Alkaline phosphatase inhibits TNF-α and IL-6 release by freshly extracted human leukocytes in the absence of LPS: a promising therapeutic candidate

Lunan Qin[1], Lin Song[2], Shuyin Wu[1], Chenzhe Gao[1], Tianqi Liu[1], Yuanyuan Jiang[1], Jiayou Cui[3], Xiuxiu Dai[3], Zhenyu Cong[1], Zihui Zhang[1], Fangyuan Zhao[4], Jie Li[1], Bao Shuang[1], Qifei Wang[1], Jessica Hui[3], Zhonglin Liu[5], and Mizhou Hui[1]

[1]Northeast Agricultural University
[2]Qingdao University of Science and Technology
[3]Shaoxing HuiHui Technology Inc
[4]Qingdao Agricultural University
[5]University of Arizona Arizona Health Sciences Center

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Abstract

BACKGROUND AND PURPOSE The use of human intestinal alkaline phosphatase (IAP) has already been validated as a novel treatment for endotoxin lipopolysaccharide (LPS)-induced inflammatory diseases, which is mediated through IAP' s ability to detoxify LPS. However, there has been little investigation into the full extent of IAP' s physiological function. This study investigates IAP's non-LPS related functions and clinical applications. EXPERIMENTAL APPROACH In this study, we use freshly extracted human leukocytes to study effects of a recombinant human intestinal alkaline phosphatase (recIAP) on secretion of TNF-a and IL-6 by the leukocytes in absence of LPS. Physiological substrates of alkaline phosphatase and their dephosphorylated products at neutral pH including ATP, ADP, AMP and adenosine were employed to investigate their effects on secretion of TNF-a by the leukocyte. KEY RESULTS We found that recIAP inhibit TNF-α and IL-6 secretion by freshly extracted human leukocyte in the absence of LPS. recIAP dephosphorylates ATP and ADP etc at physiological pH. The dephosphorylated products by recIAP including AMP and adenosine inhibit TNF-α secretion of the freshly extracted human leukocyte. CONCLUSIONS AND IMPLICATIONS Human leukocyte migrates into inflamed tissues and secretes TNF-α and IL-6. Thus, leukocyte plays an important role in inflammatory diseases. recIAP inhibits TNF-α and IL-6 secreted by freshly extracted human leukocyte in the absence of LPS. The results in this study indicate that recIAP is a promising therapeutic candidate for diseases related to leukocyte TNF-α and IL-6 secretion.

Αλκαλινε πηοσπηατασε ινηιβιτς ΤΝΦ-α ανδ ΙΛ-6 ρελεασε βψ φρεσηλψ εξτραςτεδ ηυμαν λευκοςψτες ιν τηε αβσενςε οφ ΛΠΣ: α προμισινγ τηεραπευτις ςανδιδατε

Lunan Qin[1] Lin Song[4] Shuyin Wu[2] Chenzhe Gao[2] Tianqi Liu[1] Yuanyuan Jiang[1] Jiayou Cui[3] Xiuxiu Dai[3] Zhenyu Cong[1] Zihui Zhang[1] Fangyuan Zhao[5] Jie Li[1] Bao Shuang[1] Qifei Wang [1234] Jessica Hanitta Hui[3] * Zhonglin Liu [5]* Mizhou Hui[12] *.

[1]College of Life Science, Northeast Agricultural University

[2]School of Food Science, Northeast Agricultural University

[3]Shaoxing HuiHui Technology Inc

[4]Qingdao University of Science and Technology

[5]University of Arizona.

Corresponding Author:

Mizhou Hui, *huimizhou@163.com*

Jessica Hanitta Hui, jhhui@stanford.edu

Zhonglin Liu, zliu@email.arizona.edu

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CONFLICT OF INTEREST

The authors declare no competing interests for this work.

BACKGROUND AND PURPOSE

The use of human intestinal alkaline phosphatase (IAP) has already been validated as a novel treatment for endotoxin lipopolysaccharide (LPS)-induced inflammatory diseases, which is mediated through IAP' s ability to detoxify LPS. However, there has been little investigation into the full extent of IAP' s physiological function. This study investigates IAP's non-LPS related functions and clinical applications.

EXPERIMENTAL APPROACH

In this study, we use freshly extracted human leukocytes to study effects of a recombinant human intestinal alkaline phosphatase (recIAP) on secretion of TNF-a and IL-6 by the leukocytes in absence of LPS. Physiological substrates of alkaline phosphatase and their dephosphorylated products at neutral pH including ATP, ADP, AMP and adenosine were employed to investigate their effects on secretion of TNF-a by the leukocyte.

KEY RESULTS

We found that recIAP inhibit TNF-α and IL-6 secretion by freshly extracted human leukocyte in the absence of LPS. recIAP dephosphorylates ATP and ADP etc at physiological pH. The dephosphorylated products by recIAP including AMP and adenosine inhibit TNF-α secretion of the freshly extracted human leukocyte.

CONCLUSIONS AND IMPLICATIONS

Human leukocyte migrates into inflamed tissues and secretes TNF-α and IL-6. Thus, leukocyte plays an important role in inflammatory diseases. recIAP inhibits TNF-α and IL-6 secreted by freshly extracted human leukocyte in the absence of LPS. The results in this study indicate that recIAP is a promising therapeutic candidate for diseases related to leukocyte TNF-α and IL-6 secretion.

ABBREVIATIONS

AP, alkaline phosphatase; IAP, intestinal alkaline phosphatase; recIAP, human recombinant intestinal alkaline phosphatase; bIAP, bovine intestinal alkaline phosphatase; HUVECs, Human umbilical vein endothelial cell lines; WBC, white blood cells

Key words: *αλκαλινε πηοσπηατασε, ΛΠΣ, ΤΝΦ-α, ΙΛ-6, λευκοςψτε, νευτροπηιλ, ινφλαμματιον.*

What is already known

* Phase II clinical trial of recIAP in patients suffered from LPS-related disease was successful.

What this study adds

* recIAP inhibits TNF-a and IL-6 release by freshly extracted human leukocytes in absence of LPS.

What is the clinical significance

* recIAP is a therapeutic candidate for inflammatory diseases not related to LPS.

INTRODUCTION

Alkaline phosphatase (AP) has multiple tissue subtypes such as tissue non-specific AP (TNAP), intestinal AP (IAP), placenta AP (PLAP) and is widely distributed in various tissues of the human body, including cardiac blood vasculature, brain, liver and placenta and intestinal epithelial brush border (Schultz-Hector, Balz, Böhm, Ikehara, Rieke, 1993; Bell, Scarrow, 1984; Hirschmugl et al., 2018; Bell, Williams, 1979; HAGerstrand, Lindholm, Lindroth, 1976). Currently, the physiological function of IAP has not been fully elucidated. The function of alkaline phosphatase was originally thought to be involved in bone calcification (Millan, Whyte, 2016). In the early 1990s, Hui et al. used gene transfer technology to induce AP gene expression in various cell types and found that AP is likely related to pathological vascular calcification (Hui, Li, Holmyard, Cheng 1997; Hui, Tenenbaum, 1998). But the mystery surrounding the main physiological function of such a widely distributed membrane-bound enzyme continued to prevail until an original study by Dutch scientists Poelstra and Meijer first reported that IAP is capable of inactivation of endotoxin, suggesting that IAP could inhibit endotoxin-induced inflammation and might have a potential for treatment lipopolysaccharide (LPS)-related diseases (Poelstra, Bakker, Klok, Hardonk, Meijer, 1997). By knocking out the mouse IAP respective gene, Bhan and Sonoko found that animals deficient in IAP develop type 2 diabetes (Kaliannan, 2013) and hyperlipidemia (Narisawa, 2013). Clinical studies by Peters and Lukas sponsored by Dutch biopharmaceutical company AM-Pharma have already validated the use of recombinant human IAP in providing a novel approach targeting inflammation in endotoxin-associated sepsis kidney injury and colitis (Peters, 2016a; Lukas, 2010).

Leukocytes contain 55-70% neutrophils, 20-40% lymphocytes and 3-10% monocytes. Neutrophil AP activity is increased in pathological sites. A characteristic response of neutrophils to bacterial infections is the pronounced increase in AP activity. IAP is thought to be involved in gut mucosal defense, mainly through the detoxification of LPS. However, the migration of neutrophils into inflammatory tissues acts like a double-edged sword. As a primary cell type that removes invasive microorganisms, neutrophils also contribute to the pathogenesis of human inflammatory diseases by releasing excess proinflammatory cytokines, such as TNF-α and IL-6 (Peiseler, Kubes, 2019; Suzuki, 2017; Powell, Huttenlocher, 2016; Jasper, McIver, Sapey, Walton, 2019; Kovtun, Messerer, Scharffetter-Kochanek, Huber-Lang, Ignatius 2018; Williams, Chambers, 2016; Wright, Moots, Bucknall, Edwards, 2010; Suzuki, 2018; Zhang, 2019; Mortaz, Alipoor, Adcock, Mumby, Koenderman, 2018). TNF-α is a mammalian-secreted protein capable of inducing a wide variety of effects on many cell types. TNF-α is essential to the initiation course of inflammatory reactions in the body and is involved in the triggering and/or amplification of local inflammatory responses related to apoptotic cell death. Dysregulated continual synthesis of IL-6 plays a pathological effect on chronic inflammation and autoimmunity. Currently, it has been unclear if AP has a direct mechanism of inhibiting leukocytic TNF-α and IL-6 upregulation in inflammatory human diseases. This study was designed to investigate the effects of IAP on TNF-α and IL-6 secretion using our cell-based model comprised of freshly extracted human leukocytes in the presence and absence of endotoxin LPS.

