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Amyloid Precursor Protein Expression Modulates Intestine Immune Phenotype

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Abstract

Amyloid precursor protein (APP) is widely expressed across many tissue and cell types. Proteolytic processing of the protein gives rise to a plethora of protein fragments with varied biological activities. Although a large amount of data has been generated describing the metabolism of the protein in neurons, its role in regulating the phenotype of other cells remains unclear. Based upon prior work demonstrating that APP regulates the activation phenotype of monocytic lineage cells, we hypothesized that APP can regulate macrophage activation phenotype in tissues other than brain. Ileums of the small intestines from C57BL/6/J wild type and APP^{-/-} mice were compared as a representative tissue normally associated with abundant macrophage infiltration. APP^{-/-} intestines demonstrated diminished CD68 immunoreactivity compared to wild

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type mice. This correlated with significantly less cyclooxygenase-2 (cox-2), CD68, CD40, CD11c, and β III-tubulin protein levels. Peritoneal macrophage from APP^{-/-} mice demonstrated decreased *in vitro* migratory ability compared to wild type cells and diminished basal KC cytokine secretion. Whereas, APP^{-/-} intestinal macrophage had an increase in basal KC cytokine secretion compared to wild type cells. Conversely, there was a significant decrease in multiple cytokine levels in APP^{-/-} compared to wild type ileums. Finally, APP^{-/-} mice demonstrated impaired absorption and increased motility compared to wild type mice. These data demonstrate the APP expression regulates immune cell secretions and phenotype and intestinal function. This data set describes a novel function for this protein or its metabolites that may be relevant not only for Alzheimer's disease but a range of immune-related disorders.

Keywords

amyloid; inflammation; Alzheimer; macrophage; cytokine; cyclooxygenase

Introduction

Amyloid precursor protein (APP) is a single pass transmembrane protein widely expressed in a variety of cells. A large amount of interest in this protein focuses on its expression in neurons and its ability to be proteolyzed to produce the amyloidogenic peptide, A β , characteristic of amyloid plaques in the brains of Alzheimer's disease (AD) patients (Perry et al., 1989; Joachim et al., 1991). In addition, a variety of mutations in the gene coding for APP are responsible for a rare familial form of AD (Kamino et al., 1992; Tanzi et al., 1992). Therefore, many studies have examined expression and proteolytic processing of APP in central neurons in an effort to understand possible mechanisms of disease.

However, our prior studies (Sondag and Combs, 2004, 2006, 2010) as well as others (Bauer et al., 1991; Bullido et al., 1996; Vehmas et al., 2004; Spitzer et al., 2010) have demonstrated that APP is also expressed on immune cells where it appears to play a role in regulating cellular phenotype. For example, expression on monocytic lineage cells appears to regulate the ability of these cells to interact with extracellular matrix and mediate various cell-cell interactions (Austin and Combs, 2010; Sondag and Combs, 2010). In addition, APP expression increases in both macrophage and microglia in the presence of a reactive, stimulatory environment (Haass et al., 1991; Banati et al., 1994; Monning et al., 1994; Banati et al., 1995b; Banati et al., 1995a; Gehrmann et al., 1995a; Gehrmann et al., 1995b; Monning et al., 1995; Itoh et al., 2009). We have also observed that agonist antibody stimulation of APP leads to a diverse change in proinflammatory protein expression and cytokine secretion as well as release of A β peptides from monocytic cells (Sondag and Combs, 2004, 2006). Recently, others have suggested that secreted A β peptides are anti-microbial (Soscia et al., 2010) fitting well with the ability of immune cells to make the peptides upon activating ligand stimulations (Sondag and Combs, 2006; Spitzer et al., 2010). Finally, a variety of studies have demonstrated that APP metabolites and A β peptides in both their fibrillar and oligomeric forms are potent activating stimuli for macrophage, monocytes, and microglia (Klegeris et al., 1994; Yan et al., 1998; Combs et al., 1999; Yates et al., 2000; Smits et al., 2001; Yazawa et al., 2001; Ikezu et al., 2003; Uryu et al., 2003; Xiong et al.,

2004; Floden and Combs, 2006; Sondag et al., 2009; Maezawa et al., 2010). Collectively, these data suggest that APP and its proteolytic products may serve as regulators of proinflammatory phenotype in particular immune cells.

The mammalian intestines are not only characterized by a unique enteric nervous system (Harrington et al., 2010; Tomita et al., 2010) and APP isoform expression (Yamada et al., 1989; Arai et al., 1991) but also an abundance of resident immune cell types, including macrophage necessary for monitoring resident and foreign microbial exposure (Santaolalla et al., 2010). In order to compare an environment in which neurons and macrophage are expected to abundantly express APP and a range of activating stimuli are continually being presented, we chose in this study to compare small intestine of wild type C57BL6/J and APP^{-/-} mice. Based on our previously demonstrated role for APP in regulating monocytic lineage cell function, a lack of this protein in the dynamically activated immune environment of the intestine should result in significant alteration of immune parameters and perhaps neuronal and gut function in animals that lack the protein.

Materials and methods

Materials

Anti- β -Amyloid Precursor Protein (APP) and anti-occludin antibodies was purchased from Zymed Laboratories (San Francisco, CA). Anti-mouse IgM (goat), anti-rabbit (goat), anti-goat (bovine), anti-rat (goat), and anti-mouse (bovine) horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cox-2, CD40, TLR4, CD36 and actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Elite Vectastain ABC Avidin and Biotin, Vector Vip, biotinylated anti-rabbit, anti-mouse, and anti-rat antibodies were purchased from Vector Laboratories Inc (Burlingame, CA, USA). Synaptophysin and β III tubulin antibodies were purchased from Chemicon international, Inc (Temecula, CA, USA). CD68 antibody was purchased from AbD Serotec (Oxford, UK). PSD95 and GFAP antibodies were purchased from Cell Signaling Technology Inc (Danvers, MA, USA). MAP2 antibody and MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were purchased from Sigma-Aldrich (St. Louis, MO, USA). CD3, CD11c and FATP4 antibodies were purchased from Abcam Inc (Cambridge, MA, USA). TLR2 antibody was purchased from Imgenex (San Diego, CA, USA). pTyr antibody was purchased from Millipore (Billerica, MA, USA).

Mice

APP^{tm1Dbo/J} homozygous (APP^{-/-}) mice and wild type (C57BL6/J) mice were purchased from Jackson Laboratory. Mice were provided food and water *ad libitum* and housed in a 12 hour light/dark cycle. Mice were maintained until 2 and 7 months of age then euthanized via CO₂ asphyxiation followed by cervical dislocation or cardiac perfusion. Animals were weighed prior to collection at 7 months of age. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* (eighth edition, 2010). Animal use was approved by the University of North Dakota IACUC.

Blood collection and LAL assay

At the time of perfusion, blood was collected in heparinized tubes via heart puncture and spun at 190g~7400rpm for 10min at 4°C. Plasma was then transferred to sterile centrifuge tubes which were flash frozen to be analyzed the following day for lipopolysaccharide (LPS) quantitation according to the Genscript ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit protocol (GenScript, Piscataway, NJ).

Western Blotting

At seven months, the animals were perfused with Ca²⁺/Mg²⁺ free PBS and ileum of intestine was collected, rinsed to remove lumen contents, flash frozen, pulverized and lysed using ice cold RIPA buffer (20mM Tris, pH 7.4, 150mM NaCl, 1mM Na₃VO₄, 10mM NaF, 1mM EDTA, 1mM EGTA, 0.2mM phenylmethylsulfonyl fluoride, 1% Triton, 0.1% SDS, and 0.5% deoxycholate) with protease inhibitors (AEBSF 104mM, Aprotinin 0.08mM, Leupeptin 2.1mM, Bestatin 3.6mM, Pepstatin A 1.5mM, E-64mM) and 50U/mL DNaseI (Amresco Inc, Solon, OH, USA). To remove insoluble material cell lysates were sonicated and centrifuged (14,000 rpm, 4°C, 10 min). The Bradford method (Bradford, 1976) was used to quantify protein concentrations. Proteins were resolved by 7 and 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (PVDF) for Western blotting using anti-APP, occludin, cox-2, synaptophysin, PSD95 (1:10,000), CD68 (1:2000), CD40, CD3, CD11c, GFAP (1:5000), TLR2, TLR4, CD36, FATP4, pTyr, βIII tubulin (loading control), and actin (loading control) antibodies at 1:1000 unless otherwise indicated. Antibody binding was detected with enhanced chemiluminescence (GE Healthcare, Piscataway, NJ). In some instances, blots were stripped in 0.2 NaOH, 10 min, 25°C, for reprobing. Western blots were quantified using Adobe Photoshop software. Optical densities of bands were normalized against their respective loading controls and averaged (+/-SD).

