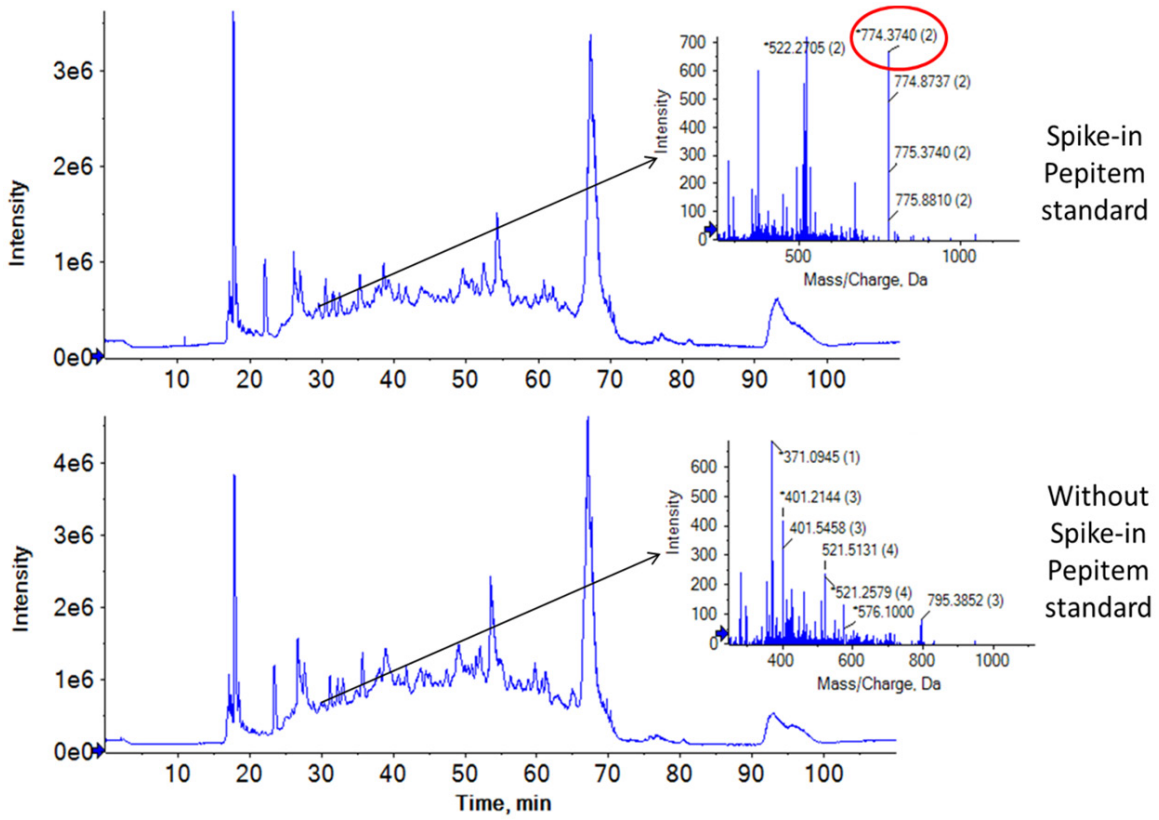

Figure 4. Extracted-ion chromatogram (XIC) and MS1 spectrum shows the mass of PEPITEM (m/z 774.37) in pooled sample (B cells, NK cells, monocytes) spiked with/without PEPITEM standard. Data generated using PeakView software.

Table 1. Summary of the number of identified peptides in each PBMCs subsets including; B cells, NK Cells, and monocyte

PBMCs subsets	Number of identified peptides	
	Unstimulated	Stimulated with Adiponectin
B cells	587	829
NK cells	847	668
Monocyte	492	605

All cells were stimulated with or without adiponectin and then subjected to LC-MS/MS and then identified using ProteinPilot Software.

The unbiased peptidomic screen for any secretomes released from NK cells or monocytes after AQ stimulation showed that neither PEPITEM nor novel peptides were identified; however, known peptides were identified after AQ stimulation, but without relevant significance. As suggested earlier, the same OXPHOS and glycolysis pathways play a role in the immune function of both NK cells and monocytes, which change their metabolic requirements after AQ stimulation [4, 26, 28].