METHODS

Expression and production of recIAP

In this study, cDNA for recombinant human intestinal alkaline phosphatase (recIAP) (Peters, 2016b; Kiffer-Moreira, 2014) was used to construct the expression vector pMH3-recIAP (Qian, 2010), which was then transferred into CHO-S cells (CVCL_7183, Life Technologies). The cells were cultured in DMEM / F12 medium containing 10% fetal bovine serum (FBS) and subjected to G418 pressure screening to obtain stable, high-expression clones. These clones were expanded and cultured by mammalian cell bioreactors in order to obtain harvest medium for purification. The harvest medium was centrifuged and passed through a pyrogen-removed cation column (SP XK50,GE Healthcare) in order to remove heteroproteins. The flow-through containing recIAP fraction was collected and passed through the pyrogen-removing anion column (Q XK50, GE Healthcare) again to collect recIAP-containing elute. All the solutions used in the purification process

were passed through a hollow fiber column (Borglong Biotechnology Co., Ltd.) to remove endotoxins The purity of concentrated recIAP solution was determined using SDS-PAGE electrophoresis and high-pressure liquid chromatography (HPLC). The recIAP solution was found to have 92.45% purity.

Detection of intestinal alkaline phosphatase activity

Alkaline phosphatase activity was measured using a phenyl disodium phosphate colorimetry kit (TE0007, Beijing Regen Biotechnology Co., Ltd.). Phenyl disodium phosphate is hydrolyzed by alkaline phosphatase under alkaline conditions to form free phenol and phosphoric acid. Under basic conditions, phenol is combined with amino antipyrine and oxidized to form red quinone structures with different shades. The absorbance was measured at 510 nm and the alkaline phosphatase activity level was calculated by colorimetric analysis. An active unit (U L^{-1}) is defined as 100 milliliters of sample at 37°C for 15 minutes of incubation with benzene disodium phosphate in order to produce 1 milligram of phenol.

Effect of recombinant human intestinal alkaline phosphatase on LPS

RecIAP was diluted to 50 U/mL with Tris-HCl (50 mmol L^{-1}, pH 6.0-8.0) buffer. 990 µl recIAP and 100µl LPS solution (Escherichia coli 0111: B4, Sigma, LPS final concentration 1µg ml^{-1}) were mixed together thoroughly and then incubated at 37°C for 3 hours. The absorbance at 545nm was measured by the TAL method (test tube Limulus amebocyte lysate kit, ec32545s, Xiamen limulus lysate Biotechnology Co., Ltd.), and the LPS content was determined using a standard curve. At physiological pH (7.4), recIAP (enzyme activity was 1, 5, 10 U ml^{-1}) was incubated with LPS (5ng ml^{-1}) at 37°C for 3 hours, and the LPS content was measured again. For the above measurements, recIAP that was inactivated at 65°C for 60 minutes was used as a blank control.

Determination of free phosphate release from AP substrate

Tris-HCl (50mmol L^{-1}, pH6.0-8.0) buffer was used to prepare 10mM of ATP, ADP, and AMP solution (ATP, Biotopped; ADP and AMP, Shanghai source leaf biology) and also to dilute recIAP (1000U ml^{-1}) to 0.5, 5.0, 50.0U ml^{-1}. 950µl of recIAP and 50µl of ATP, ADP, and AMP solutions were mixed together thoroughly, producing a final concentration of the solution is 0.5 mM, and incubated at 37°C for 60 min. In the above measurement, recIAP that was inactivated at 65 ° C for 60 minutes was used as a blank control. An inorganic phosphorus test box (C006-1-1, Nanjing Jiancheng Biotechnology Research Institute) was used to measure the content of free phosphate (Pi) released through the recIAP's effect on ATP, ADP, and AMP (0.5mM) at different pHs, ranging between 6.5-8.0. The released Pi interacts with molybdic acid to generate phosphomolybdic acid, which is then reduced to molybdenum blue and exhibits a unique absorption spectrum with a maximum peak at 660nm.

Isolation of Human venous blood leukocytes

There were 16 healthy volunteers, aged 26±5 years, whose blood collection was approved by the Medical Ethics Committee of Changchun Jiahe Surgical Hospital. We then used sucrose density-gradient centrifugation (endotoxin < 0.1EU, Tianjin Haoyang Huake Biotechnology Co., Ltd.) to separate white blood cells (WBCs) from venous blood. The samples were collected at room temperature and centrifuged at 1800 rpm for 25 minutes. The mononuclear cell layer, comprised of mostly lymphocytes with a few monocytes, and the multinuclear cell layer, comprised of mostly neutrophils with few contaminating mononuclear cells, were taken out to be mixed. Any contaminating erythrocytes were lysed and washed out. 1640 medium containing 10% FBS was used for resuspension. The morphology of the cells was observed with leukocyte staining solution, and the density was adjusted to 1×10^6 cells/ml. Blood samples from different volunteers were collected and used for each experiment in order to eliminate individual differences and ensure the study's repeatability.

Σεςρετιον οφ ΤΝΦ-α/ΙΛ-6 οφ ηυμαν λευκοςψτες

Human umbilical vein endothelial cell lines (HUVECs, RRID:CVCL_2959) were cultured in DMEM/F12 medium containing 10% FBS at 37°C until confluent. They were then detached by trypsinization and resuspended to obtain a cell density of 1×10^5 cells/ml. 100ul of the resuspended cells were added to each

well of a 96-well plate and incubated at 37°C for 24 hours. On the day of the experiment, freshly extracted human WBCs could be added to the monolayer HUVEC culture in 96-well plates in order to establish three models of cellular interaction: one with (i) only WBCs, (ii) between WBCs and HUVECs, and (iii) between WBCs, RBCs, and HUVECs (WBC: RBC= 1: 5). After the experiment was completed, a different sample of WBCs was freshly extracted from a another individual. The experiment was repeated to ensure that the experimental results were reproducible. Incremental amounts of recIAP (5, 10, 20 U ml^{-1}) were either pre-incubated with LPS for 3 hours in advance, or recIAP was added to 0.5 ng ml^{-1} LPS directly into the cell culture to stimulate WBCs. After cultured at 37°C for 24 hours, the supernatant was collected to detect TNF-α. Similarly, in an effort to examine the minimum required dosage of recIAP to elicit a response, incremental amounts of recIAP (0.5, 1.0, 2.0, 4.0U ml^{-1}) and 0.5ng ml^{-1} LPS were added to the WBCs to stimulate TNF-α secretion. The levels of TNF-α and IL-6 were measured after 24 hours of culture. In another set of experiments, 0.5ng ml^{-1} of LPS was added to either 5 U ml^{-1} of recIAP or bovine intestinal alkaline phosphatase (bIAP) (P6774-2KU, Sigma) to stimulate WBCs, and the resulting culture medium was collected after 24 hours in order to detect TNF-α and IL-6 levels. Low concentrations (0.10, 0.25, 0.50, 1.00 μM) of ATP, ADP, AMP, and adenosine were also added to the cellular interaction model containing WBCs and HUVECs. The culture medium was subsequently collected after 24 hours to determine TNF-α levels. TNF-α and IL-6 in cell culture medium were measured by use of an enzyme-linked immunoassay kit (TNF-α Elisa kit, R & D system DY210; IL-6 Elisa kit, Huamei CSB -E04638h). For specific method and procedure, refer to the manufacturer's instructions.

Statistical analysis

All data were expressed as mean ± standard deviation. The intra-assay results were compared using t-tests. $^*P < 0.05$ was considered to be statistically significant difference. Five medium samples were taken for each experiment, and each experiment was repeated at least two times, using blood samples from different individuals. Data analysis was performed using Graph Prism 6.0 software (RRID:SCR_002798, La Jolla, CA, USA).

RESULTS

Inactivation of LPS by recIAP at neutral pH

recIAP exhibited a significant effect on LPS inactivation at neutral pH7.5 (Table 1), indicating its LPS-inactivating effect in human blood and tissues. High-dose recIAP (10 U ml^{-1}) reduced the activity of LPS (5ng ml^{-1}) up to 50% within 3 hours of incubation at 37°C (Figure 1). In addition, incubation of higher-dose recIAP(50 U ml^{-1}) and higher-dose LPS(1μg ml^{-1}) at 37 for one hour reduced LPS activity by approximately 60% at pH 7.0 and 7.5, while LPS activity decreased by 76.85% at pH 8.0.