Histological Stain

At seven months, the animals were perfused with Ca²⁺/Mg²⁺ free PBS and ileum of the small intestine was collected and immersion fixed for 24hrs in 4% paraformaldehyde and cryoprotected through two successive 30% sucrose changes. A 1cm piece of ileum of small intestine was cut, then cut lengthwise and flattened for sectioning. Ileum samples were serially cryosectioned (10µm) for routine histology H & E (hematoxylin and eosin) and Alcian blue stains. For H and E stain, slides were rinsed in xylene three times for 1 min each, followed by rehydration in 2 changes of absolute alcohol for 2 min, then 95%, 80%, 70% alcohol for 1 min each, followed by a rinse in DH₂O for 1 min. Slides were then stained with Hematoxylin Regulars for 40 seconds followed by a rinse in running tap water for 10 dips, then differentiated with 3% acid alcohol for 2 dips, followed by rinse in running tap water for 10 dips. Slides were then blued in lithium carbonate (1% sodium bicarbonate) solution for 3–5 dips followed by rinsing again in running tap water before a 1 min rinse in 70% alcohol and then 5–10 seconds in Eosin. Slides were dehydrated two times each for 1 min in 95% alcohol, 100% alcohol and xylene followed by covering with permount. For Alcian blue stain, slides were placed in 100% alcohol, 95% alcohol, 70% alcohol, and DH₂O for 1 min each then mordant in 3% aqueous acetic acid for 1 min, 1% Alcian blue in 3% acetic acid pH 2.5 for 10 min followed by rinse in running water for 5–6min and rinse in

DH₂O. Slides were counterstained in nuclear fast Red 0.1% (kerechtrot) for 1.5 min then rinsed in running water for 10 min before dehydrating in 95% alcohol, 100% alcohol and xylene 2 times each for 1 minute before being covered with permount.

Immunohistochemistry

At seven months, the animals were perfused with Ca²⁺/Mg²⁺ free PBS and ileum of the small intestine was collected and immersion fixed for 24hrs in 4% paraformaldehyde and cryoprotected through two successive 30% sucrose changes. A 1cm piece of ileum of small intestine was cut, then cut lengthwise and flattened for sectioning. Ileum samples were serially cryosectioned (10µm) for immunostaining with anti-APP (1:1000), CD68 (1:500), cox-2 (1:500), occludin (1:100), MAP2 (1:500), pTyr (1:1000), and GFAP (1:1000) antibodies or respective secondary only antibodies (1:2000). Antibody binding in the intestine was visualized using the Vector VIP chromagen (Vector Laboratories, Burlingame, CA). Images were taken using an upright Leica DM1000 microscope and Leica DF320 digital camera system. Figures were made using Adobe Photoshop 7.0 software.

Cytokine Array

At seven months, the animals were perfused with Ca²⁺/Mg²⁺ free PBS and ileum of intestine was collected, flash frozen, pulverized and lysed using lysis buffer according to the manufacturer's protocol (Ray Biotech, Norcross, GA, USA). Insoluble material was removed from cell lysates via centrifugation (14,000 rpm, 4°C, 10 min). The Bradford method (Bradford, 1976) was used to quantify protein concentrations. 1mg per sample was used to probe a 40 cytokine antibody-based membrane array according to the manufacturer's protocol (Ray Biotech, Norcross, GA, USA). Optical density of dots were normalized against their respective positive controls and averaged (+/-SD).

Stool Weight

Total stool weight was measured by placing the animals in a clean empty cage and collecting all stool produced over a 1hr time period. Stool was then desiccated at 80°C overnight to determine dry weight. Stool water was calculated as the difference between these two measurements.

Macrophage Isolation and Stimulation

At 2 months, non-elicited peritoneal macrophage were rinsed from the peritoneal cavity of the collected mice with sterile PBS, spun, resuspended, and allowed to adhere to tissue culture wells for 2–3 hours in DMEM/F12 plus PSN antibiotics (0.05mg/mL Penicillin, 0.05mg/mL Streptomycin, 0.1mg/mL Neomycin (Gibco, Carlsbad, CA, USA)). Ileum of the intestines were removed from the abdominal cavity and rinsed with sterile PBS inside and outside to remove residual contents. Intestines were then rinse again with HBSS (8g NaCl, 0.4g KCl, 3.57g Hepes, 0.06g Na₂HPO₄ × 2 H₂O, 0.06 g KH₂PO₄ in 1 liter DH₂O) and then placed in sterile enzyme solution (5mM Glucose, 1.5% BSA, 5mg/mL Collagenase, 20% FBS in HBSS), minced and placed in 37°C incubator for 30 min to allow digestion to occur. Tissue was then triturated, filtered through a 70µm cell strainer, quenched with HBSS and spun for 4 min at full speed. The pellet was resuspended in HBSS, triturated and spun for

another 4 min at full speed. The pellet was resuspended in DMEM/F12 plus PSN antibiotics and 400 μ L of suspended cells was plated in another 1mL of DMEM/F12 plus PSN antibiotics for 2–3 hours to allow adherence of macrophage. Non-adherent and non-macrophage cells were rinsed from the wells using ice-cold DMEM/F12.

Enzyme-linked Immunosorbent Assay (ELISA)

Following rinses, DMEM/F12 plus PSN antibiotics with or without 25ng/mL LPS (Sigma) was added for overnight stimulation, then media was collected for quantifying secreted keratinocyte chemokine (KC), Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES), Interleukin-6 (IL-6), and Interleukin-1 beta (IL-1 β) according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA). Secretion was normalized to cell protein amount as determined by the Bradford method (Bradford, 1976) after cells were lysed in ice cold RIPA buffer with protease inhibitors, sonicated, and spun to remove insoluble material.

Migration and Proliferation Assay

After rinses, cells were removed with ice cold dissection media and gentle trituration. One set of macrophage (5,000 cells/well/96 well) had DMEM/F12 containing PSN antibiotics, 10% FBS and 5% horse serum added for 72hrs followed by an MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay to indirectly measure cellular proliferation. The media was removed and replaced with 0.1mg/mL MTT for 4 hr. The precipitated formazan was dissolved in isopropanol and absorbance read at 560nm. Another set of macrophage (5,000 cells/well) were resuspended in DMEM/F12 and transferred to transwell permeable support inserts (3 μ m, Corning Inc. Corning, NY, USA) in collagen coated 24 well dishes containing 25ng/mL LPS to measure transmigration by counting the number of migrated cells after 24 hours.

Statistical Analysis

The data were analyzed by unpaired two-tailed t-test with or without Welch correction for unequal variance as required or by a two-way ANOVA with Holm-Sidak post hoc test.

Results

Immunoreactivity differences between the ileum of APP^{-/-} and C57BL6/J control mice

In order to determine histological differences between APP^{-/-} and wild type C57BL6/J mice, the ileum of small intestine was collected from adult (7 months of age) mice and was fixed, sectioned and stained with hematoxylin and eosin and Alcian blue. Mice were collected at this age based upon prior observations that transgenic animals over-expressing mutant forms of APP demonstrate pathology in the gut at 6 months of age. This suggested that expression or function of endogenous APP, as was our study intent, may also be involved in regulating intestinal physiology at this age (Van Ginneken et al., 2011). There were no histological structural differences between the wild type C57BL6/J and APP^{-/-} mice (Fig. 1). In order to characterize APP immunoreactivity in the intestine, wild type C57BL6/J and APP^{-/-} mice ileum of the small intestine was immunostained. Wild type mice demonstrated robust APP immunoreactivity within enterocytes and neurons and

diffuse immunoreactivity within the smooth muscle of the muscularis externa (Fig. 2). The tissue was then immunostained with a common intestine regulatory protein, cyclooxygenase-2 (cox-2) as well as the tight junction protein, occludin, to gain an impression of intestinal phenotype. Both cox-2 and occludin immunoreactivity again appeared primarily localized to the enterocyte layer with some areas of robust immunoreactivity for cox-2 in the wild type intestines compared to APP^{-/-} mice (Fig. 2). This supported the idea that APP expression regulates some aspects of intestinal immune cell behavior as well as that of enterocytes. Based upon the fact that a portion of the APP immunoreactivity was clearly localized to neurons in wild type mice, the tissue was also stained with an anti-microtubule associated protein 2 (MAP2) antibody to visualize the integrity of the enteric plexi in the intestines. As expected, wild type mouse sections demonstrated robust immunoreactivity throughout the submucosa and lamina propria while the APP^{-/-} sections had visibly less immunoreactivity (Fig. 2). This suggests that lack of APP expression also modulates neuronal integrity and likely enteric function in the small intestine. Since there was no obvious immunoreactivity for APP observed within macrophage or other cell types throughout the intestinal layers (Fig. 2) we then examined other cell types in the ileum of the small intestine. Wild type intestines demonstrated robust anti-CD68 immunoreactivity to identify macrophage while APP^{-/-} mice presented with a markedly decreased staining (Fig. 3) suggesting either decreased activation or decreased numbers in the intestine. Because the phenotype of astrocyte-like enteric glia can contribute to maintenance of gut barrier integrity and inflammatory state (Savidge et al., 2007; Cirillo et al., 2011) we next assessed activation state of these cells. Based upon the fact that a population of enteric glia are astrocytic in nature (Jessen and Mirsky, 1985) we immunostained the ileum of the small intestine with anti-glial fibrillary acidic protein (GFAP) antibodies (Fig. 3). The GFAP immunoreactivity appeared to localize to the external portion of the muscularis externa. Unlike neuron and macrophage immunoreactivities there was no visible difference in astrocyte-like immunoreactivity between the wild type and APP^{-/-} intestine (Fig. 3). To assess if APP expression resulted in tyrosine kinase activation in the ileum of the small intestine as another potential marker of immune cell macrophage activation, the ileum was stained with anti-pTyr antibodies. However, there was no obvious difference in pTyr immunoreactivity between wild type and APP^{-/-} intestines (Fig. 3).