Another potential explanation for the inability of B cells to release PEPITEM is ethnicity, which may affect AQ stimulation efficacy. AQ is mainly produced by adipose tissue and has recently attracted attention for its role in systemic inflammation. However, the mechanisms underlying these effects are poorly understood [29-31]. The SWAN study, which investigated differences in the secretory profile of adipose tissue across different ethnicities, concluded that there is a significant racial difference in the levels of circulating adipokines [32]. Nevertheless, more confirmatory experiments are required.

We also hypothesized that a potential lack of serum AQ might trigger cell resistance to acute stimulation, which explains the inability of B cells to release PEPITEM. To address this question, circulating serum level of AQ was mea-

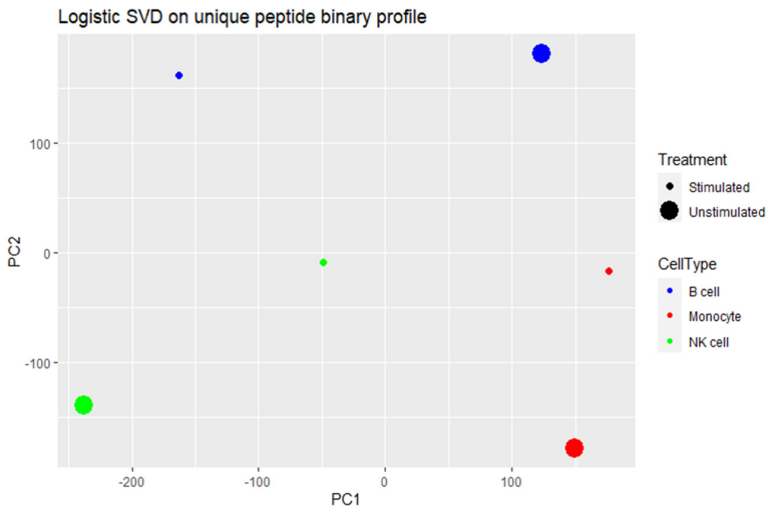

Figure 5. Logistic principal component analysis (PCA) (using "logsvd" function) of unique peptide binary profile data across different samples. "Logsvd" model converged at 299 iterations and explained 95% of the deviance with a solution of rank 3. The samples are merged on the cell type feature i.e. all samples from B cell are merged.

sured in all subjects, as shown in Figure S5 and the level was 7 μg/mL. However, the lack of Saudi Arabian-based data on serum adipokine levels did not allow us to determine normal circulating AQ levels in the Saudi Arabian population. Nevertheless, the literature suggests that AQ serum levels below 4 μg/mL are indicative of hypoadiponectinemia, which was not observed in the studied subjects [33]. Additionally, a study by Gardner et al. (2013) showed that AQ serum levels among black people and Hispanics were lower than those among Caucasian people [34]. This suggests that race-ethnic differences in circulating adipokine levels may have affected the ability of AQ to stimulate B cells to release PEPITEM in the Saudi population.

One limitation of this study is the small sample size, which may affect the generalizability of the findings. To confirm these results, future studies should include a larger and more ethnically diverse cohort. Additionally, further research should focus on validating and quantifying PEPITEM in circulation using sensitive techniques such as ELISA or LC-SRM

Conclusion

The inability of B cells to release PEPITEM after AQ stimulation is intriguing and suggests potential links to ethnicity, genetics, or environmental factors.

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Disclosure of conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abbreviations

PEPITEM, Inhibitor of Trans-Endothelial Migration; AQ, Adiponectin; ARs, Adiponectin receptors; PBMCs, Peripheral Blood Mononuclear Cell; PCA, Principal Component Analysis; SPE, Solid Phase Extraction; LC-MS/MS, Liquid Chromatography tandem Mass Spectrometry; Mono, monocyte.

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References

[1] Shyer JA, Flavell RA and Bailis W. Metabolic signaling in T cells. Cell Res 2020; 30: 649-659.

[2] Zhang X, Zink F, Hezel F, Vogt J, Wachter U, Wepler M, Loconte M, Kranz C, Hellmann A, Mizaikoff B, Radermacher P and Hartmann C. Metabolic substrate utilization in stress-induced immune cells. Intensive Care Med Exp 2020; 8 Suppl 1: 28.