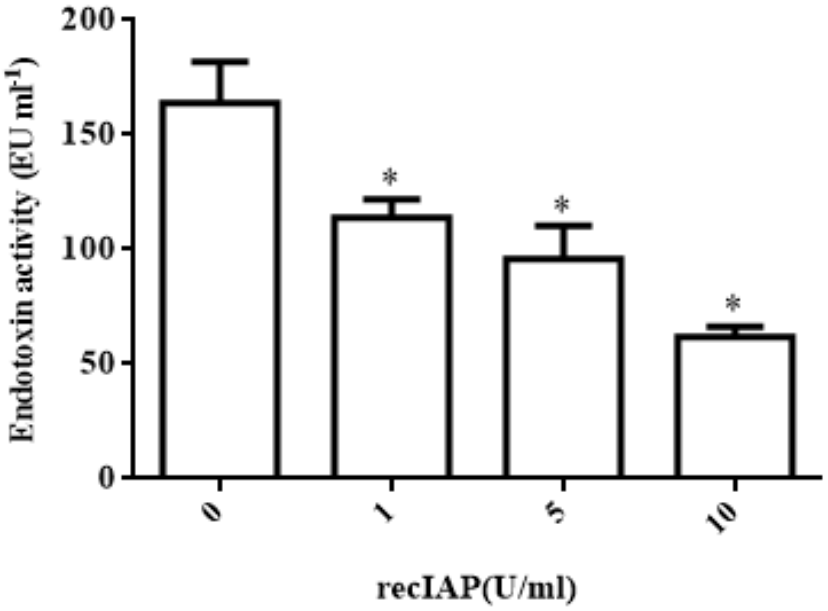


Figure1

ΛΠΣ στιμυλατες ΤΝΦ-α σεςρετιον βψ φρεσηλψ εξτραςτεδ ηυμαν λευκοςψτες

Three *in vitro* cell-interaction models (Figure 2) were employed to study the effects of LPS on TNF-α secretion by WBCs alone, or in combination with HUVECs or HUVECs and erythrocytes. In the presence of WBCs, erythrocytes and HUVECs, LPS promoted TNF-α secretion in a dose-dependent manner when it was added incrementally in the range 0.0-0.5 ng ml^{-1}.

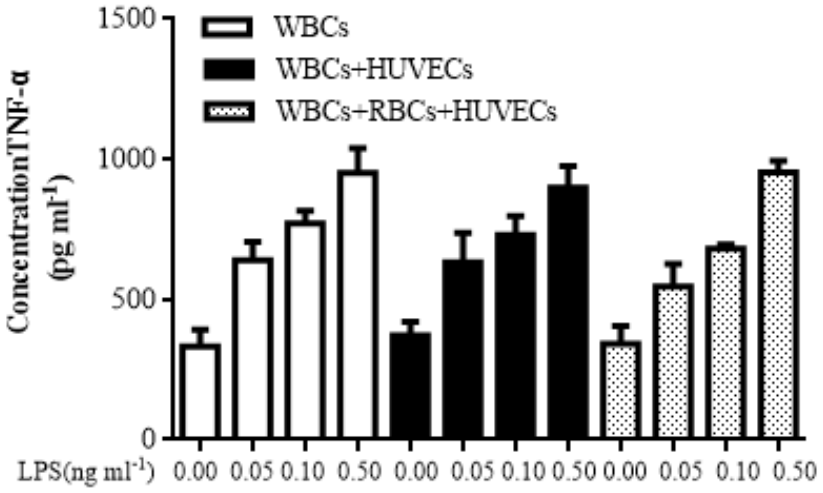
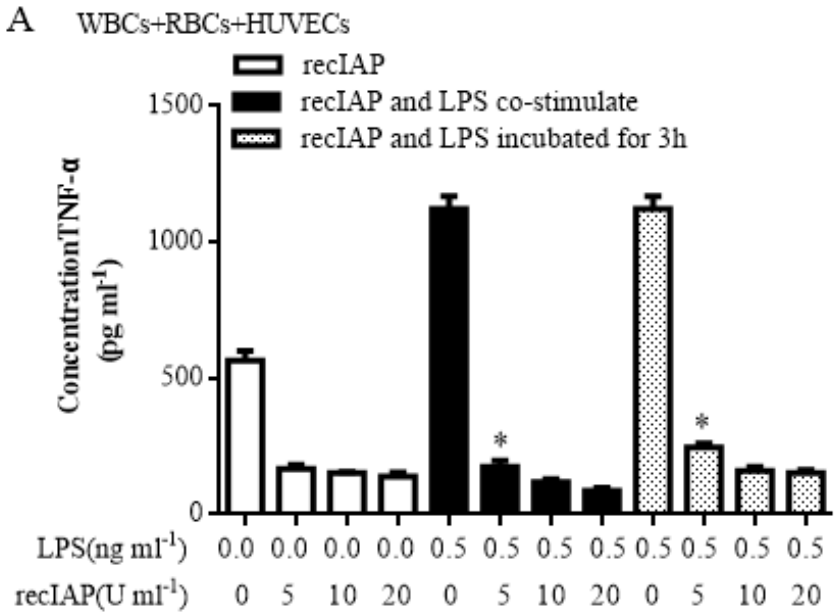


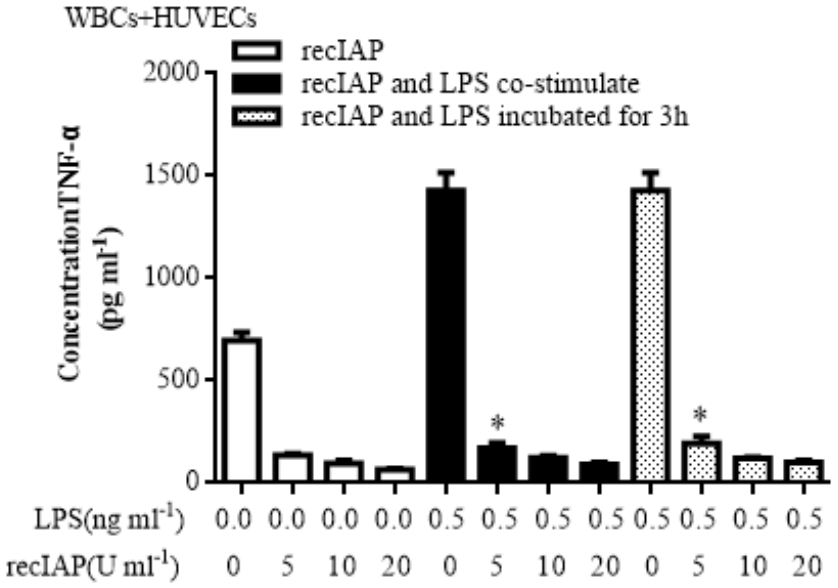
Figure 2

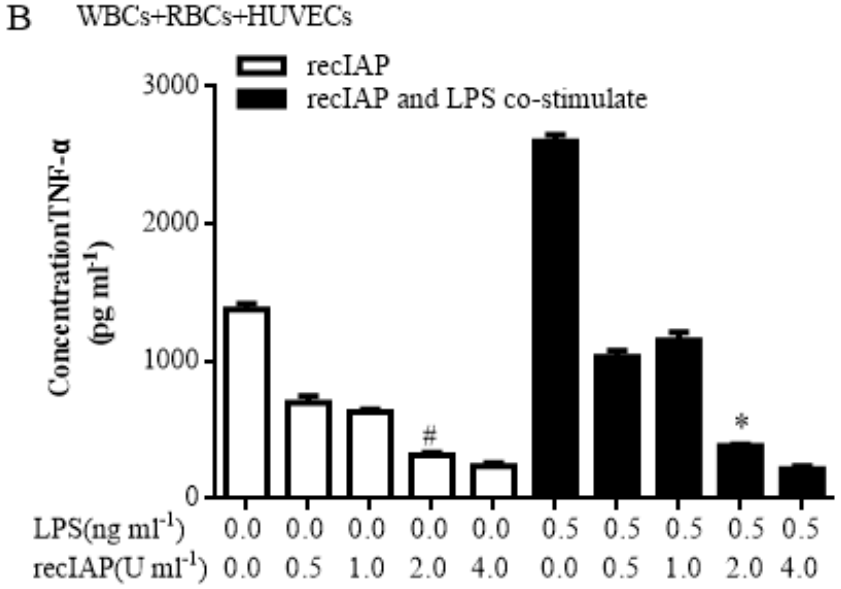
recIAP inhibits the secretion of TNF-a and IL-6 in the absence of LPS