Quantitation of protein differences in the ileum of C57BL6/J wild type and APP^{-/-} mice

Based upon the qualitative changes in immunoreactivity patterns observed, it was necessary to quantify whether there were indeed protein level changes in wild type versus APP^{-/-} intestine. Ileum samples were Western blotted to determine whether the changes observed by immunohistochemistry were significant. As expected, Western blot analysis demonstrated that cox-2 and CD68 levels were significantly decreased in the APP^{-/-} mice compared to wild type controls with no difference in occludin, GFAP, and pTyr (Fig. 4) consistent with the immunostaining observed (Fig. 2). To assess whether other immune cell types present in the intestine besides macrophage were altered in APP^{-/-} mice, Western blots for markers of T-cells (CD3), B-cells (CD40), as well as dendritic cells (CD11c) were quantified. Although there was no change in CD3 levels there was a significant decrease in CD40 and CD11C protein levels in the APP^{-/-} compared to the wild type mice (Fig. 4). As

a measure of neuronal integrity, presynaptic and postsynaptic markers were also examined by Western blot. Interestingly, the total amount of neuronal β III-tubulin protein levels was decreased between the wild type and $APP^{-/-}$ mice, with no difference in protein levels of the presynaptic (synaptophysin) or postsynaptic (PSD95) protein markers (Fig. 4). This decrease in β III-tubulin levels was consistent with the observed decrease in dendritic marker, MAP2, immunoreactivity in $APP^{-/-}$ mice (Fig. 2). A change in macrophage, B cell, and dendritic cell protein levels could correlate with altered expression of immunomodulatory receptors in the intestine. Therefore, critical pattern recognition receptor, TLR2 and TLR4, protein levels were examined as well. However there was no change in expression of either TLR between wild type and $APP^{-/-}$ ileum of the small intestine (Fig. 4). Finally, protein levels of necessary fatty acid transport proteins was assessed as an additional means of examining changes in intestinal barrier integrity. CD36 and FATP4 protein levels were analyzed, with again, no difference in expression between the wild type and $APP^{-/-}$ ileum of the small intestine (Fig. 4) suggesting again that epithelial function may be preserved in the $APP^{-/-}$ intestines.

In vitro migration, proliferation, and differentiation comparison of macrophage from $APP^{-/-}$ and C57BL6/J wild type mice

Since Western blot analysis had validated that protein differences existed in neuronal proteins and enterocyte proteins in $APP^{-/-}$ compared to wild type mice, we next sought to further examine the changes in CD68 positive macrophage immunoreactivity observed in the $APP^{-/-}$ ileums. As intestinal macrophage have previously been shown to have very different responses to LPS (Grimm et al., 1995; Rogler et al., 1998), both peritoneal and intestinal macrophage have been included in our analysis as both contribute to the immunomodulatory behavior of the ileum of the intestine. One possibility for the decrease in CD68 immunoreactivity might simply be that $APP^{-/-}$ cells were less able to migrate into the ileum. As a means of testing this idea, we isolated non-elicited peritoneal macrophage and intestinal macrophage from wild type and $APP^{-/-}$ mice at 2 months of age and compared the ability of these cells to migrate through a transwell culture insert *in vitro*. As expected, $APP^{-/-}$ peritoneal macrophage demonstrated a significantly decreased ability to migrate through the insert into the LPS containing media below compared to wild type peritoneal cells. However, there was no significant difference in the migratory behavior of wild type and $APP^{-/-}$ intestinal macrophage (Fig. 5A and B). Another possibility for decreased CD68 immunoreactivity in the $APP^{-/-}$ ileums could be due to an impaired differentiation or proliferation ability of $APP^{-/-}$ cells. As a means of assessing proliferative ability of the cells, peritoneal and intestinal macrophage from wild type and $APP^{-/-}$ animals were grown in the presence of serum for 72 hours. However, there was no significant difference in proliferative ability *in vitro* of either type of macrophage (Fig. 5C and D). To examine differentiative or activation phenotype differences between the two genotypes, collected macrophage were stimulated 24 hr in the absence or presence of the potent bacterial endotoxin LPS and media were collected to assay secretion of select cytokines. Based upon prior analysis of abundant peritoneal macrophage *in vitro* secretion, keratinocyte chemokine (KC), and interleukin-6 (IL-6) levels were quantified from the media as representative secretory factors from peritoneal and intestinal macrophage. Although there were no differences in LPS-stimulated levels between genotypes for KC cytokine secretion in the

peritoneal macrophage, the APP^{-/-} peritoneal macrophage secreted significantly less KC compared to wild type cells during basal, unstimulated conditions (Fig. 6A). LPS-stimulated wild type peritoneal macrophage had increased secretion of IL-6 compared to LPS-stimulated APP^{-/-} and untreated wild type peritoneal macrophage (Fig. 6C). The LPS-stimulated intestinal macrophage had no change in secretion of KC or IL-6, suggesting that intestinal macrophage are unresponsive to LPS (Fig. 6B and D). However, basally APP^{-/-} intestinal macrophage secreted more KC than wild type intestinal macrophage (Fig. 6B). As a follow up to the decreased migratory behavior of the APP^{-/-} peritoneal macrophage, Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES) was measured from peritoneal macrophage stimulated with and without LPS (Fig. 6E). As with the secretion of KC, LPS increased RANTES secretion with no significant difference between wild type and APP^{-/-} peritoneal macrophage (Fig. 6E). To further compare phenotype differences particularly in intestinal macrophage, interleukin-1 beta (IL-1 β) was next measured. Again as with KC and IL-6, LPS stimulation had no effect on wild type intestinal macrophage. However, LPS significantly increased secretion of IL-1 β from the APP^{-/-} intestinal macrophage as compared to the unstimulated APP^{-/-} intestinal macrophage as well as the LPS stimulated wild type intestinal macrophage (Fig. 6F). Collectively, these findings suggest that peritoneal and intestinal macrophage of APP^{-/-} mice are phenotypically different with impaired migratory ability in peritoneal macrophage compared to their intestinal counterparts. More importantly, in spite of an apparent decrease in numbers of CD68 positive macrophage in the ileum of APP^{-/-} mice, these cells appear, to some degree, hyperreactive basally and in response to LPS stimulation when compared to wild type intestinal macrophage.

Profile of cytokine levels in ileum of APP^{-/-} and C57BL6/J wild type mice

Based upon the *in vitro* macrophage secretory differences observed, the reduced CD68 ileum macrophage immunoreactivity, and the reduced protein levels of CD68, CD40, and CD11c it was reasonable to expect that levels of cytokines in the APP^{-/-} versus wild type ileums as a whole would also differ. To examine this possibility, ileums were collected from 7 month old animals and used to perform antibody-based cytokine arrays profiling 40 different cytokines. As expected, the APP^{-/-} ileums demonstrated significantly decreased levels of 12 different factors, CD30L, eotaxin, fas ligand, fractalkine, GCSF, IL-10, IL-12p40p70, IL-3, IL-9, MIG, MIP-1 γ , and sTNFR1, compared to wild type mice (Fig. 7). Therefore, these findings were consistent with the immunohistochemistry and further supported the notion that APP expression regulates immune cell phenotype in the intestine.