[3] Chimen M, McGettrick HM, Apta B, Kuravi SJ, Yates CM, Kennedy A, Odedra A, Alassiri M, Harrison M, Martin A, Barone F, Nayar S, Hitchcock JR, Cunningham AF, Raza K, Filer A, Copland DA, Dick AD, Robinson J, Kalia N, Walker LSK, Buckley CD, Nash GB, Narendran P and Rainger GE. Homeostatic regulation of T cell trafficking by a B cell-derived peptide is im-

paired in autoimmune and chronic inflammatory disease. Nat Med 2015; 21: 467-475.
[4] Wang Z, Guan D, Wang S, Chai LYA, Xu S and Lam KP. Glycolysis and oxidative phosphorylation play critical roles in natural killer cell receptor-mediated natural killer cell functions. Front Immunol 2020; 11: 202.
[5] Kouidhi S, Elgaaied AB and Chouaib S. Impact of metabolism on T-cell differentiation and function and cross talk with tumor microenvironment. Front Immunol 2017; 8: 270.
[6] MacIver NJ, Michalek RD and Rathmell JC. Metabolic regulation of T lymphocytes. Annu Rev Immunol 2013; 31: 259-283.
[7] Ramsay G and Cantrell D. Environmental and metabolic sensors that control T cell biology. Front Immunol 2015; 6: 99.
[8] Pålsson-McDermott EM and O'Neill LAJ. Targeting immunometabolism as an anti-inflammatory strategy. Cell Res 2020; 30: 300-314.
[9] Buckley CD and McGettrick HM. Leukocyte trafficking between stromal compartments: lessons from rheumatoid arthritis. Nat Rev Rheumatol 2018; 14: 476-487.
[10] Pezhman L, Tahrani A and Chimen M. Dysregulation of leukocyte trafficking in type 2 diabetes: mechanisms and potential therapeutic avenues. Front Cell Dev Biol 2021; 9: 624184.
[11] Yeo KP, Lim HY and Angeli V. Leukocyte trafficking via lymphatic vessels in atherosclerosis. Cells 2021; 10: 1344.
[12] Esmaili S, Xu A and George J. The multifaceted and controversial immunometabolic actions of adiponectin. Trends Endocrinol Metab 2014; 25: 444-451.
[13] Luo Y and Liu M. Adiponectin: a versatile player of innate immunity. J Mol Cell Biol 2016; 8: 120-128.
[14] Collins AJ, Foley RN, Chavers B, Gilbertson D, Herzog C, Johansen K, Kasiske B, Kutner N, Liu J, St Peter W, Guo H, Gustafson S, Heubner B, Lamb K, Li S, Li S, Peng Y, Qiu Y, Roberts T, Skeans M, Snyder J, Solid C, Thompson B, Wang C, Weinhandl E, Zaun D, Arko C, Chen SC, Daniels F, Ebben J, Frazier E, Hanzlik C, Johnson R, Sheets D, Wang X, Forrest B, Constantini E, Everson S, Eggers P and Agodoa L. United States Renal Data System 2011 Annual Data Report: atlas of chronic kidney disease & end-stage renal disease in the United States. Am J Kidney Dis 2012; 59 Suppl 1: A7, e1-e420.