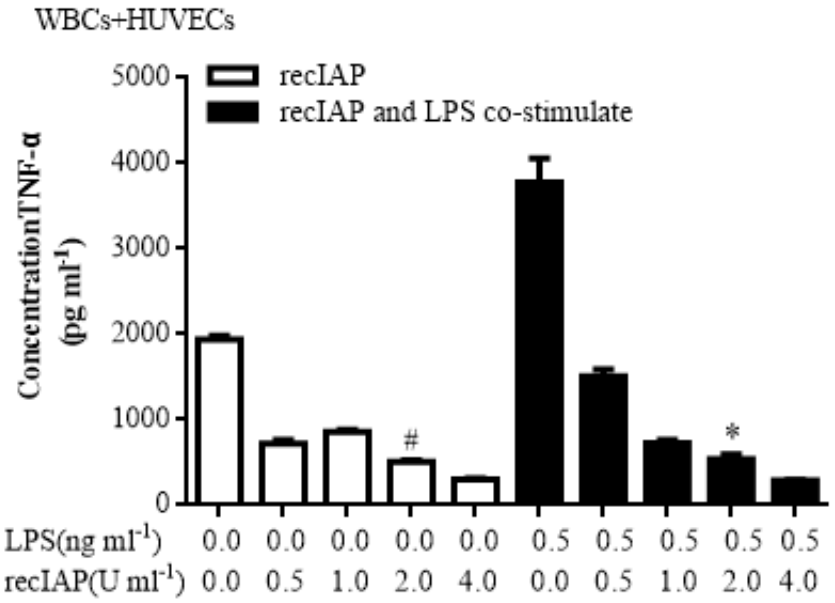
For each experiment, we examined three conditions: (i) when recIAP alone was added to the cell culture (ii) when recIAP and LPS were added simultaneously to the cell culture, and (iii) when LPS and recIAP were pre-incubated together during a separate procedure for 3 hours before being added to the cell culture (Figure 3). Our results demonstrate that recIAP has an inhibitory effect on TNF-α generated by freshly extracted human leukocytes which occurs in a dose-dependent manner. Specifically when the WBCs were subject to conditions (ii) or (iii), the results showed that relatively similar TNF-α levels were secreted regardless of whether recIAP was added to the WBCs at the same time as LPS (Figure 3A black bar), or recIAP was pre-incubated with LPS for 3 hours separately before being added to the WBCs (Figure 3A gray bar). Because we expect that in condition (iii), the recIAP will neutralize the effect of LPS when they are pre-incubated together in a separate solution, this key result suggests that recIAP is able to inhibit leukocyte secretion of TNF-α independent of LPS presence. Additionally,the rest of the results seen in Figure 3ABC further support the finding that recIAP directly inhibits leukocyte secretion of TNF-α and IL-6 in the absence of LPS.

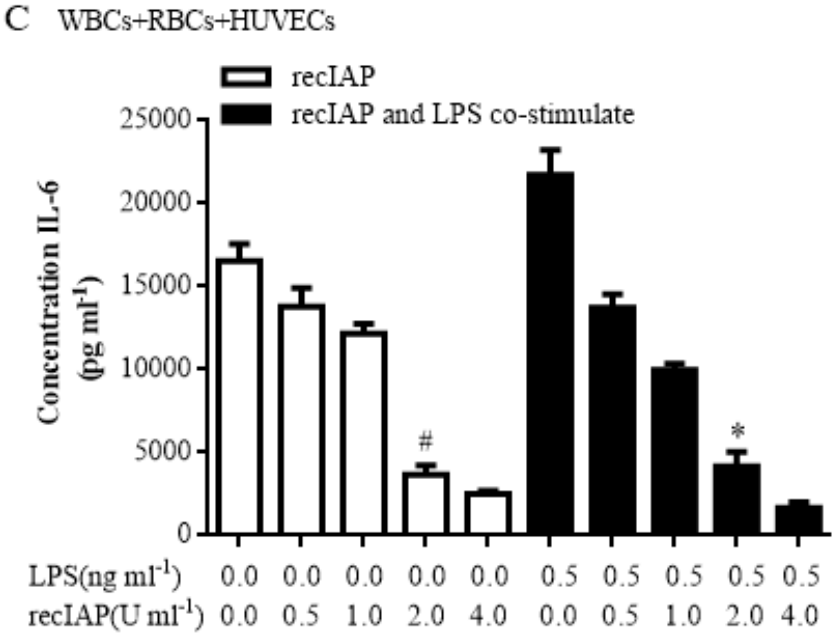
To determine the minimum effective dose of recIAP, we found that recIAP (2U ml^{-1}) inhibits the secretion of TNF-α by leukocytes both in the presence of LPS (Figure 3B black bar) and in the absence of LPS(Figure 3B white bar). Furthermore, recIAP (2U ml^{-1}) also inhibits the secretion of IL-6 by leukocytes in the presence of LPS (Figure 3C black bar) and in the absence of LPS (Figure 3C white bar,), indicating that recIAP also significantly reduces IL-6 secretion both in the presence or absence of LPS.