Differences in intestinal motility and absorption in APP^{-/-} and C57BL6/J mice

A combined change in levels of multiple cytokines, proinflammatory and regulatory proteins, numbers of macrophage, and finally neuronal synaptic markers all suggest that the function of APP^{-/-} intestines would differ significantly compared to wild type control animals. In order to address intestinal function, mean stool production and water content were compared between the genotypes. Although this was not exclusively an assessment of small intestine behavior, APP^{-/-} animals generated significantly more stool per time period with increased water content (Fig. 8A). This suggested increased motility and decreased absorption in the intestine of APP^{-/-} mice, which correlated with reduced body mass in

these mice (8B). This suggests that APP^{-/-} may have reduced weight gain ability due to increased motility and reduced absorption. Moreover, the adverse consequence of decreased absorption in the smaller APP^{-/-} mice likely has a significant effect on overall physiology. As an additional means of assessing absorption, plasma levels of LPS were quantified from the blood of C57BL6/J and wild type mice. Trace amounts of LPS are known to be taken up into the blood from the intestines during normal absorption of fatty acids (Amar et al., 2008; Ghoshal et al., 2009). As now predicted, APP^{-/-} mice demonstrated significantly less LPS in their plasma compared to wild type mice again suggesting impaired intestinal absorptive function (Fig. 8C).

Discussion

We have demonstrated that APP expression has profound regulatory effects on protein expression in a range of small intestine cells including neurons, CD68 positive macrophage, and enterocyte epithelial cells. This represents, to the best of our knowledge, the first demonstration of a function for this protein in this manner. Indeed, a scarcity of data exists regarding the function of APP in general. Based upon the quantified differences in multiple cytokine expression levels as well as changes in intestinal motility and absorption it appears that APP has a role in diverse tissue types. More importantly, mutations in the gene coding for APP or altered processing of APP could have effects in other tissues, such as the intestine, in addition or uniquely from any observed in the brain.

It is important to point out that at this point it is not clear whether lack of APP on one or multiple cell types is responsible for the changes in cytokine profile, enterocyte protein expression or motility and absorption observed. Also, in spite of the robust changes in macrophage behavior and cytokine levels in APP^{-/-} versus wild type mice, little detectable APP immunoreactivity was observed in macrophage-like cells in ileums of wild type mice. Based upon prior evidence of clear APP expression in monocytic lineage cells (Bauer et al., 1991; Bullido et al., 1996; Sondag and Combs, 2004; Vehmas et al., 2004; Sondag and Combs, 2006, 2010; Spitzer et al., 2010) it is likely that the level of expression of APP in macrophage was simply not high enough to be detectable by the immunostaining procedure employed. Indeed, prior work from others has demonstrated in the related brain macrophage cell type, microglia, that APP expression is upregulated when the cells are provided the appropriate stimulatory environment (Haass et al., 1991; Banati et al., 1994; Monning et al., 1994; Banati et al., 1995b; Banati et al., 1995a; Gehrmann et al., 1995a; Gehrmann et al., 1995b; Monning et al., 1995; Bullido et al., 1996; Vehmas et al., 2004; Itoh et al., 2009). In this fashion, it is likely that APP expression still directly regulates macrophage phenotype in the intestine in spite of the limitations in detection using the visible light chromagen method. One intriguing hypothesis is simply that macrophage numbers are limited due to impaired ability of APP^{-/-} cells to migrate into the submucosa and lamina propria. For instance, our prior work demonstrated that APP expression is required on both monocytes and endothelial cells for cell-cell interaction to occur necessary for monocytic adhesion required for activities such as diapedesis through the vasculature (Austin et al., 2009; Austin and Combs, 2010). It is possible that APP^{-/-} macrophage are limited in the intestine due to decreased influx through the vasculature. The impaired migratory ability of APP^{-/-} macrophage supports this notion.

On the other hand, it is also quite possible that APP and its proteolytic metabolites expressed on other cells, such as neurons or enterocytes are normally responsible for some level of CD68 positive macrophage numbers in the intestine. The robust detection of APP immunoreactivity that localized to enterocytes and neurons is entirely consistent with the fact that neurons particularly in the brain express large amounts of APP compared to other tissue types (Schmechel et al., 1988; Yamada et al., 1989; LeBlanc et al., 1991) and prior expression of at least the A β peptide, has been detected in rodent enterocytes (Galloway et al., 2007; Galloway et al., 2008; Galloway et al., 2009; Pallegage-Gamarallage et al., 2009). This suggests that lack of APP metabolites from neurons or enterocytes may lead to diminished stimulation of intestinal macrophage. Indeed, we as well as others have demonstrated in a plethora of studies that different multimeric forms of A β peptide or other APP fragments can directly stimulate immune cells such as monocytes, macrophage, and microglia to alter their phenotype (Klegeris et al., 1994; Yan et al., 1998; Combs et al., 1999; Yates et al., 2000; Smits et al., 2001; Yazawa et al., 2001; Ikezu et al., 2003; Uryu et al., 2003; Xiong et al., 2004; Floden and Combs, 2006; Sondag et al., 2009; Maezawa et al., 2010). In this scenario, limited CD68 immunoreactivity in the APP^{-/-} intestine could be a consequence of not only loss of macrophage APP function but also loss of APP metabolite-stimulated signaling of macrophage by neurons or enterocytes. Indeed, a lack of APP-dependent activation of immune cells or a lack of A β -dependent stimulation of immune cells in the ileum could possibly explain not only the decrease in CD68 protein levels but also CD40 and CD11c as well as the dramatic decrease in multiple cytokines in APP^{-/-} intestines. This scenario suggests that perhaps APP or its metabolites offer some sort of basal or inducible stimulatory signal to help regulate gut function. However, as the premise of the current study was to first document a novel change in intestinal immune phenotype in APP^{-/-} animals, further mechanistic dissection of the precise reason for changes in immune cell numbers or activation states remains a future goal. In fact, assessing changes in intestinal immune cell behavior as a consequence of APP expression and metabolism might be best addressed in the context of including animals that over-express human mutant APP characteristic of AD. Provided that expression of the transgene occurs in the relevant cell types in the intestine, models such as these might offer insight into consequences of over-expression of APP or elevated A β levels on gut function and immune phenotype. More importantly, this might offer insight into consequences of mutant APP expression in the gut as it relates to AD. It has already been demonstrated that A β immunoreactive plaques accumulate in the intestines of AD patients (Joachim et al., 1989) although evidence of enteric neuron loss is not established (Shankle et al., 1993; Van Ginneken et al., 2010). A role for APP or its proteolytic products in intestine function would provide not only insight into the immunoregulatory role of APP and its metabolites but also begin characterization of disease-relevant changes in a tissue other than the brain.

Although the most straightforward clinical application of our data set is to extrapolate to conditions of AD in which mutant APP has a known role in the biology of disease, it is interesting to speculate that APP may ultimately have a role in modulating immune cell behavior in conditions other than AD. For instance, the changes in CD68 immunoreactivity observed in APP^{-/-} mice combined with the cytokine profile changes suggests that systemic immune changes exist in these animals. We have only examined peritoneal macrophage and

intestinal macrophage from the ileum in this study since our prior work has demonstrated a role for APP in monocytic lineage cell behavior (Sondag and Combs, 2004, 2006, 2010). However, unlike the brain, the gut is home to a diverse population of dynamically changing and regulated immune cells that will likely be affected by any alteration of macrophage behavior. Indeed the decreased protein levels of CD40 and CD11c in the ileum suggest that levels or activation state of other immune cells such as B cells and dendritic cells are also altered in the in APP^{-/-} mice.

Furthermore, for simplicity and proof-of-concept we have limited this assessment to the ileum of the small intestine base upon the fact that it contains immune cell infiltrates and a robust enteric nervous system. However, future efforts to generate a comprehensive profile of all immune cell changes not only in the ileum but throughout the digestive tract particularly the large intestine would offer cell and region specific information that could be correlated with plasma and other organ changes in cytokine and immune cell differences in APP^{-/-} versus wild type mice.

The change in cox-2 protein levels in APP^{-/-} mice certainly supports the idea that APP expression regulates gut function. A host of studies have documented a role for cox-2 and its prostaglandin products in negatively regulating intestinal motility (Cong et al., 2007; Takechi et al., 2009; Fairbrother et al., 2011; Nylander, 2011; Shi et al., 2011). In addition, cox-2 activity positively regulates intestine macrophage infiltration in a rodent model of sepsis (Osterberg et al., 2006) perhaps offering an additional mechanism explaining why APP^{-/-} mouse CD68 positive macrophage immunoreactivity was attenuated compared to wild type mice. Therefore the decrease in cox-2 protein levels observed in the APP^{-/-} mice is entirely consistent with the increased motility and decreased water absorption observed in the collected stool samples. A change in absorptive capacity was further demonstrated by the significantly decreased levels of absorbed LPS in the APP^{-/-} mice. The uptake of bacterial flora-derived LPS into plasma of rodents and humans as a consequence of lipid absorption is a well-characterized phenomenon (Amar et al., 2008; Ghoshal et al., 2009). Although this is not necessarily the only source for plasma LPS uptake, it correlates well with prior work and suggests that APP^{-/-} intestines may also have impaired lipid absorption. In addition, although we did not detect significant differences in occludin protein levels between genotypes it is quite feasible that the integrity of the gut epithelial barrier is impaired in the APP^{-/-} mice. Further work to provide additional measures of gut motility, barrier integrity, and absorption will better resolve the differences in gut function dependent upon expression of APP.