[15] Jorgetti V, dos Reis LM and Ott SM. Ethnic differences in bone and mineral metabolism in healthy people and patients with CKD. Kidney Int 2014; 85: 1283-1289.
[16] Ramos PS, Shedlock AM and Langefeld CD. Genetics of autoimmune diseases: insights from population genetics. J Hum Genet 2015; 60: 657-664.
[17] Subramanian A, Adderley NJ, Gkoutos GV, Gokhale KM, Nirantharakumar K and Krishna MT. Ethnicity-based differences in the incident risk of allergic diseases and autoimmune disorders: a UK-based retrospective cohort study of 4.4 million participants. Clin Exp Allergy 2021; 51: 144-147.
[18] Barhoumi T, Alghanem B, Shaibah H, Mansour FA, Alamri HS, Akiel MA, Alroqi F and Boudjelal M. SARS-CoV-2 coronavirus spike protein-induced apoptosis, inflammatory, and oxidative stress responses in THP-1-like-macrophages: potential role of angiotensin-converting enzyme inhibitor (Perindopril). Front Immunol 2021; 12: 728896.
[19] Buck MD, Sowell RT, Kaech SM and Pearce EL. Metabolic instruction of immunity. Cell 2017; 169: 570-586.
[20] Lercher A, Baazim H and Bergthaler A. Systemic Immunometabolism: challenges and opportunities. Immunity 2020; 53: 496-509.
[21] O'Neill LA, Kishton RJ and Rathmell J. A guide to immunometabolism for immunologists. Nat Rev Immunol 2016; 16: 553-565.
[22] O'Neill LA and Pearce EJ. Immunometabolism governs dendritic cell and macrophage function. J Exp Med 2016; 213: 15-23.
[23] Rosser EC and Mauri C. The emerging field of regulatory B cell immunometabolism. Cell Metab 2021; 33: 1088-1097.
[24] Doughty CA, Bleiman BF, Wagner DJ, Dufort FJ, Mataraza JM, Roberts MF and Chiles TC. Antigen receptor-mediated changes in glucose metabolism in B lymphocytes: role of phosphatidylinositol 3-kinase signaling in the glycolytic control of growth. Blood 2006; 107: 4458-4465.
[25] Waters LR, Ahsan FM, Wolf DM, Shirihai O and Teitell MA. Initial B cell activation induces metabolic reprogramming and mitochondrial remodeling. iScience 2018; 5: 99-109.
[26] Keppel MP, Saucier N, Mah AY, Vogel TP and Cooper MA. Activation-specific metabolic requirements for NK Cell IFN-γ production. J Immunol 2015; 194: 1954-1962.
[27] Robert AA, Al Dawish MA, Braham R, Musallam MA, Al Hayek AA and Al Kahtany NH. Type 2 diabetes mellitus in Saudi Arabia: major challenges and possible solutions. Curr Diabetes Rev 2017; 13: 59-64.
[28] Oren R, Farnham AE, Saito K, Milofsky E and Karnovsky ML. Metabolic patterns in three types of phagocytizing cells. J Cell Biol 1963; 17: 487-501.
[29] Klöting N and Blüher M. Adipocyte dysfunction, inflammation and metabolic syndrome. Rev Endocr Metab Disord 2014; 15: 277-287.