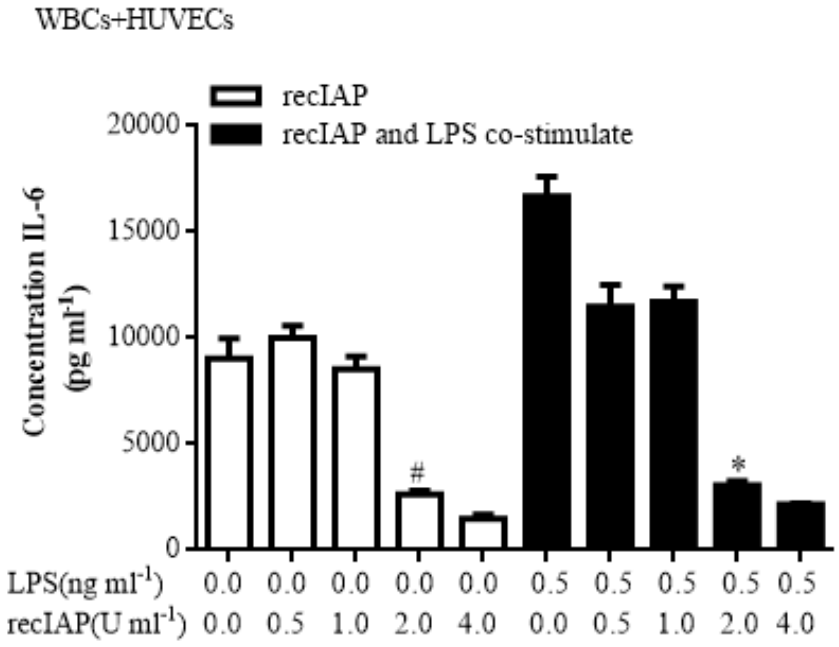








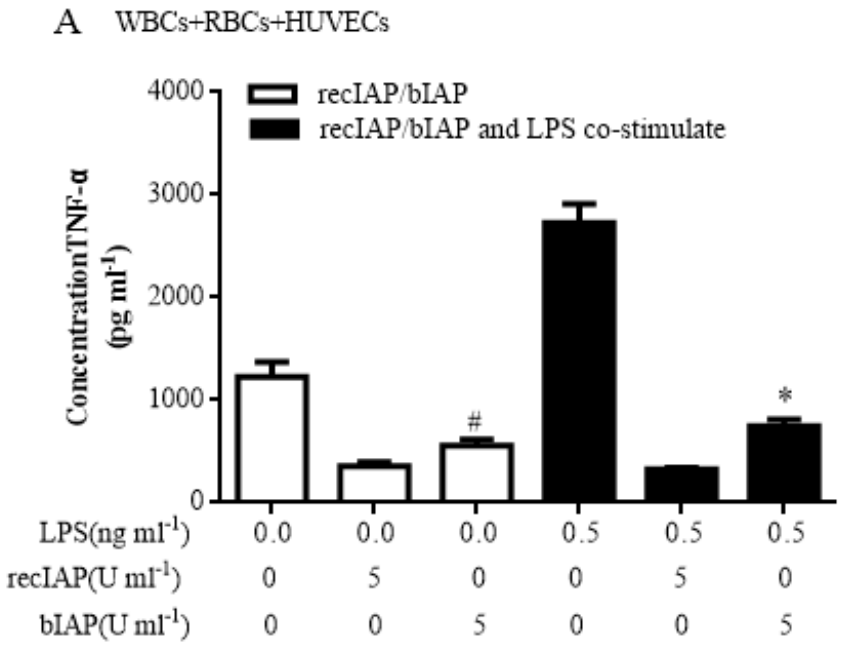


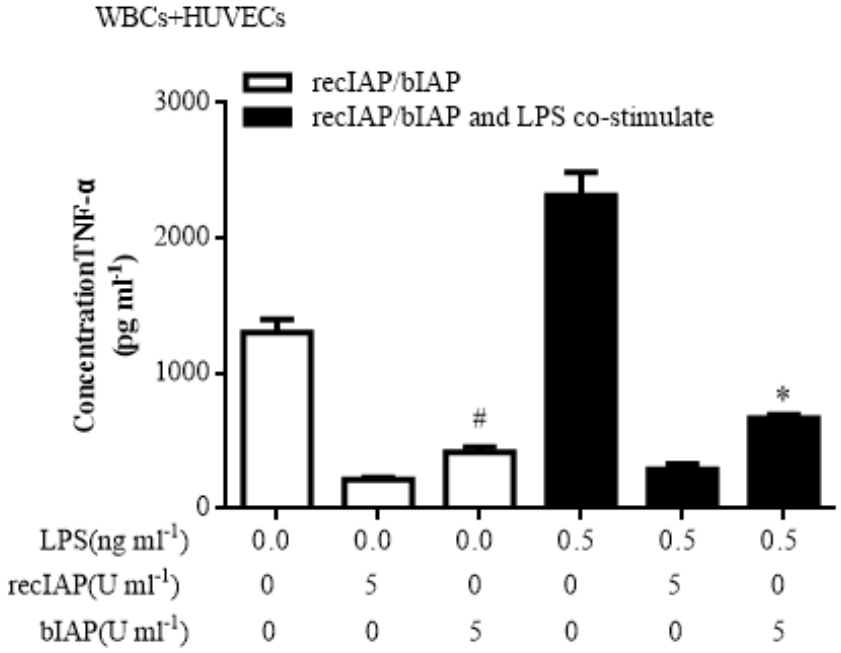


.**Figure 3**

βΙΑΠ, ας ωελλ ας ρεςΙΑΠ, ινηιβιτ τηε σεςρετιον οφ ΤΝΦ - α ανδ ΙΛ-6

To study the effect of extracted bovine intestinal alkaline phosphatase (bIAP) on leukocyte secretion of TNF-α and IL-6, bIAP was subjected to the same cell culture models as recIAP. The results showed that bIAP (5U ml^{-1}) significantly inhibit human leukocytic secretion of TNF-α and IL-6 in the presence of LPS (0.5ng ml^{-1}) (Figure 4 black bar). bIAP also exhibited a significant inhibitory effect on TNF-α and IL-6 in the absence of LPS (Figure 4 white bar). The inhibitory effect of bIAP and recIAP on TNF-α and IL-6 secretion which has been demonstrated in our experiment reflects the *in vivo* function of IAP expressed on the surface of intestinal mucosa to act in an anti-inflammatory capacity against fecal endotoxin content released by *E. coli* (Poelstra, Bakker, Klok , Hardonk, Meijer, 1997; Kaliannan, 2013; Narisawa, 2003; Peters, 2016a; Lukas,2013).





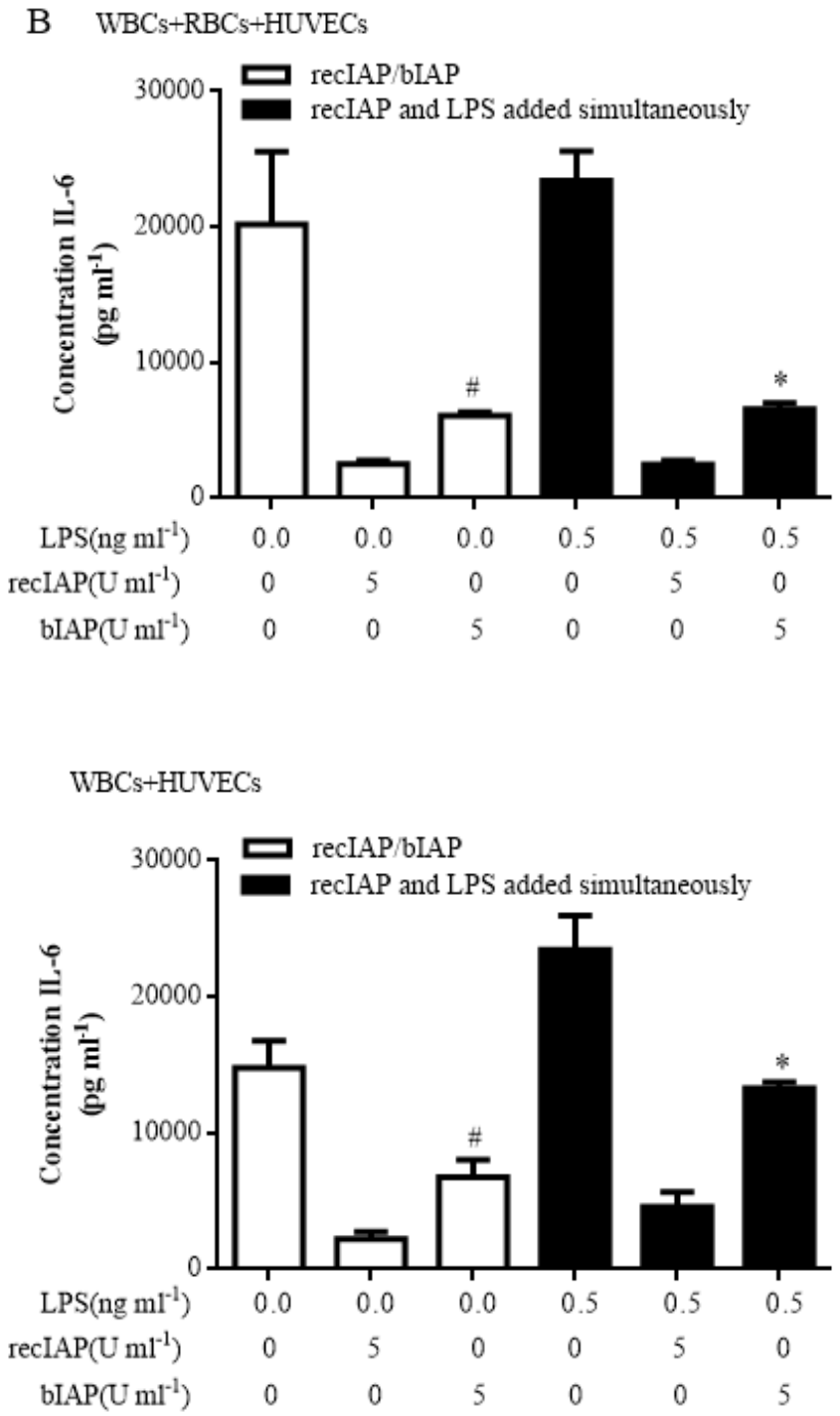
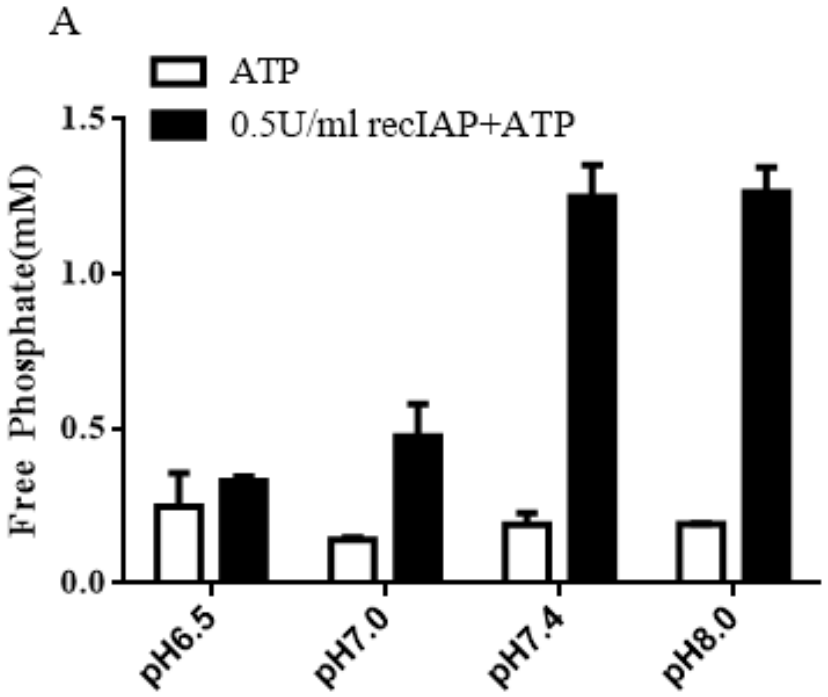