Another interesting difference between APP^{-/-} and wild type ileums was the decreased staining for MAP2 and the decrease in β III-tubulin protein levels in the APP^{-/-} mice. Based upon the role of the enteric nervous system in regulating absorption, secretion, and motility in the intestine it is feasible that the decrease in β III-tubulin levels reflects a dysfunction of the enteric nervous control of the intestine. Future work examining more refined assessment of intestinal function will offer insight into this question. This may have nothing to do with enterocyte or immune cell function but instead be the consequence of loss of neuronal APP expression. Certainly, prior work examining the brains of APP^{-/-} mice has demonstrated a similar decrease in MAP2 immunoreactivity (Seabrook et al., 1999). Moreover, a number of

studies have implicated a role for APP expression in regulating both presynaptic and postsynaptic integrity in the central and peripheral nervous system (Seabrook et al., 1999; Yang et al., 2005; Priller et al., 2006; Hoe et al., 2009; Wang et al., 2009; Lee et al., 2010; Weyer et al., 2011).

It is important to point out that we compared intestinal and peritoneal macrophage in isolation when examining cytokine secretory phenotypes. Although this analysis clearly demonstrated differences not only between peritoneal and intestinal macrophage, it also verified that intestinal macrophage from APP^{-/-} mice are unique from their wild-type counterparts. Perhaps the more physiologically relevant comparison was derived from the cytokine array comparison of wild type and APP^{-/-} ileums. This offered a broad screen of multiple cytokine changes that are a reflection of the more physiologically relevant multi-cell environment *in vivo*. Taking into account that the decreased levels of multiple cytokines in APP^{-/-} ileums are still only a snapshot into the dynamic immune changes among multiple cell types that regulate the gut, the array nevertheless does offer an interesting speculative opportunity for hypothesizing how APP may have a role in diverse intestinal conditions. For instance, it has been previously shown that CD30L plays a critical role in controlling inflammatory bowel syndrome in mice by increasing production of T-cells (Sun et al., 2008). Eotaxin mediates colonic eosinophilic inflammation (Waddell et al., 2011) and fas ligand has been shown to sequester double negative T cells in the gut epithelium (Hamad, 2010). Fractalkine can regulate increased IL-6 and TNF α release in the intestine (Niess and Adler, 2010) and IL-12 production is increased by T-cells in irritable bowel disease (Uza et al., 2011). Therefore, since APP^{-/-} mice have decreased levels of these multiple cytokines they may have decreased risk for inflammatory gut disease such as irritable bowel disease which could be investigated further. The decrease in GCSF could explain why APP^{-/-} mice also have decreased dendritic cell marker protein, CD11c, as GCSF is the key factor in differentiating monocytes into dendritic cells (Metcalf, 1985). IL-10, an anti-inflammatory cytokine, may be decreased due to the decrease in T-cell and macrophage expression as IL-10 has been shown to be a potent suppressor of macrophage and T-cells functions *in vitro* (Kuhn et al., 1993). MIP-1 γ may also be decreased due to the decrease in macrophage and dendritic cell numbers or activation as it is constitutively expressed in these cells (Mohamadzadeh et al., 1996; Haddad and Belosevic, 2009). IL-9 has been shown to promote IL-13 up-regulation of innate immune receptors (Steenwinckel et al., 2009). Therefore the decrease in both IL-9 and IL-13 observed is probably protective in these APP^{-/-} mice as IL-13 has been shown to exert detrimental effects on epithelial barrier function by increasing epithelial cell apoptosis, derangement of tight junction integrity and acts as a key effector cytokine in ulcerative colitis (Heller et al., 2005). MIG has also been shown to be concomitantly expressed in ulcerative colitis (Egesten et al., 2007). TNF α has been implicated in the pathogenesis of Crohn's disease, and by selectively blocking TNFR1 the severity of TNBS-induced colitis in rats is ameliorated (Yin et al., 2011). Again, the collective decrease in the multiple cytokines suggests that absence of APP attenuates gut inflammatory response perhaps influencing the susceptibility of these mice to gut inflammatory disease.

We ultimately expect that a number of conditions involving immune cell behavior in the gut, such as irritable bowel syndrome and Crohn's disease may well be altered in the absence of APP expression. Moreover, a role for APP in regulating immune responses may extend well beyond the gut to include conditions of immune dysfunction as varied as asthma and respiratory allergies since immune cells within the gastrointestinal system are not confined to this organ system but are free to migrate throughout the body. Future work to identify the extent of various immune cell phenotype changes dependent upon specific functions of APP or its metabolites will validate the role of this protein not only during normal physiology but a variety of diseases.

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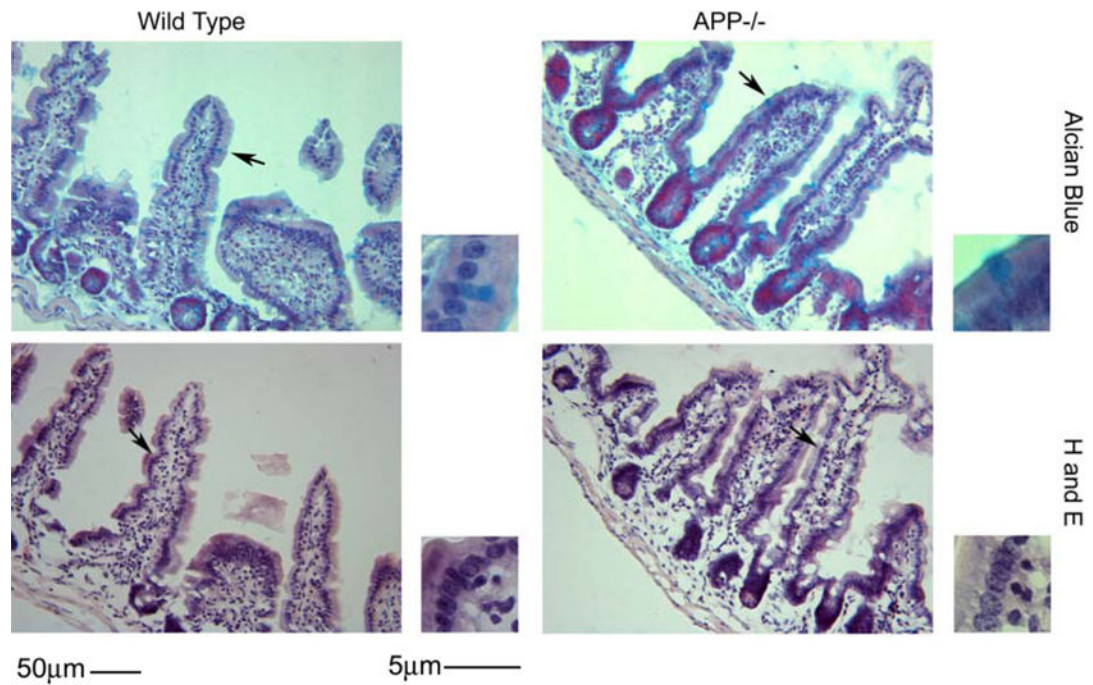


Fig. 1.

Histology of the small intestine was similar in both $APP^{-/-}$ and wild type controls. Ileum tissue samples were collected from C57BL6/J wild type and $APP^{-/-}$ mice, fixed in 4% paraformaldehyde, serially sectioned, and stained using routine histology H & E (hematoxylin and eosin) and Alcian blue. Arrows indicate the location of the region of interest shown as an enlarged inset to the right of each panel. Representative images from 5 animals per condition are shown.