[30] Hollington M. The reception of charles dickens in Europe. Bloomsbury Publishing; 2013.
[31] Zatterale F, Longo M, Naderi J, Raciti GA, Desiderio A, Miele C and Beguinot F. Chronic adipose tissue inflammation linking obesity to insulin resistance and type 2 diabetes. Front Physiol 2020; 10: 1607.
[32] Khan UI, Wang D, Sowers MR, Mancuso P, Everson-Rose SA, Scherer PE and Wildman RP. Race-ethnic differences in adipokine levels: the Study of Women's Health Across the Nation (SWAN). Metabolism 2012; 61: 1261-1269.
[33] Im JA, Kim SH, Lee JW, Shim JY, Lee HR and Lee DC. Association between hypoadiponectinemia and cardiovascular risk factors in non-obese healthy adults. Metabolism 2006; 55: 1546-1550.
[34] Gardener H, Crisby M, Sjoberg C, Hudson B, Goldberg R, Mendez AJ, Wright CB, Rundek T, Elkind MS and Sacco RL. Serum adiponectin in relation to race-ethnicity and vascular risk factors in the Northern Manhattan Study. Metab Syndr Relat Disord 2013; 11: 46-55.

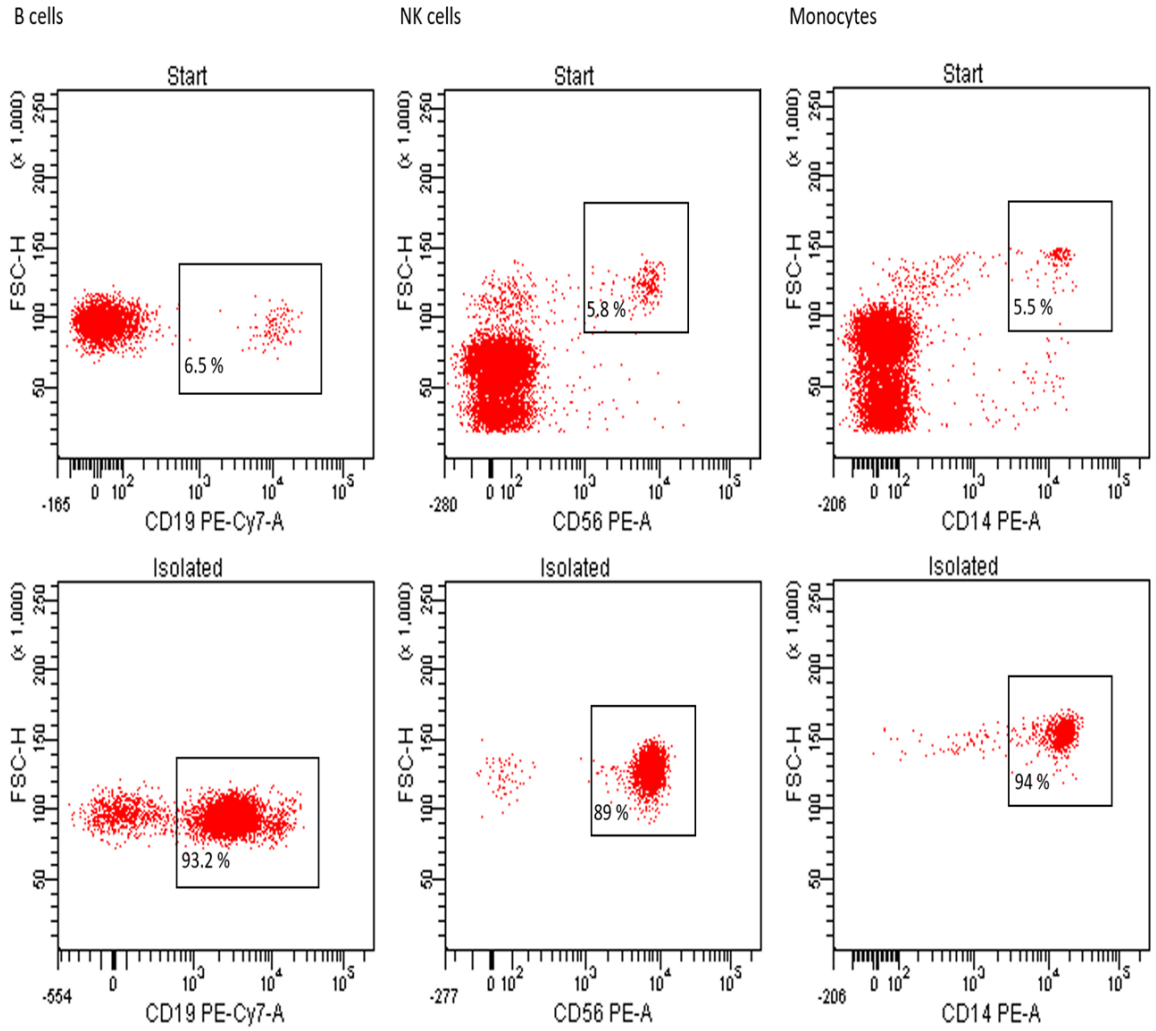


Figure S1. Representative dot plots of purity of isolated populations. Flow cytometry analysis was performed to characterize the isolated population. PBLs were isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. A CD19 antibody was used to identify B cells, CD56+ for natural killer (NK) cells, and CD14+ for monocytes. Data are means ± SEM (N = 3) and the differences between groups were determined by using an independent sample t-test. Probability values of less than 0.05 were considered significant (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001).