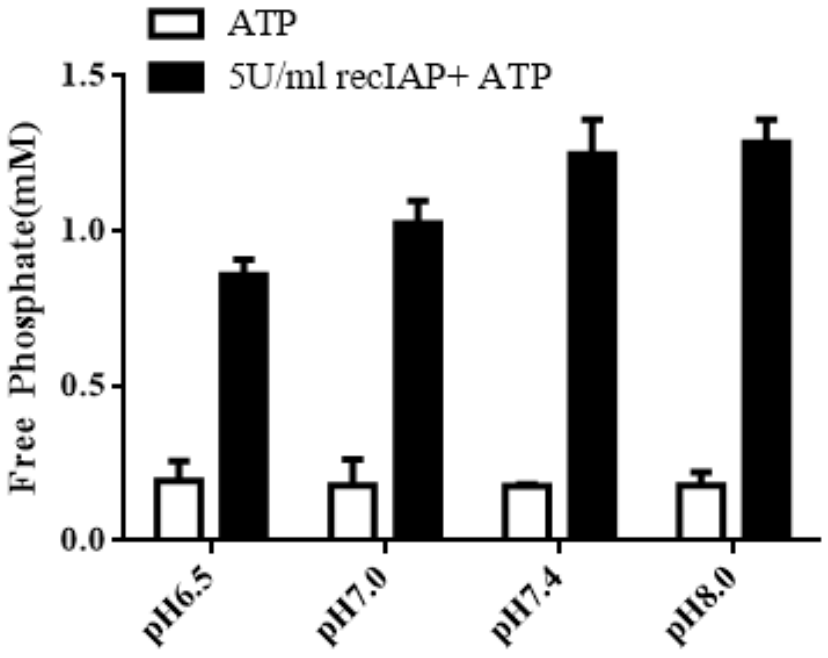
Figure 4

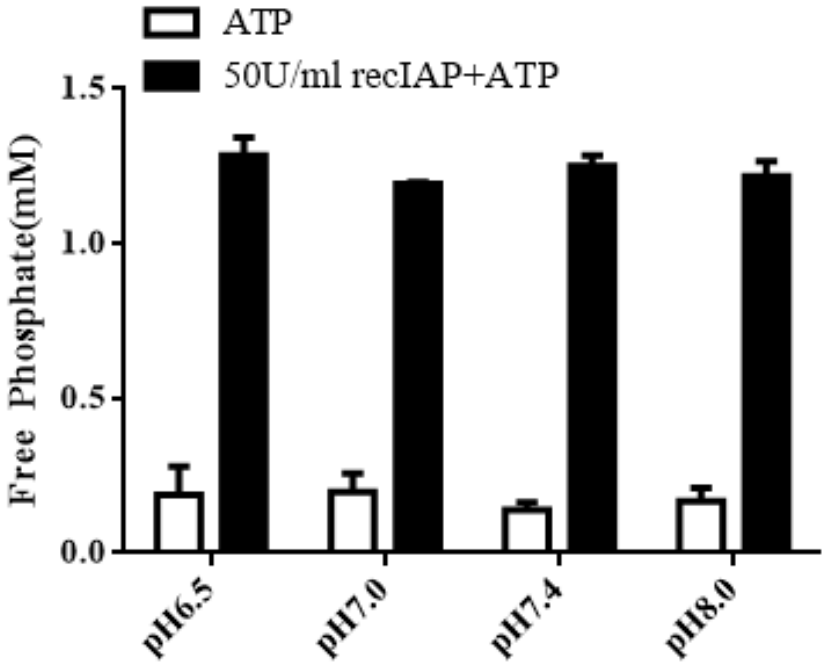
ΡεςΙΑΠ πλαψς α ρολε ιν ΤΝΦ-α σεςρετιον τηρουγη ΑΤΠ δεπηοσπηορψλατιον

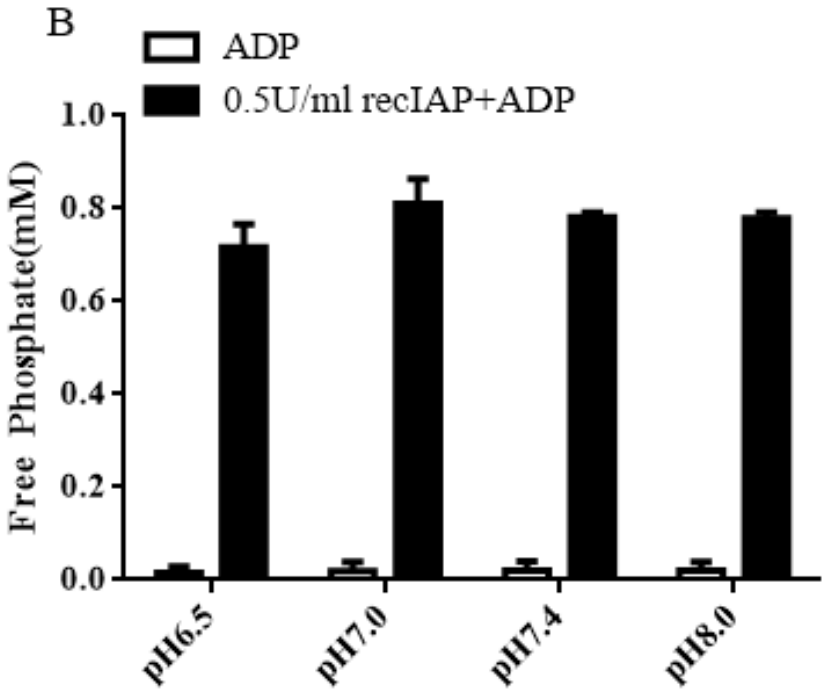
Since the use of recIAP in treating endotoxin-associated colitis (Lukas, 2010) has already been validated

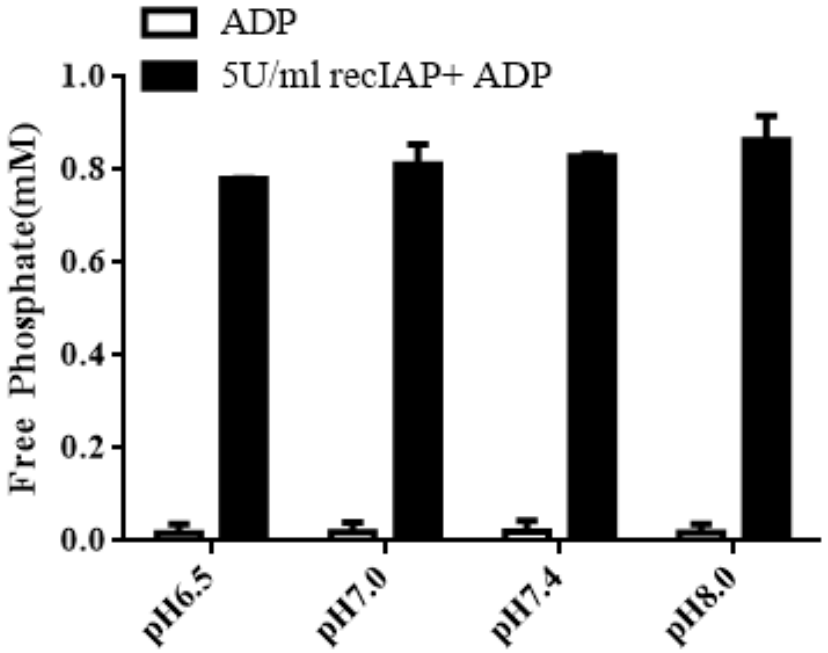
by clinical trials sponsored by AM-Pharma, this suggests that recIAP's ability to dephosphorylate ATP is intimately related to the enzyme's ability to suppress local ATP-induced inflammation generated by intestinal microorganisms (Lalles, 2014). The results of this study indicate that recIAP dephosphorylates ATP, ADP, and AMP in a dose-dependent manner and produces the products ADP, AMP, and adenosine, respectively (Figure 5 ABC). This study also finds that the ATP dephosphorylation products AMP and adenosine inhibit TNF-α secretion by human leukocytes (Figure 6 CD).