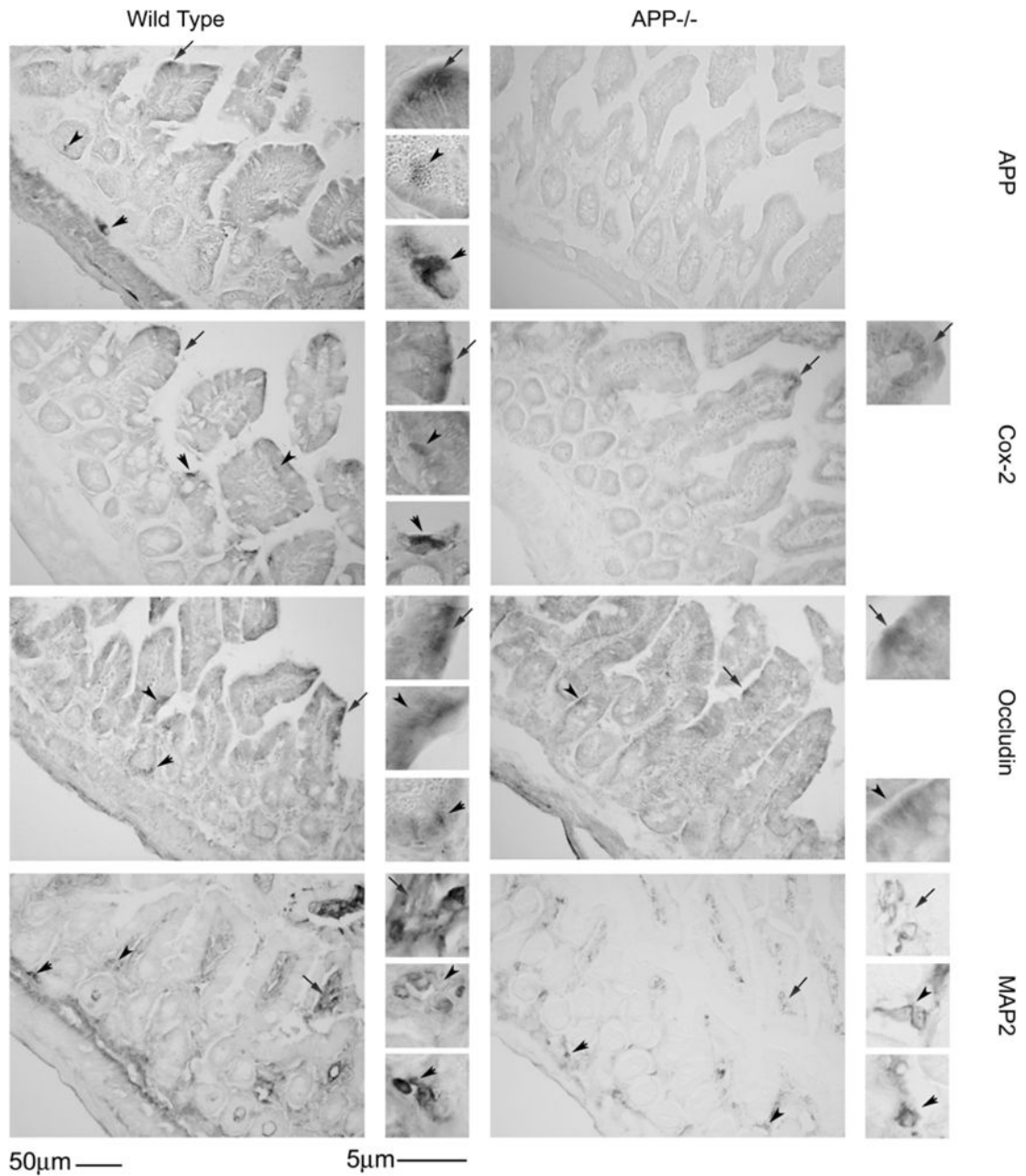


Fig. 2. Small intestine immunoreactivity for cox-2 and MAP2 decreased in APP^{-/-} mice compared to wild type controls with no change in occludin. Ileum tissue samples were collected from C57BL/6/J wild type and APP^{-/-} mice, fixed in 4% paraformaldehyde, serially sectioned, and immunostained. Tissue sections were immunostained using anti-APP, cox-2, occludin and MAP2 antibodies and antibody binding was visualized using Vector VIP as the chromagen. Arrows indicate the location of immunoreactivity shown as an enlarged inset to the right of each panel. Representative images from 5 animals per condition are shown.

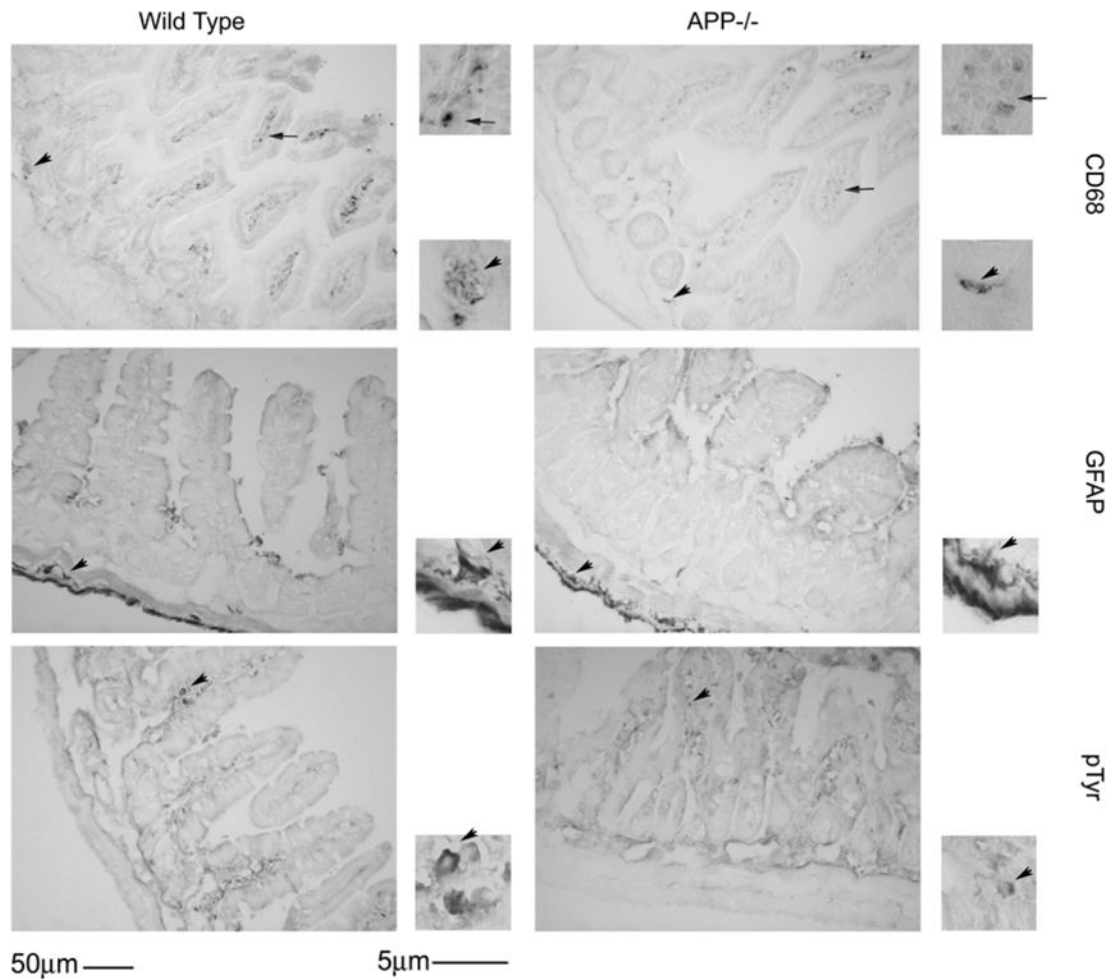


Fig. 3. Small intestine immunoreactivity for CD68 decreased in APP^{-/-} mice compared to wild type controls with no change in GFAP or pTyr. Ileum tissue samples were collected from C57BL/6J wild type and APP^{-/-} mice, fixed in 4% paraformaldehyde, serially sectioned, and immunostained. Tissue sections were immunostained using anti-CD68, GFAP and pTyr antibodies and antibody binding was visualized using Vector VIP as the chromagen. Arrows indicate the location of immunoreactivity shown as an enlarged inset to the right of each panel. Representative images from 5 animals per condition are shown.