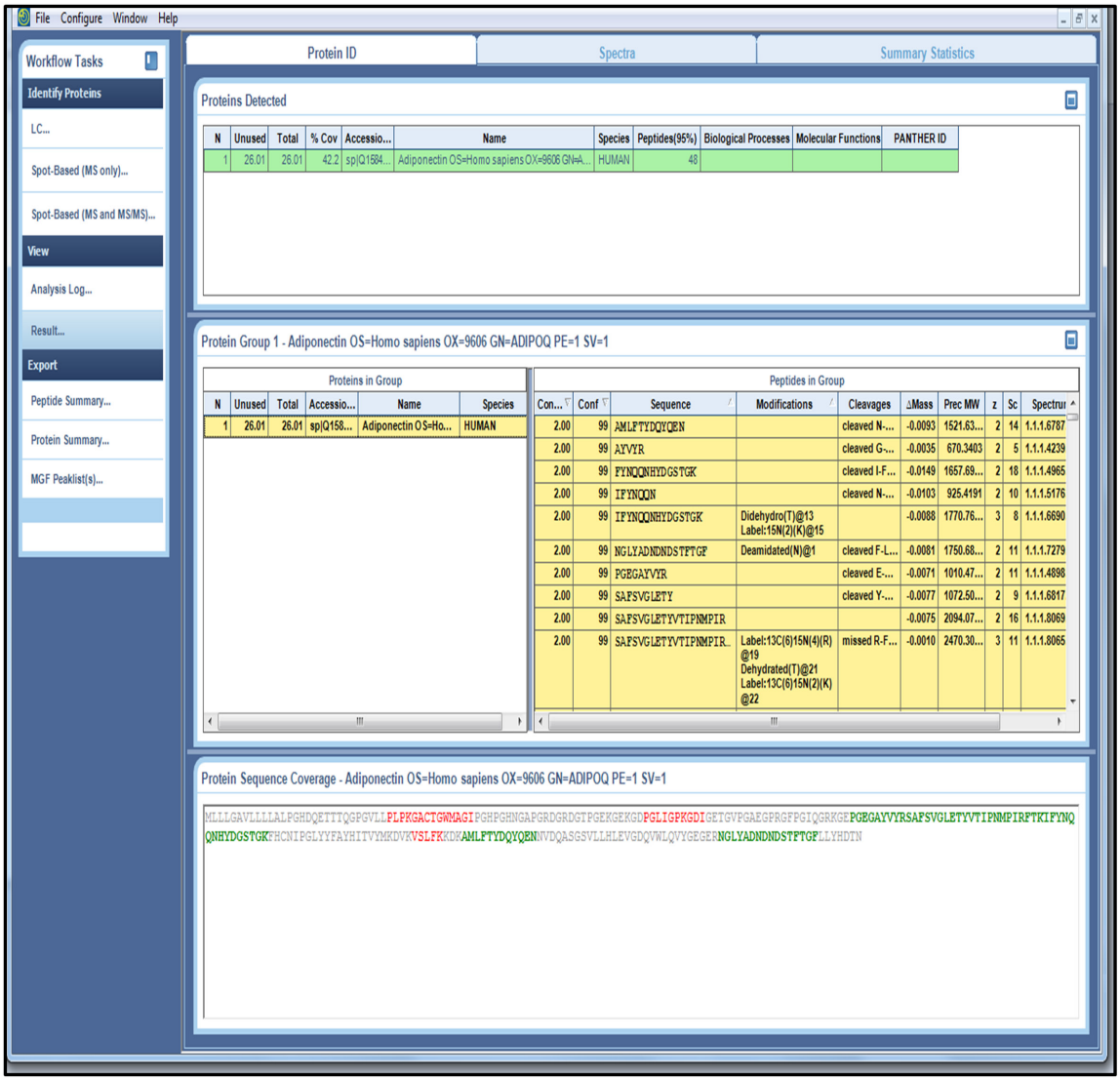
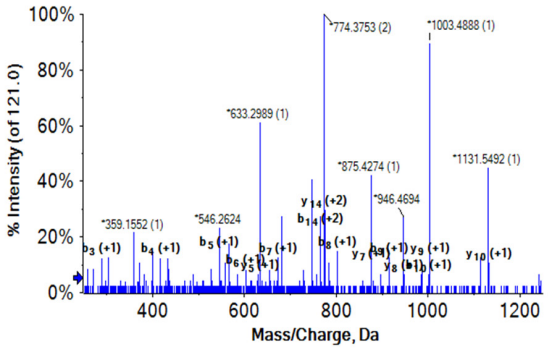


Figure S2. The identification of Adiponectin (AQ) by LC-MS/MS. Screenshot from ProteinPilot Software.



	Symbol	Res. Mass	# (N)	b	y	# (C)
	S	87.03203	1	88.03930	*1548.71362*	14
	V	99.06841	2	187.10772	1461.68159	13
	T	101.04768	3	**288.15540**	1362.61318	12
▶	E	129.04259	4	**417.19799**	1261.56550	11
	Q	128.05858	5	**545.25657**	**1132.52291**	10
	G	57.02146	6	**602.27803**	**1004.46433**	9
	A	71.03711	7	**673.31515**	**947.44287**	8
	E	129.04259	8	**802.35774**	**876.40575**	7
	L	113.08406	9	**915.44180**	747.36316	6
	S	87.03203	10	**1002.47383**	**634.27909**	5
	N	114.04293	11	1116.51676	547.24707	4
	E	129.04259	12	1245.55935	433.20414	3
	E	129.04259	13	1374.60195	304.16155	2
	R	156.10111	14	*1530.70306*	175.11895	1

Figure S3. Screenshot form Peakview software shows MS2 spectrum, the fragments ions of PEPITEM and Fragments mapping in pooled sample (B cells, NK cells, monocytes) spiked with PEPITEM standard. Data generated using Peak-View software.