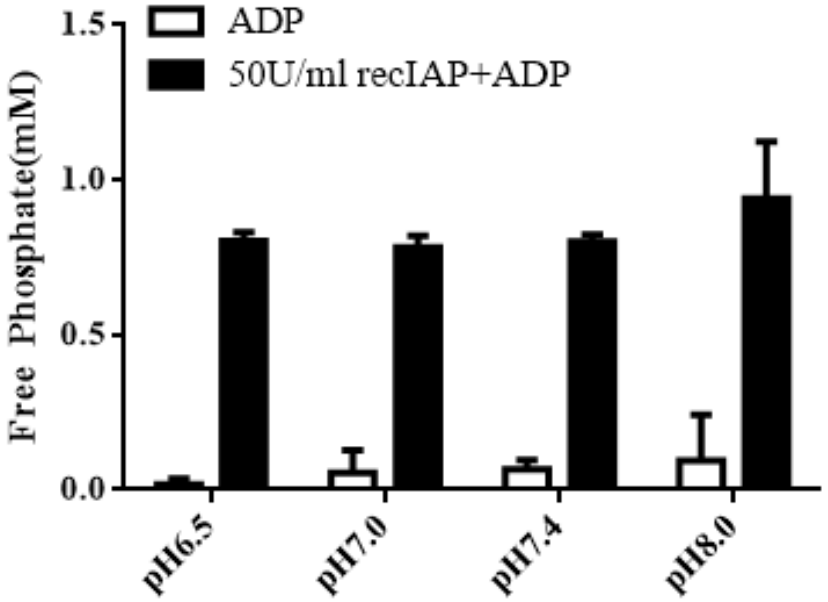


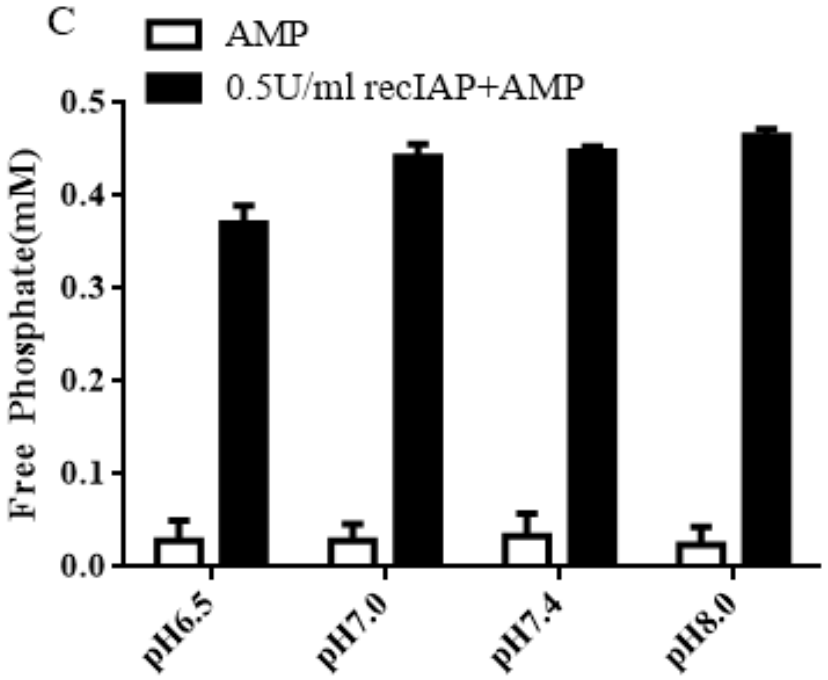












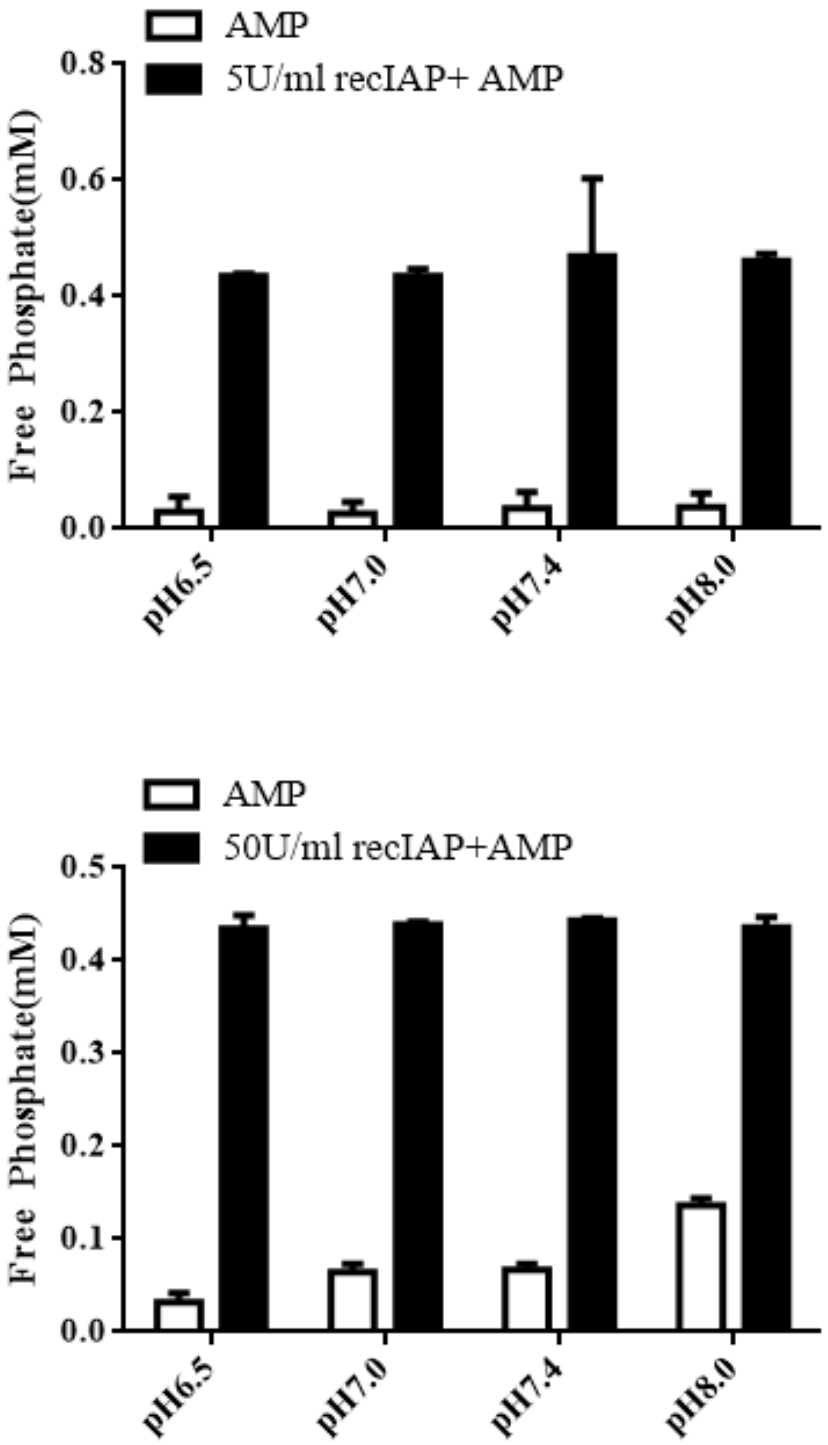
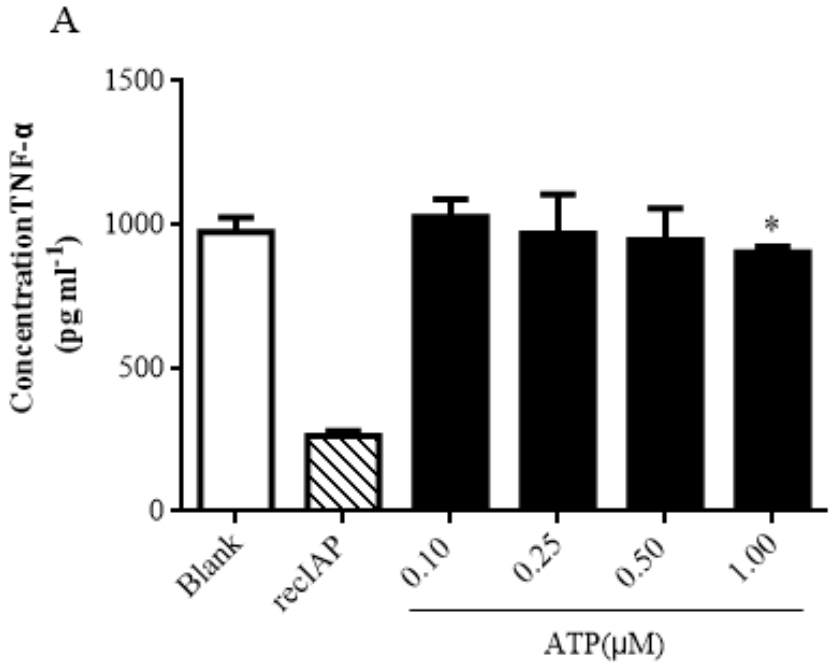
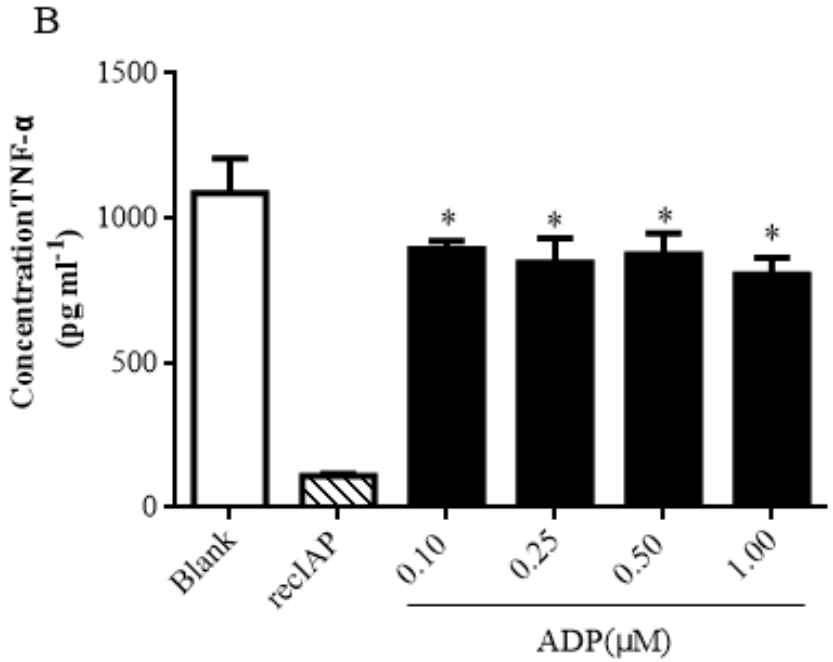