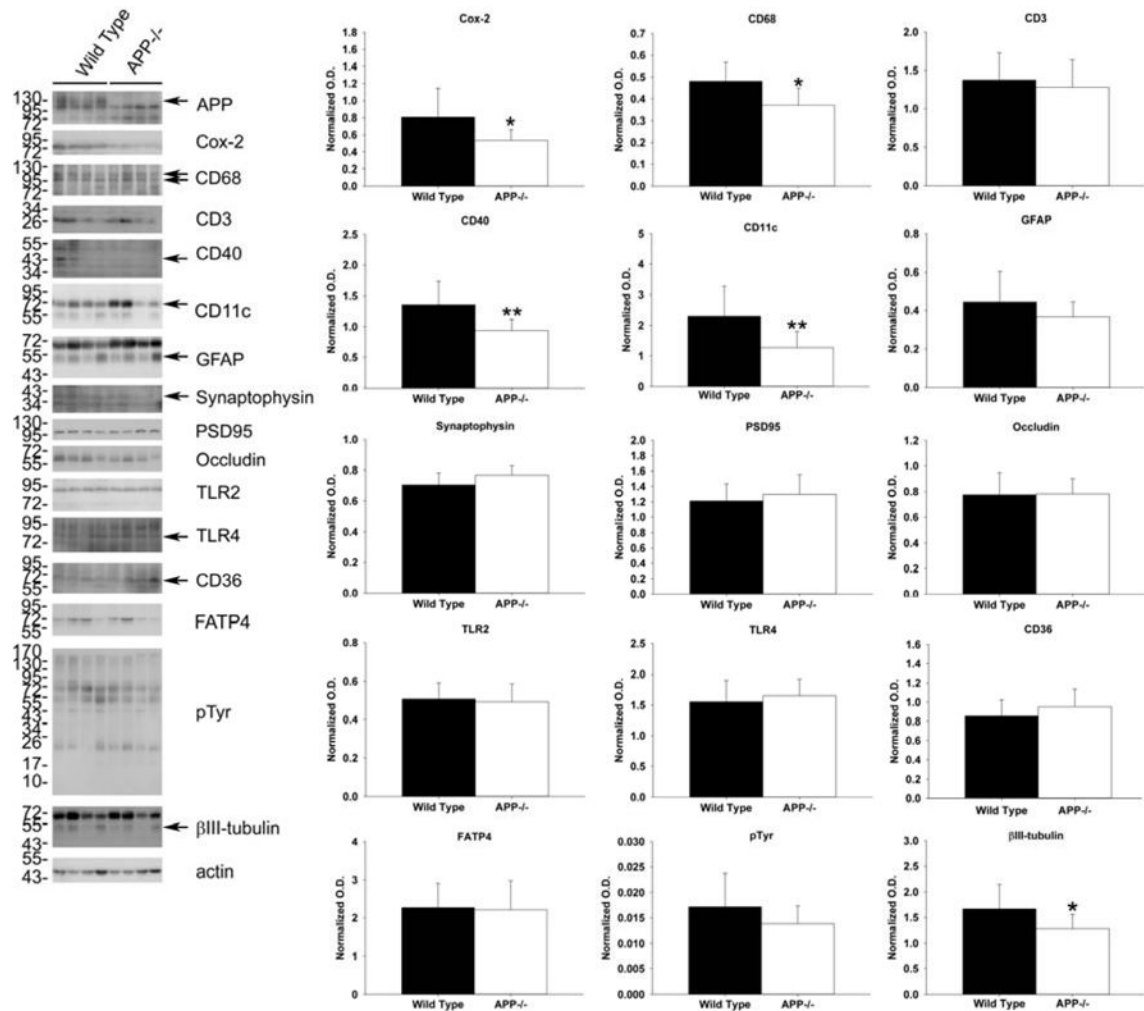


Fig. 4. Western blot analysis of cox-2, CD68, CD40, CD11c and βIII-tubulin demonstrated decreased protein levels in the small intestine of APP^{-/-} compared to wild type mice. Ileum of the small intestine was collected from APP^{-/-} and C57BL6/J wild type mice. The tissue was lysed, resolved by 10–15% SDS-PAGE and Western blotted using anti-CD68, CD3, CD40, CD11c, GFAP, TLR2, TLR4, CD36, FATP4, pTyr synaptophysin, PSD95, APP, cox-2, occludin, βIII tubulin (neuronal loading control), or actin (general loading control) antibodies. Antibody binding was visualized by chemiluminescence. Blots from 4 of the animals for each strain are shown. Optical densities of the Western blotted ileum proteins were normalized against their respective actin (cox-2, CD68, CD3, CD40, CD11c, GFAP, TLR2, TLR4, CD36, FATP4, pTyr and occludin) or βIII tubulin (synaptophysin and PSD95) loading controls and averaged (+/-SD) from 10 animals per each condition *p < 0.05.

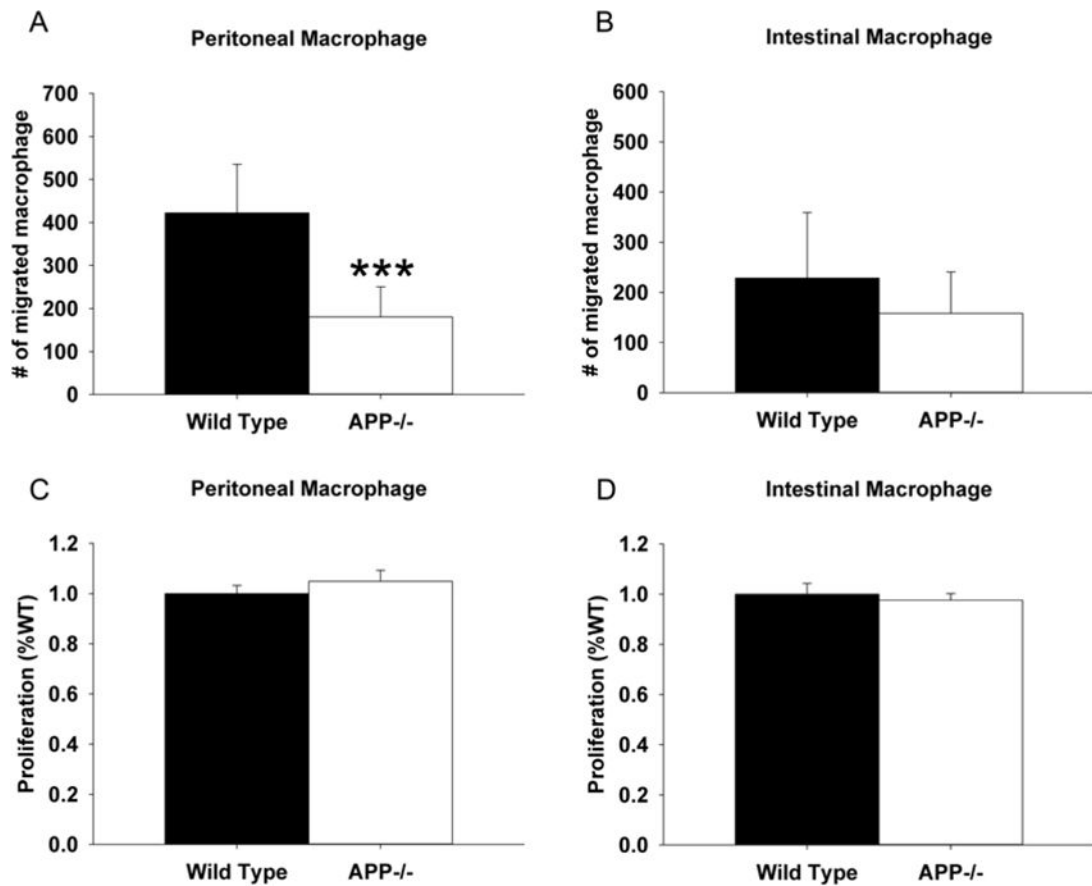


Fig. 5. Peritoneal macrophage from APP^{-/-} mice demonstrated significantly decreased migratory ability compared to wild type controls. Non-elicited peritoneal macrophage and intestinal macrophage were isolated from APP^{-/-} and C57BL6/J control mice. (A) For migration assays, macrophage were grown in serum-free DMEM/F12. Cells were added to the top of transwell permeable support inserts placed into collagen coated dishes containing 25ng/mL LPS for 24hr in order to measure migration into the bottom well as assessed by the number of migrated cells. (B) For proliferation assays, macrophage were grown in DMEM/F12 with 10% FBS and 5% horse serum for 72 hours. MTT reduction assays were used as an indirect measure of cellular number to quantify proliferation. Results are mean (+/-SD) from six animals per condition ***p<0.05.