Table S1. Peptides identification in all cells samples including biological and technical replicates, provided as separate Excel sheet

Table S2. Unique peptides identified in at least two biological subjects in B, Monocytes, and NK cells

	B Cells	Monocytes	NK cells
Unstimulated			KVSFLSALEEYTKKLNT
			AMFKVGPEADKYRLTYAY
	GSAGHWTSESSVSG		AHKSEVAHRFKDLGEENFKALVLIA
			QVLPWLKEKLQDEDLGFL
			EEAGARVQQNVPSGTDTGD
Stimulated			AGHWTSESSV
	AGPTGTGESKCPLMVKVLD		HKSEVAHRFKDLGEENFKAL
	PDPAKSAPAPKKGSKK	STGKTFPGFFSPMLGEFVSETESRGS	HKSEVAHRFK
	SEAEDASLLSFM		WESASLL
			GEYKFQNA

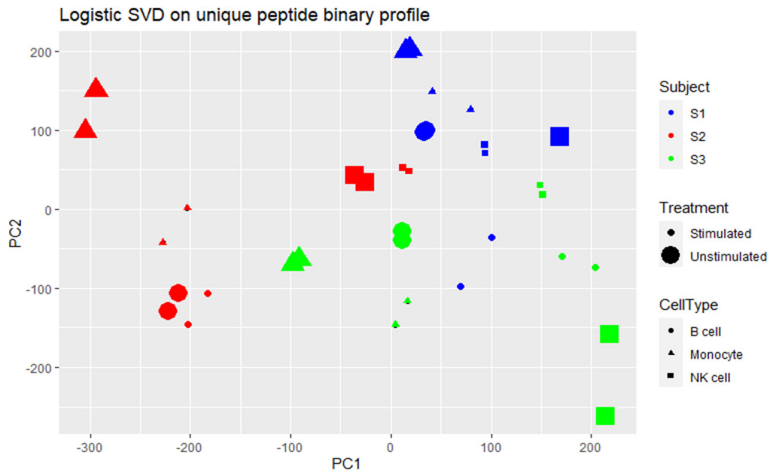


Figure S4. Logistic principal component analysis (PCA) (using "logsvd" function) of unique peptide binary profile data across different samples. "Logsvd" model converged at 510 iterations and explained 53.9% of deviance with a solution of rank 3. The samples with the same shape and color and size are technical replicates.

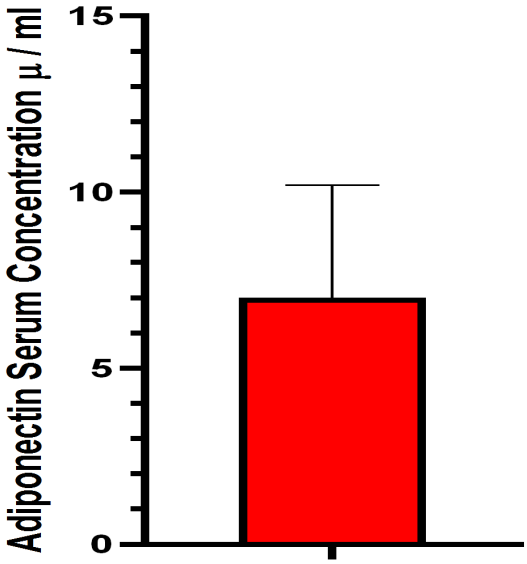


Figure S5. Circulating serum adiponectin concentration µg/ml. Acrp30 Quantikine ELISA kit was used to measure the Human Total Adiponectin serum concertation. Serum collected in in yellow top chemistry tubes was isolated from fresh blood samples. Data are means ± SEM (N = 3). Data are means ± SEM (N = 3), and the differences between groups were determined by using an independent sample t-test. Probability values of less than 0.05 were considered significant (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001).