Figure 5





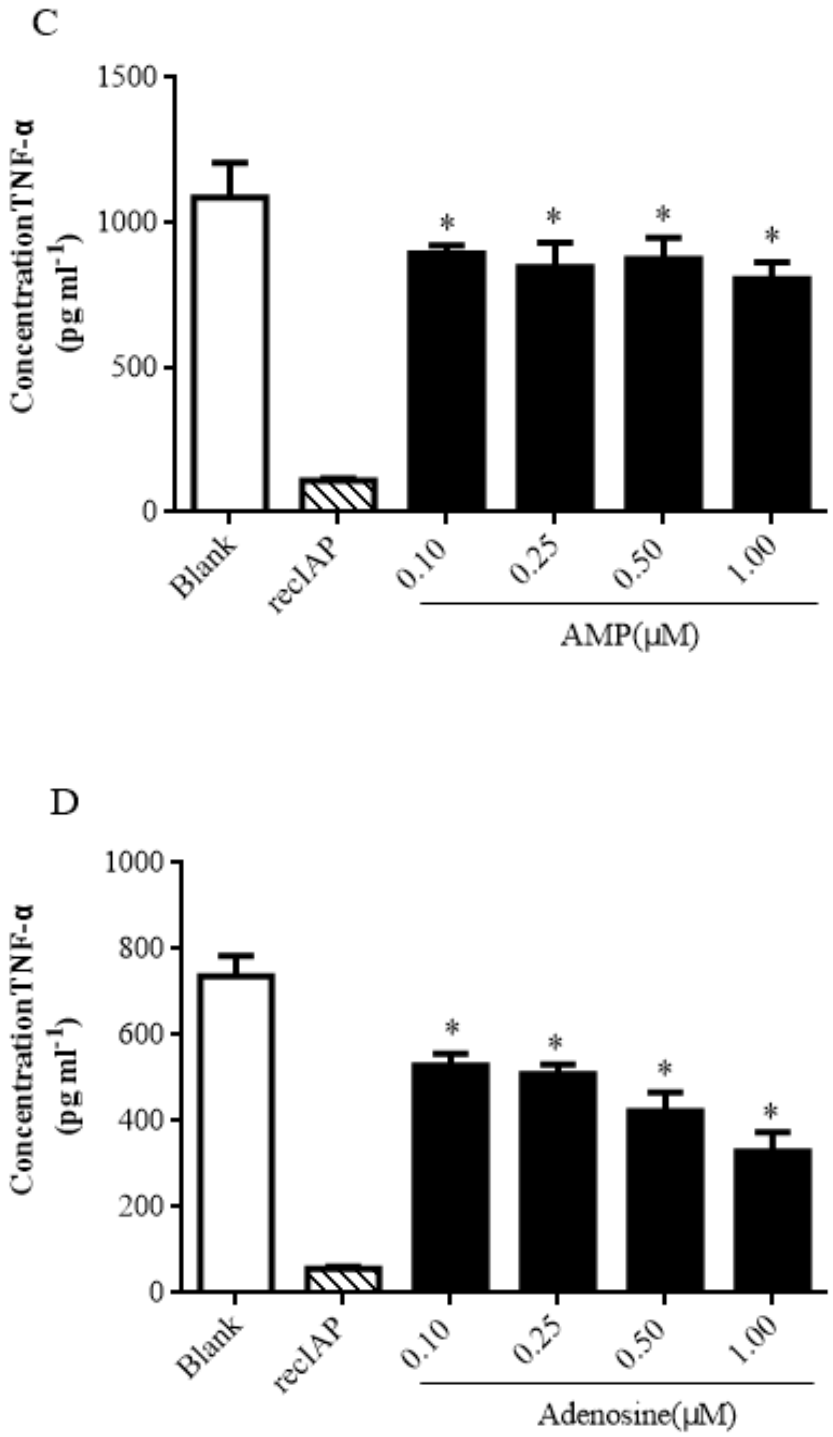


Figure 6

DISCUSSION

A large number of studies have shown that leukocytes play an important role in inducing human inflammatory diseases (Peiseler, Kubes, 2019; Suzuki, 2017; Powell, Huttenlocher, 2016; Jasper, McIver, Sapey, Walton, 2019; Kovtun, Messerer, Scharffetter-Kochanek, Huber-Lang, Ignatius 2018; Williams, Chambers, 2016; Wright, Moots, Bucknall, Edwards, 2010; Suzuki, 2018; Zhang, 2019; Mortaz, Alipoor, Adcock, Mumby, Koenderman, 2018). The migration of leukocytes into inflamed tissues behaves like a double-edged sword, not only help to remove invasive microorganisms and other foreign entities, but also contribute significantly to the pathophysiology of inflammatory diseases. In this study, the effect of intestinal alkaline phosphatase on TNF-α and IL-6 production by freshly extracted human leukocytes was successfully investigated in the presence and absence of endotoxin LPS using our cell-based model (Figure 2 and 3). LPS stimulates human leukocytes (mainly human neutrophils) and increases the production of TNF-α and IL-6. We demonstrated that IAP can effectively inactivate LPS at neutral pH 7.5 (Table 1). Interestingly, IAP inhibited leukocytic TNF-α secretion to a similar extent regardless of whether LPS and recIAP were added simultaneously to leukocytes or LPS was incubated with recIAP in advance before being added to leukocytes. This result suggests that IAP can inhibit TNF-α secretion not only by first inactivating the effect of LPS when the endotoxin is present, but also by directly acting upon leukocytes. Therefore, our finding that the IAP inhibits TNF-α and IL-6 secretion in freshly extracted human leukocytes in presence and absence of LPS indicates a therapeutic potential of the IAP for the treatment of diseases related to dysregulated production of TNF-α /IL-6 in leukocytes. In other words, the IAP administration (Peters, 2016a) can be used to treat not only LPS-related diseases such as sepsis-related, renal injury, but also diseases related to upregulation of TNF-α /IL-6. Our key finding greatly extends the application of the IAP as therapeutics to a much broader therapeutic space and disease landscape (Poelstra, Bakker, Klok , Hardonk, Meijer, 1997; Peters, 2016a; Lukas,2010; Peters, 2016b). In addition, our freshly extracted leukocyte-based assay is a promising quality control bioactivity assay for the commercialization of IAP injectable drugs (Kaliannan, 2013; Peters, 2016a; Mortaz, Alipoor, Adcock, Mumby, Koenderman, 2018; Peters, 2016b; Kiffer-Moreira, 2014; Qian, 2010).

AM-Pharma is currently manufacturing and investigating recIAP injections in clinical trials that are aimed to treat LPS-related diseases, such as sepsis-related renal injury and colitis (Peters, 2016a). It was reported that AM-Pharma's recIAP injection has a safe injection range for humans between 250, 500, and 1000U kg^{-1}(Peters, 2016a). If it is assumed that an average person's body weight is 70 kg, each treatment approximately requires a dose of 12,500, 25,000, or 50,000 U (Peters, 2016a). In this study, the effective dose of recIAP that inhibits TNF-α and IL-6 was 2.5-5U ml^{-1}, which when scaled up to a person with an average weight of 70kg (5L blood, 3L plasma) translates into 7,500- 15,000U for each treatment. For reference, AM-Pharma's recIAP has a concentration of 7036U (11.26mg ml^{-1} per injection) (Peters, 2016b) and has a specific activity of 625U/mg and purity of 99%. The specific activity of recIAP used in this study is 578 U mg^{-1}, which is close to that of AM-Pharma's recIAP injection. Thus, the recIAP used in our study proves to be cost-effective within the range of known human-safe dosing(Peters, 2016a; Peters, 2016b).

In addition to inhibiting LPS activity (Figures 3-A and B) (Poelstra, Bakker, Klok, Hardonk, Meijer, 1997), IAP might also act on TNF-α secretion through ATP dephosphorylation (Peters, 2018; Peters,2015). IAP dephosphorylates its substrate ATP and generates ADP, AMP and adenosine. This study found that in the absence of LPS, IAP inhibits TNF-α and IL-6 secretion through dephosphorylating ATP, ADP, AMP in order to generate adenosine. Our experiments demonstrated that adenosine and AMP at least partially inhibit human leukocytic secretion of TNF-α (Figure 6C and D) (Trautmann, 2009). However, it is possible that intestinal alkaline phosphatase also dephosphorylates other substrates, such as degraded cellular substances GTP, CTP, TTP, UTP, other nucleic acids, etc. For example, the rapid degradation of RNA can generate an ample source of dephosphorylation targets for IAP. Moreover, this study cannot exclude the possibility that particular cell surface receptors or signaling pathways which utilize phosphorylation are specifically targeted by alkaline phosphatase and subsequently affect the production of TNF-a and IL-6 by human leukocytes(Labugger, Organ, Collier, Atar, Van Eyk, 2000).

In conclusion,we found that IAP can inhibit TNF-α and IL-6 secreted by freshly extracted human leukocytes in the absence of endotoxin LPS. IAP is a promising injectable anti-inflammatory drug candidate for treatment of human diseases with dysregulated human leukocyte infiltration and TNF-α/ IL-6 production.

We further found that intestinal alkaline phosphatase inhibits leukocyte TNF-α and IL-6 secretion through dephosphorylating ATP, ADP, AMP, and other substrates besides LPS. The leukocyte-based cellular model developed in this study can act as a promising bioactivity assay for quality control to be used for the commercialization of an injectable IAP drug.

AUTHOR CONTRIBUTIONS

M.H., J.H.H., Z.L., and L.Q. wrote the manuscript; M.H., J.H.H., Z.L., L.Q., and L.S. designed the research; L.Q., S.W., C.G., Z.C., and Z.Z. performed the research; L.S., B.S., F.Z., and J.L. analyzed the data; L.Q., L.S., T.L., J.C., X.D., Y.J., and Q.W. contributed new reagents/analytical tools. All authors reviewed the manuscript.

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