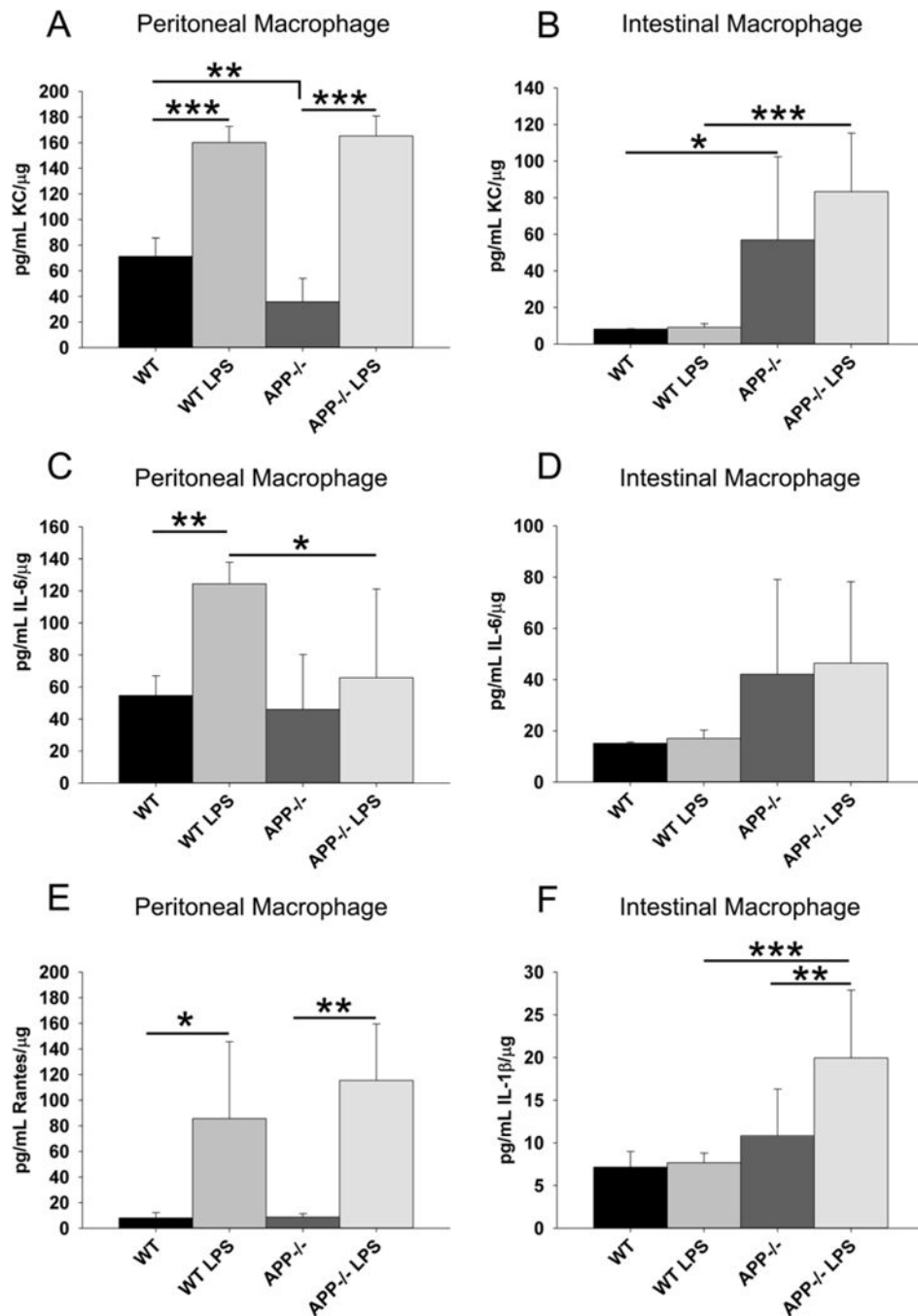
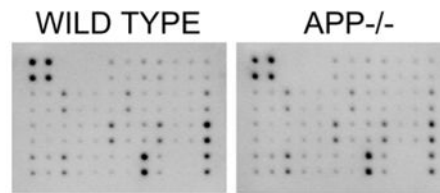
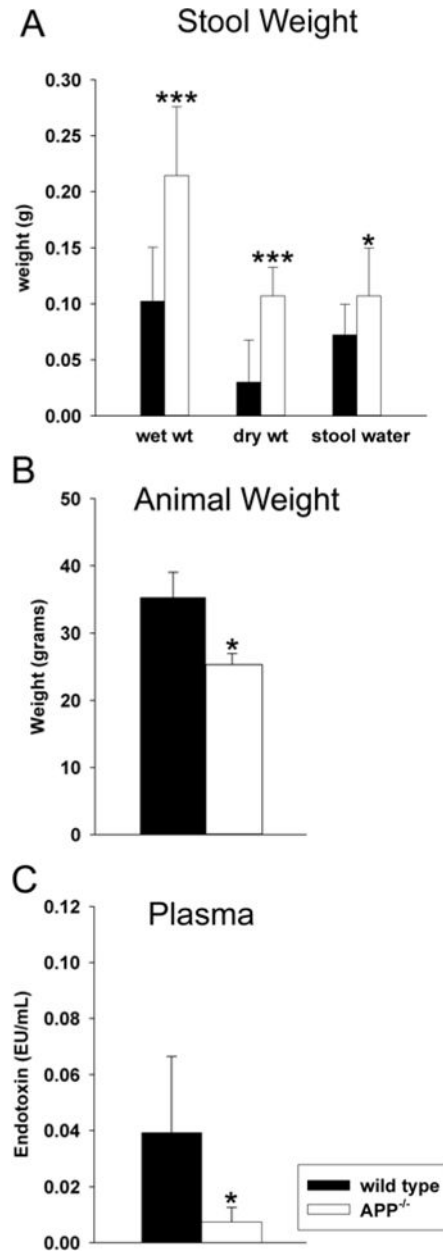


Fig. 6. $APP^{-/-}$ and wild type peritoneal macrophage demonstrated different cytokine secretory profiles compared to intestinal macrophage. Non-elicited peritoneal macrophage and intestinal macrophage were isolated from C57BL6/J wild type and $APP^{-/-}$ mice. Macrophage were stimulated with or without 25ng/mL LPS, 24hr in serum-free DMEM/F12. Media was collected to quantify basal and stimulated secretion of KC, IL-6, RANTES and IL-1 β . The results are displayed as mean (\pm SD) from 6 animals in each group * p <0.05, ** p <0.01, *** p <0.001.



	WT	APP ^{-/-}
BLC	0.98±0.06	0.94±0.04
CD30L	0.86±0.06	0.78±0.03*
Eotaxin	1.02±0.05	0.93±0.03*
Eotaxin-2	1.06±0.08	0.95±0.18
Fas Ligand	0.82±0.05	0.69±0.07*
Fractalkine	0.93±0.09	0.76±0.11*
GCSF	0.94±0.10	0.76±0.12*
GM-CSF	1.02±0.14	0.94±0.05
IFN gamma	0.93±0.09	0.85±0.03
IL-1 alpha	1.11±0.09	1.02±0.04
IL-1 beta	0.87±0.15	0.75±0.01
IL-2	0.84±0.10	0.78±0.01
IL-3	0.78±0.05	0.70±0.03*
IL-4	1.18±0.07	1.13±0.08
IL-6	0.80±0.05	0.73±0.08
IL-9	0.83±0.07	0.75±0.05*
IL-10	0.74±0.06	0.65±0.06*
IL-12p40p70	0.81±0.07	0.72±0.06*
IL-12p70	1.33±0.15	1.15±0.16
IL-13	0.99±0.12	0.96±0.02
IL-17	0.82±0.10	0.76±0.01
I-TAC	0.85±0.10	0.82±0.02
KC	0.84±0.09	0.83±0.05
Leptin	0.80±0.08	0.79±0.05
LIX	1.11±0.05	1.10±0.05
Lymphotactin	0.85±0.05	0.82±0.07
MCP-1	0.98±0.05	0.95±0.08
MCSF	1.43±0.07	1.32±0.13
MIG	0.82±0.07	0.73±0.05*
MIP-1alpha	0.90±0.10	0.84±0.03
MIP-1gamma	1.28±0.10	1.12±0.14*
RANTES	1.24±0.20	1.12±0.05
SDF-1	0.97±0.12	0.99±0.07
TCA-3	1.11±0.10	1.12±0.08
TECK	0.84±0.11	0.88±0.07
TIMP-1	0.75±0.07	0.77±0.07
TIMP-2	0.79±0.07	0.83±0.09
TNF alpha	0.75±0.08	0.70±0.07
sTNFRI	1.36±0.07	1.24±0.05*
sTNFRII	0.98±0.10	0.95±0.11

Fig. 7. Small intestine of APP^{-/-} mice demonstrated altered levels of multiple cytokines compared wild type controls. Ileum from C57BL/6/J wild type and APP^{-/-} mice were collected, lysed, and used in an antibody cytokine array. APP^{-/-} mice demonstrated decreased CD30L, eotaxin, fas ligand, fractalkine, GCSF, IL-10, IL-12p40p70, IL-3, IL-9, MIG, MIP-1 γ , and sTNFRI compared to wild type mice. A representative array for one animal from each genotype is shown. Optical densities of normalized dot intensities from 6 animals in each group are displayed as mean (+/-SD). *p<0.05

**Fig. 8.**

APP^{-/-} mice demonstrated intestinal motility and absorption differences compared to wild type mice. To compare overall intestinal absorption and motility, (A) water and stool production, (B) animal weight and (C) uptake of LPS were quantified from C57BL6/J wild type and APP^{-/-} mice. (A) To assess stool production and water absorption, wet and dry stool weights and the differences (water content) were collected over 1hr. Data from 13 animals in each group are displayed as mean (+/-SD). (B) To assess animal weight difference between strains, mice were weighed prior to collection. (C) To quantify uptake/transport of intestinal LPS into blood, whole blood was collected, plasma was separated and

the LAL Endotoxin assay was performed according to manufacturer protocol. Data from 5 animals in each group are displayed as mean (\pm SD). * $p < 0.05$, *** $p < 0.001$.